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An investigation of oxidative stress in skeletal muscle

By

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Thesis submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College Dublin.

Submitted April 2005

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'When I'm travelling through the Land of Oz in my new Takuro Spirit, I drink Nozz-A-La!' he proclaimed. 'It fills me up but never fills me out! It makes me happy to be a man! It makes me know God! It gives me the outlook of an angel and the balls of a tiger! When I drink Nozz-A-La, I say "Gosh! Ain't I glad to be alive!"

– Eddie Cantor Dean, eloquently expressing, in his characteristically unique way, the benefits of caffeinated beverages.

(Wizard and Glass, The Dark Tower).

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ii. *Acknowledgements*

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III. *Declaration*

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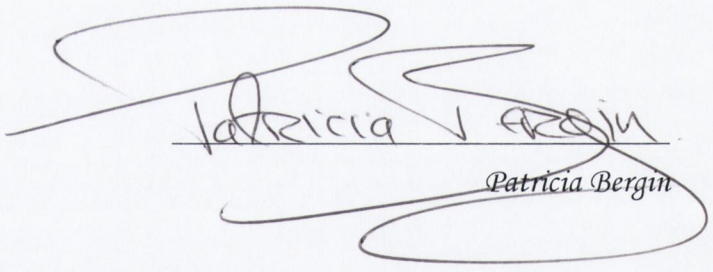

Patricia Bergin

iv. *Abstract*

Reactive oxygen species (ROS) are produced as a by-product of oxidative phosphorylation in mitochondria and via various cellular enzyme systems. ROS production is buffered by cellular antioxidant defences, which include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH), vitamin C & E. In certain circumstances, such as exercise and aging, the production of ROS in skeletal muscle increases significantly. Without adequate antioxidant defences, an oxidative stress can occur that induces damage and/or modification to cellular organelles and can result in altered cell function. In skeletal muscle, ROS have been shown to oxidize critical proteins involved in excitation-contraction coupling and the cross-bridge power stroke, which directly affects skeletal muscle force output. Deterioration in the structure and function of skeletal muscle with age, as measured by muscle atrophy, in conjunction with a diminished ability to maintain force and a loss in overall muscle strength, has been partly attributed to an increase in basal levels of ROS and reduced antioxidant defences.

The objective of this thesis was to investigate the effects of oxidative stress in skeletal muscle. To accomplish this, a number of different experimental protocols were designed where skeletal muscle isometric contractile parameters were assessed *in vitro*, in the presence or absence of an oxidative stress (as simulated by exogenous application of hydrogen peroxide (H_2O_2)). Changes in muscle mass, fibre cross-sectional area and diameter, fibre type composition and antioxidant levels were also assessed. These parameters were measured in young, adult and aged skeletal muscle throughout the course of this thesis. Dietary intervention, in which supra-nutritional doses of vitamin C & E were administered daily to adult rats for 2 months, was used to evaluate the effectiveness of vitamin supplementation in enhancing antioxidant defences and delaying ROS-induced reductions in muscle function.

Results from this thesis confirm that skeletal muscle structure and function is altered in aged animals, as verified by shifts in muscle fibre type proportions (in favour of type I and/or type IIa fibres), altered isometric contractile characteristics, reductions in maximum isometric force and a diminished ability to sustain force over time. More

importantly, it was shown that in the presence of a supramaximal dose of H_2O_2, PO in young and aged EDL was reduced to the same extent over time, whereas aged SOL resisted the decline in P_O more effectively than young SOL. These responses were attributed to changes in antioxidant status and/or changes in fibre type distribution. To confirm this hypothesis, age-related changes in antioxidant activities and fibre distributions were assessed in a number of age groups (1, 2, 10 & 22 months) and a variety of hind limb muscles with different fibre type proportions.

Age-related changes in antioxidant levels were observed (SOD, CAT, GPx, GSH and vitamin C) that depended on muscle type. Some of these alterations appeared to have been related to changes in fibre type distribution with age, where increases in CAT activity in aged SOL and increases in total SOD activity in aged EDL occurred concomitantly with increases in type I and/or IIa fibre distribution. Results suggest that the enhanced ability of aged skeletal muscle to cope with oxidative stress may be due, in part, to the age-related enhancement in antioxidant defences compared to young muscles. These results help to explain why force was affected more by an oxidant challenge in young skeletal muscle, as an improved antioxidant capacity in aged skeletal muscle could resist H_2O_2-induced alterations more effectively.

Results from the diet intervention study show that vitamin supplementation afforded skeletal muscle an enhanced level of protection against H_2O_2-induced modification. The reduction in P_O over time in vitamin-treated EDL, SOL and diaphragm muscles in the presence of an oxidant challenge was not as great as that in control muscles. It was also shown that antioxidant capacity was improved by the supplementation protocol, such that higher total SOD, CAT, GSH, vitamin C and FRAP were measured in vitamin-treated muscles, organs and plasma.

Results obtained from this thesis confirm that oxidative stress affects force production in skeletal muscle. More importantly, this thesis shows that age did not further diminish the reduction in force in the presence of an oxidant challenge compared to young muscles, whereas dietary supplementation in adult rats delayed the decline in force compared to control muscles. Collectively, these observations support the existence of a redox balance for optimal skeletal muscle contractile function, and show that contractile function can be modified by perturbations of the cellular redox balance. Therefore, in both aged and

vitamin-treated skeletal muscle, enhanced antioxidant defences afforded an enhanced level of protection above that of control muscles.

v. *List of abbreviations*

ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BSA	Bovine serum albumin
BRU	Bioresources unit
$[Ca^{2+}]_i$	Intracellular calcium concentration
CAT	Catalase
CF	Cystic fibrosis
CCL_3	Trichloromethyl
COPD	Chronic obstructive pulmonary disease
CRB	Cellular redox balance
CRC	Cellular redox curve
CSA	Cross sectional area
Cu/Zn SOD	Copper/zinc superoxide dismutase
DETAPAC	Diethylenetriaminepentaacetic acid
dH_2O	Distilled water
ddH_2O	Double distilled water
DHPR	Dihydropyridine receptors
DNA	Deoxyribonucleic acid
DTC	Dinitrophenylhydrozine/thiourea/copper
DTNB	Dithiobis-(2-nitrobenzoic acid)
EC coupling	Excitation contraction coupling
EDL	Extensor digitorum longus
EPR	Electron paramagnetic resonance
ESR	Electron spin resonance
EDTA	Ethylenediamenetetraacetic acid
ETC	Electron transport chain
$FADH_2$	Flavin adenine dinucleotide
G-6-P	Glucose-6-phosphate dehydrogenase
GPx	Glutathione peroxidase
GR	Glycolytic lobe of the gastrocnemius
GSH	Reduced glutathione

GSSG	Oxidised glutathione
GSSG-R	Glutathione reductase
GT	Whole gastrocnemius muscle
GW	Oxidative lobe of the gastrocnemius
HMP	Hexose monophosphate
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
Hz	Hertz
$L^{\cdot}$	Carbon centred lipid radical
L_f	Optimum fibre length
L_O	Optimum length
$LOO^{\cdot}$	Peroxyl radical
LOOH	lipid hydroperoxide
MnSOD	Manganese superoxide dismutase
MPO	Myeloperoxidase
$NADH_2$	Nicotinamide adenine dinucleotide
NADP	β-nicotinamide-adenine dinucleotide phosphate
NADPH	β-nicotinamide-adenine dinucleotide phosphate (reduced)
$NaHCO_3$	Sodium hydrogen carbonate
NBT	Nitroblue tetrazolium
$O_2^{\cdot -}$	Superoxide
$OH^{\cdot}$	Hydroxyl radical
$ONOO^{\cdot}$	Peroxynitrite
PL	Plantaris
PLA_2	Phospholipase A_2
P_O	Maximum isometric tetanic force
P_t	Isometric twitch force
P_t/P_O	Twitch-tetanus ratio
PUFA	Polyunsaturated fatty acid
$R^{\cdot}$	Free radical

$RO^{\cdot}$	Alkoxyl
$RO_2^{\cdot}$	Peroxyl
ROS	Reactive oxygen species
½RT	Half-relaxation time
RYR	Ryanodine receptor
SEM	Standard error of the mean
SH1	Sulfhydryl group located on the myosin head
SOD	Superoxide dismutase
SOL	Soleus
SR	Sarcoplasmic reticulum
TCA	Trichloroacetic acid
TTP	Time to peak tension
T-tubule	Transverse tubule
XO	Xanthine oxidase

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Chapter 1.

Introduction

1.1. Free radicals

Free radicals are low molecular weight molecules that contain an unpaired electron on an outer electron shell, and are often represented by a mark or dot (e.g. the hydroxyl radical, $OH^{\cdot}$, Bergendi *et al*, 1999). Since electrons are more stable when paired, radicals are generally more reactive than non-radicals and can alter/damage various biological molecules, such as lipids (Ylä-Herttuala, 1999), proteins (Stadtman & Levine, 2000) and DNA (Marnett, 2000). Some free radical sources in biological organisms include: the electron transport chain in mitochondria and non-mitochondrial enzyme systems such as: membrane-bound β-nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases, nitric oxide (NO) synthases, xanthine oxidase, cyclooxygenase, lipooxygenase and cytochrome *P*-450) (Brotto & Nosek, 1996, Diaz *et al*, 1998, Bejma & Ji, 1999, Favero, 1999, Nethery *et al*, 1999, Reid 2001). Table 1 shows some examples of free radicals.

Name	*Formula*	*Characteristics*
Hydrogen atom	H	Simplest free radical
Trichloromethyl	CCL_3	Carbon-centred radicals
Thiyl	$RS^{\cdot}$	Unpaired electron on sulphur
Peroxyl, alkoxyl	$RO_2^{\cdot}$, $RO^{\cdot}$	Product of organic peroxides
Reactive oxygen species	$O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$	Radicals derived from O_2
Reactive nitrogen species	NO, $NO_2^{\cdot}$	Formed *in vivo* from L-arginine.

Table 1.1. Examples of free radicals. Adapted from Baskin & Salem, 1997.

1.2. Reactive oxygen species

Reactive oxygen species (ROS) represent a group of oxygen centred free radicals that includes the superoxide anion ($O_2^{\cdot-}$) and the hydroxyl radical ($OH^{\cdot}$), but also intermediates in free radical reactions, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). The parent molecule in ROS production is $O_2^{\cdot-}$ (Sjodin *et al*, 1990, Jenkins, 1993, St-Pierre *et al*, 2003, Brand *et al*, 2004). All aerobic organisms produce $O_2^{\cdot-}$ as a by-product of mitochondrial electron transport chain (ETC) reactions during oxidative phosphorylation (Turrens & Boveris, 1980, Nordberg and Arner, 2001, Liu *et al*, 2002,

Chen *et al*, 2003). Under physiological conditions, the rate of ROS generation in a biological tissue is closely related to its oxygen consumption because the majority of ROS are produced in mitochondria, the cellular site of oxidative phosphorylation (Chance *et al*, 1979, Laughlin *et al*, 1990, Cadenas & Davies, 2000).

1.2.1. ROS sources in skeletal muscle

1.2.1.1. The mitochondria

Mitochondria produce energy in the form of adenosine triphosphate (ATP) (Cadenas & Davies, 2000, Brand *et al*, 2004). Electrons from reduced substrates (nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$)) are passed through electron carriers (complex I-IV in the ETC), which pump electrons across the mitochondrial inner membrane, ultimately forming H_2O in complex IV (Wilson *et al*, 1974, Liu *et al*, 2002). The electron motive force set up by this pumping drives electrons back through ATP synthase in the inner membrane, forming ATP from ADP and phosphate (Brand *et al*, 2004). These electron carriers are arranged spatially and are organised into 4 distinct complexes (Figure 1.1, Liu *et al*, 2002).

In the ETC, oxygen molecules (95-99%) remain bound to electron carriers until fully reduced (Chance *et al*, 1979). Due to electron leakage, each complex in the ETC can potentially pass an electron to O_2 to produce $O_2^{\cdot-}$, so the remaining 1-5% leaves the ETC as $O_2^{\cdot-}$. Previous studies with isolated mitochondria have detected two main ROS-forming sites; the flavin mononucleotide (FMN) group in complex I and the ubiquinone sites (Q_0 & Q_i) in complex III (Turrens & Boveris, 1980, Turrens *et al*, 1985, Degli Esposti, 2002, Liu *et al*, 2002). Complex I has been shown to produce the majority of $O_2^{\cdot-}$ in mammalian mitochondria *in vitro* (Chen *et al*, 2003, Brand *et al*, 2004), with $O_2^{\cdot-}$ generation occurring primarily on the matrix side of the inner mitochondrial membrane in skeletal muscle (Figure 1.1, Lee & Wei, 1997, St-Pierre *et al*, 2002, Miwa *et al*, 2003). Although complex I generates larger quantities of $O_2^{\cdot-}$, production by complex III is potentially more damaging to the mitochondria because the $O_2^{\cdot-}$ produced here is directed away from the antioxidant defences of the mitochondrial matrix and into the intermembrane space (Figure 1.1, Chen *et al*, 2003).

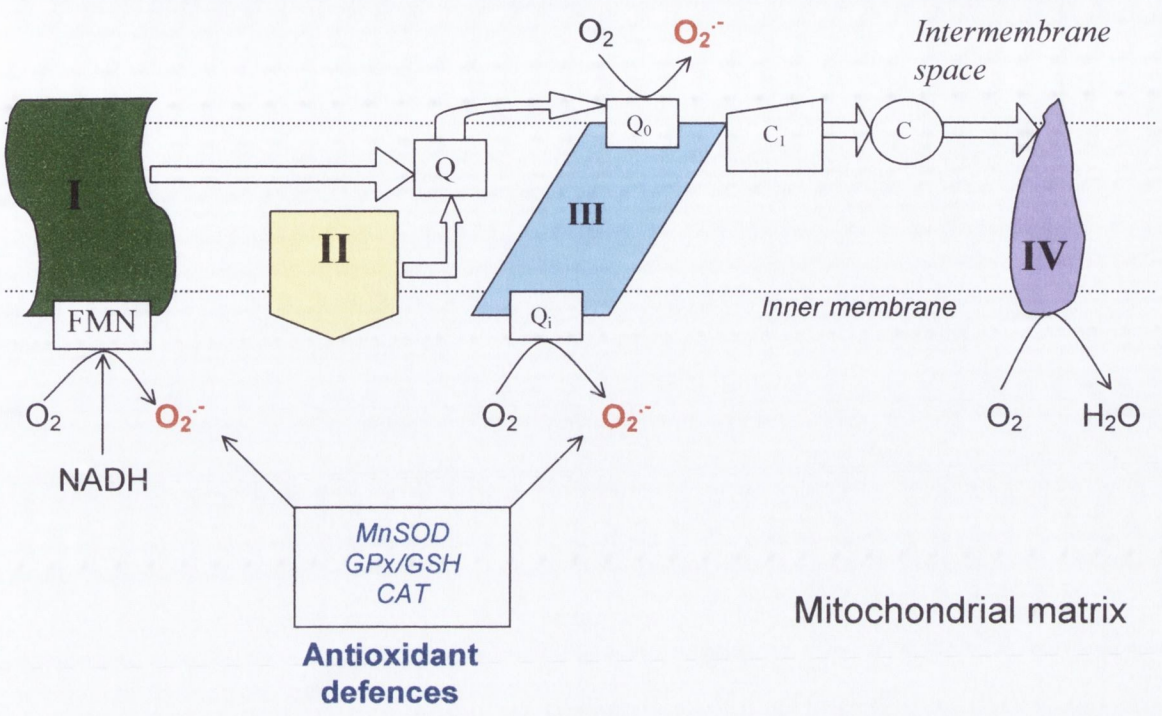


Figure 1.1. The electron transport chain showing the sites of production of ROS in complex I and III in mitochondria.

The flavin mononucleotide (FMN) site of complex I and the ubiquinone (Q_i) site of complex III release ROS into the matrix, toward mitochondrial antioxidant defences. In contrast, the ubiquinone (Q_0) site of complex III releases ROS toward the intermembrane space, away from matrix defences. Adapted from Chen *et al*, 2003.

As $O_2^{\cdot-}$ cannot penetrate lipid membranes, it remains in the intermembrane space where it can induce alterations to mitochondrial proteins and affect the function of the organelle (Cardoso *et al*, 1999, Hamilton *et al*, 2001). In contrast, complex I releases $O_2^{\cdot-}$ directly into the mitochondrial matrix in close proximity to the antioxidant defence systems (Figure 1.1, St-Pierre *et al*, 2002). Here it is quickly dismutated by manganese superoxide dismutase (MnSOD) to H_2O_2, which diffuses across mitochondrial membranes causing little (if any) damage to the organelle (Boveris & Cadenas, 1975). Experiments using MnSOD knockout mice demonstrated that mitochondrial ROS generation is a physiologically significant process *in vivo* as rats died within their first week of life (Lebovitz *et al*, 1996, Melov *et al*, 2000). This also confirms the importance of MnSOD in maintaining normal function in mitochondria-rich organs.

1.2.1.2. Non-mitochondrial sources

(i) Xanthine oxidase

$O_2^{\cdot-}$ can be produced in muscle as a by-product of the xanthine oxidase-catalysed conversion of hypoxanthine to uric acid (Equation 1.1, McCord & Fridovich, 1969). Cytosolic xanthine oxoreductase (XOR) exists cellularly as a dehydrogenase (XD) or an oxidised form (XO). Under physiological conditions, XD is the main form of XOR in cells and it uses NAD^+ for electron transfer, resulting in the formation of NADH. In contrast, XO uses O_2, which can produce $O_2^{\cdot-}$ (Equation 1.1, Kuppusamy & Zweier, 1989).

$$\text{hypoxanthine} + H_2O + 2O_2 \xrightarrow{XO} \text{uric acid} + 2O_2^{\cdot-} + 2H \qquad (1.1)$$

Expression of XO has been shown in both the peripheral and respiratory muscles of rodents and in skeletal muscles of humans (Hellsten Westing, 1993, Hellsten *et al*, 1997). Production of $O_2^{\cdot-}$ by XO significantly increases during ischemia-reperfusion injury (McCord, 1985, Smith *et al*, 1989, Zimmerman & Granger, 1994) and during a loss of Ca^{2+} homeostasis in cells, which has been shown to occur in oxidative stress due to oxidation of Ca^{2+}-ATPase pumps (Ragusa *et al*, 1996, Reid, 2001). However, it has been suggested that $O_2^{\cdot-}$ production by XO may not play a significant role in contraction-related ROS generation in humans compared to other sources (Dorion *et al*, 1993).

(ii) Neutrophils

Polymorph neutrophils (PMN) can produce hypochlorous acid (HOCl) in a reaction that involves the myeloperoxidase (MPO)-catalysed enzymatic oxidation of chloride ions (Cl^-) by H_2O_2 (Equation 1.2, Babior *et al*, 1984, Bast *et al*, 1991).

$$H^+ + Cl^- + H_2O_2 \xrightarrow{MPO} HOCl + H_2O \qquad (1.2)$$

$O_2^{\cdot-}$ is released by neutrophils alongside MPO, which dismutates to H_2O_2 and in turn serves as the substrate for the MPO-catalysed formation of HOCl (Equation 1.2, Winterbourn *et al*, 2000), which can then damage proteins and lipids in cell membranes. PMN infiltration of skeletal muscle has been shown to increase after exercise in humans (Suzuki *et al*, 1996, Saxton *et al*, 1999). It has been shown that oxidative stress can be

important in the initiation and propagation of the acute muscle inflammatory response following exercise (Tiidus, 1998, Callahan *et al*, 2001, Langen *et al*, 2001).

(iii) Other sources

In skeletal muscle, other sources of ROS include the cytosolic enzyme aldehyde oxidase (Chance *et al*, 1979) and the arachidonic acid-cyclooxygenase pathway (Kuehl & Egan, 1980). However, the biological importance of these sites has not yet been established in skeletal muscle (Reid, 2001). Skeletal muscle sarcoplasmic reticulum membranes are known to contain NADPH-dependent enzyme systems that can generate ROS (Duncan & Rudge, 1988). It is possible that increases in membrane metabolic activity could trigger generation of ROS during muscle contraction via this pathway.

1.2.2. The ROS cascade

As discussed (Section 1.2.1), $O_2^{\cdot-}$ production accounts for approximately 1-5% of total O_2 consumption in resting skeletal muscle (Equation 1.3) (Halliwell, 1994, Clanton *et al*, 1999).

$$O_2 + e^- \rightarrow O_2^{\cdot-} \qquad (1.3)$$

It has been estimated that humans produce up to 1.72kg of $O_2^{\cdot-}$ per year (Halliwell, 1994). $O_2^{\cdot-}$ is generated by a number of different biological pathways and, most importantly, is a precursor for other ROS and free radicals. It has been shown to react with nitric oxide (NO), forming the reactive nitrogen species peroxynitrite ($ONOO^{\cdot}$, Equation 1.4).

$$O_2^{\cdot-} + NO \rightarrow ONOO^{\cdot} \qquad (1.4)$$

$ONOO^{\cdot}$ has been implicated in the inhibition of the mitochondrial ETC (Lipton *et al*, 1993, Squadrito & Pryor, 1998).

Although it can react with a number of cell constituents, $O_2^{\cdot-}$ has a lower reactivity for a given biological substrate than its products (Lavelle *et al*, 1973, Kellogg & Fridovich, 1977). $O_2^{\cdot-}$ dismutates naturally to form H_2O_2, which can traverse the hydrophilic

membrane of the mitochondria and pass into the cytoplasm of the cell (Equation 1.5, Sjodin *et al*, 1990, Yu, 1994).

$$2O_2^{\cdot -} \xrightarrow[2H^+]{e^-} H_2O_2 + O_2 \qquad (1.5)$$

Although H_2O_2 itself is not a free radical, it plays an important role in ROS-induced alterations in cells due to its high diffusion capacity (Yu, 1994). In skeletal muscle cell cultures, elevated levels of H_2O_2 have been measured following intense muscle contraction, suggesting that a large quantity of H_2O_2 formed intracellularly can diffuse through cellular membranes into the extracellular space (Silveira *et al*, 2003). H_2O_2 is also a strong oxidant (Lavelle *et al*, 1973, Stocks & Dormandy, 1971) and previous studies have assessed its effects on skeletal muscle injury after exercise, excitation-contraction coupling and ROS-induced modulation of force production (Favero *et al*, 1995, Oba *et al*, 1996, Posterino & Lamb, 1996, Plant *et al*, 2001, Reid, 2001).

Once H_2O_2 enters the cytoplasm, it can be converted to $OH^{\cdot}$ by the transitional metal-dependent Fenton reaction (Equation 1.6), which involves the conversion of a moderately reactive substance (H_2O_2) into an extremely reactive ROS ($OH^{\cdot}$) (Halliwell & Gutteridge, 1991, Yu, 1994, Lloyd *et al*, 1997, Kourie, 1999).

$$H_2O_2 + Cu^+ / Fe^{2+} \rightarrow OH^{\cdot} + OH^- + Cu^{2+} / Fe^{3+} \qquad (1.6)$$

$O_2^{\cdot -}$ plays an important role in connection with the Fenton reaction by oxidizing transitional metal ions, thus maintaining adequate levels available to participate in the reaction (Equation 1.7).

$$Cu^{2+} / Fe^{3+} + O_2^{\cdot -} \rightarrow Cu^+ / Fe^{2+} + O_2 \qquad (1.7)$$

Due to its strong reactivity, $OH^{\cdot}$ induces more oxidative damage to cells than any other ROS (Cadenas & Davies, 2000). $OH^{\cdot}$ oxidizes sulfhydryl groups on cysteine residues to produce disulfide bonds, which may affect protein function (Kowaltowski & Vercesi, 1999), and reacts with the lipid constituents of cellular membranes to initiate membrane lipid peroxidation (Section 1.2.3, Chance *et al*, 1979). It has been suggested that the high

permeability of muscle cells to H_2O_2 and NO may constitute an important defence mechanism against the formation of intracellular $OH^{\cdot}$ and $ONOO^{\cdot}$ (Silveira *et al*, 2003, Patwell *et al*, 2004). In these studies, H_2O_2 and NO extracellular release were assessed by measuring dichlorofluorescin (DCFH) oxidation (a fluorescent probe for ROS) and increases in the levels of nitrite and nitrate in the solution bathing the muscle cells being tested. A marked 105% increase in the oxidation of DCFH, and 100% increase in nitrate and nitrite levels were observed after intense electrical stimulation when compared to baseline values.

1.2.3 Lipid peroxidation

The radical chain reaction of lipid peroxidation is a continuous physiological process that can alter essential cell functions by affecting membrane stability resulting in membrane rupture and cell death (Chance *et al*, 1979, Fraga *et al*, 1987). Lipid peroxidation is initiated by a free radical ($R^{\cdot}$) when a hydrogen atom is abstracted from the bis-allylic site (methylene group [$-CH_2-$] between double bonds of a polyunsaturated fatty acid (PUFA)) to give a carbon-centred lipid radical ($L^{\cdot}$) (Equation 1.8). This lipid radical reacts with oxygen to form a peroxyl radical ($LOO^{\cdot}$) (Equation 1.9).

$$-LH + R^{\cdot} \rightarrow L^{\cdot} + RH \qquad (1.8)$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \qquad (1.9)$$

The peroxyl radical reacts with an adjacent PUFA side chain (L'H) to form another lipid radical ($L'^{\cdot}$) and a lipid hydroperoxide (LOOH, Equation 1.10), which propagates the lipid chain reaction (Frei, 1994).

$$LOO^{\cdot} + L'H \rightarrow LOOH + L'^{\cdot} \qquad (1.10)$$

It has been shown that lipid peroxidation occurs following exercise and is involved in several disease states, including cancer, cardiovascular diseases and in the degenerative processes associated with aging, as measured by significant increases in thiobarbituritic acid reactive substances (Ames *et al*, 1993, Jayachandran *et al*, 1996, Chiaradia *et al*, 1998).

1.2.4. ROS & skeletal muscle

It has been demonstrated in both *in vitro* and *in vivo* preparations, using reduction of cytochrome-c (used as a measure of $O_2^{\cdot-}$) and fluorescence of various redox sensitive probes, that resting skeletal muscle produces $O_2^{\cdot-}$ (Reid *et al*, 1992, Supinski *et al,* 1997, Diaz *et al*, 1998, Clanton *et al*, 1999, Stofan *et al*, 2000, Reid, 2001). Evidence of $O_2^{\cdot-}$ production in skeletal muscle has also been produced by electron spin (ESR) and electron paramagnetic resonance (EPR) techniques (Masuda *et al*, 2003). An increase in EPR signals in rat hind limb skeletal muscle follows a single bout of fatiguing exercise (Davies *et al*, 1982) and, similarly, an increase in ESR signals in the diaphragm of rats has been shown when diaphragm workload was increased (Diaz *et al*, 1993). Other studies have infused contracting muscles with salicylate to measure the formation of 2,3-dihydrobenzoic acid (an indicator of $OH^{\cdot}$ formation) with high performance liquid chromatography (HPLC) (Fantone & Ward, 1982, Diaz *et al*, 1993).

Specific ROS that are produced by skeletal muscle have been identified by previous studies, and include: $O_2^{\cdot-}$ (Reid *et al*, 1992a & 1992b, McArdle *et al*, 2001), $OH^{\cdot}$ (O'Neill *et al*, 1996, McArdle *et al*, 2004), NO (Balon & Nader, 1994) and H_2O_2 (Silveira *et al*, 2003). It has also been suggested that the pattern of release of ROS in skeletal muscle is dependent on the frequency of stimulation used to cause contraction. Myotubes electrically stimulated to contract with low and high frequencies produced $O_2^{\cdot-}$ and NO, and also led to an increase in extracellular $OH^{\cdot}$ levels. Increasing the frequency of stimulation increased NO generation and $OH^{\cdot}$ levels, but had no significant effect on the levels of $O_2^{\cdot-}$ produced (Silveira *et al*, 2003, Patwell *et al*, 2004). The relationship between ROS and skeletal muscle will be developed later (Section 1.5.4 & 1.7).

1.3. Oxidative stress

ROS production can increase significantly and is dependent on the physiological state of the tissue (Clanton *et al*, 1999, McArdle *et al*, 2002). In resting skeletal muscle, ROS production should approximately equal ROS removal by the antioxidant defence systems (Figure 1.2*(a)*). When ROS levels exceed endogenous antioxidant defences due to: increased production of ROS with a normal antioxidant capacity (Figure 1.2*(b)*), normal ROS production in the presence of a reduced antioxidant capacity (Figure 1.2*(c)*) or as a combination of both (Figure 1.2*(d)*), a state of oxidative stress is produced (Chow, 1979,

Halliwell, 1997, Sies, 1997). Oxidative stress, resulting from any of the physiological states described in figure 1.2, may occur during exercise, fatigue, aging and various pathophysiological conditions such as cancer, septic shock and respiratory failure (Brotto & Nosek, 1996, Clanton *et al*, 1999, Cadenas & Davies, 2000, Finkel & Holbrook, 2000, Polidori *et al*, 2001, Inayama *et al*, 2002). However, the presence of oxidative stress does not automatically imply oxidative damage. ROS-induced damage can only be verified by the direct assessment of the markers of oxidative stress. Nevertheless, a large body of literature has clearly shown that oxidative stress impairs skeletal muscle contractile performance (Barclay & Hansel, 1991, Reid *et al*, 1992a, Supinski *et al*, 1995).

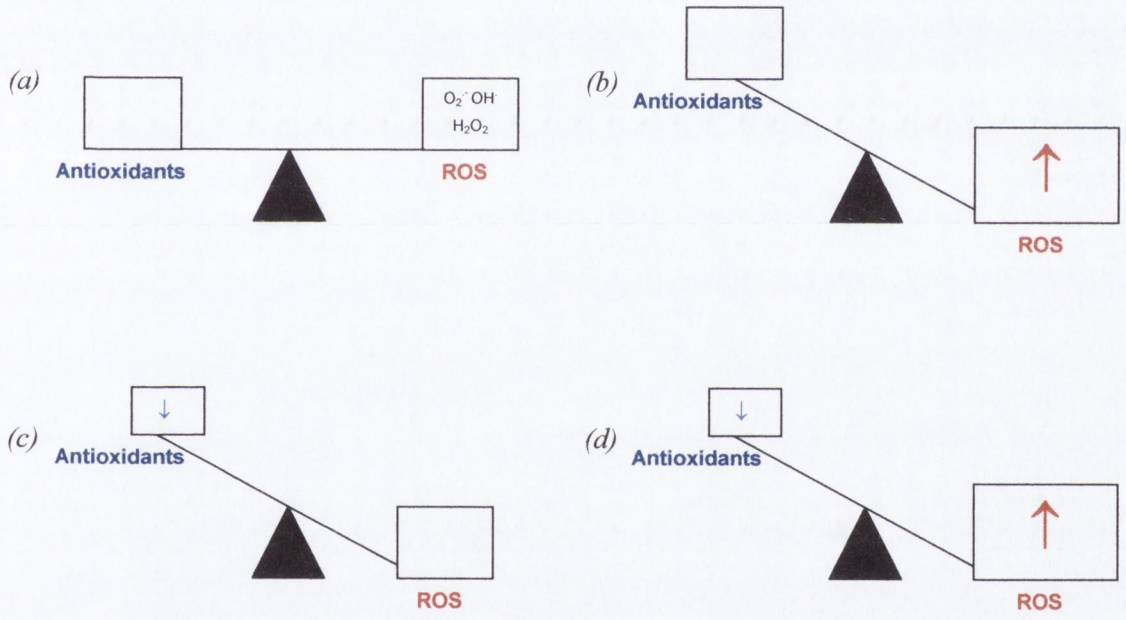


Figure 1.2. Different physiological states by which oxidative stress can occur.

↑ - increase, ↓ - decrease. *(a)* At rest, ROS production ($O_2^{\cdot-}$, H_2O_2 & $OH^{\cdot}$) is buffered by antioxidant defences (CAT, SOD, GPx) so that production approximately equals removal. *(b)* Despite an adequate antioxidant capacity, a marked increase in ROS may overwhelm antioxidant defences to produce oxidative stress. *(c)* Oxidative stress may occur due to reduced antioxidant defences without an increase in ROS production. *(d)* A worst-case scenario is seen when there is a reduction in antioxidant capacity in conjunction with an increase in ROS production. Adapted from Deaton & Marlin, 2003.

Exercise is associated with an increase in the production of ROS in skeletal muscle (Jackson *et al*, 1985, Chiaradia *et al*, 1998). Using ESR spectroscopy, Davies *et al*, 1982 were the first to show that strenuous exercise increased ROS production in rat skeletal muscles. Subsequently, fatiguing contractions were shown to increase the rate of ROS generation in the rat diaphragm (Reid *et al*, 1992a & 1992b, Kolbeck *et al*, 1997). However, this increase in ROS is not solely attributed to increased $O_2^{\cdot-}$ production in the

ETC as a by-product of oxidative phosphorylation. Following exercise of high intensity, ROS levels in skeletal muscle are amplified due to the additive effect of ROS release from infiltrating neutrophils and macrophages (Suzuki *et al*, 1996, Sacheck & Blumberg, 2001). A redistribution of blood flow also occurs with exercise, such that hypoxia and reoxygenation is induced in some tissues, which can have an additive effect to the increase in $O_2^{\cdot-}$ production by XO as previously discussed (Section 1.2.1). Both glutathione depletion (Sen *et al*, 1992) and an increased GSSG/GSH ratio (Lew *et al*, 1985), which will be discussed later (Section 1.4.2), have been measured after an acute bout of exercise, which suggests that during exercise, not only is ROS production increased but a concomitant decrease in antioxidant defences also occurs, similar to Figure 2*(d)*.

Exposure of peripheral or diaphragm muscle strips to exogenously applied ROS increases fatigue rates, as measured by significant decreases in force (Barclay & Hansel, 1991, Lawler *et al*, 1991, Diaz *et al*, 1993, Supinski *et al*, 1997), whereas antioxidants such as N-acetylcysteine, CAT, SOD and dimethylsulphoxide ($OH^{\cdot}$ scavenger) attenuate the rate of fatigue development *in vitro* (Diaz *et al*, 1994, Khawli & Reid, 1994). A significant inverse correlation has also been observed between the impairment of force generation in the diaphragm and the amount of $O_2^{\cdot-}$ released during fatiguing contractions (Reid *et al*, 1992a). These results confirm that increased ROS levels can compromise skeletal muscle function and will be developed further in Section 1.7.

It has been suggested that age-related deteriorations in cell structure and function are caused by a shift in the ROS-antioxidant balance in favour of ROS, resulting in the production of an oxidative stress (Rikans & Hornbrook, 1997, McArdle *et al*, 2002). Skeletal muscle aging has been associated with a diminished ability of antioxidant systems to convert ROS into more inert species, which results in an oxidative stress (Ji, 1993). This has been confirmed in aged skeletal muscle, as measured by significant increases in lipid peroxidation, protein and DNA oxidation, an increase in oxidized glutathione concentrations and altered antioxidant enzyme activities (Ji & Fu, 1992, Starnes *et al,* 1989, Zainal *et al*, 2000). This will be developed further in Section 1.8.

1.4. Antioxidant defences

ROS activity is tightly regulated by the presence of enzymatic and non-enzymatic antioxidant systems that are located in most mammalian cells (Fernandez & Videla, 1996). Antioxidants reduce/prevent any potential ROS-induced damage while simultaneously enhancing ROS scavenging (Reid *et al*, 1993, Heunks & Dekhuijzen, 2000, Miquel, 2002), and have been defined as '*substances that, when present at low concentrations compared with those of oxidizable substrates, significantly delay or prevent oxidation of those substrates by forming less reactive radicals or by quenching ROS reactions*' (Halliwell & Gutteridge, 1995). Enzymatic antioxidants mainly deal with ROS located intracellularly, being absent or present in small amounts extracellularly, whereas non-enzymatic antioxidants act in both the intra- and extracellular spaces, although they contribute proportionately more extracellularly (Colven & Pinnell, 1996). A large number of interacting antioxidant systems are involved in preventing/limiting oxidative damage (Ji *et al*, 1992, Yu, 1994, Nordberg & Arner, 2001), and include;

1. Interaction with oxidants and oxidizing agents by vitamin C and glutathione
2. Scavenging of free radicals and singlet oxygen by vitamin E, vitamin C and β-carotene
3. Dismutation of $O_2^{\cdot-}$ by superoxide dismutases to hydroperoxides
4. Reduction of hydroperoxides by catalase and glutathione peroxidases
5. Binding of transitional metals by various chelators

The antioxidant defence system represents a complex network with interactions, synergisms and specific tasks for a large number of antioxidants (Nordberg & Arnér, 2001, Polidori *et al*, 2001). For this reason, not all aspects of the antioxidant defence system can be discussed in this thesis. Antioxidants that are expanded upon and analyzed throughout the course of this thesis were chosen due to their level of importance in maintaining a balance between ROS production and removal and also the degree of interaction that exists between these antioxidants in ROS removal, which is expanded on in Section 1.4.4.

1.4.1. Antioxidant enzymes

Antioxidant enzymes degrade ROS and are essential for cell survival (Bejma & Ji, 1999, Clanton *et al*, 1999). Inhibition of antioxidant enzyme activity leads to the arrest of cell mitosis and cell death (Kasapoglu & Özben, 2001). It has been repeatedly shown that antioxidant enzyme levels in skeletal muscle and other tissues depend on a number of factors, including age, sex and species (Ji *et al*, 1992, Hollander *et al*, 2000, Pansarasa *et al*, 2000, Masuda *et al*, 2003, Gündüz *et al*, 2004), and also vary according to the metabolic properties of the tissue; such that antioxidant enzyme levels are higher in skeletal muscle fibres that predominantly use oxidative phosphorylation for energy production (Laughlin *et al*, 1990, Ji *et al*, 1992, Pansarasa *et al*, 1999, Liu *et al*, 2000). This will be discussed in more detail later (Section 1.6.2 & 1.8.5). The location and interaction of the different antioxidant enzymes can be seen in Figure 1.3. The main antioxidant enzymes active in the detoxification of ROS within the body are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). These antioxidant systems work in conjunction with each other to maximize the removal of ROS from both intra- and extracellular regions and will be discussed in detail below.

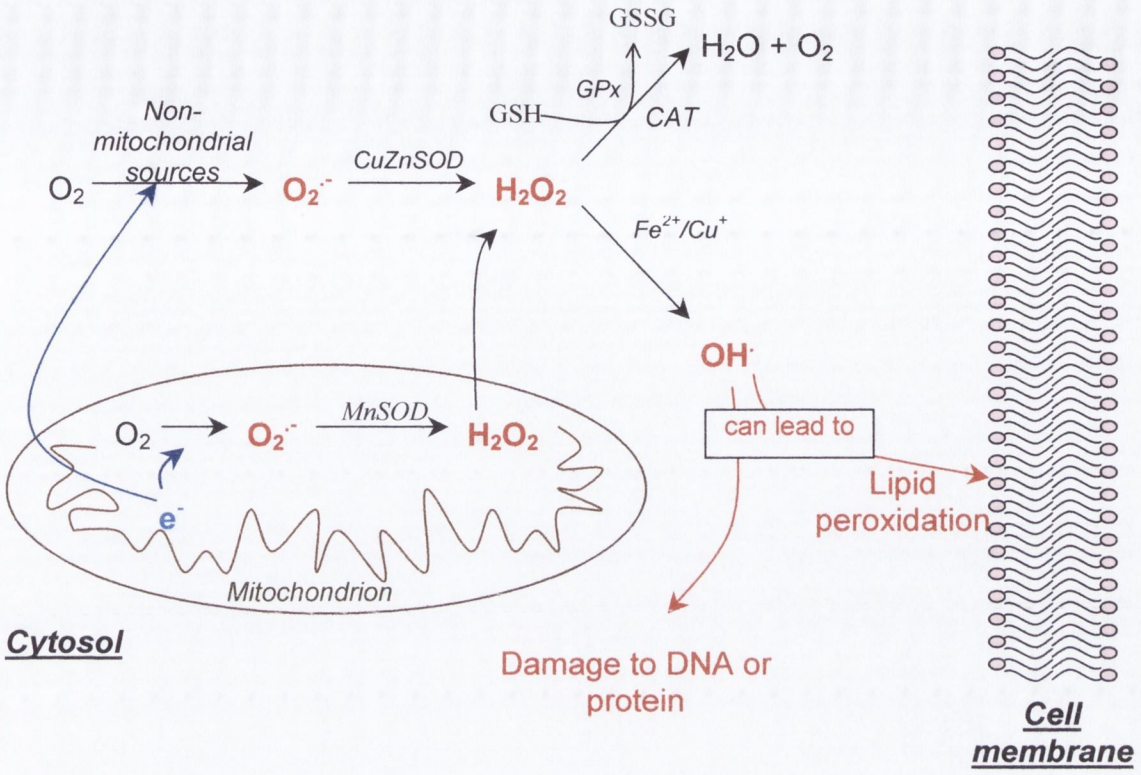


Figure 1.3. Location and interactions between antioxidant enzymes in the mitochondrion and surrounding cytosol.

Manganese superoxide dismutase (MnSOD) acts as the first line of defence against ROS by dismutating $O_2^{\cdot-}$ to H_2O_2, thereby accelerating its removal from the mitochondrion. H_2O_2 removal is achieved by the combined actions of catalase (CAT) and glutathione peroxidase (GPx). H_2O_2 can be converted to $OH^{\cdot}$ by the Fenton reaction (Fe^{2+}/Cu^{+}). In the absence of adequate antioxidant defences, $OH^{\cdot}$ production can result in lipid peroxidation and damage to DNA and proteins. Adapted from Nordberg & Arnér, 2001.

1.4.1.1. Superoxide dismutase

Superoxide dismutase (SOD) accelerates the dismutation of $O_2^{\cdot-}$ to H_2O_2 at a rate 4 times faster than natural dismutation (Equation 1.11, Yu, 1994, Hollander *et al*, 2000).

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \xrightarrow{\text{Superoxide Dismutase}} H_2O_2 + O_2 \qquad (1.11)$$

H_2O_2 produced by this pathway can either diffuse out of the mitochondrion and be converted to H_2O and O_2 by cytosolic catalase (Equation 1.12), or alternatively, if the H_2O_2 remains within the mitochondrion, it is converted to H_2O by mitochondrial GPx (Panfili *et al*, 1991). SOD acts as the first line of defence against ROS due to its interaction with $O_2^{\cdot-}$ (Figure 1.3, Van Loon *et al*, 1986) and has copper, zinc (Cu,Zn SOD), manganese (MnSOD) and iron (Fe-SOD) isoforms (Reid *et al*, 2001). Although

catalytic mechanisms appear the same, gene expression, protein turnover and regulatory properties of the 3 isoenzymes are different (Ohno *et al*, 1994).

MnSOD is responsible for the removal of $O_2^{\cdot-}$ generated on the matrix side of the inner mitochondrial membrane (Figure 1.1 and 1.3, Baizan *et al*, 1999). The importance of SOD in mammals has been demonstrated using transgenic mice deficient MnSOD, which died within their first week of life (Melov *et al*, 2000) whereas Cu,Zn SOD transgenic mice show no abnormalities until exposed to traumatic injury (Matés *et al*, 1999). Administration of SOD *in vitro* reduces the contraction-associated increase in cytochrome *c* reduction by $O_2^{\cdot-}$ in the diaphragm (Reid *et al*, 1992a, Stofan *et al*, 2000).

1.4.1.2. Catalase

In mammalian cells, catalase (CAT) is found predominantly in subcellular locations, such as peroxisomes (Nordberg & Arner, 2001), but is also located in microsomes and the cytosol of hepatocytes (Yu, 1994). CAT's primary role is to dehydrate H_2O_2 (Equation 1.12, Heunks & Dekhuijzen, 2000), but is also associated with peroxidase activity ($ROOH^{\cdot}$) in the presence of hydrogen donors, such as methanol and ethanol (AH_2) (Equation 1.13, Matés *et al*, 1999).

$$2H_2O_2 \xrightarrow{Catalase} O_2 + 2H_2O \qquad (1.12)$$

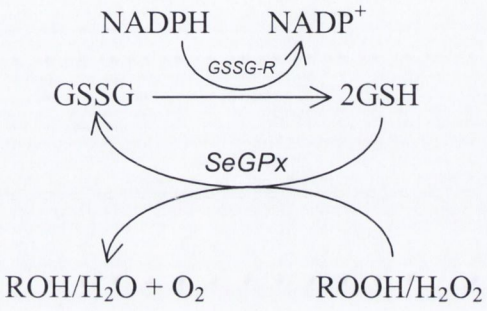
$$ROOH^{\cdot} + AH_2 \xrightarrow{Catalase} H_2O + ROH + A \qquad (1.13)$$

The survival of rats exposed to 100% O_2 increased when they were pre-treated with liposomes containing high levels of SOD and CAT (Matés *et al*, 1999). Where SOD reduces $O_2^{\cdot-}$ concentrations in both the mitochondrion and cytosol by accelerating dismutation to H_2O_2 (Equation 1.11), CAT reduces $OH^{\cdot}$ formation by lowering the concentrations of H_2O_2 available to participate in the Fenton reaction (Equation 1.6, Figure 1.3).

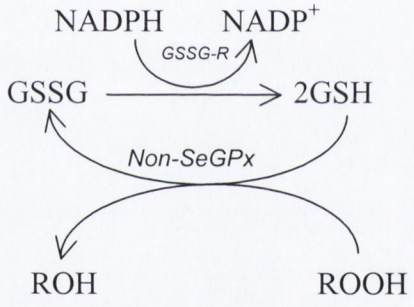
1.4.1.3. Glutathione peroxidase

Until 1976, it was believed that a single selenoprotein, the glutathione-hydrogen peroxide oxidoreductase protein, exclusively expressed glutathione peroxidase activity (Equation 1.14, Paglia & Valentine, 1967). This selenoprotein (SeGPx) used organic

hydroperoxides (ROOH) and H_2O_2 as substrates. However, a second enzyme of smaller molecular weight was later identified in selenium (Se)-deficient livers that also exhibited GPx activity (Burk *et al*, 1978). This enzyme used organic hydroperoxides, but not H_2O_2, as a substrate and was referred to as selenium independent GPx (non-SeGPx) (Equation 1.15). These GPx isoforms are found in different cellular locations, where SeGPx is localised within the cytosol and non-SeGPx is associated with lipid membranes (Ragusa *et al*, 1996).



(1.14)



(1.15)

GPx activity levels differ between species with regard to the peroxide specificity of the enzyme (Di Ilio *et al*, 1983) and in the proportion of SeGPx to non-SeGPx (Carmagnol *et al*, 1983). Although SeGPx shares the substrate H_2O_2 with CAT, evidence has suggested that SeGPx removes H_2O_2 generated by peroxisomal oxidases (Oshino *et al*, 1975), whereas CAT dehydrates H_2O_2 generated by SOD in the mitochondria and cytosol (Oshino & Chance, 1977). The GPx system is essential in skeletal muscle where oxidative stress and lipid peroxidation increase significantly with increased work, exercise, age and disease (Watanabe *et al*, 1993, Powers & Lennon, 1999, Supinski *et al*, 1999).

1.4.1.4. The thioredoxin family

Thioredoxins (Trx) are proteins with oxidoreductase activity that repair oxidized sulfhydryl groups, remove H_2O_2, and act as radical scavengers (Holmgren, 2000). Expression of Trx is induced by oxidative stress and results in an increase in the reduction of intracellular proteins and other biomolecules, which is believed to act as a preventative mechanism against ROS-induced damage (Taniguchi *et al*, 1996). Trx also modulates the signal transduction properties of ROS by reducing intracellular disulfides that have been oxidized by ROS and by lowering ROS levels directly (Nakamura *et al*, 1997). Trx is also involved in regeneration of dehydroascorbic acid to vitamin C (Figure 1.4, Section 1.4.4, Nordberg & Arner, 2001).

A role for glutaredoxin (Grx) has been implicated in the protection and repair of proteins and non-protein thiols, and its function overlaps that of the Trx family. Grx can be reduced by GSH and is capable of reducing GSH-mixed protein disulfides formed by oxidative stress (Holmgren, 2000). Peroxiredoxins (Prx) have been classified as antioxidant enzymes that protect protein and lipids against oxidant-induced damage by directly reducing peroxides. Prx are found in the cytosol, mitochondria, peroxisomes and plasma, and are thought to be involved in signalling pathways regulating apoptosis and cell proliferation, differentiation and gene expression. (Kim *et al*, 1988, Chae *et al*, 1999). The thioredoxin and glutaredoxin enzyme systems may contribute to antioxidant activity, however, these systems are still very novel and their roles in skeletal muscle have not been well defined.

1.4.2. Antioxidant molecules

Vitamin C, vitamin E, uric acid, β-carotene and other carotenoids are categorised as antioxidant molecules (Dekkers *et al*, 1996). Other molecules with ROS-scavenging capabilities include glutathione and the micronutrient elements zinc and selenium (Polidori *et al*, 2001). Glutathione, vitamin C and uric acid are all important hydrophilic antioxidants, whereas vitamin E is the major lipophilic antioxidant and, as such, is present in membranes and lipoproteins (Burton *et al*, 1982, Frei, 1994).

1.4.2.1. Glutathione

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is one of the most abundant short-chain peptides in the body and is a major source of non-protein thiols (Ji *et al*, 1992, Favero, 1999, Heunks & Dekhuijzen, 2000). It is synthesized by γ-glutamyl cysteine synthase, which uses glutamate and cysteine, in combination with glycine (catalysed by glutathione synthase) to form glutathione (Dringen *et al*, 2000). Glutathione functions as a sulfhydryl buffer, but also detoxifies compounds either by conjugation reactions catalysed by glutathione transferase (Di Ilio *et al*, 1983) or directly in the SeGPx-catalysed reaction with H_2O_2 (Equation 1.14, Ji *et al*, 1992). One of the primary functions of GSH in the body is to serve as a substrate for GPx and as a scavenger of H_2O_2 (Meister & Anderson, 1983).

Intracellular glutathione metabolism is regulated by complex pathways (Meister, 1995). Reduced glutathione (GSH) scavenges H_2O_2 resulting in the formation of oxidized glutathione (GSSG), a pathway that is catalysed by SeGPx (Equation 1.16, Gohill *et al*, 1988, Jenkins, 1988, Ji, 1995). To maintain the cellular glutathione balance, GSSG is converted back to GSH by the NADPH-dependent flavoenzyme glutathione reductase (GSSG-R) (Equation 1.17). This reaction activates the transfer of hydrogen ions (H^+) from the hexose monophosphate (HMP) shunt pathway of glucose metabolism (G-6-P) to $NADP^+$ in the presence of glucose-6-phosphate dehydrogenase (G-6-P*ase*) (Equation 1.18, Nordberg & Arnér, 2001). GSSG-R maintains the equilibrium between GSH and GSSG, in favour of GSH (Meister, 1995).

$$H_2O_2 \xrightarrow[2GSH \quad GSSG]{SeGPx} 2H_2O \qquad (1.16)$$

$$GSSG \xrightarrow[NADPH \quad NADP^+]{GSSG\text{-}R} 2GSH \qquad (1.17)$$

$$NADP^+ \xrightarrow[G\text{-}6\text{-}P \quad HMP]{G\text{-}6\text{-}Pase} NADPH \qquad (1.18)$$

In mammalian cells, GSH concentrations are in the millimolar range whereas the concentration of GSSG is 2 or 3 orders of magnitude lower (Lew *et al*, 1985). Therefore, the ratio of GSH to GSSG is maintained at a relatively high level (range 100:1) (Ji *et al*, 1992, Pansarasa *et al*, 2000, Reid & Durham, 2002). The concentrations and ratio of reduced glutathione (GSH) versus oxidized glutathione (GSSG) can undergo dynamic changes under various physiological and pathological conditions, such as exercise, inflammatory lung processes, coronary disease, cancer and aging, and are often documented as sensitive measures of oxidative stress (Sies *et al*, 1980, Sies & Akerboom, 1984, Halliwell & Gutteridge, 1985, Deneke & Fanburg, 1989, Ji *et al*, 1992, Baskin & Salem, 1997). As GSH scavenges ROS and becomes oxidized, an elevated level of the oxidized form of glutathione (GSSG) may be considered a marker of oxidative stress and is calculated according to equation 1.19 to provide an index of the redox state of cells (Benzi *et al*, 1988).

$$\left([\mathrm{GSH}] + 2[\mathrm{GSSG}]\right) / \left(2[\mathrm{GSSG}] \times 100\right) \qquad (1.19)$$

1.4.2.2. Vitamin C (ascorbic acid)

Vitamin C (ascorbic acid) is a ubiquitous, water-soluble antioxidant found in the plasma and the cytosol of cells (May, 1999, Griffiths & Lunec, 2001). Vitamin C intercepts ROS in the aqueous phase of plasma before they can react with and oxidize lipoproteins (Frei *et al*, 1988, Polidori *et al*, 2004). Due to the absence of L-gulono-γ-lactone oxidase, the final enzyme in ascorbate synthesis, human beings are scorbutic and have no ability to synthesize ascorbate (Colven & Pinnell, 1996, Griffiths & Lunec, 2001). The total ascorbic acid pool in humans has been estimated at 1500-2500mg, with a daily turnover of 45-60mg (Fain, 2005). Therefore, the minimal daily dietary requirement to combat vitamin C deficiency in humans has been set at 40-60mg/day (Levine *et al*, 2001).

Vitamin C intake is dependent on absorption in the ileum by an energy driven, Na^+-dependent transport process, which becomes saturated when oral intake exceeds 180mg/day (Rose & Bode, 1993, Tsukaguchi *et al*, 1999). A vast range of ROS ($OH^{\cdot}$, peroxyl radicals, $O_2^{\cdot-}$ and HOCl), RNS ($ONOO^{\cdot}$) and antioxidant-derived radicals (α-tocopheroxyl and urate radical) are scavenged and/or neutralized/recycled by vitamin C (Sies & Stahl, 1995, Naidoo & Lux, 1998, Carr & Frei, 1999). Vitamin C has been shown to be the first antioxidant oxidized in the presence of ROS, even in mitochondria where

concentrations are low (Vatassery, 1995). Once scavenging/recycling has taken place, vitamin C is oxidized to dehydroascorbic acid (DHA) in two 1-electron steps with the release of H^+ (Figure 1.4, Halliwell, 1996). The intermediate radical produced, the ascorbyl radical, has low reactivity, which helps to prevent further oxidation of various biomolecules.

Short- and long-term vitamin C supplementation in humans has been shown to significantly increase plasma ascorbate concentrations and dose-dependently improve the resistance of plasma to lipid peroxidation *ex vivo* (Polidori *et al*, 2004). Vitamin C supplementation has been shown to increase the concentration of GSH and the activities of SOD, CAT and GPx in cardiac muscle of guinea pigs, which are unable to synthesise vitamin C similar to humans (Chatterjee *et al*, 1975, Rojas *et al*, 1994). Additional evidence supporting the beneficial effects of supra-nutritional doses of vitamin C include the diminished alcohol-induced oxidative stress response measured in the liver of guinea pigs administered vitamin C over a period of 30 days (Suresh *et al*, 1999). However, vitamin C can act as a pro-oxidant if present in high concentrations and in the presence of transition metals (Aust *et al*, 1985). An increase in a mutagenic lesion, 8-oxoadenine (a marker of ROS-induced DNA damage) following vitamin C supplementation (500mg/day) has been measured in humans (Podmore *et al*, 1998).

A number of factors adversely affect vitamin C concentrations. Smoking is associated with ROS-induced damage to tissue and organs (Pryor & Stone, 1993), and a smoking-related depletion of plasma ascorbic acid has been previously demonstrated (Tribble *et al*, 1993). Vitamin C deficiency also induces collagen abnormalities due to it's role in the synthesis of collagen (Halliwell, 1994), which helps to explain the clinical symptoms of scurvy, including: abnormal dentine production and loss of teeth, vessel wall damage and bleeding and skin changes related to keratin abnormalities (DeLuna *et al*, 2003). Males who live alone, chronic alcoholics and smokers, and individuals on a restrictive/poor diet are at high risk of low vitamin C intake (Kallner *et al*, 1981, Jacob *et al*, 1988, Malmauretet *et al*, 2002).

1.4.2.3. Uric acid

Uric acid is produced from the breakdown of purine compounds (Becker, 1993) and functions as an antioxidant by binding to free iron, thus preventing the Fenton reaction

and scavenging ROS such as HOCl and $OH^{\cdot}$ (Sevanian *et al*, 1991). It has been proposed that uric acid functions as an antioxidant in plasma (Ames *et al*, 1981) and is responsible for 30-65% of the peroxyl radical-scavenging capacity of plasma (Waynes *et al*, 1987). It is then oxidized to the urate radical, which is either recycled or oxidized further to form allantoin, oxonic acid or parabanic acid (Becker, 1993). The presence of allantoin in biological samples indicates oxidative consumption of uric acid and reflects an increase in ROS levels (Maples & Mason, 1988).

1.4.2.4. Vitamin E (α-tocopherol)

Vitamin E was discovered over 80 years ago (Evans & Bishop, 1922) and is the generic name given to a group of biologically active lipid-soluble compounds the tocopherols and tocotrienols, of which α-tocopherol is the most abundant in the plasma of humans (Di Mambro *et al*, 2003, Choi *et al*, 2004). The most active form of vitamin E is α-tocopherol, which accounts for approximately 90% of the activity of vitamin E in tissues, and it is located in the hydrophobic region of cell membranes (Machlin *et al*, 1982). It has been suggested that vitamin E stabilises the cell membrane against ROS-induced lipid peroxidation through the interaction of its phytyl side chain with the polyunsaturated fatty acid (PUFA) groups of phospholipids (Valk & Hornstra, 2000). It is because of this association with the cell membrane and its ability to act directly with ROS that vitamin E is regarded as an important dietary antioxidant in biological systems (Erin *et al*, 1984, Packer *et al*, 1991, Coombes *et al*, 2001).

When α-tocopherol reacts with lipid peroxyl radicals ($LOO^{\cdot}$) a lipid hydroperoxide (LOOH) and a tocopheroxyl radical is produced (Equation 1.20, Suga *et al*, 1984).

$$LOO^{\cdot} + \alpha - \text{tocopherol} \rightarrow LOOH + \alpha - \text{tocopheroxyl radical} \qquad (1.20)$$

This reaction helps to reduce/prevent the propagation of lipid peroxidation (Equation 1.10, Fraga *et al*, 1987, Dekkers *et al*, 1996, Wang & Quinn, 2000, Azzi *et al*, 2003). In the tissues of vitamin E-deficient animals, an increase in the occurrence of lipid peroxidation has been observed, suggesting that vitamin E plays a role as a physiological antioxidant based on its chemical properties (Suga *et al*, 1987).

Vitamin E is absorbed by the lymphatic system (Machlin & Gabriel, 1983). After oral administration, vitamin E combines with mixed bile salt micelles in the small intestine to form a fine emulsion before moving across the epithelial cell membrane (Gallo-Torres, 1970). It is incorporated into a lipoprotein unit, a chylomicron, which is too large to move through the pores of the blood capillaries and instead is passed into the lymphatic vessels (Julianto *et al*, 2000). Examination of individual plasma level profiles show that absorption of vitamin E is biphasic in nature (Julianto *et al*, 2000), which is likely to reflect the need for mobilization of other components of the chylomicrons in the absorption process.

Supplementation with vitamin E significantly reduces ROS production in skeletal muscle, as measured by reduced ESR signals and decreases in lipid and protein oxidation products (Factor *et al*, 2000, Gatellier *et al*, 2000). As the main site of ROS production in the cell, mitochondria have the highest concentration of vitamin E, most of which is located in the inner membrane (Bjorneboe *et al*, 1991). Supplementation with vitamin E has been shown to significantly reduce H_2O_2 production in the mitochondria of liver and skeletal muscle and dose-dependently lower the rates of mitochondrial H_2O_2 output (Chow *et al*, 1999). An acute bout of exercise does not seem to significantly affect vitamin E content in tissues, which suggests that physiological levels of vitamin E are adequate protection against exercise-induced ROS generation (Gohill *et al*, 1987, Tiidus *et al*, 1993). However, the protective margin may be relatively small since vitamin E concentration decreases significantly in rat skeletal muscle, liver and heart after chronic exercise (Packer *et al*, 1989, Tiidus *et al*, 1993). Vitamin E has also been shown to regulate $O_2^{\cdot-}$ levels in neutrophils (Ando *et al*, 1996) and monocytes (Cachia *et al*, 1998).

Vitamin E deficiency results in increased oxidative stress in peripheral skeletal muscle as indicated by an increased ESR signal, increased lipid peroxidation of cell membranes, altered GSH/GSSG redox status and early fatigue in the diaphragm during resistance breathing in rats (Davies *et al*, 1982, Anzueto *et al*, 1993, Tiidus & Houston, 1994, Urano, 1998). Disruption of mitochondrial ultrastructure is one of the earliest pathological events observed in the skeletal muscle of vitamin E-deficient animals (Thomas *et al*, 1993), which also leads to a marked increase in H_2O_2 production in skeletal muscle mitochondria (Chow *et al*, 1999). Rats with vitamin E deficiency demonstrated exacerbated muscle ROS production and excessive lipid peroxidation and

mitochondrial dysfunction after an acute bout of exhaustive exercise compared with vitamin E adequate rats (Davies *et al*, 1982). Synthesis of XO is also markedly increased in the skeletal muscle of vitamin E-deficient rabbits (Catignani *et al*, 1974) and liver of vitamin E-deficient rats (Masugi & Nakamura, 1976), which in turn can enhance $O_2^{\cdot-}$ production as discussed previously (Equation 1.1, Section 1.2.1).

1.4.2.5. Coenzyme Q

Coenzyme Q or ubiquinone is a redox-active, lipophilic substance present in the hydrophobic interior of the phospholipid bilayers of virtually all the cellular membranes (Miles *et al*, 2005). It consists of a quinine head attached to a chain of isoprene units numbering 9 or 10 in the various mammalian species. The quinone head can assume three different redox states: ubiquinone (the fully oxidized form); ubisemiquinone (the partially reduced form, also a free radical); and ubiquinol (the fully reduced form).

Coenzyme Q plays multiple roles in cells (Turunen *et al*, 2004). Its quinone form transfers electrons in the mitochondrial electron transport chain from complexes I and II to complex III (Figure 1.1). In accordance with it being an essential component of the mitochondrial electron respiratory chain, total coenzyme Q concentration is 1.5-5 fold higher in mitochondria than in tissue homogenates. Second, the quinol form of coenzyme Q acts as a potent antioxidant in the inner mitochondrial membrane where it inhibits lipid peroxidation by scavenging free radicals and/or reducing α-tocopheroxyl radicals to α-tocopherol. Lipid solubility, efficient continuous regeneration and involvement in the initiation and propagation steps of lipid peroxidation explain why coenzyme Q is considered a highly efficient antioxidant against ROS produced in biological membranes. Finally, the third established function is that autoxidation of its semiquinone form is an intracellular source of $O_2^{\cdot-}$ and H_2O_2.

Coenzyme Q tissue content is highest in organs with increased energy requirements, e.g. cardiac and skeletal muscle. Coenzyme Q supplementation has been shown to attenuate oxidative stress in rat skeletal muscle, as indicated by lower protein carbonyls in mitochondria and an increase in GSH/GSSG, suggesting an elevation in reducing potential (Kwong *et al*, 2002). Therefore, coenzyme Q supplementation may be beneficial in lowering *in vivo* oxidative stress by tipping the pro-oxidant/antioxidant balance towards antioxidants.

1.4.4. Interactions between antioxidants

Antioxidant molecules have the ability to interact with one another (Figure 1.4, Packer *et al*, 1972, Niki *et al*, 1982, Packer *et al*, 2001, Urso & Clarkson, 2003). Vitamin C (ascorbic acid) can reduce the vitamin E radical (α-tocopheroxyl) to regenerate vitamin E (Figure 1.4, May, 1999, Umegaki *et al*, 2000). The resulting vitamin C radical (ascorbyl radical) is stable and almost unreactive and can be enzymatically reduced back to vitamin C by the NADPH system, by TrxR, by dismutation reactions or by the GSH system (Figure 1.4, Colven & Pinnell, 1996, Dekkers *et al*, 1996, May *et al*, 1997). *In vitro* studies have demonstrated that vitamins C & E work together (Niki *et al*, 1995, Benzie & Strain, 1999) and *in vivo* studies report an increase in vitamin E status with vitamin C supplementation in human subjects (Hamilton *et al*, 2000), guinea pigs (Liu & Lee, 1998), rats (Tanaca *et al*, 1997) and rabbits (Castellini *et al*, 2000). A chronologically distinct response in plasma vitamin C and vitamin E ingestion has been measured, suggesting that the effect of vitamin C ingestion may have passed by the time vitamin E-associated effects have begun (Choi *et al*, 2004), which could help to explain differences in vitamin C and E concentrations measured between studies.

Mitochondria can also regenerate vitamin C from its oxidised form, helping to maintain vitamin levels in the mitochondria and the cytoplasm (Li *et al*, 2002). Coenzyme Q also has the ability to reduce the vitamin E radical (α-tocopheroxyl) to regenerate vitamin E (Kwong *et al*, 2002). It has been shown that vitamin C also recycles the urate radical, which is produced when uric acid scavenges ROS (Naidoo & Lux, 1998). In erythrocytes and in the aqueous portion of the cell, GSH is one of the major reductants of dehydroascorbic acid and works synergistically with vitamin C to protect the cell from oxidative damage (Winkler *et al*, 1994, Meister, 1995). Vitamin E can also be recycled by GSH (Sacheck & Blumberg, 2001, Gosker *et al*, 2004).

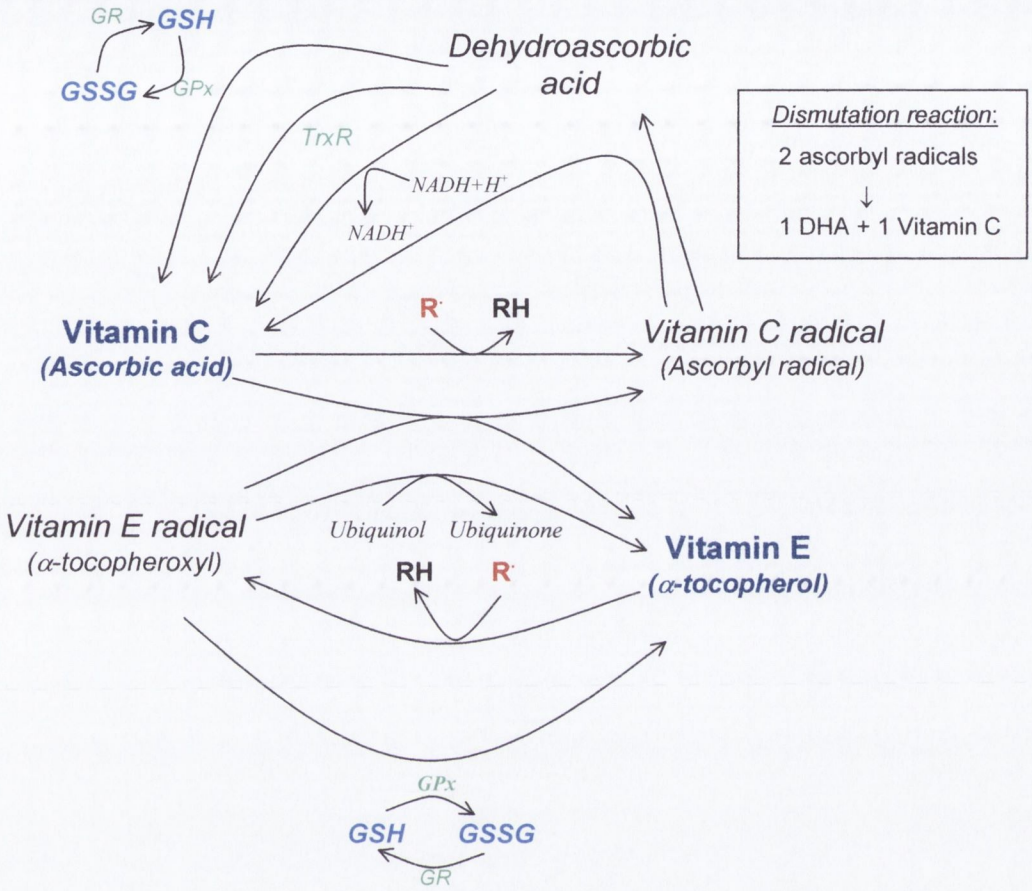


Figure 1.4. A representative diagram of the interplay between various antioxidant molecules.

Scavenging of a radical ($R^{\cdot}$) by vitamin C or E results in them becoming radicals. The vitamin E radical *(α-tocopheroxyl)* is recycled back to vitamin E (α-tocopherol) by vitamin C (ascorbic acid) (Packer *et al*, 1979). In this reaction, vitamin C radicals (*ascorbyl radicals*) are formed, 2 of which can spontaneously dismutate to produce vitamin C and dehydroascorbic acid. TrxR and the NADH system can reduce dehydroascorbic acid to vitamin C. Interactions between the GSH system and vitamins E & C are also represented. Adapted from Nordberg & Arnér, 2001.

1.5. Skeletal muscle

1.5.1. Anatomy

Skeletal muscle is a very heterogeneous tissue, composed of different muscle fibre types, and has a clear striation pattern of light and dark bands (Figure 1.5, Lieber *et al*, 1991).

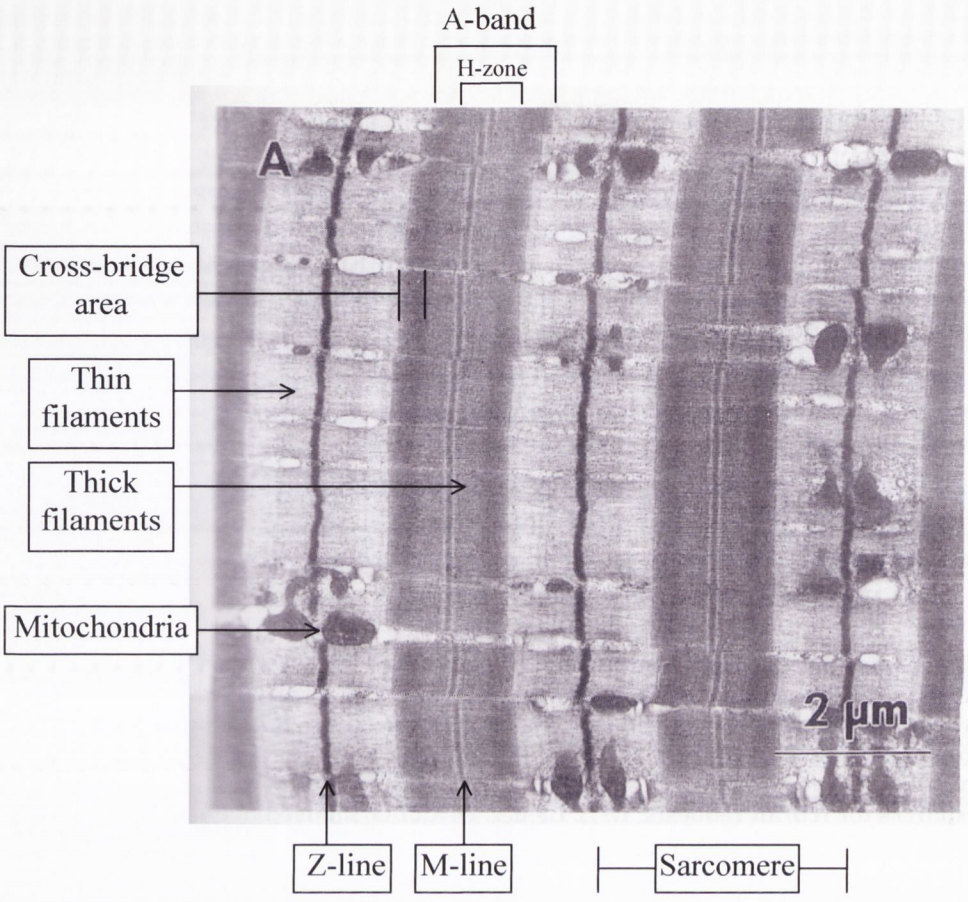


Figure 1.5. Longitudinal electron micrograph of rabbit tibialis anterior muscle fibre.

The striation pattern produced by the arrangement of thick and thin filaments (Lieber *et al*, 1991). Thick filaments are connected together by M-lines. The A-band spans the length of the thick filaments along with the portions of thin filaments that overlap on both ends of the thick filaments. The H-zone is an area that thin filaments do not reach, only the central portion of the thick filaments is found here.

The light band consists of thin actin filaments and the darker band contains both thick myosin and thin actin filaments (Hansen & Huxley, 1953). Each myosin molecule consists of 2 identical subunits projecting out at one end forming the myosin head, each of which contains an actin binding and a myosin ATPase site (Herzog & Ait-Haddou, 2002). Actin is the primary structural protein in the thin filament, which is also composed of tropomyosin and troponin. Tropomyosin and troponin are often referred to as regulatory proteins because of their role in covering or exposing the binding sites for cross-bridge interaction between actin and myosin (Patchell *et al*, 2005).

The sarcoplasmic reticulum (SR) is a membranous structure located in skeletal muscle and contains terminal cisternae, which store Ca^{2+} (Dulhunty *et al*, 2002). The survival of cells is dependent upon Ca^{2+} homeostasis in the cytosol, and in skeletal muscle this is

achieved by the combined actions of Ca^{2+} release channels and Ca^{2+}-ATPase pumps located on the SR membrane (Figure, 1.6, Franzini-Armstrong & Protasi, 1997). Total SR Ca^{2+} concentration has been determined in the millimolar (mM) range per muscle fibre and represents the driving force for Ca^{2+} release into the sarcoplasm where intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is in the nanomolar (nM) range (Fryer *et al*, 1995, Fryer *et al*, 1996).

Excitation of skeletal muscle via sarcolemmal depolarization induces biphasic changes in $[Ca^{2+}]_i$, with an initial rise of myoplasmic Ca^{2+}, due to Ca^{2+} release from the lumen of the SR through Ca^{2+} release channels, followed by a subsequent decrease as a result of several mechanisms among which is the reaccumulation of Ca^{2+} into the SR via the Ca^{2+}-ATPase pump (Fleischer & Inui, 1989, Ishii *et al*, 1998). Sarcolemmal depolarization in skeletal muscle activates specialized membrane L-type voltage-sensitive Ca^{2+} channels (dihydropyridine receptor, DHPR) on transverse (T)-tubules (membranous perpendicular extensions of the surface membrane that penetrate the muscle fibre). In response to this depolarization, DHPRs open the Ca^{2+} release channels in the SR (Flucher & Franzini-Armstrong, 1996, Wu & Hamilton, 1998), which initiates a flux of Ca^{2+} into the sarcoplasm (Aghdasi *et al*, 1997).

1.5.2. Ryanodine and dihydropyridine receptors

Since the discovery that a plant alkaloid, ryanodine, was a specific ligand for the Ca^{2+} release channel on the SR membrane, this Ca^{2+} release channel is now commonly referred to as the ryanodine receptor (RYR) (Fleischer *et al*, 1985). The RYR of skeletal muscle is a tetramer comprised of four 565kDa RYR subunits (Marks, 1996) that is activated by micromolar concentrations of Ca^{2+}, caffeine and ATP and is inhibited by Mg^{2+}, procaine and ruthenium red (Kasas *et al*, 1999). The RYR is located near the DHPR on the T-tubule (Eu *et al*, 1999), and plays a central role in excitation-contraction coupling by releasing the Ca^{2+} required for muscle contraction. It has been proposed that DHPRs are physically coupled to the RYR of SR (Franzini-Armstrong & Nunzi, 1983, Rios *et al*, 1991) such that morphological (Block *et al*, 1988) and physiological data (Simon & Hill, 1992) have suggested a model in which 4 DHPRs interact with a single RYR molecule (Figure 1.6). As not every RYR is associated with a DHPR, increased levels of cytosolic Ca^{2+} stimulate other RYR not directly linked to DHPR via a positive, feed forward mechanism called Ca^{2+}-induced Ca^{2+} release (Heunks & Dekhuijzen, 2002). Signalling

between DHPR and RYR is reciprocally interactive, conformational changes in the DHPR causes Ca^{2+} release through the RYR (orthograde signalling), and the RYR induces an enhanced Ca^{2+} current through the DHPR (retrograde signalling) (Nakai *et al*, 1996, 1998).

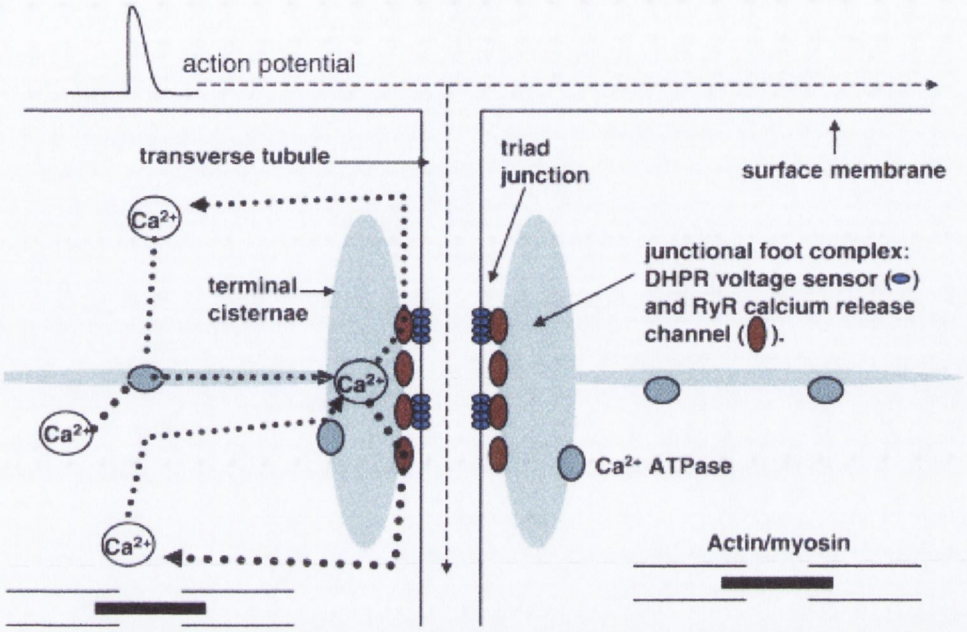


Figure 1.6. Membrane systems and proteins involved in excitation-contraction coupling.

An action potential propagates along the surface and T-tubule membranes. It is transmitted across the junction that is formed between DHPRs on the T-tubule and the RYR on the terminal cisternae of the SR. A tetrad of four DHPRs oppose every second RyR. The contraction/relaxation cycle is terminated when Ca^{2+} is taken back into the SR by the Ca^{2+}-ATPase pump. Adapted from Dulhunty *et al*, 2002.

Endogenous sulfhydryl (SH) groups localised to the SR RYR (Trimm *et al*, 1986, Marengo *et al*, 1998) and DHPR (Campbell *et al*, 1996) are thought to play a significant role in their regulation. Oxidation of these SH groups to disulfide bonds have been consistently shown to trigger the opening of the RYR, while reduction of the disulfide bond induces closure of the channel (Abramson & Salama, 1989, Aghdasi *et al*, 1997, Zable *et al*, 1997). By studying isolated SR from skeletal muscle fibres, it has been suggested that ROS affect the function of these proteins by oxidizing critical thiol groups present on the RYR, DHPR and Ca^{2+}-ATPase pumps to induce Ca^{2+} release and inhibit Ca^{2+} reuptake, respectively (Meszaros *et al*, 1996, Aghdasi *et al*, 1997). At millimolar concentrations, H_2O_2 induces Ca^{2+} release from the SR by increasing the open probability of the RYR channel activity (Favero *et al*, 1995, Oba *et al*, 1996, Oba *et al*, 1998, Plant *et al*, 2002). This will be expanded in Section 1.7.

1.5.3. Excitation-contraction coupling & cross-bridge activity

Excitation-contraction (EC) coupling is defined as the signal transduction process that occurs at the SR junction, and involves depolarization of the plasma membrane and the T-tubules (Shirokova *et al*, 1998, Wood & Slater, 2001), followed by a charge movement corresponding to the voltage sensing of the DHPR, and finally release of Ca^{2+} from the SR through the RYR (Figure 1.6, Catterall, 1991, Melzer *et al*, 1995). Increased $[Ca^{2+}]_i$ enhances Ca^{2+} binding to the troponin complex, which induces a conformational change that increases the availability of myosin binding sites on actin and facilitating cross-bridge cycling (Ashley *et al*, 1991). Thin filaments are pulled inward relative to stationary thick filaments by cross-bridge activity in a process called a power stroke, which leads to force production in skeletal muscle (Hainaut & Duchateau, 1989, Herzog & Ait-Haddou, 2002). Once contraction has occurred, the SR Ca^{2+}-ATPase pump is responsible for muscle relaxation by transporting cytosolic Ca^{2+} into the lumen of the SR. In theory, ROS could alter muscle function by interacting with any of the proteins involved in EC coupling and/or cross-bridge activity.

1.5.4. ROS and contraction

It has been shown that ROS can reduce the amplitude of action potentials in ventricular myocytes of Wistar rats and lead to a progressive but complete loss of the remaining resting potential, due to an inhibition of the fast sodium current (Courtois *et al*, 1998). This could have a downstream effect on Ca^{2+} release due to reduced DHPR activation and RYR opening. H_2O_2 inhibits chloride channels of the SR in skeletal muscle, which may also affect the basal membrane potential of skeletal muscle cells (Kourie, 1999). EC coupling in skeletal muscle may also be altered via changes in the redox state of SR RYR (Salama *et al*, 1992). In resting muscle, the RYR open probability is significantly enhanced by exposure to 10μM H_2O_2 (Oba *et al*, 2002). Alterations in Ca^{2+}-ATPase pump function have also been recorded in the presence of ROS (Scherer & Deamer, 1986). Following prolonged incubation with H_2O_2, SR Ca^{2+} reuptake has been shown to decrease, whereas passive Ca^{2+} leak through the RYR increases (Posterino & Lamb, 1996, Oba *et al*, 1998, Zhang *et al*, 1999, Oba *et al*, 2002). Supraphysiological levels of $[Ca^{2+}]_i$, resulting from conformational changes in Ca^{2+} pumps and channels as described above, can activate Ca^{2+}-activated neutral proteases, leading to a conversion of XDH to

XO, which in turn catalyzes a reaction that produces $O_2^{\cdot-}$ (Equation 1.1, Baker & Austin, 1989).

Some studies have argued that the proteins most sensitive to oxidative modification are not those responsible for Ca^{2+} release, but those involved in cross-bridge cycling including actin, myosin, troponin and other myofibrillar proteins (Reid, 2001). Possible targets for ROS-induced modulation of the proteins involved in cross-bridge activity include hyper-reactive thiol groups present on the myosin head (Perkins *et al*, 1997), sulfhydryl groups in myosin (Crowder & Cooke, 1984) and reactive cysteine residues present on the troponin complex (Chong & Hodges, 1982). ROS can also affect other physiological processes important in skeletal muscle function, such as: mitochondrial respiration through competitive interaction with the oxygen-binding site of cytochrome oxidase (Cleeter *et al*, 1994), and alterations in insulin-dependent glucose uptake in muscle fibres (Roberts *et al*, 1997).

1.6. Skeletal muscle fibres

1.6.1 Fibre type

Skeletal muscle is a very heterogeneous tissue, which undergoes considerable differentiation during development to produce a functionally and phenotypically distinct population of fibres in the adult muscle (Bárány *et al*, 1998, Hilber *et al*, 1999, Pette *et al*, 1999, Bottinelli & Reggiani, 2000). This plasticity is based on the fact that multigene and alternative transcript splicing create multiple thick and thin filament protein isoforms to cover large ranges of functional properties (Wada *et al*, 2002). During development, skeletal muscle fibres evolve into slow- and fast-twitch fibres under the control of motor neuron electrical activity and muscle cell lineage (Desphay *et al*, 2001). It has been suggested that the main signal responsible for the differentiation is probably the discharge frequency (Buchthal & Schmalbruch, 1980). It has been shown that fast-twitch muscle fibres submitted to chronic low-frequency electrical input acquire many, although not all, of the properties of slow-twitch muscle fibres (Buonanno & Fields, 1999), and the inverse transition has also been observed in response to unweighting of the antigravity slow-twitch muscles (Talmadge, 2000). The expression of voltage-gated sodium channels also differs between slow- and fast-twitch muscle fibres (Desaphy *et al*, 2001). These channels are important in determining the upstroke as well as the refractory period of the action

	Physical Characteristics			Fibre type distribution (%)				
Muscle	*Age/mass*	*Rat Strain*	*Sex*	*Type I*	*Type IIa*	*Type IIb*	*Type IId/x*	*References*
EDL	3 months	Brown-Norway	Male	7	12	26	51	Dammeijer *et al*, 2000
SOL	4 months	Sprague Dawley	Male	81	18		<1	Higashiura *et al*, 1999
Plantaris	180g	Sprague Dawley	Female	5	11	38	46	Roy *et al*, 1997
Gastrocnemius	6 months	Fischer 344	Male	10	7	83		Larkin *et al*, 2003
Diaphragm	350-360g	Sprague Dawley	Female	40		34	27	Metzger *et al*, 1985

Table 1.2. Muscle fibre type distribution (%) among the hind limb and diaphragm muscles of the rat.

potential. Therefore the density of available sodium channels in the sarcolemma influences the firing pattern of muscle fibres, which in turn contributes to the phenotypic feature of myofibres.

The existence of specific myofibrillar protein isoforms in different fibre types adds to skeletal muscle heterogenicity, and these fibre types are categorised according to their myosin heavy chain (MHC) isoforms (Brooke & Kaiser, 1970, Hämäläinen & Pette, 1993, Hilber *et al*, 1999, Bottinelli & Reggiani, 2000, Pansarasa *et al*, 2002). One slow fibre type, type I, and three fast fibre types: types IIa, IIb and IId/x, containing MHC isoforms MHCI, MHCIIa, MHCIIb and MHCIId/x, respectively, have been categorised in limb muscles of animal and human models (Schiaffino & Serrano, 2002). These fibres differ in oxidative enzyme (succinate dehydrogenase and citrate synthase) and mitochondrial concentrations, which are higher in type I (oxidative) and IIa (fast-oxidative) fibres, when compared to type IIx (fast-intermediate) and IIb (fast-glycolytic) fibres (Laughlin *et al*, 1990, Danieli-Betto *et al*, 2000). Fibre type distribution within skeletal muscle varies according to age, gender and species, but the most commonly quoted fibre distributions of 4 hind limb muscles and the diaphragm can be seen in Table 1.2.

1.6.2. ROS and muscle fibre types

Antioxidant protection within muscle fibres is proportional to oxidative metabolism; such that muscles composed largely of type I and IIa muscle fibres contain greater antioxidant defences than muscles composed mainly of type IIb and IId/x fibres (Jenkins & Tengi, 1981). It has been repeatedly shown that the activities of SOD, CAT and GPx are higher in type I and IIa fibres compared to type IIb fibres (Jenkins, 1988, Ji *et al*, 1992, Lawler *et al*, 1993, Powers *et al*, 1994, Oh-ishi *et al*, 1995, Hollander *et al*, 2000). GSSG-R activity also follows a similar distribution, such that higher GSSG-R activity has been measured in type I fibres (Ji *et al*, 1992, Lawler & Powers, 1998). In rat skeletal muscle, it has been demonstrated that there can be as much as a 15-fold difference in GPx activity, and a 3- to 4-fold difference in GSSG-R and CAT activities between SOL, a muscle composed of type I and IIa fibres, and the vastus lateralis, a muscle composed largely of type IIb fibres (Ji *et al*, 1992). These findings are consistent with data produced in the gastrocnemius muscle, where type I fibres in the SOL muscle and type IIa fibres in the oxidative portion of the gastrocnemius had higher SOD, GPx and CAT compared

with type IIb fibres in the predominantly glycolytic portion of the gastrocnemius (Laughlin *et al*, 1990). In skeletal muscle, GSH has also been correlated with oxidative capacity, such that GSH concentration is higher in type I and IIa than type IIb muscle fibres in both rodent and human models (Ji *et al*, 1992, Pansarasa *et al*, 1999).

1.6.3. Changes in fibre type

Skeletal muscle is a remarkably adaptive tissue that is capable of changing its morphological, physiological and biochemical properties in response to a variety of stimuli/perturbations (Ji, 2002). Various signal transduction pathways that rely on external stimuli, changes in intracellular enzyme activity and/or gene expression accomplish these adaptations. Functional overload of the rat plantaris by removal of its major synergists (the SOL and gastrocnemius muscles) resulted in hypertrophy and a shift in the contractile, biochemical and metabolic properties toward those observed in a slow twitch muscle (Roy *et al*, 1991). On the basis of gel electrophoresis analyses, functional overload also induced increases in the percentage composition of type I and IIa fibres with concomitant decreases in type IIb fibre distribution (Noble & Pettigrew, 1989, Kandarian *et al*, 1992, Diffee *et al*, 1993, Fauteck & Kandarian, 1995). Nineteen weeks of heavy resistance training also caused a decrease in the percentage of type IIb and an increase in type IIa fibres in human muscle biopsies, as determined by qualitative histochemical analysis of myofibrillar ATPase activity (Adams *et al*, 1993). Type II fibre loss (specifically type IIb) is a hallmark of skeletal muscle aging (Pansarasa *et al*, 2002), with a general shift from type IIb to type IIa initially, and eventually to type I fibres (Pansarasa *et al*, 1999). Changes in fibre type distribution are likely to reflect an adaptive response to increase the number of type I fibres in muscles, which appear to be better equipped to deal with the oxidative stress measured with exercise and aging. This will be discussed further in Section 1.8.4.

1.7. ROS and skeletal muscle

1.7.1. ROS production in skeletal muscle

It has been well established that skeletal muscle produces ROS (Reid *et al*, 1992a, Jackson *et al*, 1985, Phung *et al*, 1994). However, despite elaborate antioxidant protective mechanisms (Section 1.4), ROS still react with cellular membranes, lipids and proteins within skeletal muscles (Abramson & Salama, 1989, Kourie, 1999). The possible roles of

ROS on skeletal muscle function have been investigated, and it has been clearly demonstrated that ROS are involved in maintaining optimum skeletal muscle contractility and can modulate contractile function (Barclay & Hansel, 1991, Salama *et al*, 1992, Zhang *et al*, 1999, Reid, 2001, Oba *et al*, 2002, Reid & Durham, 2002). Previous studies have shown that maximum isometric strength (P_O) is sensitive to both endogenously produced and exogenously applied ROS and ROS scavengers (Plant *et al*, 1998, Diaz *et al*, 1998, Zhang *et al*, 1999, Plant *et al*, 2001, Reid & Durham, 2002). Treatment with exogenous H_2O_2 increases submaximal force production in intact single muscle fibres (Andrade *et al*, 1998) and maximal force production in intact skeletal muscles (Plant *et al*, 2001). Conversely, scavenging ROS by exogenous application of CAT to the solution bathing a muscle *in vitro*, leads to a reduction in P_O (Reid *et al*, 1992a, Andrade *et al*, 1998). Antioxidant enzymes SOD and CAT have also been shown to shorten time to peak tension (TTP) and half-relaxation times (½RT), and decrease twitch (P_t) and submaximal tetanic forces (Reid *et al*, 1993). Subsequently, it was suggested that a relationship exists between the muscle redox state (the balance between oxidants and reductants) and force production (Reid, 2001), such that changes in the cellular redox balance may alter P_O by inducing changes in the structural conformation of SR proteins.

1.7.2. The cellular redox balance

The continuous interaction between ROS and the cellular environment in skeletal muscle determines the 'cellular redox balance' (CRB) and refers to the oxidative/reductive state of different redox pools (Arrigo, 1999). It is a dynamic condition that fluctuates from a reductive to an oxidative state as cells can regulate their own redox balance to a certain extent (Chance *et al*, 1979). Force production in skeletal muscle is affected by oxidation and reduction of the CRB and these responses have been modelled as a bell shaped continuum of contractile function called the cellular redox curve (CRC). The CRC predicts that an intracellular redox state exists that is optimal for force production and assumes that the redox state in skeletal muscle is a physiologically regulated variable in which muscle fibres balance ROS production against antioxidant buffering (Reid, 2001). Data produced by contractile function studies have also suggested that the CRC in resting skeletal muscle is slightly reduced, which is not optimal for maximum force production (Figure 1.7, Andrade *et al*, 1998, Diaz *et al*, 1998, Reid, 2001, Lamb & Posterino, 2003). Therefore, according to the CRC, P_O can only be produced in skeletal muscle after it has been exposed to an oxidizing agent that is capable of shifting the redox balance from a

slightly reduced to a more oxidized, optimal position (Reid, 1996). Application of H_2O_2 *in vitro* can increase P_O by up to 20% from baseline values in single muscle fibres and whole muscle (Andrade *et al*, 1998, Plant *et al*, 2001). However, it has been clearly shown that this relationship is concentration and time-dependent and muscle-specific (Plant *et al*, 2001, Lamb & Posterino, 2003, Posterino *et al*, 2003).

Incubation with H_2O_2 has been shown to initially cause a potentiation in P_O, followed by a reduction in force over and above that of the initial potentiation (Andrade *et al*, 1998). Therefore, because force increases in response to incubation with an oxidant (H_2O_2), the baseline redox balance in skeletal muscles is reduced (● for the EDL and ○ for the soleus, Figure 1.7). Exposure to H_2O_2 oxidizes the CRB, causing a shift to the right of this baseline state, which causes force to increase (◆, Figure 1.7).

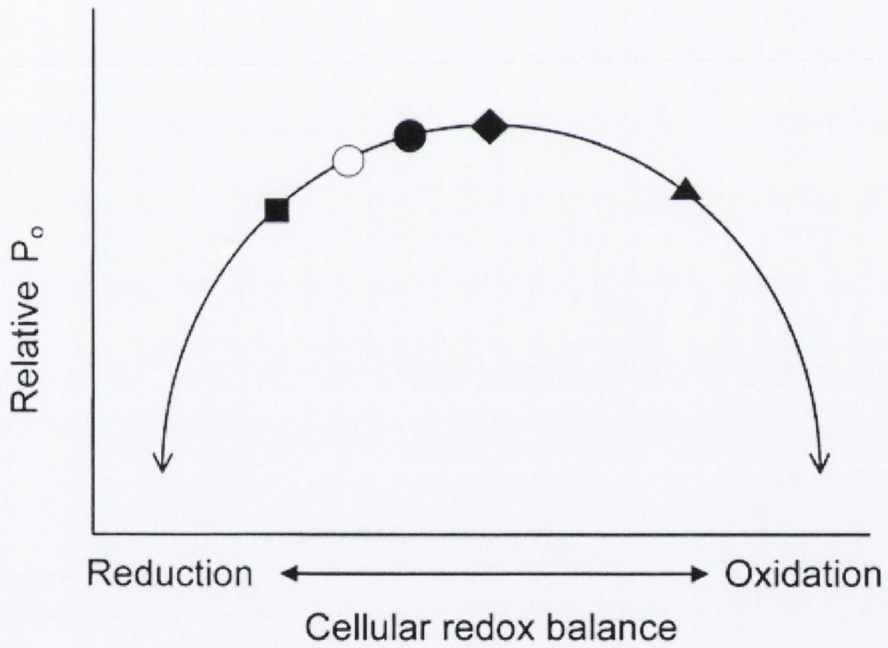


Figure 1.7. Relative P_O as a function of cellular redox balance for EDL and SOL.

The CRB in EDL (●) and SOL (○) at rest is slightly reduced, which is not optimal for maximum force production (P_O). After exposure to an oxidant, the CRB shifts to a more oxidized state, which is a more optimal position for production of P_O (◆). Prolonged exposure to H_2O_2 shifts the CRB to a highly oxidized state, where P_O is reduced (▲), whereas exposure to a reductant shifts the CRB to a reduced state, where P_O is also diminished (■) (Plant *et al*, 2001).

As exposure to H_2O_2 continues, or in situations of oxidative stress, the skeletal muscle CRB becomes progressively more oxidized, moves past the optimum position for force production and ultimately leads to a reduction in P_O (▲, Figure 1.7). Application of a supramaximal dose of H_2O_2 would produce a similar response, as the point of potentiation

would be bypassed leading to direct reductions in force over time. Conversely, P_O in resting skeletal muscle *in vitro* is compromised following exogenous application of an antioxidant or reductant, which takes place due to a shift in the CRB to a more reduced state that is further away from the redox state necessary for optimum force production (■, Figure 1.7, Reid *et al*, 1993, Clanton *et al*, 1999).

The additive effect of ROS generated from endogenous sources during muscle contraction, in conjunction with exogenously applied oxidants, accelerates the oxidation of the CRB leading to direct reductions in P_O. This response is analogous to the decline in P_O *in vitro* observed in skeletal muscle with exercise and fatigue (Reid *et al*, 1993). When dithiothreitol, a reducing agent, is applied to a muscle bath *in vitro* with fatigued diaphragm strips, a 10% improvement in P_O is observed compared to untreated fatigued muscle strips (Diaz *et al*, 1998). In this case, dithiothreitol returns the CRB from an oxidized to a more reduced state in the fatigued muscle strips, which is closer to the optimum of the CRC leading to a restoration in force.

1.7.3. Protein sulfhydryls and Ca^{2+} changes

Alterations in the CRB can affect protein sulfhydryl bonds, and ultimately $[Ca^{2+}]_i$. It has been shown previously that ROS modify skeletal muscle proteins by oxidizing sulfhydryl (SH) bonds on cysteine residues to form disulfide bonds (Favero *et al*, 1995, Andrade *et al*, 1998, Plant *et al*, 2001). Disulfide bond formation causes conformational changes that can regulate the biological activity of proteins (Vogt, 1995, Diaz *et al*, 1998, Winterbourn & Metodiewa, 1999). These changes can affect channel conductivity and/or alter other important contractile proteins in skeletal muscle (Abramson, 1987, Brotto & Nosek, 1996, Callahan *et al*, 2001, Lamb & Posterino, 2003), leading to increased Ca^{2+} release by the SR RYR and decreased activity of the SR Ca^{2+}-ATPase pump in isolated membrane vesicle preparations following application of H_2O_2 (Salama *et al*, 1992, Favero *et al*, 1995, Oba *et al*, 1996, 1998, Favero, 1999).

Exposure of intact single skeletal muscle fibres and muscle fibre bundles to millimolar concentrations of H_2O_2 initially causes potentiation of twitch force, but there is a decline and even abolition of force production with longer exposure (Reid *et al*, 1993, Oba *et al*, 1996, Murrant *et al*, 1999). It had been suggested that the initial potentiation of twitch force was due to an oxidation effect on the DHPR, resulting in a direct increase of Ca^{2+}

release through the RYR (Oba *et al*, 1996), and/or due to direct oxidation of the RYR in the SR (Reid *et al*, 1993). However, exposure to H_2O_2 (100-300μM) in isolated fast-twitch mouse fibres caused potentiation of submaximal tetanic force responses without any increase in cytoplasmic Ca^{2+} concentrations (Andrade *et al*, 1998). An increase in the Ca^{2+} sensitivity of the contractile apparatus was suggested instead.

A small number of critical sulfhydryls near the active site on the Ca^{2+}-ATPase pump have been shown to regulate enzyme activity and, hence, pump function (Scherer & Deamer, 1986). Reductive stress inhibits the Ca^{2+}-ATPase pump via these regulatory sulfhydryls, which must be oxidized for ATP hydrolysis to proceed (Daiho & Kanazawa, 1994). ROS-induced oxidative stress also affects these sulfhydryls and slows the reuptake of Ca^{2+} into the SR (Grover *et al*, 1997, Morris & Sulakhe, 1997). It has been directly demonstrated, using SR vesicles incorporated into planar lipid bilayers and Ca^{2+} flux measurements, that the skeletal muscle RYR channel is sensitive to singlet oxygen (Stuart *et al*, 1991), H_2O_2 (Favero *et al*, 1995, Oba *et al*, 1996) and HOCl (Trimm *et al*, 1986). Cytoplasmic application of 1.5–3mM H_2O_2 to SR vesicles increases the open probability of RYR (Oba *et al*, 1996), and also increases their sensitivity to activation by Ca^{2+}-induced Ca^{2+} release as previously discussed (Section 1.5, Eager *et al*, 1997, Oba *et al*, 2002, Plant *et al*, 2002, Posterino *et al*, 2003). It is possible that the normal mechanism of controlling Ca^{2+} release is altered by oxidation, which not only affects SH groups but also can reduce the inhibitory effect of cytoplasmic Mg^{2+} in isolated RYR's (Donoso *et al*, 2000). It has been shown that ROS produced during repeated muscle contractions reduced P_O by oxidizing critical SH groups on the RYR and Ca^{2+}-ATPase pump, resulting in altered $[Ca^{2+}]_i$ (Diaz *et al*, 1998). Disulfide bond formation is reversible upon exposure to a reductant, such as dithiothreitol (DTT) (Aghdasi *et al*, 1997, Plant *et al*, 2001). Thus P_O depends on the fine balance between these 2 opposing effects, which provides a possible mechanism for direct redox modulation of T-tubule DHPR, SR RYR and Ca^{2+}-ATPase pump activity in skeletal muscle.

1.7.4. Factors affecting the CRB

Muscle size, total antioxidant capacity and the redox sensitivity of regulatory proteins within skeletal muscle are important determinants of the response of P_O *in vitro* to an oxidant challenge (Plant *et al*, 2001). The effects of H_2O_2 on muscle contractility are muscle dependent, with a greater potentiation in P_O in SOL than EDL (Plant *et al*, 2001,

Plant *et al*, 2002). Muscle specific responses to redox manipulation have been explained by differences in antioxidant levels (Section 1.6.2), such that muscles composed predominantly of type I and IIa muscle fibre types are more resistant to the effects of ROS-modulated force production (Heunks & Dekhuijzen, 2000). As antioxidant levels are less in EDL than SOL, it is closer to the apex of the CRC (●, Figure 1.7), such that a more immediate potentiation, followed by a reduction in P_O has been observed in the EDL upon exogenous application of an oxidant *in vitro*. However, the larger antioxidant levels in SOL delay, rather than completely prevent, any effects an oxidant has on P_O, such that a decrease will eventually be measured with time (Plant *et al*, 2001).

The effect an exogenously applied oxidant has on aged skeletal muscle is likely to be very different to that in young muscle due to the oxidized state of the CRB, which occurs due to a combination of increased ROS production, altered antioxidant activity and increased sensitivity of regulatory proteins to oxidative modification (Figure 1.8, Lawler *et al*, 1997, Ji *et al*, 1998, Bejma & Ji, 1999, Hollander *et al*, 2000, Nordberg & Arnér, 2001, Reid, 2001). This will be developed in Section 1.8.

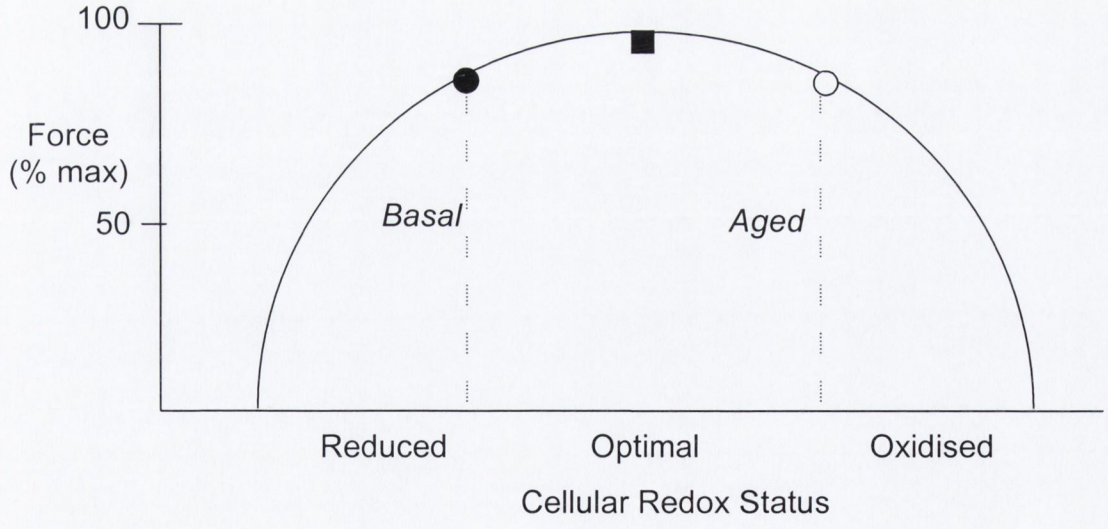


Figure 1.8. Redox sensitivity of P_O in aged skeletal muscle.

The curve depicts the biphasic response of P_O as muscle redox status is altered. ■ Optimum CRB for maximum force production in skeletal muscle. ● Proposed status of healthy adult muscle under basal conditions. ○ Proposed status of aged muscle. Adapted from Reid & Durham, 2001.

1.8. Aging

1.8.1. Sarcopenia

Aging is a complex physiological process involving morphological, biochemical and functional changes in single cells and in whole organisms (Harman, 2001, Carmeli *et al*, 2002). It is characteristically described as a time-dependent, functional decline in a cell's capacity to withstand stress (McArdle *et al*, 2002), resulting in a progressive imbalance of the control regulatory systems of the organism (Kasapoglu & Özben, 2001). Therefore, aging can be attributed to two independent biological processes: the loss of functionality, and the loss of resistance or adaptability to stress. The central nervous system and skeletal muscle are particularly vulnerable to age-related dysfunction and pathology because both contain post-mitotic cells that are liable to accumulate oxidative damage over time, and both consume large amounts of O_2 (Finkel & Holbrook, 2000, Pansarasa *et al*, 2000). The nature of the biochemical events that lead to the attenuation of various physiological process during aging in skeletal muscle still remains to be clearly understood. During the past 150 years, many factors and variables have been studied in skeletal muscle, but the basic observation of Quetelet (1835) remains that a significant reduction in muscle strength is observed in humans between 30 & 80 years of age (Larsson *et al*, 1979, Young *et al*, 1985).

Skeletal muscle wasting in aged individuals is often referred to as 'sarcopenia of old age' (Carmeli *et al*, 2002), a generic term used to describe the overall loss in mass, strength and quality of skeletal muscle (Zainal *et al*, 2000, Lynch, 2002). Decreases in whole skeletal muscle strength and size are features commonly associated with old age (Larsson *et al*, 1979, Lexell *et al*, 1983, Frontera *et al*, 1991) and cross-sectional studies have shown that the decrease in muscle mass starts towards the end of the fifth decade of life in humans (Janssen *et al*, 2000), which corresponds to the onset of force decline (Narici *et al*, 1991). By the age of 70, the cross-sectional area (CSA) of skeletal muscle decreases by up to 30% in humans when compared to the same muscles in younger individuals, with a subsequent reduction in muscle strength of 30-40% (Porter *et al*, 1995). This decline in strength continues to fall at a rate of 1-2% per year (Skelton *et al*, 1994). Many reasons have been proposed for the underlying mechanisms for sarcopenia, some of which include: a reduction in mediating factors involved in the activation of progenitor myoblasts (Carlson, 1995); denervation of muscle fibres (Lexel *et al*, 1988);

decreased/altered muscle protein synthesis (Viner *et al*, 1999, Çakatay *et al*, 2003); changes in muscle fibre type (Pansarasa *et al*, 2002); alterations in antioxidant enzyme activities (Rikans & Hornbrook, 1997) and increased ROS production (Leeuwenburgh *et al*, 1998, Nordberg & Arnér, 2001).

1.8.2. Age-associated oxidative stress

1.8.2.1. Free radical theory of aging

The free radical theory of aging suggests that ROS are responsible for the functional deterioration associated with aging (Harman, 1956). Modern thinking interprets the free radical hypothesis in terms of oxidative stress, and it has been reformulated to propose that aging is caused by a shift in the balance between pro-oxidative (ROS) and anti-oxidative processes (antioxidant enzymes and molecules) in the direction of the pro-oxidative state (Rikans & Hornbrook, 1997, McArdle *et al*, 2002). One of the fundamental assumptions of the free radical theory is that the free radicals that contribute to the aging process are derived directly or indirectly from O_2. This idea is re-enforced by an increase in the oxidation rate of dichlorofluorescin (DCFH), a synthetic probe for $O_2^{\cdot-}$ (Reid *et al*, 1992b), in skeletal muscle of 25-month-old rats (Bejma & Ji, 1999), demonstrating that aged muscle does indeed produce higher levels of ROS compared to younger muscles.

Animal experiments have shown that older muscles are injured more easily following eccentric contractions, as measured by a larger decrease in isometric strength compared to young muscle (Zerba *et al*, 1990), and regenerate more slowly, which has been suggested to reflect the environment for regeneration provided by the old host (Carlson & Faulkner, 1989). Hydroperoxide levels in skeletal muscle have been shown to increase with age, as measured by increased GPx activity in aged skeletal muscle (Lawler & Demaree, 2001), and increased production of H_2O_2 and lipid peroxidation is a consistent finding with aging in virtually every tissue (Yu, 1994). The literature on human skeletal muscle strongly supports an age-associated increase in oxidative damage to proteins, lipids and DNA (Meccoci *et al*, 1999, Pansarasa *et al*, 1999, Pansarasa *et al*, 2000, Fano *et al*, 2001). ROS-mediated lipid peroxidation has been implicated as a causative agent in the degenerative process of aging due to the damage it causes to cell walls and components (Ames *et al*, 1993). Protein in aged skeletal muscle is more sensitive to oxidative stress (Radak *et al*, 2002), as confirmed by a significant increase in protein carbonyl levels with

age (Gianni *et al*, 2004). Higher levels of 8-hydroxydeoxyguanosine (indicative of oxidative damage to DNA) have been measured in human skeletal muscle from older individuals (Gianni *et al*, 2004).

1.8.2.2. Mitochondrial free radical theory of aging

The free radical theory of aging has been extended to include the fact that ROS production enhances the mutation rate of DNA, and more specifically of mitochondrial DNA (mtDNA), particularly in the brain and skeletal muscle (Cardoso *et al*, 1999, St-Pierre *et al*, 2002). Oxidative damage causes impairment of mitochondrial function and morphology with aging, as observed by a decline in membrane potential and increases in peroxide production and organelle size, which may accelerate skeletal muscle aging due to intrinsic apoptosis (Sastre *et al*, 2003). In support of the mitochondrial theory of aging, a progressive relationship has been established between increasing age and the accumulation of mtDNA damage products, deletions and mutations in skeletal muscle (Hayakawa *et al*, 1991, Lee *et al*, 1993, Melov *et al*, 1995, Wanagat *et al*, 2001, Yarovaya *et al*, 2002).

Given the proximity of mtDNA and several functional mitochondrial proteins to the primary ROS generators in the mitochondria (Complex I & III) (Liu *et al*, 2002, Brand *et al*, 2004), these molecules are at a greater risk of oxidative damage, which may potentially lead to further age-associated mitochondrial dysfunction. mtDNA is particularly susceptible to oxidative damage and mutation because it lacks protective histones and an effective repair system to prevent/reduce ROS-induced damage (Richter, 1988, Bowling *et al*, 1993, Miquel, 1998). From these findings, it has been suggested that the increase in ROS production during aging is likely, to some extent, result from the functional abnormalities induced by ROS in mitochondria, such that ROS production is increased even more in aged mitochondria (Miquel, 2002, Gianni *et al*, 2004). The damage to mtDNA induced by age-related oxidative stress could also be induced by defective ETC proteins, reduced ETC activity, and enhanced production of ROS (Hamilton *et al*, 2001). Bejma & Ji (1999) have provided direct evidence of an increase in resting ROS levels in isolated mitochondria from aged skeletal muscle, as measured by an increase in the rate of dichlorofluorescin oxidation. Other studies have re-enforced the hypothesis that aged skeletal muscle mitochondria accumulate significant oxidative damage as shown by increases in both protein carbonyl levels, thiobarbituric acid reactive

substances and decreases in protein sulfhydryl (SH) groups (Lass *et al*, 1998, Kowaltowski & Vercesi, 1999).

1.8.3. Contractile function

1.8.3.1. Muscle strength

Aging is associated with a progressive loss of motor function, a slowing of muscle movements and a decline in muscle strength (Thompson, 1994, Larsson & Ansved, 1995). There are mixed reports about the effects of age on P_O, with some studies reporting a decrease with aging (Brooks & Faulkner, 1988, Klitgaard *et al*, 1989), some reporting increases (Eddinger *et al*, 1986), whereas others showed that P_O remained unchanged with age (Fitts *et al*, 1984). The reasons for these discrepancies are likely explained by differences in the strains of rats or mice used, whether the animals were old enough to show age-related changes (Brown & Hasser, 1996), and the fact that aging is thought to affect specific types of muscles, motor units and muscle cells differently (Larsson & Ramamurthy, 2000). Observations on P_O developed by whole muscles of rodents studied *in vitro* support the premise that a decrease in P_O with age is not unique to humans, (Larsson & Edström, 1986). In 26-month-old male Wistar rats, the EDL developed 24% less force than that from 5-month-old rats (Gutmann & Carlson, 1976). In a similar comparison of SOL muscle, a deficit of 19% was recorded between 6-month and 20-24 month-old rats (Larsson & Edström, 1986). These results have been reinforced by reports of a 20% reduction in EDL & SOL specific muscle force from aged rodents *in vitro* (Brooks & Faulkner, 1988). Progressive muscle fibre denervation and the loss of fast motor units have been implicated as mechanisms responsible for the age-related decrease in P_O as discussed (Section 1.8.4) (Einsiedel & Luff, 1992, Faulkner *et al*, 1995). In 27-29 month old male Fischer 344 rats, ~15% of the observed deficit in specific force was explained by the presence of non-innervated muscle fibres (Urbanchek *et al*, 2001).

The absence of an age-related change in the Ca^{2+}-free rigor forces of EDL and SOL permeabilized muscle fibres suggests that the decrease in maximum force observed in aged skeletal muscles is not likely due to a decrease in the total number of cross-bridges participating in contraction (Plant & Lynch, 2001). No difference in the number of interacting sites between the actin and myosin filaments of permeabilized fibres from young and old rats leaves the possibility of age-related changes to the intrinsic properties of muscle fibres in either the force output per cross-bridge, or in EC coupling processes

(Renganathan *et al*, 1997). Alterations in EC coupling have also received support as a mechanism likely responsible for the changes in muscle contractility with age (Margreth *et al*, 1999). Proteins involved in EC coupling are sensitive to oxidant-induced modification (Section 1.5.4), and will be discussed below.

1.8.3.2. EC coupling proteins

Age-related alterations in the proteins involved in EC coupling contribute to the changes in muscle contractility (Margreth *et al*, 1999) and include: a reduction in Ca^{2+} release due to DHPR-RYR uncoupling (Delbono *et al*, 1995); impairment of SR Ca^{2+} pump function (Narayanan *et al*, 1996); abnormalities in the regulation of RYR (Damiani *et al*, 1996) and a decreased turnover of Ca^{2+}-ATPase and RYR (Ferrington *et al*, 1998). An age-related reduction in the speed of contraction has been observed before the onset of severe muscle wasting, as measured by lower rates of ATP-supported Ca^{2+} uptake by aged skeletal muscle SR compared with adult muscle (Narayanan *et al*, 1996). The data strongly suggest that impairment in SR Ca^{2+}-ATPase pump function, due to uncoupling of ATP hydrolysis from Ca^{2+} transport, contributes to the age-associated slowing of relaxation in the soleus muscle. In rodents, age-related slowing of muscle contractions has been reported in muscle cells expressing both the type I and IIa MHC isoforms (Li & Larsson, 1996, Larsson *et al*, 1997, Thompson & Brown, 1999). It has been shown that DHPR-RYR uncoupling at the T-tubule-SR triadic junction results in an absolute reduction in SR Ca^{2+} release in response to sarcolemmal depolarization and, consequently, a reduction in contractile strength in aged skeletal muscle due to alterations in the voltage-gated SR Ca^{2+} release mechanism, decreases in myoplasmic Ca^{2+} elevation in response to sarcolemmal depolarization, reduced Ca^{2+} supply to contractile proteins (Renganathan *et al*, 1997). Decreased conformational stability of the SR Ca^{2+}-ATPase pump in skeletal muscle of old rats has also been reported (Ferrington *et al*, 1997), which has been attributed to a significantly slower turnover in this protein in aged muscle, leading to an increase in the potential for post-transitional oxidative modifications over time (Ferrington *et al*, 1998).

1.8.4. Muscle fibre type

Aging is associated with a decline in the mechanical function of skeletal muscle (Coggan *et al*, 1992), which is explained, in part, by a decline in the ability of aged muscle to reinnervate or alter patterns of fibre type expression following normal denervation-

reinnervation processes occurring throughout the lifespan of the animal (Larkin *et al*, 2003). Previous studies have demonstrated that the process of reinnervation by type I fibres is faster than that of type IIb fibres (Lowrie & Vrbova, 1984), and preferential reinnervation of type I fibres following nerve repair has been observed, which leads to an increase in type grouping of type I fibres in reinnervated muscle (Yoshimura *et al*, 1999). Thus, if during the lifespan of the animal, denervation-reinnervation is a normal ongoing process, it is expected that there will be an increase in the type grouping of type I fibres. The decrease in the proportion of type IIb fibre types in skeletal muscle with age has also been attributed to a decrease in physical activity (Campbell *et al*, 1973). With age there is an increase in denervation in type II fibres (Larkin *et al*, 2003). Without the trophic effect of a motor neuron, type II muscle fibres atrophy and die (Ozawa, 1989). Therefore, in order to reduce/prevent muscle fibre loss, type I motor neurons form collateral branches, which innervate type II fibres. Since motor neuron firing patterns determine fibre type, type II fibres are converted to type I fibres, thus leading to an increase in type I fibre distribution with age at the expense of type II fibres. Age-associated increases in fibre grouping of type I fibre populations have been measured (Larkin *et al*, 2003), and it has been shown that fiber type-grouping alters the ability of the muscle to smoothly and efficiently contract when stimulated, which may explain some of the age-associated changes in muscle function.

The age-related reduction in muscle volume and CSA has been attributed to a decrease in the total number of individual muscle fibres within a whole muscle and/or atrophy of the remaining fibres (Faulkner *et al*, 1995, Thompson, 1994). However, in the rat, fibre CSA has been shown to remain relatively constant throughout the aging process until 36 months, at which time a significant decrease in CSA occurred (Thompson & Brown, 1999). Type IIb fibres show a greater age-related decrease in both fibre number and size (Pansarasa *et al*, 2002) compared to type I fibres, whose numbers in muscle are affected little or may increase with aging (Larsson *et al*, 1979, Lexell *et al*, 1988, Trappe *et al*, 1995, Kirkendall & Garrett, 1998). The medial gastrocnemius muscle of male Fischer 344 rats showed an increase in both the number of type I fibres and the percentage area of type I and IIa fibres, which occurred concomitantly with a decrease in both the number and percentage area of type IIb fibres in aged (24 months) compared to young controls (2 months) (Larkin *et al*, 2003).

1.8.5. Antioxidant defences and aging

The influence of aging on skeletal muscle antioxidant defences has been investigated extensively, and has been associated with a reduced capacity of the enzymatic and non-enzymatic antioxidant systems to convert ROS into more inert species. However, a close examination of the literature indicates that age-related antioxidant changes are varied, such that age-related increases, decreases and/or no change in antioxidants are measured in the same skeletal muscle in different studies.

Total SOD activity has been shown to increase (Lammi-Keefe *et al*, 1984, Ji *et al*, 1990, Leeuwenburgh *et al*, 1994, Ji *et al*, 1998) or remain unchanged with age (Vertechy *et al*, 1989, Lawler *et al*, 1993, Jayachandran *et al*, 1996). Decreases in total SOD activity have also been measured, however a reduction in activity was accompanied by simultaneous increase in one of the SOD isoforms (MnSOD or Cu,Zn SOD) (Oh-Ishi *et al*, 1995, Pansarasa *et al*, 1999, 2000, Hollander *et al*, 2000). An age-related increase in MnSOD activity could be as important as an increase in total SOD because of its role in $O_2^{\cdot-}$ removal from the mitochondria (Section 1.2.1), which is essential due to age-related increases in $O_2^{\cdot-}$ production (Section 1.8.2), and its importance in maintaining normal function in mitochondria-rich tissues (Lebovitz *et al*, 1996, Melov *et al*, 2000). As MnSOD is located in the mitochondria and Cu,Zn SOD is found in the sarcoplasm (Lawler & Powers, 1998), the divergent responses observed in the aforementioned studies could be related to differences in the microenvironments in the mitochondria and sarcoplasm as a result of aging.

The most consistent age-associated alteration in antioxidant enzyme activity has been observed in CAT and GPx, where levels have been shown to increase with age (Ji *et al*, 1990, Hammeren *et al*, 1992, Luhtala *et al*, 1994, Ji *et al*, 1998). However, this increase in activity with age is muscle specific (Oh-ishi *et al*, 1995) and dependent upon the degree of aging in animal models (Lawler & Demaree, 2001), such that CAT and GPx activities were higher in aged SOL but not EDL, and only increased in the gastrocnemius muscle after 18 months. It has been argued that increases in antioxidant enzyme activity observed with aging represents an attempt to compensate for the increased production of hydroperoxides (Ji *et al*, 1990). Therefore, it is likely that an increase in GPx (Hammeren *et al*, 1992) and CAT (Ji *et al*, 1990, Luhtala *et al*, 1994) represents an increased capacity to dehydrate hydroperoxides to H_2O and O_2 in skeletal muscle. However, no age-

associated change or decreases in CAT and GPx in various rat and human skeletal muscles have also been measured (Pansarasa *et al*, 1999, Hollander *et al*, 2000, Gündüz *et al*, 2004).

Vitamin C concentrations are reduced in aged skeletal muscle (Patnaik, 1968, Jayachandran & Panneerselvam, 1995, Jayachandran *et al*, 1996, Van der Loo *et al*, 2003). Age-associated decreases in vitamin C may occur because it is increasingly used to regenerate the vitamin E radicals produced by increased ROS scavenging, and is itself involved in ROS scavenging (Figure 1.4, Van der Loo *et al*, 2003). As discussed previously, vitamin C is the first antioxidant oxidized under certain physiological conditions, even in mitochondria where concentrations are low (Vatassery, 1995).

GSH concentrations also increase in aged rat skeletal muscle (Bejma & Ji, 1999), which when combined with higher GPx levels, suggests that aged skeletal muscles are well equipped to deal with the age-related increase in hydroperoxide levels (Yu, 1993, Lawler & Demaree, 2001). However, human skeletal muscle GSH levels have been shown to remain unchanged with age (Leeuwenburgh *et al*, 1994, Pansarasa *et al*, 1999). Also, GSH concentrations were significantly lower in aged skeletal muscle taken from 24-month-old rats (Jayachandran *et al*, 1996). Studies assessing age-related changes in human blood samples also observed no changes in GSH or vitamin C concentrations (Kasapoglu & Özben, 2001).

1.9. Summary

The information reviewed in this introduction has shown that ROS ($O_2^{\cdot-}$, H_2O_2, HOCl and $OH^{\cdot}$) have the ability to modulate contractile function and force output in skeletal muscle by primarily inducing conformational changes to proteins involved in EC coupling and the cross-bridge power stroke. Antioxidant defences, comprised of enzymes (SOD, CAT and GPx) and low molecular weight molecules (GSH and vitamin C & E), can counter ROS-induced modifications to a certain degree, but can become compromised in situations where ROS production is enhanced and/or antioxidant defences are reduced, such as exercise and aging. This physiological state is called oxidative stress, and is characterised by the imbalance produced between ROS and antioxidants in favour of ROS. Oxidative stress can have implications on skeletal muscle function, such that the maximum force producing ability of skeletal muscle is significantly reduced in the presence of high levels of ROS due to excessive oxidation of the proteins involved in contraction.

An increase in resting ROS levels has been consistently observed in aged skeletal muscle compared to younger muscles. These findings demonstrate that aged skeletal muscle exists in a more permanent state of oxidative stress and that age-associated alterations in skeletal muscle structure and function, as measured by muscle atrophy, reduced force output and altered antioxidant defences, are likely to have occurred partly as a result of this oxidative state. A strategy that has been utilized to improve antioxidative defences in skeletal muscle, and ultimately lower ROS levels, is the administration of antioxidants, such as selenium, α-lipoic acid, vitamin C and E. Significant incorporation of low molecular weight antioxidants into skeletal muscle mitochondrial membranes and lipids has been measured as a result of dietary supplementation, which has coincided with reductions in ROS concentrations in skeletal muscle. These findings could have important implications for aged skeletal muscle, such that administration of antioxidants could improve overall structure and function by lowering ROS levels, through enhanced antioxidant scavenging, and stabilizing cell membranes, leading to a reduction in lipid peroxidation and cell damage.

1.10. Objectives

From the information reviewed in the introduction, the main objectives of this thesis are to:

1. Determine the effect of aging and oxidative stress on skeletal muscle structure and function.
    i. Assess reductions in muscle mass and changes in fibre type composition with age
    ii. Measure age-related differences in a number of isometric contractile characteristics
    iii. Determine the effect of an oxidant challenge on force production in aged skeletal muscle
2. Assess age-associated alterations in antioxidant capacity.
    i. Measure antioxidant enzymes and low molecular weight molecules in hind limb skeletal muscle from a range of age groups
    ii. Determine if age-related changes are dependent on muscle type, and more specifically on fibre type distribution in whole muscle
3. Measure the effects of antioxidant supplementation on skeletal muscle function and antioxidant defences.
    i. Assess isometric contractile characteristics
    ii. Determine the effect of an oxidant challenge on force production in vitamin-treated and control skeletal muscle
    iii. Establish whether long term vitamin supplementation has a beneficial effect on antioxidant defences

From these studies, 2 main sets of hypotheses can be investigated. Firstly, it was hypothesized that aging in skeletal muscle would:

1. Cause a significant degree of muscle atrophy independent of muscle type
2. Cause a shift in fibre proportion in favour of type I and/or IIa fibre types
3. Increase contraction times in both EDL and SOL muscles
4. Reduce maximum isometric tetanic force irrespective of muscle type

5. Be more sensitive to an oxidant challenge *in vitro* due to already elevated levels of ROS
6. Have altered antioxidant levels compared to younger muscles due to a higher type I and/or IIa fibre proportions

Secondly, it was hypothesized that antioxidant supplementation would:

1. Cause an increase in tissue and plasma antioxidant levels
2. Have no effect on resting isometric contractile characteristics
3. Preserve force in the presence of an oxidant challenge due to an improvement in antioxidant defences

Chapter 2.

Materials and Methods

2.1. Materials

2.1.1. Chemicals

The full names and addresses of the sources listed below are given in Appendix i.

Materials	Source
Acetic acid	Sigma
Adenosine 5-triphosphate	Sigma
Ammonium persulfate	Sigma
L-ascorbic acid	Lennox/Sigma
BioRad	BioRad laboratories
Bovine serum albumin	Sigma
Calcium Chloride	Sigma
Catalase	Sigma
Chrome alum	Sigma
Copper (II) sulfate pentahydrate	Sigma
Creatine hydrate	Sigma
Cumene hydroperoxide	Sigma
Cupric sulfate	Sigma
Diethylenetriaminepentaacetic acid	Sigma
N,N'-dimethyl-9,9'biacridinium dinitrate	Sigma
2,4-dinitrophenylhydrazine	Sigma
5,5'-dithio-bis(2-nitrobenzoic acid)	Sigma
Ethanol	Sigma
Ethylenediaminetetraacetic acid	Sigma
Ferric tripyridyltriazine	Sigma
Ferrous sulfate	Sigma
Formaldehyde	Sigma
Gelatin	Sigma
D-Glucose	Sigma
Glutathione (reduced)	Sigma
Glutathione reductase	Sigma
Glycerol anhydrous	Sigma

Glycine	Sigma
Hydrochloric acid	Lennox
Hydrogen peroxide	Sigma
Iron (III) chloride hexahydrate	Sigma
Isopentane	BDH
β-mercaptoethanol	Sigma
Magnesium sulfate heptahydrate	Aldrich
Methanol	Sigma
Nicotinamide adenine dinucleotide phosphate	Sigma
Nitroblue tetrazolium	Sigma
Potassium bichromate	Sigma
Potassium carbonate	Aldrich
Potassium chloride	Sigma
Potassium hydroxide	Sigma
Potassium phosphate dibasic	Sigma
Potassium phosphate monobasic	Sigma
Potassium thiocyanate	Sigma
Sodium acetate trihydrate	Aldrich
Sodium chloride	Sigma
Sodium dodecyl sulfate	Sigma
Sodium hydrogen carbonate	AnalaR®
Sodium hydroxide	Sigma
Sodium salicylate	Sigma
Sodium phosphate	Sigma
Sodium succinate	Sigma
Sodium thiosulfate	Sigma
Sulphuric acid	Sigma
Superoxide dismutase	Sigma
Thiourea	Sigma
Triton-X_{100}	Sigma
Trizma base	Sigma
Vitamin E acetate	Aldrich
Xanthine	Sigma
Xanthine oxidase	Sigma

2.1.2. Animals

All animals used in the following studies were cared for in accordance with the guidelines set out by the Department of Health and Children based on the Cruelty to Animals Act, 1876 and the European Community Directive, 86/609/EC. Male Wistar rats were used throughout the course of this investigation and were obtained from purpose-bred stocks within the Bioresources unit, Trinity College Dublin, Ireland. Animals were housed in groups of 2-3 within the animal holding facility in the BRU under controlled environmental conditions (21°C ± 1°C; 12 hour light/dark cycle). Food (standard laboratory chow, Red Mills Ltd., Appendix ii) and water were available *ad libitum* to all animals unless otherwise specified.

2.2. Methods

The following methods will be used at various times in the studies to be described throughout this thesis. Any further or additional descriptions of study design and protocols will be outlined in the appropriate chapters where required. There are 5 main sets of procedures used for the duration of this thesis:

1. Tissue collection
2. Contractile function tests
3. Tissue storage
4. Biochemical antioxidant assays
5. Muscle fibre typing

2.2.1. Tissue collection

2.2.1.1. Anaesthesia and euthanasia

Unless stated otherwise, all animals were anaesthetised with an intra-peritoneal injection of sodium pentobarbital (60mg/kg body weight, Sagatal, Rhône Mérieux Ltd, Essex, UK). Supplemental doses were given to ensure that animals remained unresponsive to tactile stimuli. The absence of a pedal reflex was used to confirm deep anaesthesia and if needed a further top-up dose was administered. Animals were euthanised by cervical dislocation followed by immediate removal of the heart.

2.2.1.2. Tissue dissection

Following sacrifice, the fast-twitch extensor digitorum longus (EDL) and the predominantly slow-twitch soleus (SOL) muscles of the lower hind limb were rapidly dissected out. Proximal and distal tendons of each muscle were exposed and carefully dissected free and intact from the point of origin to insertion. In Chapter 4, plantaris (PL) and gastrocnemius (GT) were also dissected. In experiments where the diaphragm muscle was being assessed (Chapter 5), the entire muscle, including spinal cord and ribs, was dissected free and rinsed thoroughly in Krebs solution to remove any blood.

Following dissection, each muscle was blotted on filter paper (Whatman® no.1, Whatman® International Ltd., England) and transferred to a glass container filled with Krebs-Henseleit solution (composition in mM: 118 NaCl, 4.75 KCl, 1.18 KH_2PO_4, 1.18 $MgSO_4.7H_2O$, 24.8 $NaHCO_3$, 2.5 $CaCl_2$, 11 D-Glucose) made with double distilled H_2O (ddH_2O) at room temperature. pH was maintained at 7.4 by bubbling with carbogen, a gas mixture of 95% O_2 and 5% CO_2 (BOC Gases, Manchester). From this point on, Krebs-Henseleit solution bubbled with carbogen at room temperature will be referred to as Krebs solution unless stated otherwise.

Individual muscles were placed in a glass Petri dish, filled with Krebs solution, for further dissection to remove all visible fat, blood vessels and connective tissue. GT, a large bilobed muscle, was further divided into gastrocnemius white (GW, glycolytic lobe of GT) and gastrocnemius red (GR, oxidative lobe of GT). The whole diaphragm was placed in a glass Petri dish for further dissection to remove connective tissue, fat and the majority of the rib cage. In contractile function experiments, some of the rib was left for the attachment of silk suture. Following dissection, the diaphragm was placed in a glass container filled with Krebs solution.

2.2.1.3. Blood collection

Prior to sacrifice, a 5ml blood sample was taken from the carotid vein of all CON and VIT animals used in Chapter 5, which was injected into a vacutainer lined with EDTA (BD Vacutainer Systems, Plymouth, UK). Samples were centrifuged at room temperature for approximately 10 minutes at 8000 $rev.min^{-1}$, following which aliquots of plasma were removed and either frozen immediately for the later determination of total antioxidant

capacity and antioxidant levels, or were mixed with 5% TCA for the later analysis of vitamin C and glutathione levels and placed in a -20°C freezer.

2.2.2. Contractile function methods

2.2.2.1. Tissue preparation

In experiments designed to assess the contractile function of the hind limb muscles, tendons at each end of the EDL and SOL were tied securely with braided, non-absorbable silk suture (Silkam ®, Germany) in a glass Petri dish, where muscles were continually bathed in Krebs solution. Sutures did not impinge on any muscle fibres. Small loops were left for attachment to a force-recording apparatus. Once completed, muscles were placed back into a glass beaker with Krebs solution until required for contractile experiments. All muscles removed from animals were tested within 4 hours as the viability of the tissue may be compromised beyond this period of time (Van Breda *et al*, 1990).

Where the contractile properties of the diaphragm were being assessed, the diaphragm was cut down the middle with dissection scissors from sternum to spinal cord, through the tendinous tissue and crural muscle, resulting in 2 hemidiaphragms (Figure 2.1). A muscle strip from the ventral costal region of each hemidiaphragm (average width; 5-6mm, as measured with Vernier callipers) was dissected out from the central tendon to the attachment point at the rib (Region 5 & 6, Figure 2.1).

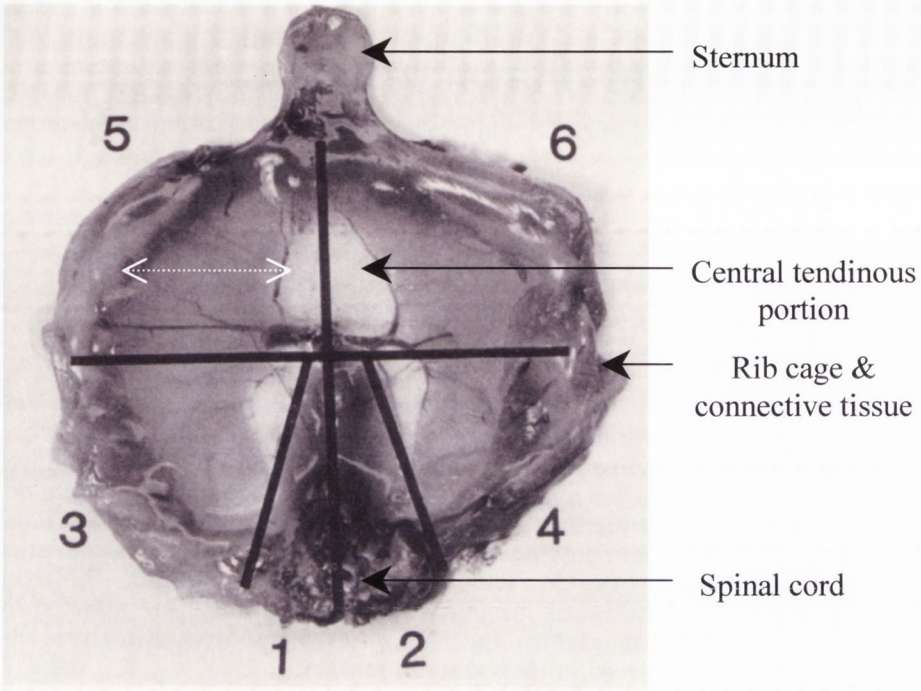


Figure 2.1. Illustration of the rat diaphragm showing regional divisions of the muscle.

Numbers 1-6 represents left crural (**1**), right crural (**2**), left dorsal costal (**3**), right dorsal costal (**4**), left ventral costal (**5**) and right ventral costal (**6**) segments of diaphragm, respectively (Metzger *et al*, 1985). The white dotted line indicates fibre orientation in the ventral costal region and the direction in which muscle dissection took place.

The central tendinous portion of the ventral section was tied securely with silk suture, as described above for the hind limb muscles. Another loop was loosely tied around the attachment area of the diaphragm to the rib cage. Muscle was left attached to the bone to stabilize the silk suture into place without compromising constituent fibres. The ventral muscle strip was then transferred to a muscle bath, while the remainder of the diaphragm was kept bathed in Krebs solution (~ 45 mins). Once the first set of experiments was completed, a second segment was dissected from the ventral costal region of the opposite hemidiaphragm. Pilot studies performed showed that the contractile properties of ventral costal strips are unaffected by the 45 min incubation period (Appendix iii), which has also been reported previously (Metzger *et al*, 1985).

The ventral costal region of the diaphragm was chosen for contractile experiments due to muscle fibre orientation, which run horizontally from the central tendon directly to the rib cage (Figure 2.1). Dissection through this area reduces the proportion of muscle fibres cut and damaged during preparation of muscle sections for contractile experiments and this maximises the contribution of functional fibres to the measurement of force in these

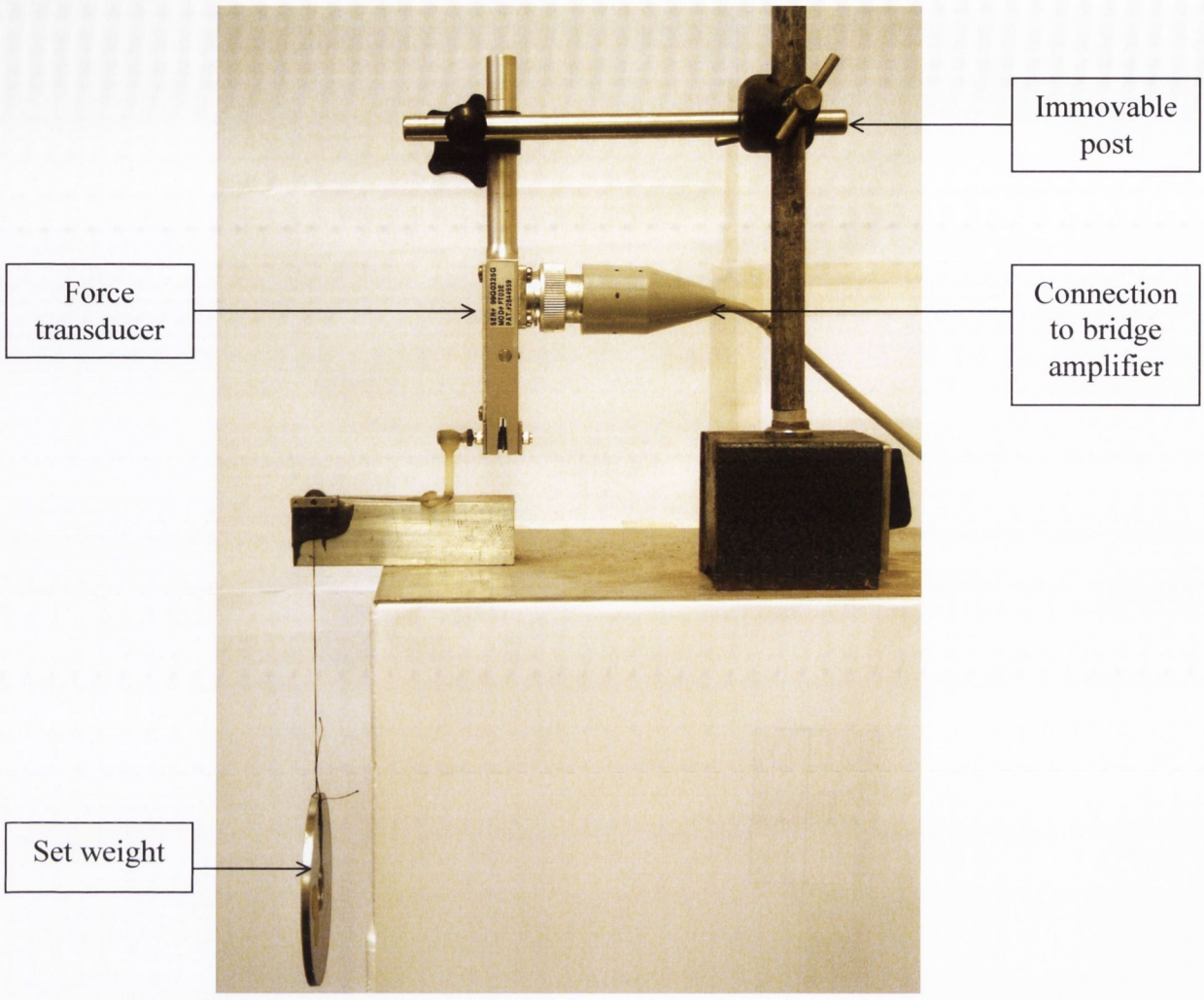


Figure 2.2. Calibration set up used prior to each testing session.

The metal plate has been developed to allow calibration to occur in the same alignment as the force transducer would be in the muscle bath during testing.

In trial runs prior to the testing protocol, it was noted that the absolute forces produced by diaphragm sections were less than that of the hind limb muscles in both twitch and tetanic contractions. Therefore, a more sensitive recording was required to obtain accurate readings of baseline and maximum values from the traces produced. To increase the sensitivity of the force transducer, internal springs were replaced within the transducer from 1kg/mm for the hind limb muscles to 0.2kg/mm for diaphragm sections. This altered the maximum working range and increased the sensitivity of the reading produced.

(iii) Equipment set-up

Each whole muscle or muscle strip was aligned horizontally and tied directly between the force transducer and an inflexible post, held stable by a micromanipulator. Platinum plate

electrodes were positioned on either flank of the muscle to provide a means of direct stimulation of the muscle being tested. Twitch contractions were elicited by single square-wave pulses generated by a stimulator (Grass S44, Quincy, USA). The trace of each contraction was recorded at high speed so that the profile of each contraction could be accurately analysed (100 samples/s, 200ms/division). Once it was established that the equipment set-up was working effectively, the signal was passed through an amplifier (Model CE 1000A, Crown instruments, USA) to increase and sustain current intensity to a sufficient level, ensuring that all muscle fibres were recruited during a maximal tetanic contractions (Figure 2.3).

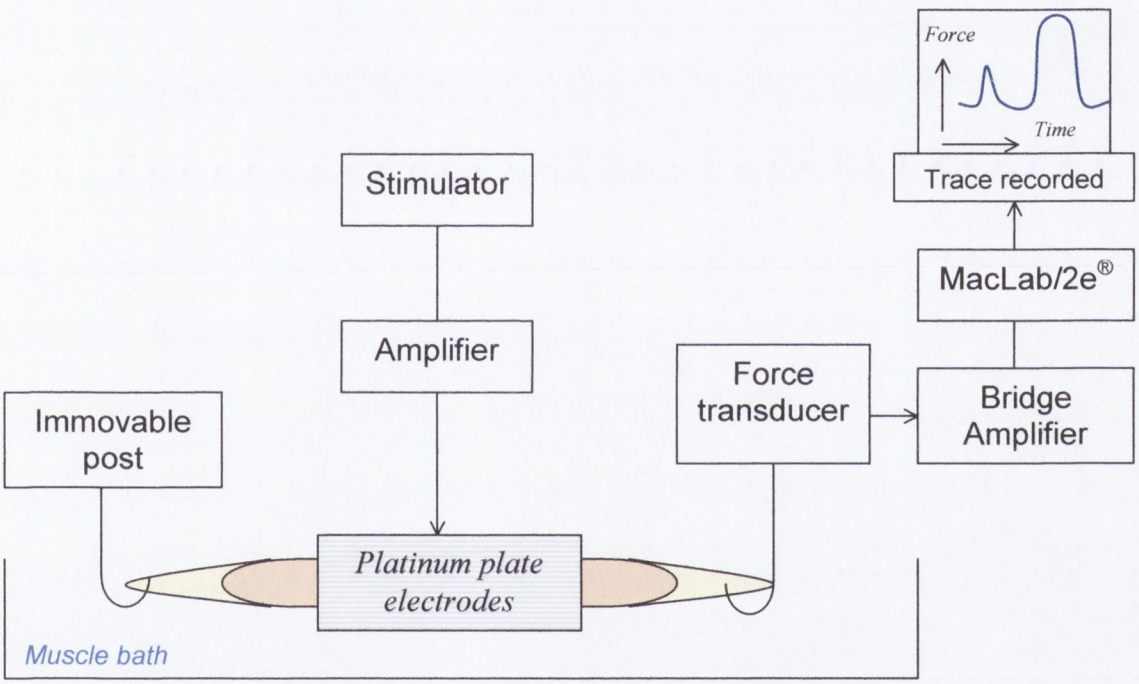


Figure 2.3. Schematic representing the arrangement of equipment throughout contractile function experiments.

(iv) Optimum length (L_O)

Following 1Hz stimulations, muscle length was adjusted from an initial slack position using a micromanipulator, at a ratio of no greater than 1mm per stimulation, until a point was achieved at which maximal twitch tension was generated. This length was designated L_O (the length at which maximum force was achieved in response to a 1Hz stimulation) and the point of P_t (maximum twitch tension) (Rassier *et al*, 1999). This length was held for all subsequent isometric measurements. A rest period of 2 minutes was given following each twitch contraction to allow complete recovery of the muscle following the

stimulation and to prevent muscle fatigue, which may have affected subsequent measurements.

(v) Parameters measured

Time to peak contraction (TTP) and one half-relaxation time (½RT) was recorded for each P_t. TTP was defined as the time required to generate maximum force during a single twitch and ½RT was defined as the time for maximum force to fall by 50% after a single twitch (Figure 2.4). P_t/P_O ratio was determined from the measurements taken.

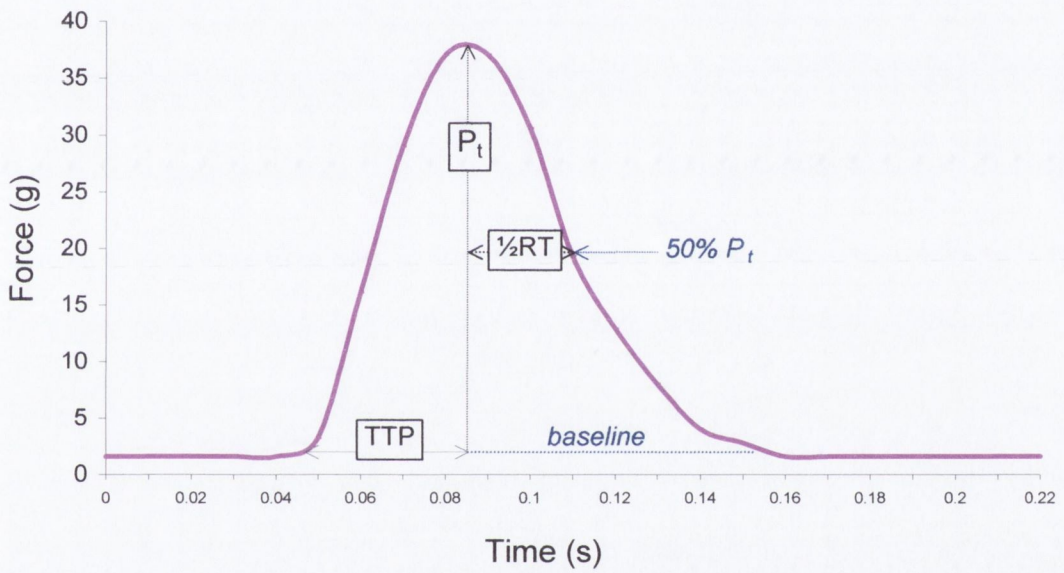


Figure 2.4. Twitch profile recorded from the EDL, showing P_t, TTP & ½RT.

Mean contraction (P_t/TTP) and relaxation velocities (($½P_t$)/½RT) were measured from the average gradient produced by the twitch profile during each contractile stage as set out according to Nakahata *et al*, 2001 (Figure 2.5).

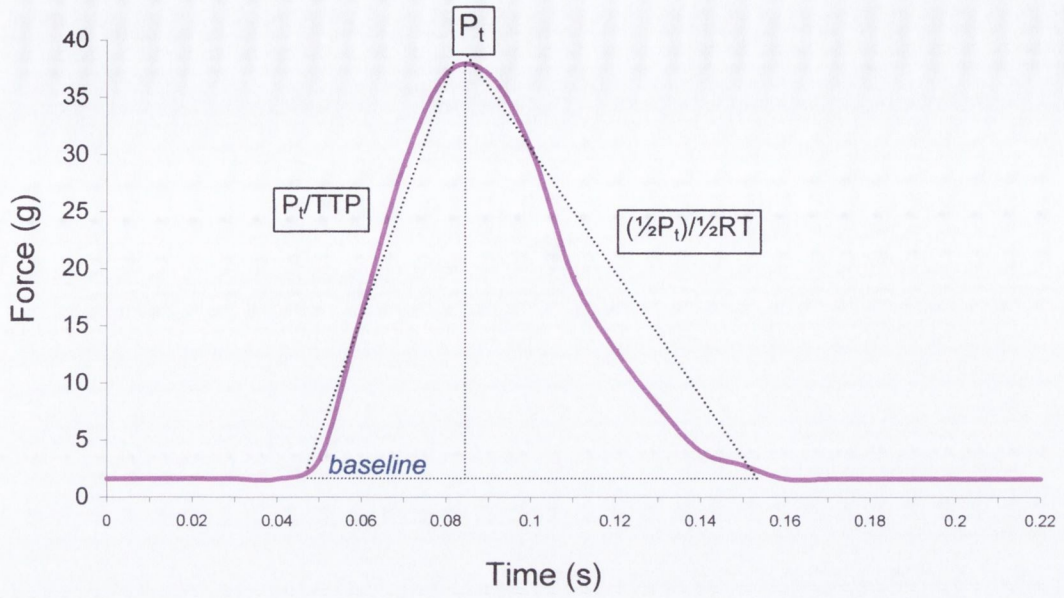


Figure 2.5. Calculation of contraction (P_t/TTP) and relaxation ((½P_t)/½RT) velocities from EDL twitch profile.

Once L_O was established, maximum tetanic force (P_O) was recorded by stimulating each muscle tetanically for a period of approximately 1s for the EDL and the diaphragm, and 2s for the SOL as described below.

2.2.2.3. Equipment settings

Single square-wave pulses of supramaximal voltage, with a duration of 0.5ms and a delay of 0.2ms were used for each muscle tested, as established by pilot experiments. Force-frequency relationships were completed to ensure that each muscle was producing maximum force during tetanic contractions (Appendix iv). Results established frequencies of 70Hz for the EDL, 50Hz for the SOL and 100Hz for the diaphragm. These settings have been shown to produce P_O consistently in both hind limb (Plant *et al*, 2001) and diaphragm muscles (Diaz *et al*, 1998, Coombes *et al*, 2001, Nakahata *et al*, 2001).

2.2.2.4. Experimental protocol

After establishing P_t, the contractile function of hind limb and diaphragm muscles to 30 mins incubation in Krebs solution, with H_2O_2 (O) or without (C), was investigated. Whole muscle and muscle strips were stimulated maximally to produce a tetanic contraction every 5 mins for 30 mins. Rest periods of 5 mins between contractions were given to allow the muscle being tested to completely recover from each contraction. Immediately

following the first maximum tetanus, a supramaximal dose of H_2O_2 (160mM, from a 30% stock) was added directly to the bath in O muscles only.

The effect of H_2O_2 on force production in diaphragm muscle sections has not yet been investigated. As the diaphragm is a sheet of muscle that has been cut through, as opposed to a whole and intact muscle, the response to incubation with H_2O_2 may differ to that produced by hind limb muscles. Any H_2O_2 concentration that is too high could affect function in diaphragm sections too rapidly, thus limiting the ability to clearly examine the muscle for the effects of oxidative stress. A dose-response relationship was established in diaphragm segments prior to testing on CON and VIT diaphragm sections in Chapter 5 (Appendix v) to determine which concentration of H_2O_2 would produce a similar response as that previously seen in the hind limb (Chapter 3).

2.2.2.5. Force conversion

For all isometric contractile experiments performed in the EDL and SOL, specific force ($kN.m^{-2}$) was determined based on an estimation of total cross sectional area (Equation 2.1), where density = 1.06 mg/mm^3 (density of mammalian skeletal muscle), L_O = optimum muscle length and L_f = fiber length. Optimum fibre length (L_f) was determined by multiplying L_O by previously measured L_f:L_O ratios of 0.44 for EDL and 0.71 for SOL muscles (Brooks & Faulkner, 1988).

$$\text{Force } (kN.m^{-2}) = \left(\frac{\left(\text{Force (g)} \times 9.8 / 1000 \right)}{\left(\text{Mass (g)} / \text{Density } (mg/mm^3) \times L_O \text{ (cm)} \times L_f \right)} \right) \times 10 \qquad (2.1)$$

Specific force for diaphragm experiments was normalized for muscle cross-sectional area and expressed in 2 ways to correspond with published data: 1) by applying the absolute force recorded from the diaphragm in grams to equation 2.2 (Close, 1972); 2) absolute force was then converted to the standard unit of force ($kN.m^{-2}$) (Equation 2.3).

$$\text{Force } (g.cm^{-2}) = \frac{\text{Force (g)} \times L_O \text{ (cm)} \times \text{Density } (mg/mm^3)}{\text{Mass (g)}} \qquad (2.2)$$

$$\text{Force (kN.m}^{-2}) = \left(\frac{\left(\text{Force (g)} \times \frac{9.8}{1000} \right) \times L_O \text{ (cm)} \times \text{Density (mg/mm}^3)}{\text{Mass (g)}} \right) \times 10 \qquad (2.3)$$

Similar specific force obtained from diaphragm sections have been recorded previously (Reid *et al*, 1998, Supinski *et al*, 2000, Nakahata *et al*, 2001).

2.2.2.6. Hydrogen peroxide

H_2O_2 was used in the contractile function tests to induce an oxidative state/medium in the bath because externally applied H_2O_2 can be added to experimental preparations in precise quantities, is hydrophilic and can cross cell membranes (Beckman & Freeman, 1986). It is also relatively stable in the absence of transition metals (Rush *et al*, 1990). H_2O_2 can decompose to produce $OH^{\cdot}$ via the Fenton reaction, however the level of $OH^{\cdot}$ generated in the absence of iron is very small (Josephson *et al*, 1991).

A supramaximal dose of 160mM H_2O_2 was used throughout this thesis to assess the ability of different groups of skeletal muscle to withstand oxidative stress-induced reductions in force over time. This concentration was designated as a supramaximal dose following analysis of data from previous studies. The ability to resist changes in force in the presence of a supramaximal dose of H_2O_2 gives a measure of the oxidative stability of muscles being tested and allows comparisons of the effects of different conditions on this parameter.

2.2.3. Tissue storage

All muscles and viscera were weighed prior to storage (Oertling balance, model no. R20, London, England, accurate to 0.1mg). In experiments where viscera were removed, a portion of the left ventricular wall was excised from the heart, weighed and frozen in isopentane cooled in liquid nitrogen. The central lobe of the liver was also frozen. Immediately following dissection and/or contractile experiments, all muscles were snap frozen in isopentane, cooled in liquid nitrogen, at a length approximately equivalent to L_O, and stored at -80°C for later analysis of antioxidant characteristics and fibre typing.

2.2.4. Antioxidant assays

2.2.4.1. Homogenization protocol

Tissues were thawed, blotted dry on filter paper and divided into a small and a large piece. Each segment was weighed and placed into approximately 8 volumes of Krebs solution. The smaller portion was placed in Krebs solution containing 5% trichloroacetic acid (TCA, stock concentration; 0.3M) for the later determination of total glutathione and vitamin C concentrations. All solutions and samples were kept chilled on ice. Each piece of tissue was homogenized thoroughly using a Powergen 125 homogenizer for approximately 1 minute at 20,000 rpm (Fisher Scientific, Leicestershire, UK).

2.2.4.2. Protein quantification

The protein concentration of each homogenate was determined using a colourimetric assay technique based on the Bradford protein assay (Bradford, 1976). The protein assay is a dye-binding assay based on the differential colour change of a coomassie blue dye (BioRad, Hertfordshire, UK) in response to various concentrations of protein.

(i) Standard and sample preparation

Serial dilutions of a stock solution of bovine serum albumin (BSA; 1000µg/ml) were used as standards in the protein assay to produce a standard curve of absorbance against protein concentration in the range of 7.81-1000µg/ml. ddH_2O was used as a blank. Diluted aliquots of stock tissue homogenate were prepared with Krebs solution to ensure that each sample fit on the standard curve. Following thorough mixing to ensure even protein distribution, 20µl of the standards and diluted samples were added to a 24-well plate (Starsted, Ireland) in duplicate. The dye reagent (BioRad) was provided as a 5-fold concentrate; therefore 1 volume of dye reagent concentrate was diluted with 4 volumes of ddH_2O and was filtered prior to use in the standard assay procedure. 1ml of diluted BioRad was then added carefully to each well to avoid excess foaming, which is suggested to affect colour yields.

(ii) Analysis

Standards and samples were allowed to develop for 5 - 10 mins on a rocking machine (Gyro-Rocker STR9, Stuart Scientific, UK). 200µl from each well was added to a 96-well

plate (Starsted, Ireland) and absorbance was measured at 600nm on a multiwell plate reader (EIA Multiwell reader, Sigma Diagnostics, Dorset, UK). Standards (μg/ml) were plotted against mean absorbance at 600nm (Appendix vii). A straight line of best fit was calculated for the points on the standard curve. The protein concentration of each sample was then determined from sample absorbencies according to the standard absorbance versus protein relationship. Aliquots of the stock homogenate were equalized to 2mg/ml of protein. Stock and equalized samples were then immediately stored at -80°C for later biochemical analysis.

2.2.4.3. Catalase activity

(i) Background

Catalase activity was measured spectrophotometrically according to a ferrithiocyanate reaction (Cohen *et al*, 1996). As catalase decomposes H_2O_2 (Equation 2.4), its activity is commonly assayed by recording the rate of disappearance of H_2O_2 colourimetrically. In this assay, H_2O_2 disappearance is measured with added ferrous ions (Fe^{2+}) and potassium thiocyanate (KSCN) (Equation 2.5).

$$2H_2O_2 \xrightarrow{CAT} O_2 + 2H_2O \qquad (2.4)$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + 2OH^{\cdot} \xrightarrow{KSCN} \text{Colour change} \qquad (2.5)$$

KSCN and Fe^{3+} form a red coloured complex with a peak in the 450-480nm range, which remains stable at room temperature for several hours. When an increasing amount of CAT is added to the system, the rate at which H_2O_2 decomposes is accelerated, leading to a subsequent decrease in the reaction of Fe^{3+} and KSCN, and a reduction in colour intensity is observed.

(ii) Method

Samples equalized for protein (2mg/ml) were thawed and a 1:10 volume ratio of Triton-X_{100} (5% stock solution) to sample was prepared. Triton-X_{100} increases CAT activity by solubilizing any CAT bound to intracellular organelles (Cohen *et al*, 1996). Samples were mixed thoroughly, then centrifuged at 14,000 rpm for 5 min at 4°C to sediment unbroken cells and cellular debris. An aliquot of the resulting supernatant (100μl) was added to an

eppendorf containing 800µl of phosphate buffer (final concentration: 8mM). The reaction was initiated by addition of 100µl H_2O_2 (diluted from stock 30% H_2O_2, final concentration in eppendorf: 6mM), followed by gentle mixing (low speed vortex).

At 2 and 10 min intervals, 100µl aliquots of the reaction mixture were added to a tube containing 0.3M H_2SO_4 (4ml) and 2mM ferrous sulphate ($FeSO_4.7H_2O$; 1ml) at room temperature. H_2SO_4 stops the reaction shown in equation 2.4. Samples were inverted gently to ensure that the solutions had been mixed thoroughly and the reaction had ceased completely. Colour was developed by addition of 400µl KSCN (Equation 2.5; final concentration: 0.2mM). All tubes were covered with aluminium foil, as the colour developed from the assay is photosensitive and fades with exposure to light. Samples were again gently inverted. An aliquot of 200µl from each tube was transferred to a 96-well plate. Absorbance was read at 450nm. All samples were measured in triplicate.

(iii) Analysis

Results were expressed in terms of the first-order reaction rate constant (k) and protein concentration (mg) (Equation 2.6):

$$\text{Catalase enzyme units} = k.\text{mg protein}^{-1} = [\ln(A1/A2)/t]/\text{protein (mg)} \qquad (2.6)$$

where: ln is the natural log, $A1$ and $A2$ are the measured mean absorbance of triplicate samples at the two time points (2 & 10 mins) and t is the time differential between the two points (8 minutes in the selected samples)

The greater the difference in absorbance between 2 and 10 minutes, the larger the concentration of CAT in the sample being analysed. Results are expressed as enzyme units ($\times 10^{-3}$) per mg of protein.

2.2.4.4. Total superoxide dismutase activity

Two methods were used to measure total SOD activity in various samples collected throughout the course of this thesis. The first involved a spectrophotometric technique as described in (a) below. This method was used in Chapter 4 to analyse total SOD activity in rat skeletal muscle taken from animals of different ages. However, this method was

very time-consuming. Therefore, a microtitre chemiluminescence assay outlined in (b) below was used for subsequent analysis of total SOD activity in rat tissue samples collected from a dietary intervention study as described in Chapter 5. Comparison of the results produced by running samples through both the spectrophotometric and chemiluminescence assay revealed differences in total SOD activity. Following subsequent analysis (Appendix vi), these differences were attributed to an inherent property of the assay technique as opposed to a real variation in enzyme levels.

(i) Spectrophotometric assay

(a) Background

Total SOD activity was measured using the method described by Beauchamp & Fridovich, 1971. Xanthine and xanthine oxidase were used to generate a $O_2^{\cdot -}$ flux (Equation 2.7, Kuppusamy & Zweier, 1989). In the absence of skeletal muscle homogenate, nitroblue tetrazolium (NBT) is reduced by $O_2^{\cdot -}$ to blue formazen. This can be followed at 560nm using a spectrophotometer. The rate of NBT reduction in the absence of tissue was used as a reference rate for any background activity (Equation 2.8.1). When increasing amounts of SOD are added to the system, the rate of NBT reduction is progressively inhibited as $O_2^{\cdot -}$ dismutates via SOD to H_2O_2, which is then decomposed by CAT in the reaction mixture (Equation 2.8.2).

$$\text{Xanthine} \xrightarrow{\text{Xanthine oxidase}} O_2^{\cdot -} \tag{2.7}$$

$$\text{Xanthine} \xrightarrow{\text{Xanthine oxidase}} O_2^{\cdot -} + \text{NBT} \rightarrow \text{Blue formazen} \tag{2.8.1}$$

$$\downarrow_{SOD}$$

$$2H_2O_2 \xrightarrow{CAT} O_2 + 2H_2O \tag{2.8.2}$$

(b) Method

Protein-equalized samples were thawed and centrifuged at 14,000 rpm for 5 min at 4°C to sediment unbroken cells and cellular debris. Serial dilutions of supernatant were prepared with phosphate buffer (50mM) according to the following ratio: pure homogenate, 1:1, 1:2, 1:3, 1:5, 1:10 and 1:20. An aliquot (100µl) of each dilution was added to 800µl of the reaction mixture (final concentration: 1mM DETAPAC; 1 unit catalase; 5.6×10^{-5}M NBT; 0.1mM xanthine; pH 7.8) in a 2ml cuvette. To initialise the reaction, 100µl of

xanthine oxidase was added to the cuvette, which was then gently vortexed to ensure thorough mixing of the contents. Potassium phosphate buffer (final concentration: 5mM) was used as a blank to establish the background rate. The change in absorbance at 560nm was measured every 10 seconds for 8 minutes using a spectrophotometer. Samples were measured in duplicate.

(c) Analysis

The absorbance at 560nm of the blank and each sample dilution was plotted against time (Appendix vii). The slopes produced were added to equation 2.9, which determined each sample slope as a percentage of the reference rate of NBT reduction when SOD activity is absent (blank slope) and was expressed as % inhibition.

$$\% \text{ inhibition} = (100 - (\text{sample slope}/\text{blank slope}) \times 100) \qquad (2.9)$$

The percentage inhibition of NBT reduction for each sample slope was plotted against the protein concentration (μg/ml) of the corresponding dilutions. One unit of activity was defined as the amount of protein necessary to decrease the rate of reduction of NBT by 50% (Figure 2.6, Spitz & Oberley, 1989).

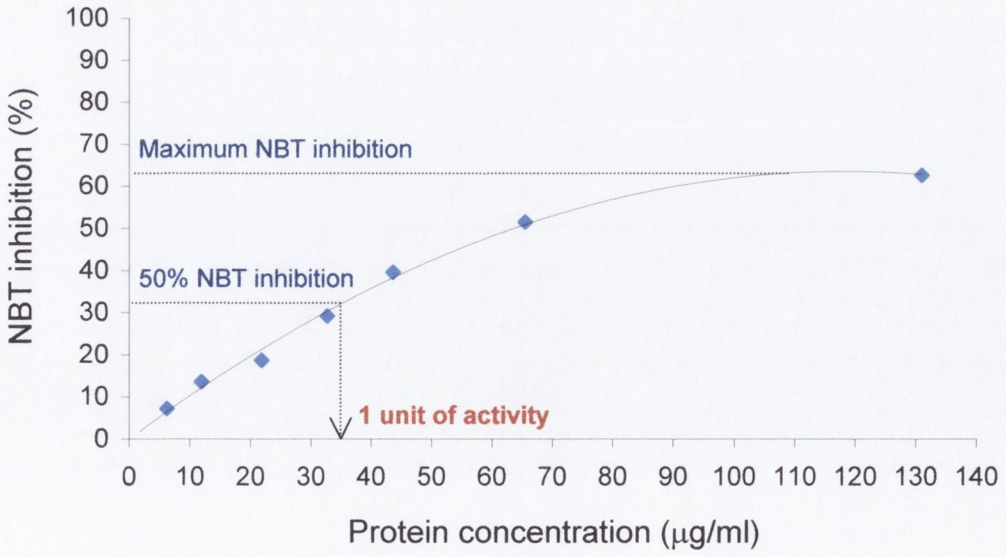


Figure 2.6. Example of the relationship produced by a plot of % inhibition versus protein concentration (μg/ml).

◆ % Inhibition produced by various dilutions of sample homogenate as calculated by equation 2.9.

The value recorded for 1 unit of activity was converted to express results as units of SOD/mg protein.

(ii) Chemiluminescence assay

(a) Background

In this series of experiments, a highly sensitive method of quantification of total SOD activity was used, utilising the chemiluminescence (CL) of lucigenin, which has specific sensitivity to $O_2^{\cdot-}$ (Corbisier *et al*, 1987). Total SOD activity was given by the percentage inhibition of the chemiluminescent reaction produced in the oxygen-xanthine-xanthine oxidase-lucigenin system (Equation 2.10), in which SOD is in competition with lucigenin for $O_2^{\cdot-}$.

$$\text{Sample/Standard} + \text{xanthine} + O_2 \xrightarrow{XO} O_2^{\cdot-} \begin{cases} \xrightarrow{\text{Lucigenin}} \text{Light signal} \\ \xrightarrow{SOD} O_2 + H_2O \end{cases} \qquad (2.10)$$

(b) Method

The standard CL assay was performed in 0.1M glycine-NaOH buffer containing 1mM EDTA and 1mM sodium salicylate at a pH 9.0. Xanthine was diluted in a glycine buffer to a concentration of 25μM. Xanthine oxidase was diluted in the same buffer, but in the presence of lucigenin. The final concentrations of lucigenin and xanthine oxidase in each test were 0.1mM and 4mU/test, respectively. One unit of xanthine oxidase is defined as the amount of enzyme needed to convert 1µmol of xanthine in uric acid with formation of 2µmol of $O_2^{\cdot-}$ at 25°C and pH 7.4. Purified SOD and samples were diluted in 10mM phosphate buffer, pH 7.4. The assay was initiated by addition of 120µl xanthine to 120µl xanthine oxidase-lucigenin solution in the presence of 20µl of sample/standard. This ensured that $O_2^{\cdot-}$ formation and dismutation occurred simultaneously. The light signal produced by each sample was measured 30s after initiation of the reaction by xanthine with a chemiluminescence plate reader (Fluroskan, Ascent, Labsystems, Finland).

(c) Analysis

The relationship between SOD standards (range: 0.0064 - 4U/ml SOD activity) and chemiluminescence measured after 30 seconds was plotted and used as a reference for sample SOD concentrations. ddH_2O was used a blank to determine background chemiluminescence. Contrary to previous spectrophotometric assays, there is no linear section in the standard curve produced due to the low levels of $O_2^{\cdot -}$ produced in the assay (Figure 2.7).

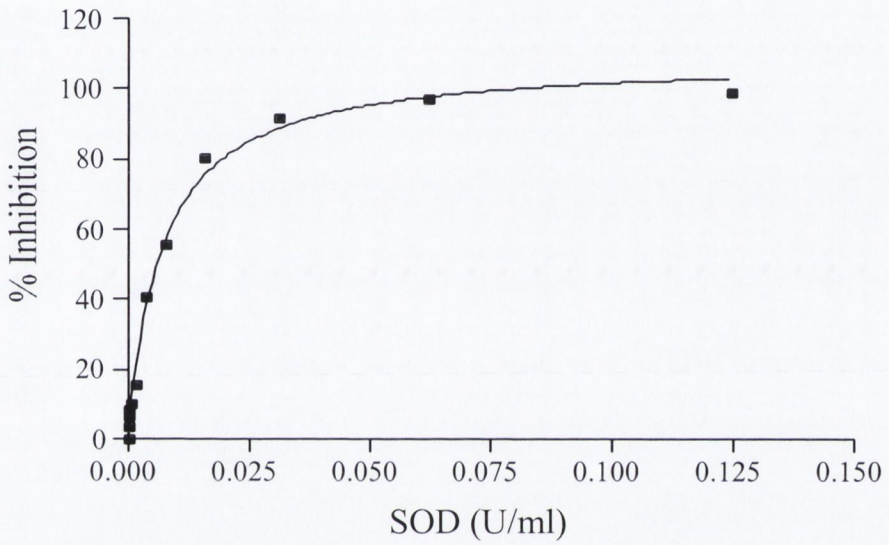


Figure 2.7. Example of the relationship produced by CL inhibition (%) versus SOD concentration (U/ml).

The percentage of CL inhibition by samples and standards was calculated according to equation 2.11.

$$\% \text{ inhibition} = \frac{CL_{blank} - CL_{sample}}{CL_{blank}} \times 100 \qquad (2.11)$$

The curve was adjusted by an Eadie-Hofstee mathematical equation (Equation 2.12, Corbisier *et al*, 1987).

$$[SOD] = [k_2/(k_1 - i\%)] \times i\% \qquad (2.12)$$

From this equation, k_2 represents the concentration of SOD that inhibits CL by 50%, k_1 is the maximum inhibition by SOD, *i%* is the percentage of CL inhibition, and [SOD] is the concentration of SOD in the test. Results were expressed as units of SOD/mg protein.

2.2.4.5. Total glutathione concentration

Two methods following the same principle were used to measure total glutathione levels in tissue collected from various studies. The first method, outlined in (a) below, followed absorbance in samples over time on a spectrophotometer and was used in Chapter 4. This method was time consuming. Therefore, the second technique used a microtitre plate technique, described in section (b) below, where absorbance was recorded on a multiwell plate reader. This method was adapted for use in the dietary intervention study described in Chapter 5.

(i) Spectrophotometric assay

(a) Background

GSH rapidly oxidizes non-enzymatically to GSSG at pH > 7. Therefore, biological samples must be acidified quickly to reduce this pathway (Anderson, 1985). Acid treatment also inactivates γ-glutamyl transpeptidase, a catalyst in the following reactions that can also decrease the levels of both GSH and GSSG.

$$\text{GSH + amino acid} \leftrightarrow \text{γ-Glu-amino acid + CysH-Gly} \qquad \text{(transpeptidation)}$$

$$\text{GSH + } H_2O \rightarrow \text{Glu + CysH-Gly} \qquad \text{(hydrolysis)}$$

$$\text{GSH + GSH} \rightarrow \text{γ-Glu-GSH + CysH-GSH} \qquad \text{(autotranspeptidation)}$$

Therefore, 5% TCA was added to solutions prior to homogenisation of muscle and viscera.

The DTNB-GSSG recycling assay for total glutathione (GSH + GSSG) is a sensitive and specific enzymatic procedure. The accuracy of the assay is controlled by the high specificity of GSSG reductase (Griffith, 1980). GSH is oxidized by 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB) to produce GSSG with formation of 5-thio-2-nitrobenzoic acid (TNB) (Equation 2.13). GSSG reductase reduces GSSG back to GSH in the presence of β-nicotinamide-adenine dinucleotide phosphate (NADPH) (Equation 2.14). The rate of

TNB formation is measured spectrophotometrically at 412nm and is proportional to the sum of GSH and GSSG present.

$$2GSH + DTNB \rightarrow GSSG + TNB \quad (2.13)$$

$$GSSG + NADPH + H^+ \xrightarrow{GSSG\ Reductase} 2GSH + NADP^+ \quad (2.14)$$

(b) Method

A standard stock of 200μM GSH was prepared daily containing 5% TCA (stock concentration: 0.3M). Serial dilutions ranging from 200μM to 1.56μM GSH were prepared from stock. Sample buffer was used as a blank to determine background rates when no GSH was present. Equalized samples containing 5% TCA were thawed and centrifuged at 14,000 rpm for 5 min at 4°C to sediment unbroken cells and cellular debris.

Standards and the supernatant from samples (100μl) were added to 800μl of sample buffer (comprising (mM): 75 potassium phosphate buffer, 3.8 EDTA, pH 7.5) and 50μl of 10mM DTNB in a 2ml cuvette. To initialise the reaction, 100μl of GSSG-R (stock concentration: 5U/ml) was added to the cuvette. Samples were gently mixed and incubated for 1 minute at room temperature. NADPH (100μl of 2.4mM stock) was added, and each sample was gently mixed again. Absorbance was monitored every 10 seconds for 2.5 min at 412nm. Each sample was tested in triplicate. The absorbance of the various samples and standards was plotted against time (Appendix vii), and the slope of the relationship produced for each was recorded.

(c) Analysis

The slope of the absorbance versus time relationship for each standard was plotted against GSH concentration (Appendix vii). The total glutathione concentration in each sample tested was determined from the slope of the sample absorbance versus time, using the equation of the line for the relationship produced. Results were expressed as μmol/min/mg tissue.

(ii) Microtitre plate assay

(a) Background

The protocol outlined below is a microtiter plate technique, whose working principle is the same as that described in Section 2.2.4(i) above.

(b) Method

The method described in Section 2.2.4(i) was adapted for use on a 96-well plate. The previous concentrations of each solution remained the same. However, volumes were reduced to fit onto a multiwell plate. 30µl of sample/standard was added to each well, followed by 230µl of reaction mixture (sample buffer, DTNB, GSSG-R). Plates were gently shaken on a plate reader to ensure thorough mixing of solutions, and were incubated at room temperature for 1 minute. 30µl NADPH was added to initiate the reaction and the plates were shaken again. Absorbance was read every 10 seconds for 2 minutes at 405nm. Each sample was tested in duplicate.

(c) Analysis

Total GSH concentrations in the samples tested were calculated according to that explained in 2.2.4(a).

2.2.4.6. Total glutathione peroxidase activity

(i) Background

Glutathione peroxidase (GPx) enzyme activity was measured using a cyclic assay system. In this assay, oxidation of GSH to GSSG via GPx is coupled to reformation of GSH from GSSG via GSSG-R (Equation 2.15). The rate of GSH formation is measured by following the decrease in NADPH to $NADP^+$ by GSSG-R in the reaction mixture at 340nm (Paglia & Valentine, 1967).

$$2GSH + H_2O_2 \xrightarrow{GPx} H_2O + GSSG \xrightarrow[NADPH \longrightarrow NADP^+]{GSSG-R} 2GSH \qquad (2.15)$$

In this assay, total GPx activity is measured with cumene hydroperoxide. All solutions had a pH of 7.6 as the enzymatic activity of partially purified GPx peaks at pH 8.

Negligible activities are measured below pH 6 with rapid increases in the non-enzymatic rate above pH 7 (Paglia & Valentine, 1967).

(ii) Method

Protein-equalized samples were thawed and centrifuged at 14,000 rpm for 5 min at 4°C to sediment unbroken cells and cellular debris. Aliquots of standards and sample supernatant (100µl) were added to 800µl of reaction mixture (comprising in mM: 0.8 EDTA; 0.8 NaN_3; 1.2 NADPH; 0.8 GSH; 0.8 units GSSG-R) in a 2ml cuvette. NaN_3 inhibits CAT activity in the samples (Carmagnol *et al*, 1983). The non-enzymatic oxidation of GSH was determined by assay of samples with no substrate. Samples were incubated at room temperature and absorbance was monitored every 10 seconds for 5 minutes at 340nm. To initialise the reaction, cumene hydroperoxide (100µl of 1.5mM stock) was added to the cuvette. Samples were gently mixed. The conversion of NADPH to $NADP^+$ was monitored every 10 seconds for 5 minutes at 340nm. Samples acted as their own blanks (Appendix vii). Each sample was tested in triplicate.

(iii) Analysis

The relationship produced by a plot of absorbance against time with and without cumene hydroperoxide was generated for each sample (Appendix vii). A molar absorptivity of NADPH at 6.22×10^3 L mol^{-1} cm^{-1} (Andersen *et al*, 1997) was used to express results as µmol NADPH oxidised/min/mg tissue.

2.2.4.7. Vitamin C concentration

(i) Background

Methods for determining vitamin C (ascorbic acid) are numerous. Chemical analyses for the vitamin may be divided into 2 groups; the determination of the reduced form (ascorbic acid) and the determination of the oxidized form (dehydroascorbic acid) (Omaye *et al*, 1979). The latter group of analyses is usually based on the oxidation of ascorbic acid, with the subsequent formation of a hydrazone. Best results are obtained if samples are quickly stabilised with TCA. The greater stability of ascorbic acid in acid solution results from a decreased tendency for hydrolysis of the lactone ring with decreasing pH.

Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketogulonic acid (Equation 2.16).

$$\underset{(Vitamin\ C)}{\text{Ascorbic acid}} + Cu^{+} \rightarrow \underset{(Vitamin\ C\cdot)}{\text{Dehydroascorbic acid}} + \text{diketogulonic acid} + Cu^{2+} \qquad (2.16)$$

The products of this reaction are treated with 2,4-dinitrophenylhydrazine to form the derivative bis-2,4-dinitrophenylhydrazone. This compound, in strong sulphuric acid, undergoes a rearrangement (Equation 2.17), which forms a product with an absorption band that is measured at 540nm.

$$\text{bis} - 2,4 - \text{dinitrophenylhydrazone} + H_2SO_4 \rightarrow \text{colour change} \qquad (2.17)$$

The reaction is run in the presence of thiourea to provide a mildly reducing medium, which helps to prevent interference from non-ascorbic acid chromagens.

(ii) Method

Vitamin C concentration was determined by a modification of the method used by Omaye *et al*, 1979. Protein-equalized samples were thawed and centrifuged at 14,000 rpm for 5 min at 4°C to sediment unbroken cells and cellular debris. Standards were made in 95% Krebs solution and 5% TCA (stock concentration: 0.3M) and ranged from 0 to 20µg/ml ascorbic acid. To form bis-2, 4-dinitrophenylhydrazone, 0.3ml of supernatant or standard was mixed with 60µl dinitrophenylhydrazine/thiourea/copper (DTC) stock solution (comprising in mM: 50 thiourea, 0.02 $CuSO_4.5H_2O$, and 15 2,4-dinitrophenylhydrazine, brought to a total volume of 100ml with 4.5M H_2SO_4). Each sample was measured in triplicate.

Samples and standards were incubated at 37°C in a temperature controlled water bath for 3 hours. To convert bis-2, 4-dinitrophenylhydrazone to the rearranged product, which produces a colour change (Equation 2.17), 0.5ml of ice-cold 65% H_2SO_4 was added to each sample and standard and mixed well. The solutions were allowed to stand at room temperature for an additional 30 minutes. Following incubation, 100µl aliquots from each sample and standard was transferred to a 96-well plate. Absorbance was read at 540nm.

(iii) Analysis

Standard absorbance was plotted against vitamin C concentration. A straight line of best fit was calculated for the points on the standard curve (Appendix vii). Each sample's vitamin C concentration was determined from its absorbance according to the standard absorbance versus ascorbic acid relationship. Results were expressed as μg vitamin C oxidized/mg protein.

2.2.4.8. FRAP assay

(i) Background

Tests that measure the combined antioxidant power of non-enzymatic defences in biological fluids provide an index of the ability to resist oxidative damage (Popov & Lewin, 1994, Ghiselli *et al*, 1995, Uotila *et al*, 1994). Most tests of 'total antioxidant power' have measured the ability of plasma to withstand the oxidative effects of reactive species generated in the reaction mixture (Popov & Lewin, 1994, Uotila *et al*, 1994). FRAP has been used to analyze antioxidant status in humans after hyperbaric oxygen treatment (Dennog *et al*, 1999), compare the effects of different diets on plasma (Lee *et al*, 2000), study the efficiency of vitamin C in plasma (Benzie & Strain, 1999) and to compare the effects of different diets in rats (Aprikian *et al*, 2001).

A biological antioxidant has been defined as '*any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate'* (Halliwell & Gutteridge, 1995). However, unless an antioxidant prevents the generation of ROS (e.g. by metal chelation or enzyme-catalysed removal of a potential oxidant), a redox reaction still occurs. The difference is that the oxidizing species reacts with the antioxidant instead of the substrate. Therefore, the interaction between ROS and non-enzymatic antioxidants can be described as a redox reaction in which one reactive species is reduced at the expense of the oxidation of another. In this context, antioxidant power may be referred to analogously as reducing ability. Therefore, a method using reductants in a redox-linked colourimetric method offers a simple way of assessing this ability.

At low pH, when a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) form, an intense blue colour with absorption maximum at 593nm develops. In the FRAP assay, excess Fe^{3+} is used and the rate-limiting factor of Fe^{2+}-TPTZ, and hence

colour formation, is the reducing ability of the sample. FRAP values are obtained by comparing the absorbance change at 593nm in test reaction mixtures with those containing ferrous ions in known concentration. Since FRAP measures the reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ, there are some limitations to this method. Some antioxidants, such as glutathione (GSH), are not able to reduce Fe^{3+}, therefore, this test does not account for this group of antioxidants (Prior & Cao, 1999). However, the FRAP assay will be used in conjunction with other tests for GSH, thus providing a more thorough determination of the status of tissue/plasma when exposed to an oxidizing environment. When the FRAP test was performed with plasma and no Fe^{3+} added to the reaction mixture, no colour developed (Benzie & Strain, 1996). This indicates that there is no detectable free Fe^{2+} in EDTA plasma and also no detectable agent in normal EDTA plasma that can react directly with TPTZ to form the blue chromagens.

(ii) Method

This method was modified from Benzie & Strain's protocol (1996, 1999) as performed by Griffin & Bhagooli (2004). The working FRAP reagent was produced by mixing 300mM acetate buffer (pH 3.6), 10mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution and 20mM $FeCl_3.6H_2O$ in a 10:1:1 ratio just before use, and was heated to 37°C in a temperature controlled water bath. The 300mM acetate buffer was prepared by making a solution of 10mM TPTZ in 40mM HCl.

Working FRAP reagent (150μl) was added a 96-well plate. A blank reading was taken at 600nm. Standards and the supernatant of samples (20μl) were added to the 96-well plate in duplicate. A second reading was performed after 8 minutes at 600nm. The original protocol measures absorbance at 593nm, but the change can also be measured using a range from 585nm (Bub *et al*, 2000) to 600nm (Halvorsen *et al*, 2002). A time interval of 8 minutes was chosen between addition of the sample to the FRAP reagent and the second absorbance reading to allow for reaction of the samples with the ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex in the reagent. The initial blank reading for each well with just FRAP reagent was then subtracted from the final reading of FRAP reagent with sample to determine the FRAP value for each sample. The change in absorbance after 8 minutes from the initial blank reading was then compared to that of a standard that was run simultaneously. Standards of known Fe^{2+} concentrations ($FeSO_4.7H_2O$) were run

in duplicate using several concentrations ranging between 25 and 1000μM. ddH_2O was used to determine the background rate.

(iii) Analysis

A standard curve was created by plotting the FRAP value for each standard versus its concentration (Appendix vii). In the FRAP assay, the antioxidant efficiency of the sample was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration. The results were corrected for dilution and expressed in μM Fe^{2+}/mg protein.

2.2.5. Muscle fibre typing

2.2.5.1. Background

Skeletal muscle contain a combination of fibre types best suited to its activity patterns. It is possible to quantify a percentage and pattern of these fiber types within each muscle, thus allowing further understanding of various physiological parameters. Three fundamental muscle fibre types can be identified using histochemical methods (I, IIa and IIb). Histochemistry allows the activity of various enzymes to be visualised and its principles are based upon the chemical reaction of these enzymes located in thin sections of muscle fibres. The basic requirement for a histochemical assay is similar in principle to the requirement for any biochemical assay. A substrate is provided for the enzyme to be studied. An energy source is then made available, which allows the enzyme to utilize the substrate. Finally, a reaction product is linked to another product to form an insoluble precipitate that can be visualized microscopically.

The histochemical assay for succinate dehydrogenase (SDH) is used to distinguish between oxidative and less oxidative fibres. Fibres with a high oxidative capacity generate ATP via oxidative phosphorylation in the mitochondria. Therefore, muscle cells that contain more mitochondria will have a higher oxidative capacity. The SDH enzyme is located bound to cristae on the inner membrane of mitochondria, and is responsible for oxidizing succinate to fumarate in the citric acid cycle. As this reaction proceeds, succinate is oxidized, and the reduced form of NADH is produced. Therefore, in this

staining technique, succinate is the substrate, NADH is the reaction product (although a different electron acceptor is used for practical reasons) and SDH is the enzyme.

The electron acceptor is chemically reacted with nitro blue tetrazolium to visualize the reaction. This results in a speckled pattern, which corresponds to granules of diformazan, the reaction product of NBT (Kiernan, 1981), indicating the presence of mitochondria. The intensity of the pattern is proportional to the number of mitochondria and the SDH activity within them. Since all cells contain mitochondria, some degree of staining occurs in every cell in the section. The more SDH a fibre contains, the greater the intensity of the stain. Oxidative fibres have a relatively dense, purple-speckled appearance, whereas non-oxidative fibres have only a scattered paler stain. Therefore, this histochemical assay reflects the relative oxidative potential of muscle fibres.

2.2.5.2. Method

(i) Slide preparation

Slides were soaked overnight in cleaning acid to remove any impurities (100g of potassium bichromate in 850ml H_2O and 100ml 30% H_2SO_4). After immersion in the acid bath, slides were washed for several hours in cold running water, followed by 2 final rinses in ddH_2O for 30 mins each. Once slides were dry, they were immersed in a subbing solution (5g gelatine, 0.5g chrome alum in 1000ml H_2O) at room temperature. Slides were drained and dried in a dust-free environment. The subbing solution was filtered after use and stored at 2°C.

(ii) Histology

Muscles were removed from -80°C and allowed to slowly equilibrate to -20°C. Large cross-sectional pieces were cut with a surgical blade from the belly of each muscle at -20°C and mounted in Tissue-tek OCT. Once the OCT had hardened, serial 8μm cross-sections were cut on a cryostat microtome (Leica CM/1900, Leica Instruments, Nussloch, Germany). Serial sections were mounted on subbed glass slides, allowed to air-dry and then stored at -20°C until staining. During cutting, orientation of fibres was monitored by staining sections with cresyl violet.

(iii) Succinate dehydrogenase staining

Muscle sections were stained according to a technique described by Pearse, 1968 and Brooke & Kaiser, 1970. Air-dried sections were incubated in a working solution (final concentration; 50mM phosphate buffer, 50mM sodium succinate, 0.5% NBT) for approximately 70 minutes at 37°C in a temperature-controlled water bath. Test runs determined that incubation for 70 mins was necessary to produce a stain intense enough to distinguish between the 3 fibre types being assessed. Slides were then washed for 2 minutes in saline and transferred into a fixative solution (10% formal saline) for 10 minutes. Finally, slides were immersed in 15% ethanol for 5 minutes, followed by mounting in glycerine jelly.

2.2.5.3. Analysis

On each slide, 3 sections per muscle were counted to obtain an average value for fibre distribution. 5 random fields were analysed on each stained section at 20× magnification, which resulted in approximately 250 fibres per section being typed. Fibre types were determined by a colour code where type IIa fibres stained darkly, type IIb fibres stained pale and type I fibres were intermediate (Schmalbruch & Kamieniecka, 1975). Colour images demonstrating the staining intensity developed by type I, IIa and IIb fibre types in EDL, SOL and PL muscles can be seen in Appendix viii. The fibre cross-sectional area of each fibre type in a number of age groups was determined by a trace outline of individual muscle fibres, following which the cross-sectional area was calculated and expressed as μm^2. Fibre diameters were also measured and expressed as μm.

2.3. Statistical analysis

A number of statistical tests were used throughout the course of the various studies, depending on the groups of animals, experimental tests performed and parameters analysed. The statistical tests will be outlined for each study in the following chapters.

3.1.1. Aging

Aging is associated with a reduction in skeletal muscle mass, a decrease in isometric strength and an overall slowing of muscle contractions (Section 1.8, Lynch, 2002). Many reasons have been proposed for the reduction in skeletal muscle function with age (Section 1.8) including: alterations in the proteins involved in E-C coupling (RYR, DHPR and SR Ca^{2+}-ATPase pump) (Larsson & Salviati, 1989, Narayanan *et al*, 1996, Ferrington *et al*, 1997, 1998), changes in fibre type distribution (Kirkendall & Garrett, 1998, Pansarasa *et al*, 2002), reduced capacity/alterations in antioxidant enzyme and non-enzymatic activities (Ji, 1993, Rikans & Hornbrook, 1997) and increased ROS production (Leeuwenburgh *et al*, 1998, Richmonds *et al*, 1999, Nordberg & Arnér, 2001).

3.1.2. ROS and skeletal muscle

It has been established that when ROS production becomes excessive, as observed in exercise, fatigue and aging, cellular damage and/or structural modifications can occur, which have the potential to disrupt or modulate normal skeletal muscle function (Abramson & Salama, 1989, Sumien *et al*, 2003). The importance of basal levels of ROS in maintaining optimum skeletal muscle contractility has been investigated and confirmed, and addition of exogenously applied H_2O_2 can lead to an increase in submaximal force production in intact single muscle fibres and maximal force production in intact skeletal muscles (Section 1.7, Reid *et al*, 1992, 1993, Andrade *et al*, 1998, Zhang *et al*, 1999, Plant *et al*, 2001, Reid & Durham, 2002). However, the effect on P_O of a supramaximal dose of H_2O_2 *in vitro* to a muscle with elevated resting ROS levels is unknown.

3.1.3. Aims of the study

The main objective of this study is to assess the effect of an oxidant challenge (as simulated by application of a supramaximal dose of H_2O_2) on maximum isometric strength *in vitro* in young and aged EDL and SOL muscles.

It was hypothesized that aging would:

1. Alter skeletal muscle function, as measured by changes in isometric twitch parameters and reductions in tetanic force.
2. Accelerate force reductions in the presence of an oxidant challenge due to the additive effect of elevated endogenous ROS levels compared with young muscle

3.2. *Materials and Methods*

3.2.1. Materials

Young (Y, 2 months, n = 13) and aged (A, 24 months, n = 10) male Wistar rats were used in this study.

3.2.2. Methods

3.2.2.1. Tissue dissection and preparation

Following animal sacrifice, EDL and SOL from both hind limbs were dissected free and the tendons of each muscle were tied with loops for attachment to a force transducer (Section 2.2.1.2). Following hind limb dissection, the heart was removed and its mass was recorded.

3.2.2.2. Experimental protocol

The SOL and EDL muscles from each animal were split randomly into 2 groups; control (C) and treatment with H_2O_2 (O). Muscles were stimulated maximally to produce P_O every 5 minutes for 30 minutes, in the presence (YO & AO) or absence (YC & AC) of a supramaximal dose of H_2O_2 (160mM, Section 2.2.2.4). H_2O_2 was added to YO & AO following the initial P_O at 0 mins.

3.2.2.3. Tissue storage

Following the cessation of the experimental protocol, all muscles were weighed to allow estimation of cross-sectional area. Muscles were snap-frozen in isopentane cooled in liquid nitrogen, and stored at -80ºC (Section 2.2.3).

3.2.3. Statistical analysis

3.2.3.1 Changes in mass

An unpaired Student's t-test was used to compare differences in mass between young and aged muscle, heart and body mass. Changes in skeletal muscle and heart mass (as a % of

body mass) were analysed with a 2-way ANOVA to assess if a tissue-specific change in mass occurred with age. Significance was set at $P < 0.05$. Where significance was reported, *post-hoc* analysis with a Student-Newman-Keuls test was performed.

3.2.3.2. Isometric characteristics

A 2-way ANOVA assessed the effects of age and muscle type on isometric contractile characteristics (P_O, P_t, P_t/P_O, TTP, ½RT, and contraction and relaxation velocities). Absolute changes in force between young and aged muscle was also analyzed with a 2-way ANOVA. *Post-hoc* analysis with a Student-Newman-Keuls test was performed where significance was recorded.

A 3-way ANOVA was used to analyse the effects of H_2O_2 on P_O over time in young and aged skeletal muscle. A Tukey LSD *post-hoc* test was performed where significance was reported. Significance was set at $P < 0.05$.

3.3. Results

Isometric characteristics	EDL		SOL	
	Young	*Aged*	*Young*	*Aged*
P_t ($kN.m^{-2}$)	38.80 ± 1.08^{c}	39.10 ± 1.13^{b}	33.91 ± 1.18	$27.22 \pm 1.41^{***}$
P_O ($kN.m^{-2}$)	165.94 ± 3.66	$152.67 \pm 3.75^{*d}$	158.63 ± 3.65	$137.43 \pm 4.26^{***}$
P_t/P_O ratio	0.239 ± 0.005^{a}	$0.261 \pm 0.005^{**b}$	0.210 ± 0.006	0.203 ± 0.005
TTP (ms)	53 ± 3^{a}	59 ± 3^{b}	185 ± 14	202 ± 16
½RT (ms)	49 ± 2^{a}	55 ± 3^{b}	192 ± 23	$296 \pm 31^{***}$
P_t/TTP ($kN.m^{-2}.s^{-1}$)	800.88 ± 55.47^{a}	$691.91 \pm 33.44^{*b}$	222.39 ± 28.77	161.39 ± 22.31
$(½P_t)/$ ½RT ($kN.m^{-2}.s^{-1}$)	424.34 ± 28.85^{a}	376.95 ± 21.01^{b}	109.83 ± 9.26	$54.49 \pm 5.19^{+}$

Table 3.3.2. Data representing differences in contractile characteristics between young (n = 13) and aged (n = 10) EDL and SOL. P_O data in young and aged groups is a combination of the force produced by control and treated muscles, prior to incubation with H_2O_2.

* Different from young ($P < 0.05$)
** Different from young ($P < 0.01$)
*** Different from young ($P < 0.001$)
\+ Different from young ($P = 0.055$)

a Different from young SOL ($P < 0.001$)
b Different from aged SOL ($P < 0.001$)
c Different from young SOL ($P < 0.01$)
d Different from aged SOL ($P < 0.05$)

P_t – isometric twitch tension
P_O – isometric tetanic tension
P_t/P_O – twitch/tension ratio

TTP – time to peak twitch tension
½RT – one-half relaxation time

P_t/TTP – contraction velocity
$(½P_t)/$ ½RT – relaxation velocity

All results are expressed as mean ± SEM, unless indicated otherwise. Contractile function data (Section 3.3.3) was normalised to a percentage of the initial P_O. Specific forces ($kN.m^{-2}$) for each age and muscle group can be seen in Appendix ix.

3.3.1. Morphometric data

Morphometric data are shown below (Tables 3.3.1). Body, heart and muscle mass of aged rats (n = 10) were significantly heavier than young rats (n = 13) (Table 3.3.1, $P < 0.001$), however this is expected with growth.

	Young *(2 months)*	Aged *(24 months)*
Body (g)	208.92 ± 8.36	534.40 ± 14.57*
Heart (g)	0.702 ± 0.025	1.311 ± 0.066*
EDL (mg)	92 ± 0.47	240 ± 0.63*
SOL (mg)	65 ± 0.39	201 ± 0.79*

Table 3.3.1. Changes in body, heart and muscle mass between young (n = 13) and aged (n = 10) rats.

* Different from young ($P < 0.001$)

3.3.2. Isometric contractile parameters

Isometric contractile data for the EDL and SOL are shown in Table 3.3.2. A 20% reduction in P_t was observed in aged SOL compared to young SOL ($P < 0.001$), while ½RT was ~100ms longer in aged SOL ($P < 0.001$). Together these values represent a more prolonged twitch profile in aged SOL compared with young SOL. Although the relaxation velocity in aged SOL appears slower than in young SOL, this did not reach statistical significance ($P = 0.055$). These changes in P_t and ½RT were not observed for the EDL. However, a 12% reduction in contraction velocity (P_t/TTP, $P < 0.05$) and a larger P_t/P_O ratio was measured in aged EDL compared to young EDL. P_O was significantly lower in aged EDL ($P < 0.05$) and aged SOL ($P < 0.001$) compared to corresponding young groups. These results show that aging has had an effect on skeletal muscle function, independent of muscle type, which could reflect changes in fibre type distribution in favour of type I and/or IIa fibres.

Normal inter-group differences between EDL and SOL were observed in the various parameters outlined in Table 3.3.3 (Asmussen *et al*, 2003). Analysis showed that specific force during twitch contractions is larger in young EDL compared with young SOL ($P < 0.001$), whereas no difference was reported between tetanic contractions. Similarly TTP, ½RT and contraction and relaxation velocities were all faster in EDL when compared with SOL ($P < 0.01$). These differences were maintained with age except for P_O, which was larger in aged EDL compared to aged SOL ($P < 0.05$).

3.3.3. Incubation protocol

Group divisions for the contractile function protocol have been outlined previously (Section 3.2.2.6). To summarize, young (Y) and aged (A) muscles were further spilt into control muscles (YC & AC) and muscles exposed to an oxidant challenge, as simulated by exogenous application of 160mM H_2O_2 (YO & AO).

3.3.3.1. EDL force

Baseline P_O was 172.41 ± 4.89 $kN.m^{-2}$ and 160.04 ± 5.60 $kN.m^{-2}$ in YC and AC, respectively. Although P_O declined over time in both groups, P_O was significantly lower at all time points in AC when compared to YC ($P < 0.01$, Figure 3.3.1).

Prior to application of H_2O_2, P_O was 158.89 ± 5.08 $kN.m^{-2}$ and 144.49 ± 5.38 $kN.m^{-2}$ in YO and AO, respectively. Over time, force declined in both YO and AO ($P < 0.01$). However, this was time and age dependent such that the decrease in P_O was significantly greater in AO compared to YO at all time points except 30 mins ($P < 0.01$). No potentiation of force was observed in either YO or AO at a supramaximal dose of H_2O_2.

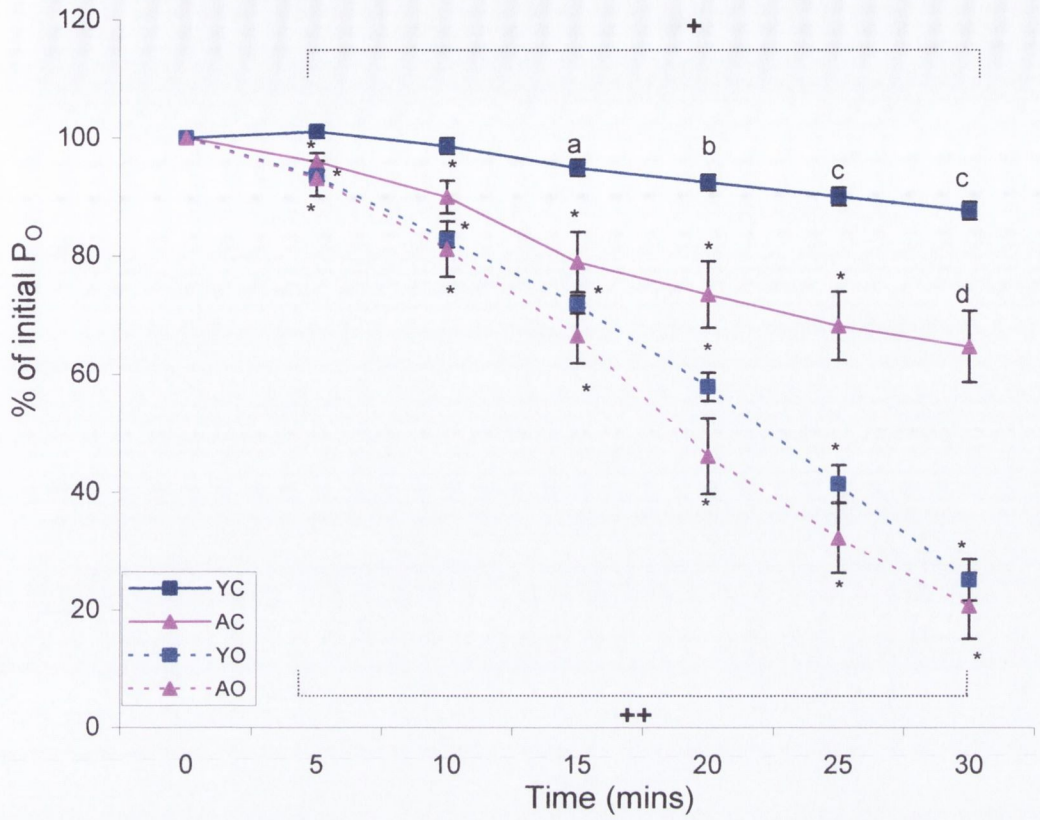


Figure 3.3.1. Force production normalized to initial P_O in Young (n = 13) and Aged (n = 10) EDL during 30 mins incubation with (YO & AO) or without (YC & AC) H_2O_2.

+ YC & YO different from AC & AO ($P < 0.01$), except at 5 and 30 mins between YO & AO

++ YO & AO different from YC & AC ($P < 0.01$), except at 5 mins for AC & AO

a Different from 0-5 mins ($P < 0.05$)
b Different from 0-10 mins ($P < 0.05$)
c Different from 0-15 mins ($P < 0.05$)
d Different from 0-20 mins ($P < 0.05$)

* Different from all preceding time points ($P < 0.05$)

It appears from the mean data presented in Figure 3.3.1 that P_O in young EDL muscles declined more over time in the presence of a supramaximal dose of H_2O_2 than aged EDL. However, since P_O declined differentially in YC and AC over time. Therefore, in order to obtain a more accurate measure of the effect of H_2O_2 on P_O the absolute magnitude of the effect of H_2O_2 on force production ($kN.m^{-2}$) in the EDL was assessed relative to untreated values in young and aged muscle at each time point. Each control muscle was matched to the treated values produced by the opposite hind limb muscle. No significant difference in the absolute difference between control and treated muscle was observed between young and aged EDL muscles at any time point (Figure 3.3.2).

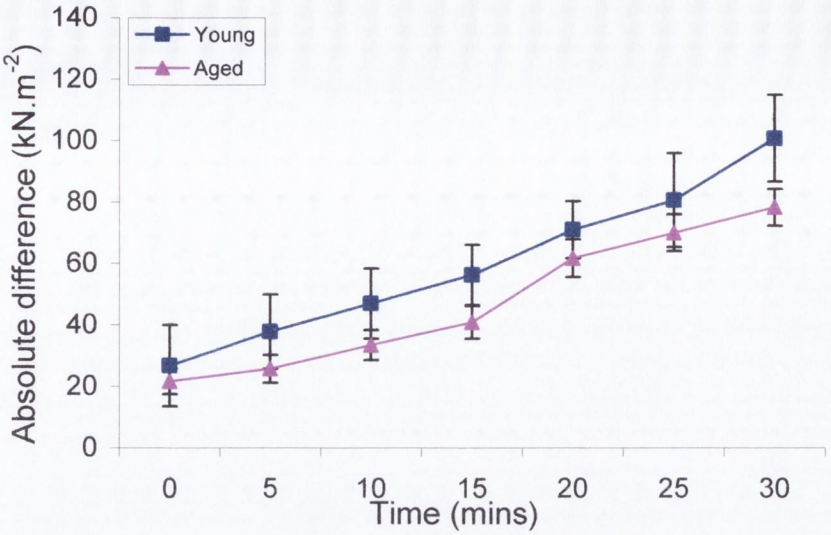


Figure 3.3.2. Absolute difference ($kN.m^{-2}$) between control and treated EDL in young (n = 13) and aged (n = 10) groups over time. No significant difference was observed at any time point between young and aged muscles.

When the absolute difference between control and H_2O_2-treated muscles was expressed as a percentage decrease from control values, no significance was measured between young and aged EDL muscles (Figure 3.3.3).

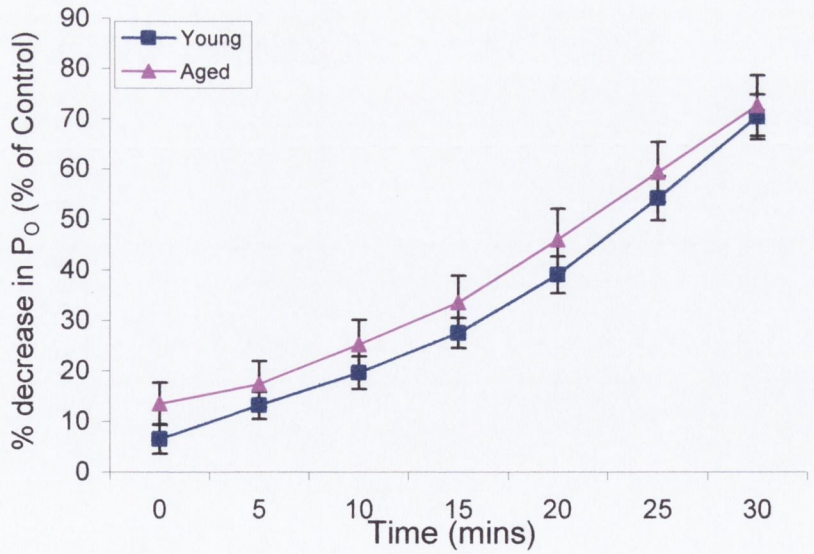


Figure 3.3.3. Percentage decrease in P_O (as a % of control values) in young (n = 13) and aged (n = 10) EDL over time. No significant differences were measured at any time point between age groups.

Therefore, results indicate that young and aged EDL groups experienced similar decreases in force over time in response to incubation with a supramaximal dose of H_2O_2. These results could indicate that, despite higher resting levels of ROS, aged muscles appear to adapted enhanced antioxidant defences which appears to have reduced the impact of H_2O_2 on various proteins involved in force production. Due to the supramaximal amount of H_2O_2 added to the bath, differences between age groups could be more apparent at lower H_2O_2 concentrations.

3.3.3.2. SOL force

Baseline P_O was 164.89 ± 4.65 $kN.m^{-2}$ and 141.73 ± 8.59 $kN.m^{-2}$ in YC and AC, respectively. P_O increased in YC at 5 mins and was maintained for the duration of the incubation protocol ($P < 0.05$, Figure 3.3.4), whereas P_O diminished in AC over time and was significantly lower than YC at all time points ($P < 0.05$).

Prior to application of H_2O_2, P_O was 152.27 ± 5.59 $kN.m^{-2}$ and 132.59 ± 4.81 $kN.m^{-2}$ in YO and AO, respectively. Over time, P_O decreased in both YO and AO ($P < 0.05$). This was only time dependent, however P_O was greater in AO than AC and YO at 10 mins ($P < 0.05$).

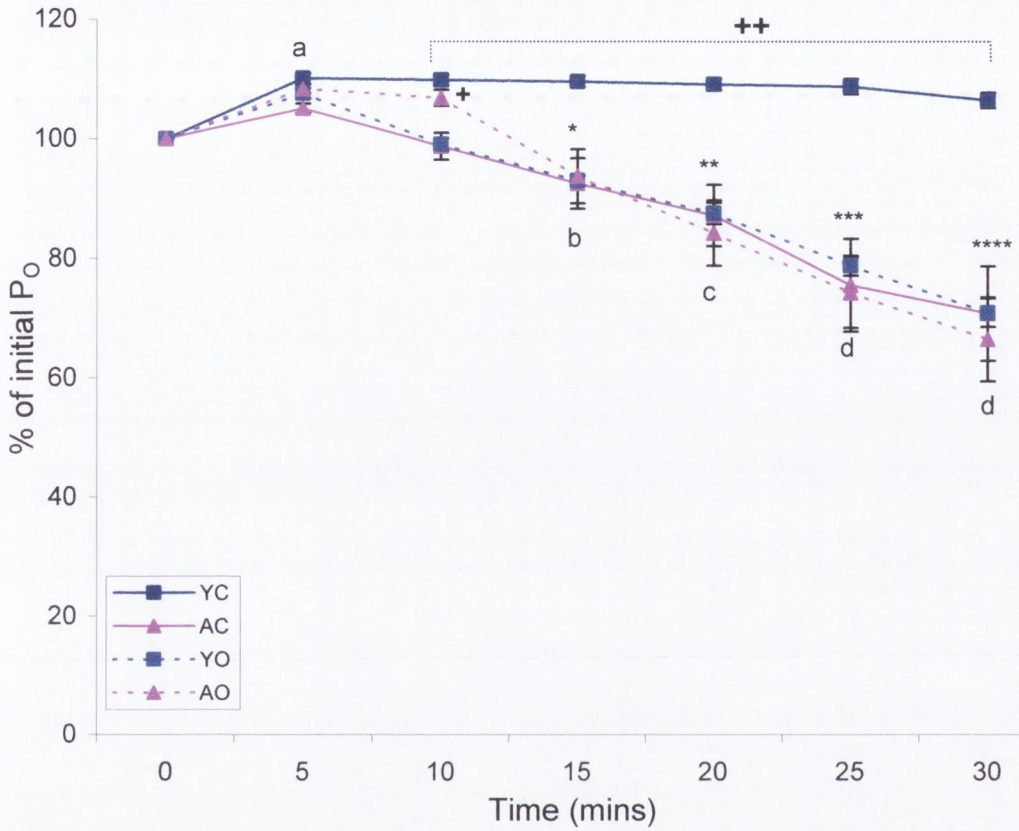


Figure 3.3.4. Force production normalized to initial P_O in young (n = 13) and aged (n = 10) SOL during 30 mins incubation with (YO & AO) or without (YC & AC) H_2O_2.

+ AO different from AC & YO ($P < 0.05$)
++ YC different from YO, AC ($P < 0.001$) and 0 mins ($P < 0.05$)

* YO different from 5 mins ($P < 0.05$)
** YO different from 0-10 mins ($P < 0.05$)
*** YO different from 0-15 mins ($P < 0.05$)
**** YO different from all preceding time points ($P < 0.05$)

a YC different from 0 mins ($P < 0.05$)
b AC different from 0-5 mins; AO different from 5-10 mins ($P < 0.05$)
c AC and AO different from 0-15 mins ($P < 0.05$)
d AC and AO different from 0-20 mins ($P < 0.05$)

Again, it appears that young muscles were more affected by the oxidative treatment than aged muscles due to the larger difference between mean values in control and H_2O_2-treated muscles. However, since P_O was reduced to different extents in YC and AC over time (Figure 3.3.4), the absolute magnitude of the effect of H_2O_2 on force production in the SOL was assessed relative to untreated values in young and aged muscle at each time point in order to obtain an accurate measure of the effect of H_2O_2 on P_O. Each control

muscle was matched to the treated values produced the opposite hind limb muscle. Significant differences were observed between young and aged muscles at 20, 25 and 30 mins, where force had decreased by a significantly larger amount in young compared to aged muscle due to the oxidative stress simulated by exogenous application of H_2O_2 (Figure 3.3.5).

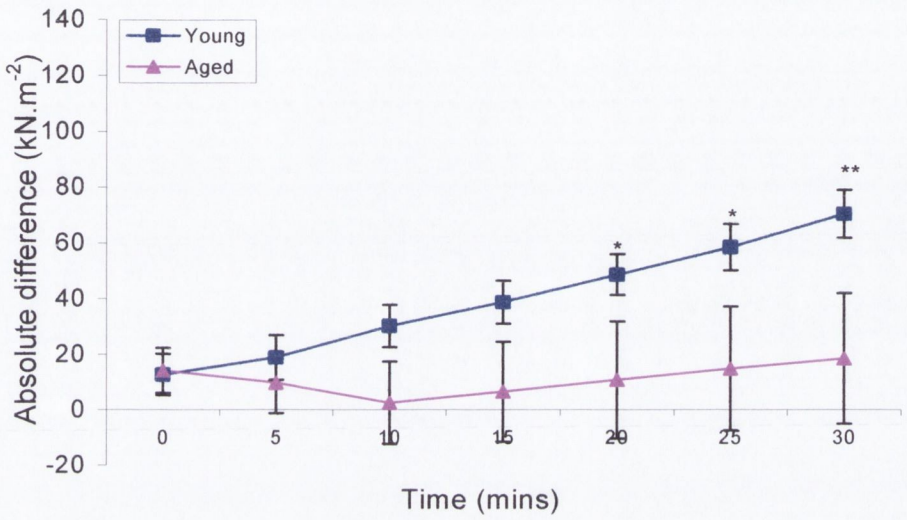


Figure 3.3.5. Absolute difference ($kN.m^{-2}$) between control and treated SOL in young (n = 13) and aged (n = 10) groups over time. * Different from young ($P < 0.05$). ** Different from young ($P < 0.01$).

When the absolute difference between control and treated muscles was expressed as a percentage decrease from control values, a significant difference was observed between young and aged SOL, clearly demonstrating that force had decreased to a greater extent in young SOL compared with aged SOL (Figure 3.3.6).

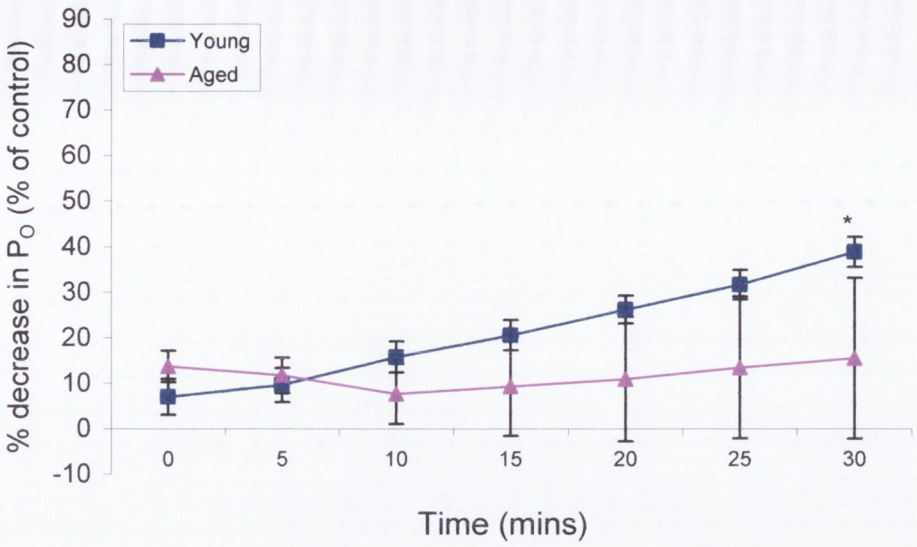


Figure 3.3.6. Decrease in P_O (as a % of control values) between young (n = 13) and aged (n = 10) SOL groups over time. * Different from young ($P < 0.05$).

These results demonstrate that aged SOL resists oxidative stress-induced reductions in force more effectively than young muscles, which could reflect differences in antioxidant defences between age groups. However, again due to the supramaximal amount of H_2O_2 added to the bath, differences between age groups could become more apparent at lower H_2O_2 concentrations.

3.4. Discussion

3.4.1. Aims of the study

The main objective of this study was to assess the effect of an oxidant challenge (as simulated by application of a supramaximal dose of H_2O_2) on maximum isometric strength *in vitro* in young and aged EDL and SOL muscles. This set of experiments were novel in that the effect of a high level of oxidation on skeletal muscle function was compared in both young and aged whole skeletal muscle, which to the author's knowledge had not been previously performed.

There were 2 main hypotheses for this study. It was hypothesized that aging would:

1. Alter skeletal muscle function, as measured by changes in isometric twitch parameters and reductions in tetanic force.
2. Cause force to decline more rapidly in the presence of an oxidant challenge due to the additive effect of endogenous ROS levels

The major findings of the present study confirm that aging is associated with:

1. An increase in twitch contraction duration of skeletal muscle, independent of muscle type (i.e. in both EDL and SOL).
2. A reduction in twitch force (P_t) in the SOL, but not EDL.
3. A reduction in specific maximal muscle force (P_O) independent of muscle type.
4. A greater reduction in P_O over time with age independent of muscle type.
5. With H_2O_2 incubation, aged muscle showed a dramatically different profile of P_O over time such that:
    i. The reduction in P_O was more dramatic in EDL compared to SOL, independent of age
    ii. A more prominent reduction in P_O was observed in young SOL compared to aged SOL.
    iii. Decreases in P_O were similar in EDL independent of age.

3.4.2. Isometric contractile characteristics

3.4.2.1. Twitch characteristics

A reduction in the speed of contraction in aged skeletal muscle has previously been shown to precede the onset of muscle wasting (Fitts *et al*, 1984, Larsson & Edström, 1986, Narayanan *et al*, 1996, Margreth *et al*, 1999). In the present study, isometric twitch durations were prolonged in aged EDL (evidenced by a reduced contraction velocity) and in aged SOL (larger ½RT) compared to young muscles (Table 3.3.2). Similar twitch profiles in EDL and SOL muscles have been recorded previously with age (Brooks & Faulkner, 1988, Narayanan *et al*, 1996, Renganathan *et al*, 1997). Therefore, despite the absence of muscle atrophy in either muscle group in the present study, changes in twitch profiles show that aged EDL and SOL had undergone some level of modification with age and if left to age further may have shown muscle atrophy.

A number of physiological processes have been implicated as causative factors for alterations in twitch profiles. An increase in connective tissue content has been observed with age, which can act as internal drag to all muscle fibres (Thompson *et al*, 1998). Also, *in vitro* motility assays have demonstrated that myosin molecules extracted from aged soleus muscle fibres have a decreased ability to move actin relative to younger rats (Höök *et al*, 1999, Höök *et al*, 2001). Alterations in the proteins involved in EC coupling could also be contributing to the changes in muscle contractility that were observed in the present study (Section 1.8.3, Delbono *et al*, 1995, Vogt, 1995, Diaz *et al*, 1998, Margreth *et al*, 1999, Winterbourn & Metodiewa, 1999). Age-associated reductions in the Ca^{2+}-sequestering activity of SR, combined with a significantly prolonged twitch duration; have been reported in aged EDL and SOL muscles (Larsson & Salviati, 1989, Narayanan *et al*, 1996). An age-related deterioration of SR Ca^{2+}-ATPase pump function contributes to the slow rate of relaxation seen in SOL with age (Ferrington *et al*, 1997, 1998). Also, reduced RYR/DHPR coupling and/or altered RYR regulation adds to changes in twitch profiles with age (Delbono *et al*, 1995, Damiani *et al*, 1996).

Age-associated changes fibre type composition in skeletal muscle with age, in favour of type I and/or type IIa fibres, has been shown previously (Alnaqeeb & Goldspink, 1987, Kovanen, 1989, Danieli-Betto *et al*, 1995, Pansarasa *et al*, 1999, Richmonds *et al*, 1999) and, more importantly, have been implicated as one of the main causative factors of

altered twitch profiles in aged skeletal muscle (Sugiura *et al*, 1992, Thompson, 1994). Type I and IIa fibres act as an internal drag on type IIb fibres when their numbers are increased with age, resulting in a slower shortening velocity in whole muscle (Thompson & Brown, 1999, Höök *et al*, 2001). Changes in fibre type with age were not assessed in the present study. However, results from Chapter 4 show that age-related increases in type I and/or IIa fibre type distributions occurred in aged muscle, which implicates alterations in fibre distribution to the changes in twitch profile observed in the present study.

3.4.2.2. Force production

In the present study, P_O was reduced by ~10% in aged EDL and ~14% in aged SOL compared with young muscles, and P_t was 20% lower in aged SOL (Table 3.3.2). The reductions in P_O observed in the present study were not as dramatic as those previously reported (~22% in SOL and ~27% in EDL) (Brooks & Faulkner, 1988, Payne *et al*, 2003). However, the rats used in these studies were 26-28 months old, which suggests that the degree of aging also determines the extent to which P_O is reduced with age. Muscle atrophy was thought to be the primary cause of strength loss in aged skeletal muscle because muscle wasting corresponded to decreases in muscle strength (Narici *et al*, 1991, Porter *et al*, 1995). However, when muscle atrophy was normalized, specific force production was still lower in aged muscles (Larsson & Edström, 1986, Brooks & Faulkner, 1988, Thompson & Brown, 1999), which agrees with the results produced in the present study.

Alterations in EC coupling, and hence $[Ca^{2+}]_i$, have received strong support as one of the main mechanisms responsible for age-related reductions in force in skeletal muscle (Narayanan *et al*, 1996, Ferrington *et al*, 1997, Margreth *et al*, 1999). The function of a number of important proteins involved in EC coupling are extensively modified in aged skeletal muscle compared to young muscle, which has been connected to the increased ROS production in skeletal muscle with aging (Ferrington *et al*, 1998, Bejma & Ji, 1999, Meccoci *et al*, 1999, Richmonds *et al*, 1999, Fano *et al*, 2001, Oba *et al*, 2002). ROS-induced conformational changes to these proteins can lead to an absolute reduction in SR Ca^{2+} release in response to sarcolemmal depolarization (Renganathan *et al*, 1997, Courtois *et al*, 1998), which ultimately reduces the number of active binding sites on actin participating in the cross-bridge power stroke resulting in a reduced force output in aged

skeletal muscle. Another target for ROS-induced modulation is the reactive cysteine residue present on the troponin complex on the actin strand (Chong, 1982). If altered by ROS, the number of sites available to participate in cross-bridge activity could be affected, leading to a reduction in force. ROS-induced oxidative modification of SH1, a highly reactive sulfhydryl group located in the catalytic domain of the myosin head (Ostap *et al*, 1995), also reduces muscle strength and decreases the shortening velocity of muscle contraction (Crowder & Cooke, 1984, Perkins *et al*, 1997). Since ROS levels were not assessed in the present study, it is not possible to say whether extensive ROS-induced modifications to various proteins in aged skeletal muscle had taken place. However, increased ROS production in aged skeletal muscle has been consistently shown in previous studies (Rikans & Hornbrook, 1997, Bejma & Ji, 1999, McArdle *et al*, 2002), therefore it is within reason to make these assumptions.

Reductions in the myofibrillar protein content per unit area of skeletal muscle, with an increase in intermyofibrillar spaces, connective tissue, fat and lipofuscin molecules have been reported in aged skeletal muscle (Borkan *et al*, 1983, Alnaqeeb *et al*, 1984, Ansved & Edstrom, 1990). These findings demonstrate that since myofibrillar content is reduced in aged skeletal muscle, the number of sites participating in the cross-bridge power stroke is less than that in young muscle, and could contribute the reduction in P_O measured in the present study. Indeed, a reduction in the number of active sites participating in the power stroke during cross-bridge activity has been confirmed in permeabilized single skeletal muscle fibres from aged animals (Eddinger *et al*, 1986, Brooks & Faulkner, 1994, Thompson & Brown, 1999, Frontera *et al*, 2000, Trappe *et al*, 2003). Since permeabilized fibres do not have intact membranes, any reductions in force directly reflect alterations in the interaction of myosin and actin filaments independent of EC coupling, changes in fibre architecture and alterations in intercellular connective tissue (Stephenson & Williams, 1981, Wilson *et al*, 1991, Frontera *et al*, 2000). Studies using high-resolution site-specific EPR spectroscopy have also confirmed that the number of force generating cross-bridges per unit area during muscle contraction is reduced with age by a fraction that is comparable to the decrease in specific tension (Lowe *et al*, 2001, 2004).

However, no difference in the force produced by fast or slow permeabilized muscle fibres, or in the number of interacting sites on filaments has also been reported in aged skeletal muscle (Larsson *et al*, 1997, Reganathan *et al*, 1997, Plant & Lynch, 2001). This

adds difficulty to the interpretation of the literature and analysis of reasons for the results produced in the present study. It is likely the difference in data presented above lies with variations in experimental conditions and in the techniques used to prepare and measure force output in single fibres (Plant & Lynch, 2001). Therefore, changes in the structure of myosin during contraction, and in the number of sites involved in cross-bridge activity, may provide a molecular explanation for the reduction in muscle specific tension observed in aged EDL and SOL in the present study.

Type IIb muscle fibres generate the highest forces of all fibre types followed by type IIa and type I fibres (Fitts & Widrick, 1996, Bottinelli *et al*, 1999). Consequently, reductions in type IIa fibre distribution in aged SOL could account for the reductions in P_t and P_O measured with age in SOL and EDL (Table 3.3.2). Although fibre typing was not performed in this study, results from Chapter 4 demonstrate clearly that increases in the distribution of type I and/or IIa fibres occurred with age in EDL, SOL and PL muscles. Therefore, it is reasonable to assume that this morphological change may be a contributing factor to the reduction in force in aged skeletal muscle.

3.4.3. Incubation protocol

In the present study, a ~10% decay in P_O over time (30 mins) was measured under control conditions *in vitro* in young EDL, but not young SOL (Figure 3.3.1 & 3.3.2). This has been shown previously (Diaz *et al*, 1998, Plant *et al*, 2001). This decline in P_O over time was more dramatic in aged muscle, such that force was reduced in both EDL and SOL by ~30% with 30 mins incubation. These results clearly demonstrate that the ability to maintain P_O in aged muscle is reduced compared to young muscle. The results of the present study also show that P_O *in vitro* is modulated by H_2O_2 and the response produced is dependent on muscle type, such that P_O in EDL is more affected by an oxidant challenge than SOL (Figure 3.3.1 & 3.3.4), which is consistent with previous studies (Reid *et al*, 1993, Oba *et al*, 1996, Murrant *et al*, 1999, Plant *et al*, 2001). More importantly, an age-specific response to incubation with H_2O_2 was observed, such that reductions in P_O over time were more prominent in young SOL when compared to aged SOL (Figure 3.3.4, 3.3.5 & 3.3.6), whereas the decrease in P_O was similar between young and aged EDL (Figure 3.3.1, 3.3.2 & 3.3.3).

A limitation to the interpretation of the effects produced with H_2O_2 with age is the fact that a supramaximal dose was applied *in vitro* which may have caused similar reductions in force no matter what age or muscle type. Previous studies have used lower doses (1-5mM H_2O_2) and measured potentiation in P_O (Andrade *et al*, 1998, Plant *et al*, 2001, Plant *et al*, 2003). However, in this study, oxidation occurred more rapidly, thus bypassing any potentiation in P_O before it could be measured. However, despite this, aged muscle still dealt with the degree of oxidative stress simulated similarly and even better than young muscle, which may reflect changes in antioxidant status.

Redox control of $[Ca^{2+}]_i$ by modification of critical SR proteins and/or redox modulation of proteins involved in cross-bridge cycling have been proposed as potential mechanisms underlying the effects of exogenously applied oxidants on skeletal muscle function (Section 1.8.3, Reid *et al*, 1993, Favero *et al*, 1995, Brotto & Nosek, 1996, Andrade *et al*, 1998, Kawakami & Okabe, 1998, Kourie, 1999, Zhang *et al*, 1999). ROS-induced modulation of these proteins can lead to alterations in $[Ca^{2+}]_i$ and affect the function of the cross-bridge power stroke (Section 1.7), leading to alterations in P_O. Previous studies have suggested that these proteins have already been oxidized to some extent in aged skeletal muscle due to the age-associated elevation in ROS production (Section 1.8.3, Delbono *et al*, 1995, Narayanan *et al*, 1996, Renganathan *et al*, 1997, Ferrington *et al*, 1998, Bejma & Ji, 1999).

It has been proposed that force production is a function of cellular redox balance and that the point of baseline redox balance is slightly reduced in unfatigued muscle (Section 1.7, Reid, 1996, Andrade *et al*, 1998, Plant *et al*, 2001, Reid, 2001). Under basal conditions, unfatigued muscle maintains a lower oxidant level than is necessary for optimal contraction, with antioxidants further reducing the CRB through ROS removal (Figure 1.7, Reid *et al*, 1993, Reid, 2001). The activities of SOD, CAT, GPx and GSH in skeletal muscle are higher in slow-twitch than fast-twitch muscle (Jenkins & Tengi, 1981, Jenkins, 1988, Laughlin *et al*, 1990, Ji *et al*, 1992, Lawler *et al*, 1993, Powers *et al*, 1994, Oh-ishi *et al*, 1995, Lawler & Powers, 1998, Pansarasa *et al*, 1999, Hollander *et al*, 2000), therefore, the CRB in slow-twitch muscle is more reduced than fast-twitch muscle, due to higher antioxidant activity (Figure 1.7), and application of an oxidant will affect P_O in fast-twitch more rapidly than slow-twitch muscle due to their respective redox states. The

results of the present study agree with this theory, as P_O in EDL was reduced more rapidly and dramatically in the presence of an oxidant challenge than SOL (Figure 3.3.1 & 3.3.4).

It has been suggested that increased ROS levels, in conjunction with an altered antioxidant defence system, place the CRB in aged skeletal muscle in an oxidized state (Figure 1.8, Andrade *et al*, 1998, Bejma & Ji, 1999, Plant *et al*, 2001, Reid, 2001, Reid & Durham, 2001, Lamb & Posterino, 2003). Where H_2O_2 incubation (1-5mM) in young muscle can potentiate P_O due to oxidation of the CRB into a more ideal redox state for production of P_O (Figure 1.7, Plant *et al*, 2001), a similar response should not be seen in aged muscle due to its starting point on the curve. In fact, H_2O_2 application *in vitro* in aged skeletal muscle should shift the CRB into an even more oxidized state ahead of young muscles, leading to more rapid reductions in force over time (Figure 1.8, section 1.7). However, such a model is inconsistent with the results obtained in the present study because force in aged muscle, independent of muscle type, was not reduced more rapidly than young muscle. Although antioxidants were not assessed in the present study, results from a subsequent study demonstrate that antioxidant defences are larger in aged SOL muscles compared with younger muscles. Therefore, although ROS production is increased in aged skeletal muscle (Bejma & Ji, 1999), an adaptation to this stress occurred in the form of enhanced antioxidant defences. Therefore, the CRB could have been similar in young and aged SOL, such that the effect of an oxidant challenge on P_O produced the same response in these muscles.

3.4.4. Conclusions

Despite the lack of muscle atrophy, this study confirms previous findings where reductions in P_O and altered isometric contractile characteristics occur in aged skeletal muscle with age. Incubation with H_2O_2 also showed that aged muscle was able to deal with an oxidative stress as effectively as young muscle, despite having higher resting levels of ROS (not assessed in this study). These results could reflect a combination of changes in fibre type distribution and antioxidant status.

Chapter 4.

The effects of aging on skeletal muscle structure and antioxidant status

4.1. Introduction

4.1.1. Effect of age on skeletal muscle structure

Skeletal muscle is a very heterogeneous tissue due to the differential distribution of fibre types (Table 1.2, Brooke & Kaiser, 1970, Hämäläinen & Pette, 1993, Hilber *et al*, 1999, Bottinelli & Reggiani, 2000, Pansarasa *et al*, 2002). Since type I and IIa fibre types have higher SOD, GPx, CAT and GSH than type IIb fibres (Laughlin *et al*, 1990, Ji *et al*, 1992, Powers *et al*, 1994, Oh-ishi *et al*, 1995, Pansarasa *et al*, 1999, Danieli-Betto *et al*, 2000, Hollander *et al*, 2000) and type IIb fibre loss and atrophy, with a concomitant increase in type I and IIa fibre distribution, is a hallmark of muscle aging (Pansarasa *et al*, 2002), age-associated changes in fibre type distribution could have a direct effect on antioxidant defences with age.

4.1.2. Antioxidant defences

ROS production stimulates antioxidant defences to reduce/prevent any ROS-induced damage/modification while simultaneously enhancing ROS scavenging (Reid *et al*, 1993, Heunks & Dekhuijzen, 2000, Miquel, 2002). Therefore, the assessment of the antioxidant defence system can be used to evaluate oxidative stress in human and animal models. Aging has been associated with increases (Lammi-Keefe *et al*, 1984, Ji *et al*, 1990, Hammeren *et al*, 1992, Leeuwenburgh *et al*, 1994, Luhtala *et al*, 1994, Ji *et al*, 1998, Bejma & Ji, 1999), decreases (Patnaik, 1968, Jayachandran *et al*, 1996, Oh-Ishi *et al*, 1995, Pansarasa *et al*, 1999, Hollander *et al*, 2000, Van der Loo *et al*, 2003) and no change (Vertechy *et al*, 1989, Lawler *et al*, 1993, Leeuwenburgh *et al*, 1994, Jayachandran *et al*, 1996, Pansarasa *et al*, 1999, Kasapoglu & Özben, 2001) in various components of the antioxidant defence system. Changes in antioxidant status are also dependent on muscle type (Oh-ishi *et al*, 1995) and the degree of aging that has occurred (Lawler & Demaree, 2001) (Section 1.8).

4.1.3. Aims of the study

The main objective of the present study is to assess the effects of age on antioxidant status in a range of hind limb skeletal muscle. Since specific antioxidant levels in skeletal muscle depend on sex, species, fibre type distribution, age and oxygen demands

(Laughlin *et al*, 1990, Ji *et al*, 1992, Pansarasa *et al*, 1999, Liu *et al*, 2000, Pansarasa *et al*, 2000, Masuda *et al*, 2003), it was hypothesized:

1. Muscles composed largely of type I fibres would have higher antioxidant levels than muscles with a higher proportion of type II fibres.
2. Age would change fibre type distribution in favour of type I and/or IIa fibres, which could have a direct effect on antioxidant status

4.2. *Materials and Methods*

4.2.1. Materials

Male Wistar rats aged 2 months (n = 7), 10 months (n = 8), and 22 months (n = 8) were used in this study. Due to the size of EDL and SOL muscles in 2-month-old rats, there was not enough protein-equalized homogenate to complete all antioxidant assays or fibre type analysis. Therefore, another group was added (1-month-old (n = 6)) to complete GSH, GPx and vitamin C assays. Stored EDL and SOL muscle from 2 and 24-month old male Wistar rats from Chapter 3 were also used to assess the effect of age on fibre type distributions and cross-sectional areas.

4.2.2. Methods

4.2.2.1. Skeletal muscle preparation

Following animal sacrifice, EDL, SOL, plantaris (PL) and gastrocnemius (GT) were dissected free (Section 2.2.1.2). GT was further divided into gastrocnemius white (GW) and gastrocnemius red (GR). Following complete dissection, muscles were snap-frozen and stored at -80°C. Prior to antioxidant assessment; muscles were homogenized in Krebs solution and equalized for protein (2mg/ml) using the Bradford protein assay (Section 2.2.4.2).

4.2.2.2. Antioxidant assays

A number of antioxidant enzymes (SOD, CAT & GPx) and antioxidant molecules (glutathione and vitamin C) were measured in this study. All assays have been described in detail previously (Section 2.2.4).

4.2.2.3. Fibre typing

EDL, SOL and PL muscle sections were incubated in various solutions to measure succinate dehydrogenase (SDH) activity (Section 2.2.5). Muscle fibres were classified as type I, IIa or IIb on the basis of SDH activity and expressed as a percentage of the total number of fibres counted in each section. Fibre cross-sectional areas and diameters were also measured in EDL and SOL muscle.

4.2.3. Statistical analysis

4.2.3.1. Antioxidant assays

Data were analyzed using a 2-way ANOVA to estimate the effect of 2 independent variables, aging and muscle type, on a dependent variable, antioxidant activity. *Post-hoc* analysis using Student-Newman-Keuls was performed where statistical significance was recorded. Significance was established as $P < 0.05$.

4.2.3.2. Changes in fibre characteristics

A 2-way ANOVA was used to test the effect of aging and muscle type on fibre type distribution in the EDL, SOL and PL and fibre cross-sectional area and diameter in the EDL and SOL. *Post-hoc* analysis using Student-Newman-Keuls was performed where statistical significant was recorded. Significance was established as $P < 0.05$.

4.3. Results

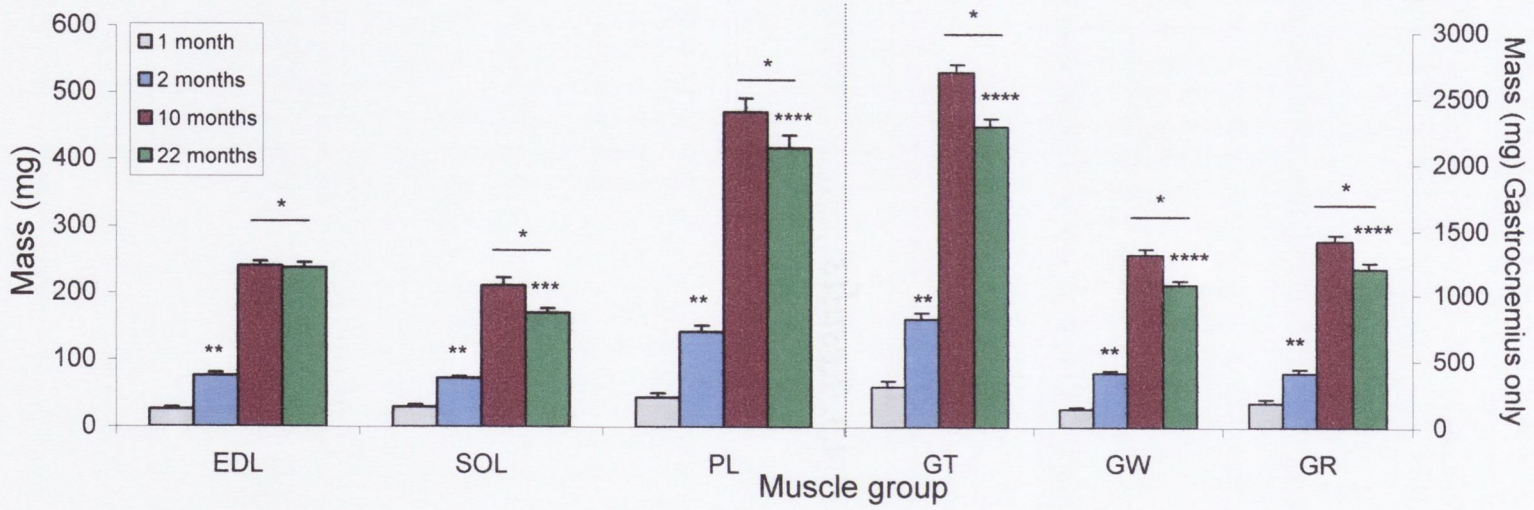


Figure 4.3.1. Change in muscle mass (mg) in the EDL, SOL, PL, GW, GR and GT at 1 (n = 6), 2 (n = 7), 10 (n = 8) and 22 (n = 8) months.

* Different from 1 and 2 months ($P < 0.001$)
** Different from 1 month ($P < 0.01$)
*** Different from 10 months ($P < 0.01$)
**** Different from 10 months ($P < 0.001$)

All results are expressed as mean ± SEM unless indicated otherwise.

4.3.1.Age-related muscle atrophy

4.3.1.1. Muscle mass

There was a main effect for muscle mass such that all muscles were significantly larger at 10 and 22 months compared to 1 and 2 months as is expected with growth ($P < 0.001$, Figure 4.3.1). No significant reductions in muscle mass were reported in EDL, SOL or PL between 10 and 22 months. However, gastrocnemius muscle mass (GR, GW & GT) was significantly lower at 22 months when compared to 10 months ($P < 0.001$) indicating a degree of age-related muscle atrophy.

As the mass of EDL, SOL and PL at each age is significantly smaller than that of the gastrocnemius, the magnitude of change between age groups is not as dramatic as that observed in the larger gastrocnemius. Therefore, the EDL, SOL and PL muscles were subsequently analysed without the gastrocnemius to minimize any impact of the larger muscle masking the changes observed in the smaller muscle. Similar reductions in muscle mass to that observed in the gastrocnemius were measured in the SOL ($P < 0.01$) and PL ($P < 0.001$) at 22 months compared to 10 months (Figure 4.3.1).

4.3.1.2. Fibre cross-sectional area and diameter

(i) EDL

Muscle fibre cross-sectional area (Figure 4.3.2) and diameter (Figure 4.3.3) was larger with age in all fibre types. No significant age-effect was observed in either parameter. The percentage increase in cross-sectional area from 2 months to 24 months was highest in type I and IIa (124% increase from 2 months) followed by type IIb (101%). Analysis of fibre diameter revealed that type I and IIa showed the largest change (50% each) followed by type IIb (43%).

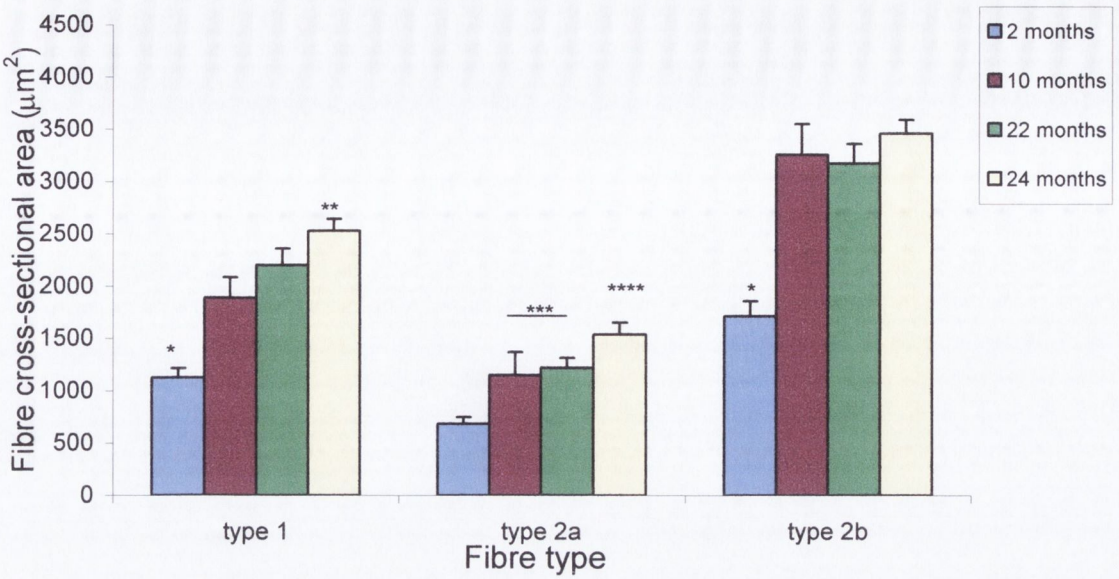


Figure 4.3.2. Fibre cross-sectional area (μm^2) in EDL at 2 (n = 13), 10 (n = 8), 22 (n = 8) and 24 (n = 10) months.

* Different from 10, 22 & 24 months ($P < 0.001$).
** Different from 10 months ($P < 0.01$).
*** Different from 2 months ($P < 0.05$).
**** Different from 2 months ($P < 0.001$).

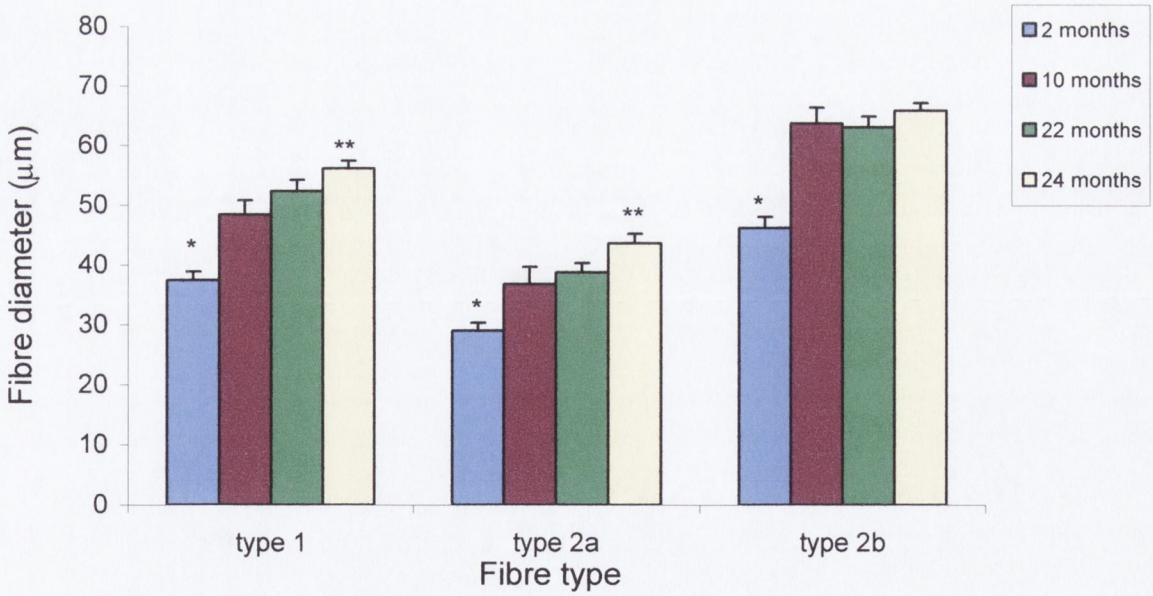


Figure 4.3.3. Fibre diameters (μm) in EDL at 2 (n = 13), 10 (n = 8), 22 (n = 8) and 24 (n = 10) months.

* Different from 10, 22 & 24 months ($P < 0.001$).
** Different from 10 months ($P < 0.05$).

Images showing changes in fibre size with age in the EDL can be seen in Figure 4.3.4.

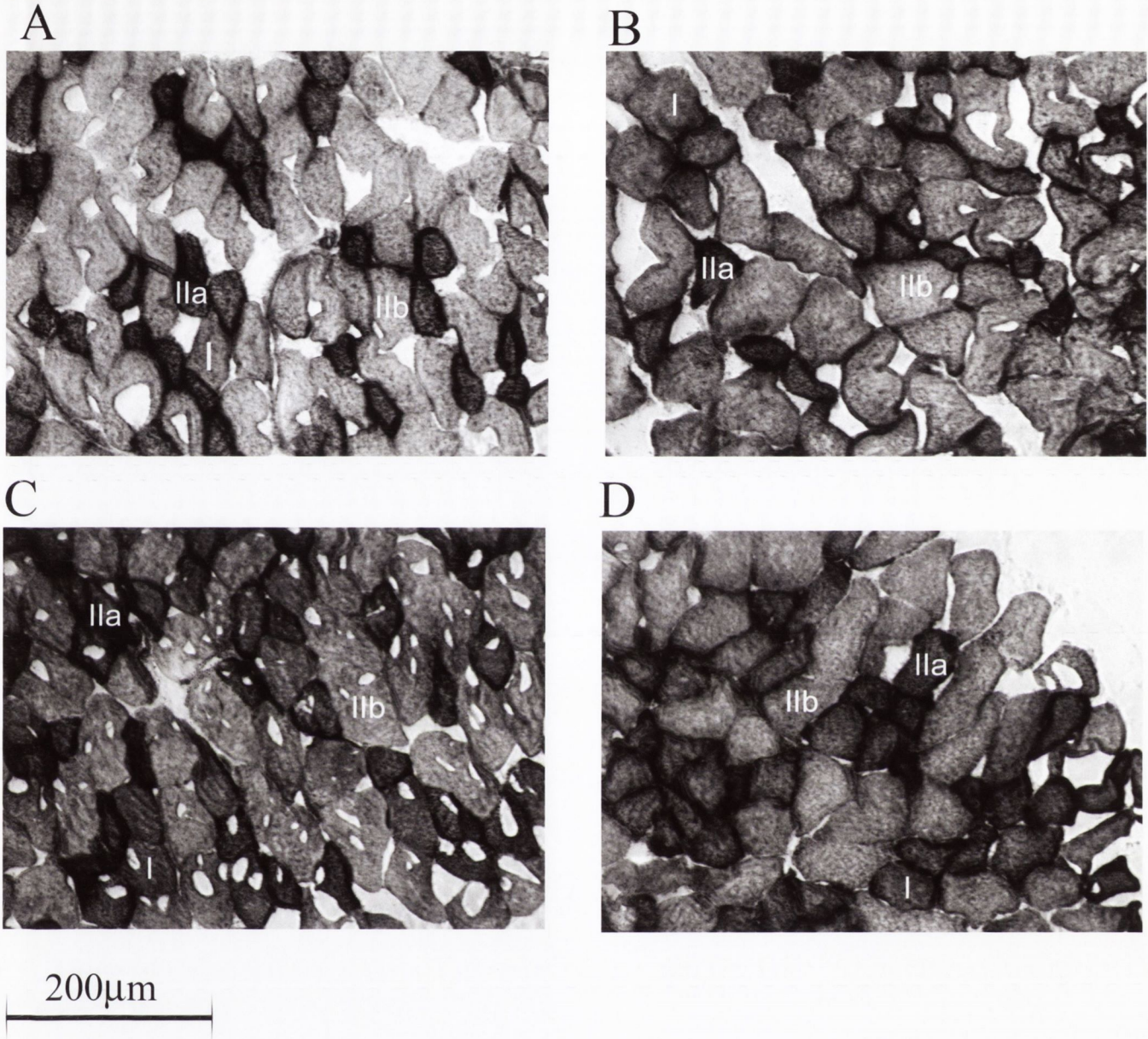


Figure 4.3.4. Change in fibre size in EDL at 2 (A), 10 (B), 22 (C) and 24 months (D) at 20X magnification.Fibre types are indicated at each age. Fibre size increased from 2 to 10 months and was maintained with age.

(ii) SOL

Muscle fibre cross-sectional area (Figure 4.3.5) and diameter (Figure 4.3.6) was larger with age in all fibre types. No significant reductions in fibre diameter or cross-sectional area were observed in any fibre type with age in SOL. The percentage increase in cross-sectional area from 2 months to 24 months was highest in type IIa (155% increase from 2 months) followed by type I (77%). Type IIa showed the largest increases (61% each) in fibre diameter, followed by type I (34%).

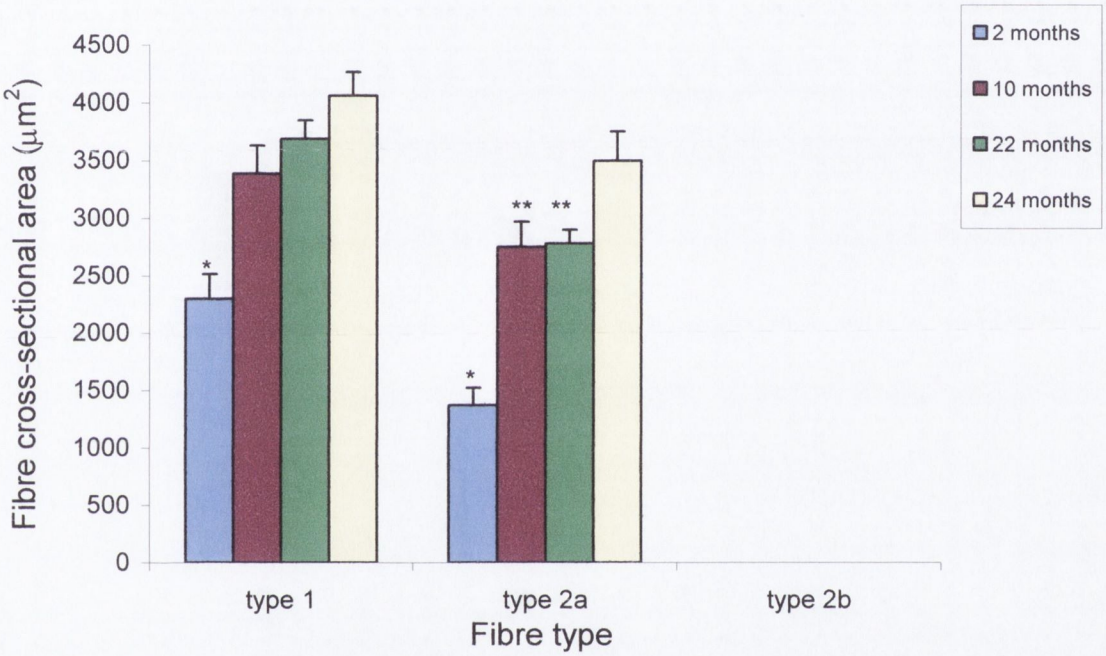


Figure 4.3.5. Fibre cross-sectional area (μm^2) in SOL at 2 (n = 13), 10 (n = 8), 22 (n = 8) and 24 (n = 10) months.

* Different from 10, 22 & 24 months ($P < 0.001$).
** Different from 24 months ($P < 0.05$).

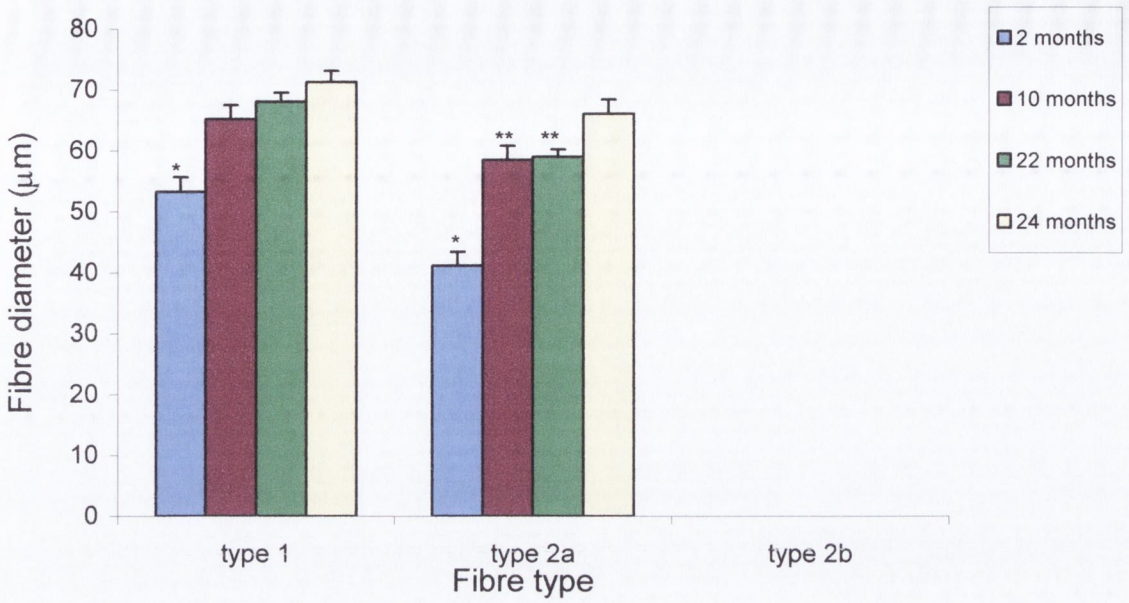


Figure 4.3.6. Fibre diameters (μm) in SOL at 2 (n = 13), 10 (n = 8), 22 (n = 8) and 24 (n = 10) months.

* Different from 10, 22 & 24 months ($P < 0.001$).
** Different from 24 months ($P < 0.001$).

Age-related increases in fibre size in SOL with age can be seen in Figure 4.3.7.

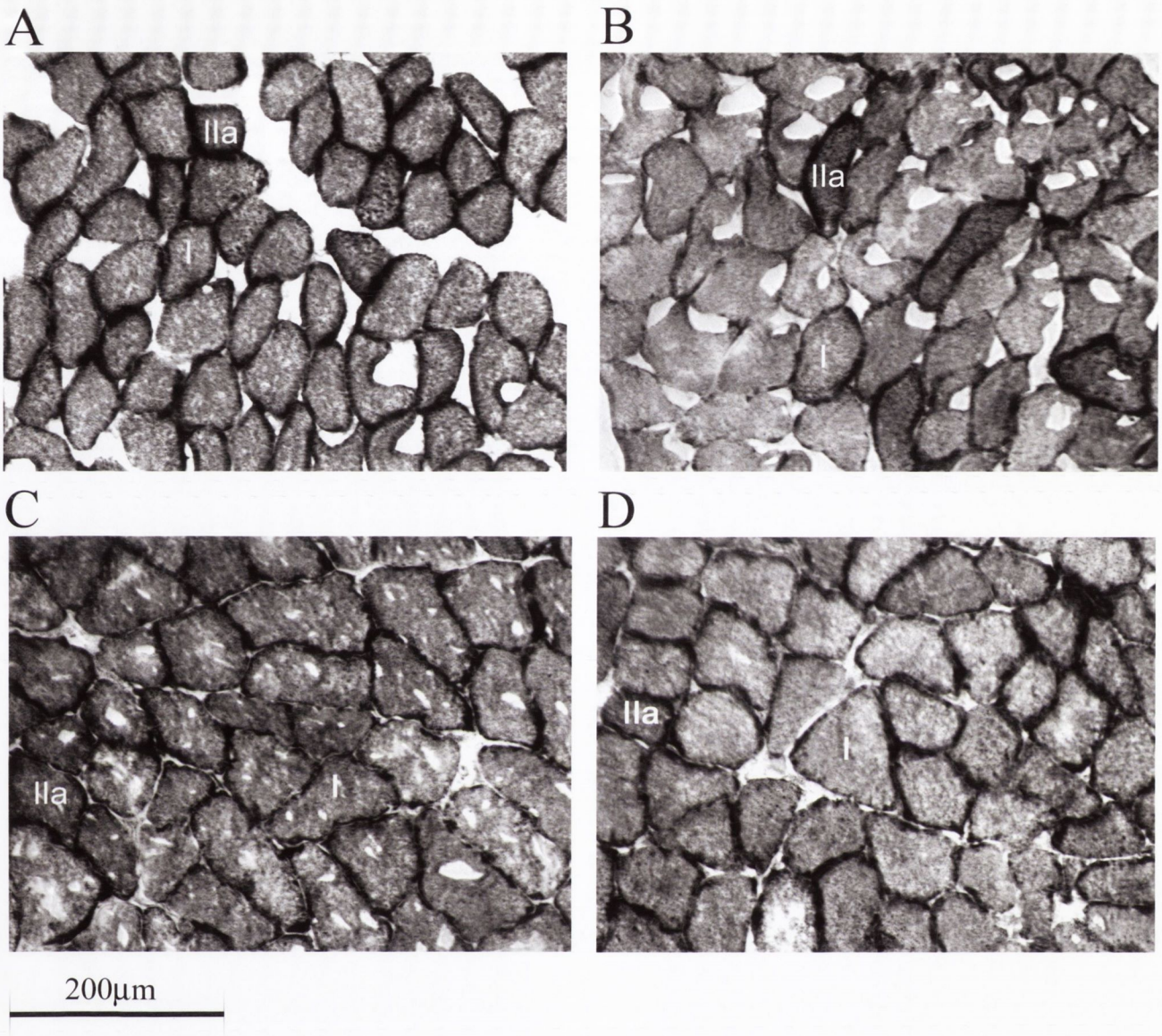


Figure 4.3.7. Change in fibre size with age in SOL at 2 (A), 10 (B), 22 (C) and 24 months of age at 20X magnification.Fibre types are indicated at each age. Fibre size increased from 2 to 10 months and was maintained.

4.3.2. Fibre type changes

4.3.2.1. Fibre distribution in EDL

At 2 months, EDL muscles were composed of 9% type I, 37% type IIa and 52% type IIb fibres (Figure 4.3.8). At 10 months, fibre type distribution changed to 12% type I ($P < 0.05$), 41% type IIa ($P < 0.05$) and 47% type IIb fibres ($P < 0.001$). At 22 months, EDL muscles were composed of 18% type I, 48% type IIa and 34% type IIb fibres, which was significantly different from 2 and 10 months ($P < 0.001$). At 24 months, this distribution shifted to 20% type I and 46% type IIa fibres, with a maintenance of 34% in the proportion of type IIb fibres. This was significantly different from 2 and 10 months ($P < 0.001$), but not 22 months. Therefore, a progressive increase in type I fibre distribution was observed with age in the EDL.

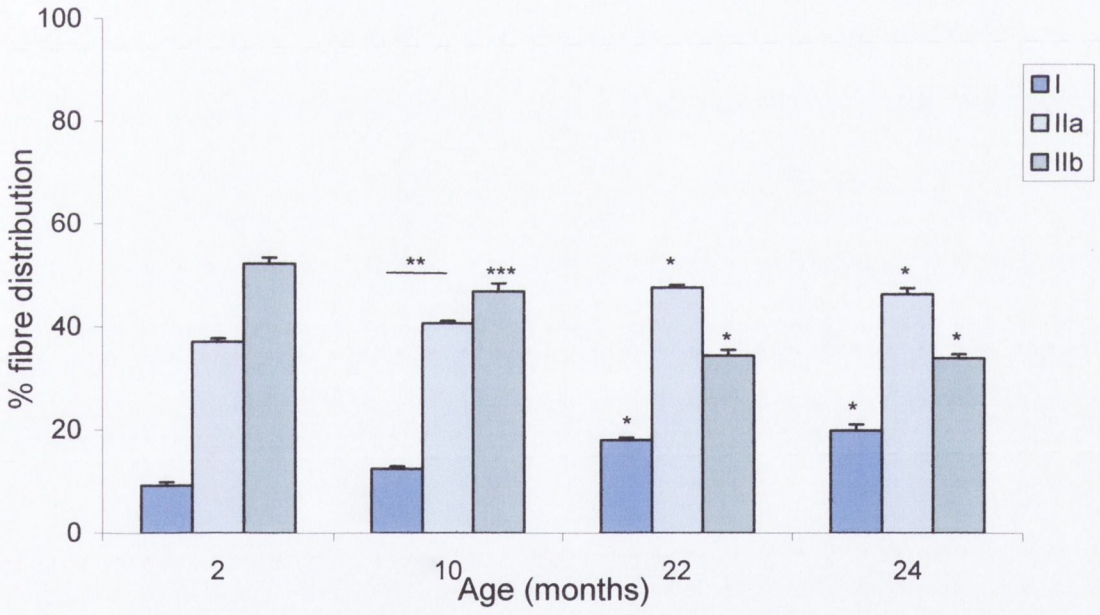


Figure 4.3.8. Fibre type distribution (%) of EDL at 2 (n = 13), 10 (n = 8), 22 (n = 8) and 24 (n = 10) months.

* Different from 2 & 10 months ($P < 0.001$)
** Different from 2 months ($P < 0.05$)
*** Different from 2 months ($P < 0.001$)

4.3.2.2. Fibre distribution in SOL

At 2 months, SOL were composed of 81% type I fibres and 19% type IIa fibres (Figure 4.3.9). At 10 months, fibre distribution changed to 75% type I and 25% type IIa fibres ($P < 0.01$, Figure 4.3.9). This distribution changed significantly at 22 months to 81% type I

fibres and 19% type IIa fibres ($P < 0.05$). At 24 months, type I fibre proportion rose to 95% ($P < 0.001$), with a reduction to 5% in type IIa fibres ($P < 0.001$). No type IIb fibres were observed in any age group in SOL. These results show that type I fibre distribution was higher in aged groups, which agrees with previous studies.

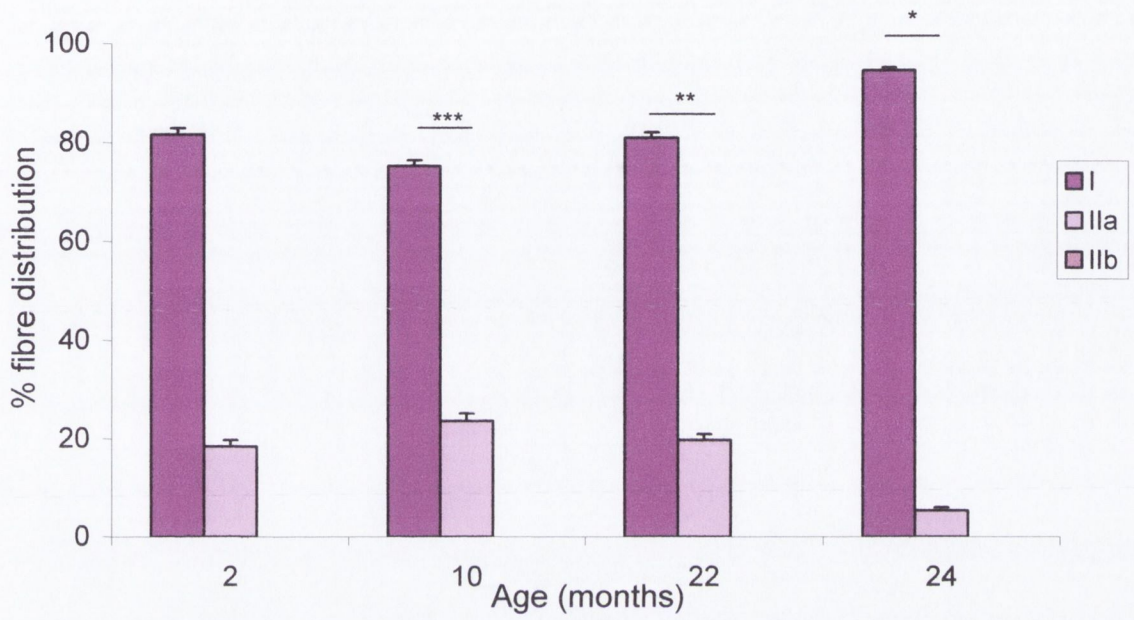


Figure 4.3.9. Fibre type distribution (%) of SOL at 2 (n = 13), 10 (n = 8), 22 (n = 8) and 24 (n = 10) months.

* Different from all ages ($P < 0.001$)
** Different from 10 months ($P < 0.05$)
*** Different from 2 months ($P < 0.01$)

4.3.2.3. Fibre distribution in PL

At 2 months, plantaris was composed of 15% type I, 44% type IIa and 41% type IIb fibres (Figure 4.3.10). At 10 months, fibre distribution was not significantly different compared to 2 months (17% type I, 42% type IIa and 41% type IIb fibres). However, at 22 months type I fibre proportion was larger than at 2 and 10 months (34%, $P < 0.001$), while type IIb was lower (28%, $P < 0.001$). Type IIa fibre proportion was larger at 22 months when compared to 2 months (38%, $P < 0.05$).

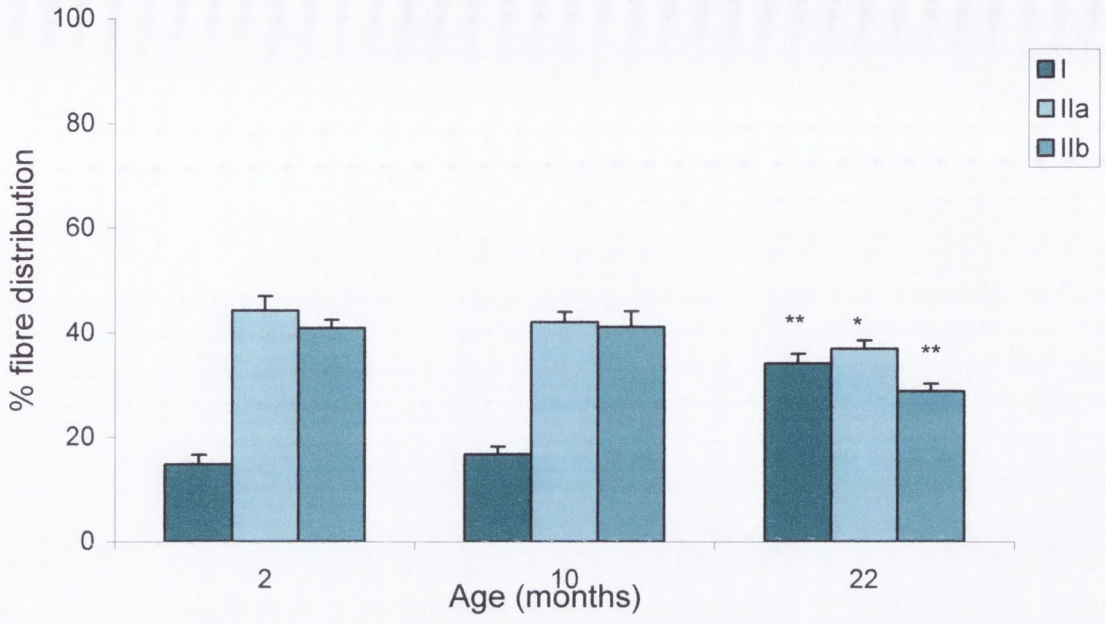


Figure 4.3.10. Fibre type distribution (%) of PL at 2 (n = 7), 10 (n = 8) and 22 (n = 8) months.

* Different from 2 months ($P < 0.05$)
** Different from 2 and 10 months ($P < 0.001$)

4.3.3. Antioxidant changes

Differences in antioxidant levels between muscles in each age group can be seen in Appendix x.

4.3.3.1. Total SOD activity

Total SOD activity was similar across all muscles. However, EDL SOD activity was 23% higher in the aged muscle (22 months) compared to 2 months ($P < 0.05$). In contrast, SOD activity in PL was lower at 22 months compared to 2 months ($P < 0.05$) (Figure 4.3.11). Total SOD activity in SOL was larger at 10 months compared to 2 months ($P < 0.01$), but was 15% lower from 10 to 22 months ($P < 0.05$). Despite this, activity levels in SOL at 22 months were still larger than at 2 months ($P < 0.01$). No age-related alterations were observed in GW or GR. The largest SOD activity was measured in SOL at 10 & 22 months compared to all other muscles at all ages ($P < 0.01$).

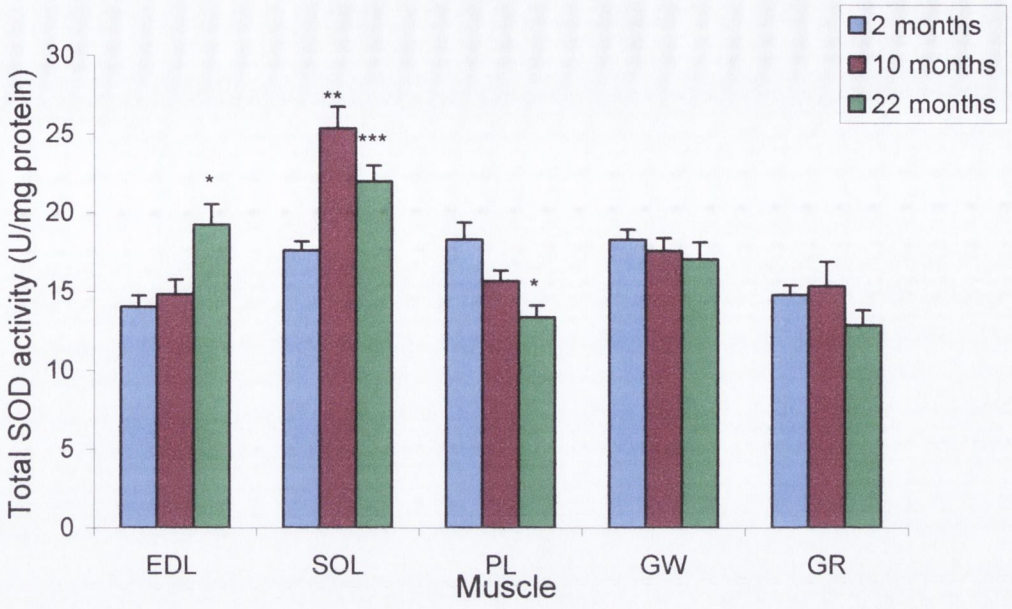


Figure 4.3.11. Effect of age and muscle type on total SOD activity (U/mg protein).

* Different from 2 months ($P < 0.05$)
** Different from 2 months ($P < 0.01$)
*** Different from 10 months ($P < 0.05$)

4.3.3.2. Catalase activity

CAT activity was similar across all muscles across all ages except in SOL. CAT activity was greater in SOL in all age groups when compared with all other muscles ($P < 0.01$, Figure 4.3.12). In addition, CAT activity was greater at 10 months compared to 2 months, and also at 22 months compared to all other age groups ($P < 0.01$). No significant age-associated changes were recorded in any other muscle.

4.3.3.3. Total glutathione concentration

Independent of muscle type, there was a main effect for age such that total glutathione concentration was greater at 1 month in all muscles compared to all other ages ($P < 0.01$, Figure 4.3.13). At 22 months, total glutathione levels were larger in SOL and GR only compared with 10 months ($P < 0.05$).

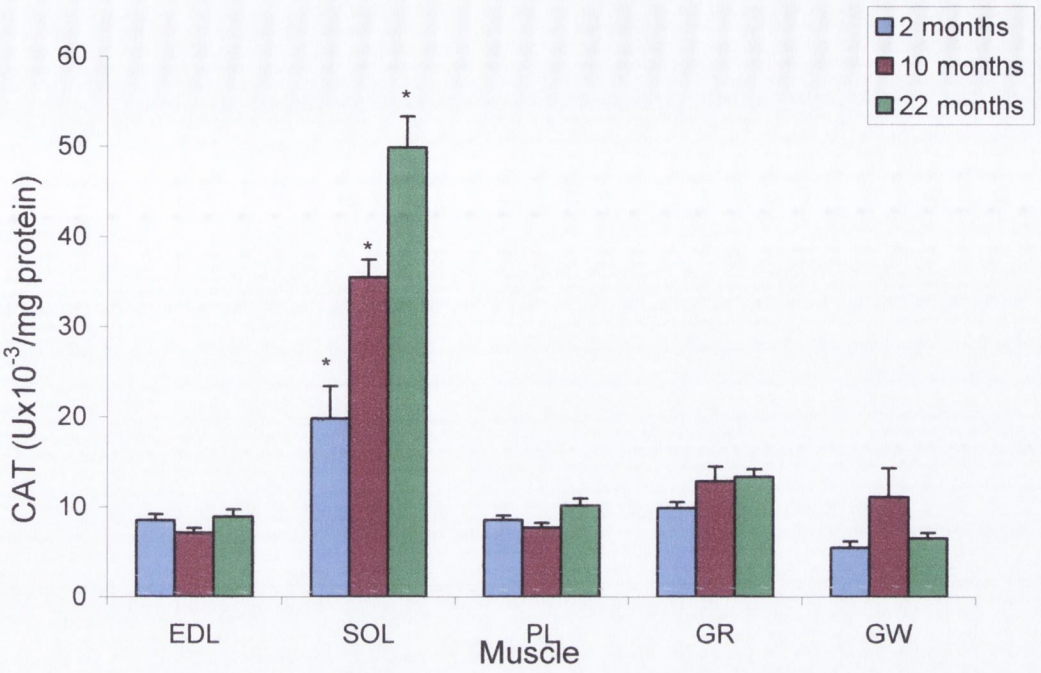


Figure 4.3.12. Effect of age and muscle type on CAT activity ($U \times 10^{-3}$/mg protein)

* Different from all age groups and all muscles ($P < 0.01$)

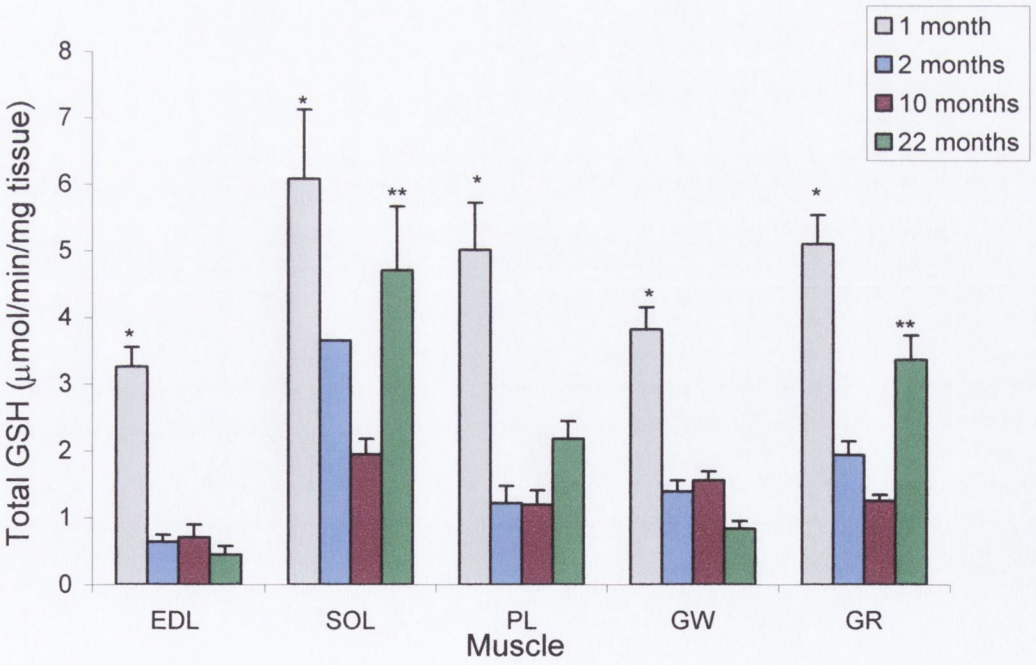


Figure 4.3.13. Effect of age and muscle type on total GSH content (µmol/min/mg tissue).

* Different from all ages ($P < 0.01$)
** Different from 10 months ($P < 0.05$)

Note: Due to a lack of remaining homogenate, the GSH value in SOL at 2 months corresponds to 1 muscle and acts simply as a representative of total glutathione concentration.

4.3.3.4. Total glutathione peroxidase activity

Total GPx activity was higher in GW only at 22 months compared to 10 months ($P < 0.05$, Figure 4.3.14). No significant differences were reported between age groups in EDL, SOL or GR. At 10 and 22 months, SOL GPx activity was greater than other muscles in those age groups ($P < 0.01$).

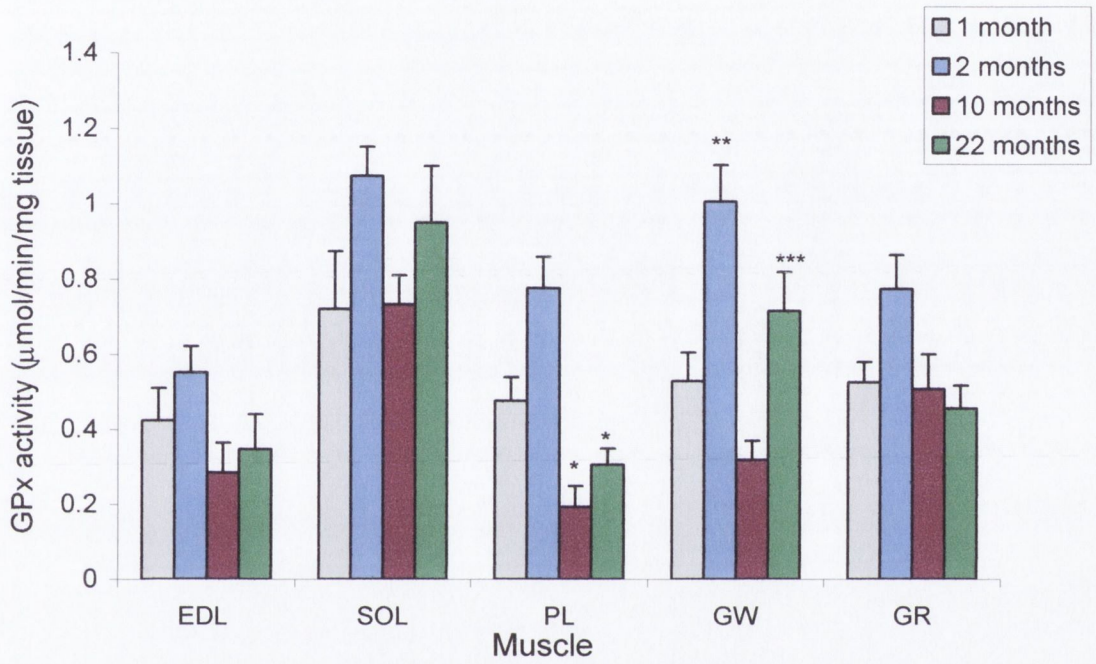


Figure 4.3.15. Effect of age and muscle type on GPx activity (µmol/min/mg tissue).

* Different from 2 months ($P < 0.05$)
** Different from 1, 10 & 22 months ($P < 0.01$)
*** Different from 10 months ($P < 0.05$)

4.3.3.5. Vitamin C concentration

Muscle-specific age-associated alterations for vitamin C can be seen in Figure 4.3.16. The highest concentrations of vitamin C in all muscles were measured in PL and GW at 1 month compared to all other ages ($P < 0.01$). No age-associated change in vitamin C content was seen in any other muscle group (Figure 4.3.15).

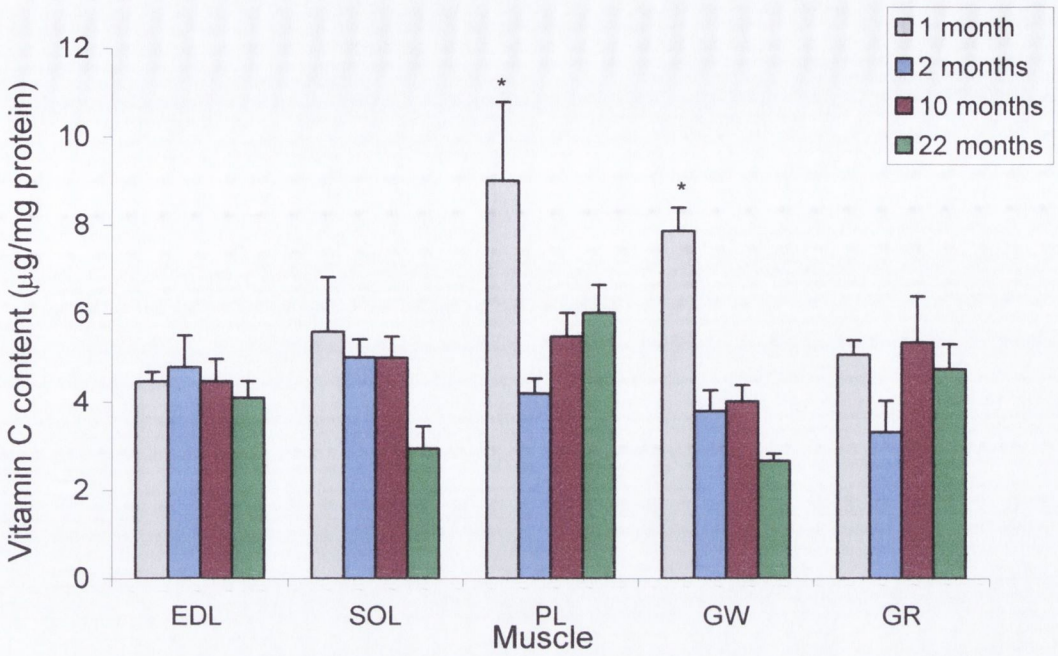


Figure 4.3.15. Effect of age and muscle type on vitamin C concentration (µg/mg protein).

* Different from all other age groups ($P < 0.01$)

4.4. Discussion

4.4.1. Aims of the study

The objective of the present study was to assess the effect of aging on antioxidant levels in skeletal muscles. A relationship, if any, between age-related changes in antioxidant status and changes in fibre type distribution was also assessed. It was hypothesized that type I fibre distribution would increase with age and that this would have an effect on antioxidant levels.

The major findings of the present study confirm that aging is associated with:

1. Increases in type I and/or IIa fibre proportions in EDL, SOL and PL.
2. Absence of fibre atrophy, independent of muscle type.
3. Alterations in antioxidant defences.

4.4.2. Muscle atrophy

Muscle atrophy is a characteristic marker of skeletal muscle aging (Section 1.8.1). No significant skeletal muscle atrophy was observed with age in EDL, SOL and PL (Figure 4.3.1). However, when these groups were analyzed separately from the gastrocnemius muscles, as previously outlined (Section 4.3.1), the reductions in mass in SOL (20%) and PL (12%) at 22 months were considered significant (Figure 4.3.1). Similar muscle wastage with age has been observed previously between 8 and 30-month-old rats (16% PL, 14% SOL) (Thomson & Gordon, 2005), and 10 and 27-month-old mice (20% SOL) (Brooks & Faulkner, 1988). In the present study, the gastrocnemius muscles had atrophied by 15-20% at 22 months when compared to 10 months (Figure 4.3.1). Comparable muscle atrophy (20%) has been shown previously in the gastrocnemius between adult (9 months) and old (24 months) Sprague-Dawley male rats (Barani *et al*, 2003). EDL muscle mass was unaffected by age (Figure 4.3.1). This finding disagrees with the literature, where age-related reductions in mass of up to 22% in the EDL have been consistently reported with aging (Gutmann & Carlson, 1979, Fitts *et al*, 1984, Larsson & Edström, 1986, Brooks & Faulkner, 1988).

No measure of body mass was taken in the rats used in the present study, therefore it is difficult to determine solely from these data whether muscle atrophy did occur or whether

muscle mass was reduced due to overall decreases in body mass with age. Analysis of the literature also indicates that muscle atrophy is a late-stage event in aging, such that significant reductions in mass are not always apparent in rats until approximately 28 months, at which point muscle atrophy becomes increasingly more prominent (Fitts *et al*, 1984, Larsson & Edström, 1986, Brooks & Faulkner, 1988, Thompson & Brown, 1999, Payne *et al*, 2003, Gallegly *et al*, 2004, McKiernan *et al*, 2004).

In order to clarify this point, age-related changes in fibre cross-sectional areas and diameters were assessed as the structural changes responsible for age-related reductions in muscle mass include a reduction in muscle volume and cross-sectional area, a decrease in the total number of muscle fibres, which appears to be irreversible, and atrophy of the remaining fibres (Lexell *et al*, 1988, Holloszy *et al*, 1991, Porter *et al,* 1995, Mecocci *et al*, 1999). In the present study, no age-related reductions in fibre cross-sectional area or diameter were observed in EDL (Figure 4.3.3, 4.3.4 & 4.3.5) or SOL (Figure 4.3.6, 4.3.7 & 4.3.8). In the rat, fibre CSA has been shown to remain relatively constant throughout the aging process until 36 months, at which time a significant decrease in CSA occurred (Thompson & Brown, 1999). Therefore, since no muscle fibre atrophy was observed, it appears that muscle atrophy had not occurred. A more likely scenario is that overall fibre numbers were reduced with increases in fibre diameter and cross-sectional area as a compensatory mechanism. A limitation to this part of the study is that muscle total cross-sectional areas and total muscle fibre numbers could not be measured. This data would have given a more complete picture of the effect of age on the muscles as it could be assessed whether or not total fibre number and muscle cross-sectional area was reduced with age.

4.4.3. Fibre type distributions

In the present study, type I and/or IIa fibre distributions were higher with age in EDL (Figure 4.3.7), SOL (Figure 4.3.8) and PL (Figure 4.3.9). These results agree with the percentage fibre type distribution and age-related changes in fibre type in EDL and SOL observed in previous studies (Larsson *et al*, 1979, Larrson & Edström, 1986, Lexell *et al*, 1988, Trappe *et al*, 1995, Kirkendall & Garrett, 1998). With age there is an increase in denervation in type II fibres. Without the trophic effect of a motor neuron, type II muscle fibres atrophy and die (Ozawa, 1989). Therefore, in order to reduce/prevent muscle fibre

loss, type I motor neurons form collateral branches to innervate type II fibres and prevent fibre loss. Since motor neuron firing patterns determine fibre type, type II fibres are converted to type I fibres, thus leading to an increase in type I fibre distribution with age at the expense of type II fibres. Although fibre typing was not performed on gastrocnemius muscles in the present study, an increase in both the number of type I fibres, percentage area of type I and type IIa fibres and a decrease in both the number and percentage area of type IIb fibres has been observed with age previously in gastrocnemius muscles from young (6 month) and aged (24 months) rats (Larkin *et al*, 2003). As age-associated changes in fibre type distribution in EDL, SOL and PL were in accordance with the literature, it is reasonable to assume that a similar shift in fibre type distribution occurred in the gastrocnemius muscle group in the present study.

Type I and type IIa muscle fibres produce most of their ATP by oxidative phosphorylation, whereas type IIb fibres predominantly use glycolysis (Bottinelli & Reggiani, 2000). Since a percentage of O_2 in mitochondria is always converted to $O_2^{\cdot-}$ (Liu *et al*, 2002, Chen *et al*, 2003), $O_2^{\cdot-}$ production is higher in muscles composed largely of these fibre types (Ji, 1993, Leeuwenburgh *et al*, 1994, Oh-Ishi *et al*, 1995, Hollander *et al*, 2000) However, it has been shown that higher SOD, GPx and CAT activities exist in these muscle fibres as a protective measure against excessive $O_2^{\cdot-}$-induced damage (Jenkins *et al*, 1984, Laughlin *et al*, 1990, Lawler *et al*, 1993, Oh-Ishi *et al*, 1995, Masuda *et al*, 2003). GSH concentrations have also been correlated with oxidative capacity, such that GSH is higher in type I and IIa than type IIb fibres (Ji *et al*, 1992, Pansarasa *et al*, 1999). Since type I and IIa fibres have higher antioxidant defences than type IIb, age-associated shifts in fibre type distribution in favour of type I and/or IIa fibres could enhance antioxidant defences in aged skeletal muscle. In the present study, higher type I fibre proportions in EDL could explain higher total SOD activity. A similar situation could be established between CAT activity in SOL and type I fibre distribution. Data from the present study may therefore suggest that an increase in type I and/or IIa fibre proportions with age could account for some of the significant increases seen in GSH in GR (Figure 4.3.4) and GPx in GW (Figure 4.3.5). However, as fibre typing was not performed in this muscle group, no definitive conclusions can be made. Also, since antioxidant levels did not increase in all muscles despite an increase in type I and/or IIa fibre distribution, it is possible that age-associated increases in ROS levels could have influenced antioxidant status independent of fibre type changes.

Reference	*Animal*	*Muscle type*	*Age (months)* Y	A	O	SOD (Total, Mn, Cu,Zn)	CAT	GPx	Glutathione (GSH & GSSG)	Vitamin C
Lawler & Demaree, 2001	Female Fischer	S & G	4	18	24			↑ G, ↔ S (nmol/min/mg protein)		
Hollander *et al*, 2000	Female Fischer	S, VL, G	8		25	↑ VL & G, ↔ S (U/mg protein)	↑ VL, ↔ G & S ($K \times 10^4$/mg protein)	↑ VL, ↔ G & S (nmol/min/mg protein)		
Gündüz *et al*, 2004	Male Wistar	S & G	9		21	↑ G, ↔ S (U/mg protein)	↑ G & S (k/g protein)	↔ G & S (U/g protein)		
Bejma & Ji, 1999	Female Fischer	VL	8		25				↔ GSH & GSSG (nmol/mg protein)	
Oh-Ishi *et al*, 1995	Male Fischer	S & E	4		24	↑ Cu,Zn , ↔ Mn (E & S) (U/mg protein)	↑ S, ↔ E ($K \times 10^{-4}$/mg protein)	↑ S, ↔ E (U/mg protein)		
Van Der Loo *et al*, 2003	Male F1	*	6		27-30					↓ (µg/g)
Kumaran *et al*, 2004	Male Wistar rats	*	Young		Aged+			↓ (µg GSH oxidized/min/mg protein)	↓ GSH ↑ GSSG (µg/ mg protein)	
Pansarasa *et al*, 1999	Human subjects	VL, RA, GM	17-91 years			↓ Total , ↑ Mn * (U/mg protein)	↔ * (nmol/min/mg protein)	↔ * (mU/mg protein)	↔ GSH ↑ GSSG * (µmol/min/mg protein)	
Kasapoglu & Özben, 2001	Human subjects	Blood samples	20-70 years			↑ (U/gHb)	↑ (k/gHb)	↓ (U/gHb)	↔ (mg/gHb)	↔ (mg/dl)
Pansarasa *et al*, 2000	Human subjects	*	17-91 years			↓ Total , ↑ Mn (U/mg protein)	↑ (nmol/min/mg protein)	↔ (mU/mg protein)	↔ GSH ↑ GSSG (µmol/min/mg protein)	
Gianni *et al*, 2004	Human subjects	VL	22		72	↑ Mn, ↔ Cu,Zn (U/mg protein)	↑ (µmol/min/mg protein)			

Table 4.4.1. Summary of age-associated alterations in antioxidant defences. Variations in units used to express activity levels and concentrations can be clearly seen.

↑ Increase, ↔ No change and ↓ Decrease with age. Y – young, A- adult, O – aged.

* Muscle type not specified. + Exact age not specified.

S – soleus, E – extensor digitorum longus, G – gastrocnemius, VL – vastus lateralis, RA – rectus abdominus, GM – gluteus maximus

Age-related shifts in fibre type help to explain the changes seen in twitch profiles and tetanic force in EDL and SOL in Chapter 3. Age-related increases in type I and/or IIa fibre proportions are partly responsible for alterations in contraction duration in aged skeletal muscle (Sugiura *et al*, 1992, Thompson, 1994). These fibre types act as an internal drag on type IIb fibres, resulting in a slower shortening velocity in whole muscle (Thompson & Brown, 1999, Höök *et al*, 2001). Type IIb muscle fibres also generate the highest forces of all fibre types followed by type IIa and type I fibres (Fitts & Widrick, 1996, Bottinelli *et al*, 1999). Consequently, the reduction in type IIa and/or IIb fibre distribution in aged SOL and EDL could account for the reductions in P_t and P_O measured (Table 3.3.3).

4.4.4. Antioxidant defences

ROS-induced skeletal muscle damage can be reduced/prevented by the stimulation of antioxidant enzymes and molecules (Section 1.4). Aging is associated with increased ROS generation (Bejma & Ji, 1999) and an increase in type I fibres (Sugiura *et al*, 1992, Thompson, 1994). Since type I fibres have higher antioxidant activities (Laughlin *et al*, 1990), an increase in antioxidant activity with age is expected. However, an increase in ROS levels also stimulates antioxidant defences, as discussed previously (Section 1.4). Therefore, since no measure of ROS generation or oxidative damage was performed in the present study, it cannot be definitively stated whether the changes in antioxidants measured were attributed to an increase in ROS and/or an increase in type I fibre distribution.

Also, despite extensive investigation on the effect of aging on antioxidant defences in skeletal muscle in both animal and human models, an age-associated increase in antioxidants does not always appear to occur (Lammi-Keefe *et al*, 1984, Ji *et al*, 1990, Leeuwenburgh *et al*, 1994, Oh-Ishi *et al*, 1995, Rikans & Hornbrook, 1997, Ji *et al*, 1998, Pansarasa *et al*, 1999, Hollander *et al*, 2000). Analysis of the literature reveals variable alterations in antioxidant levels with age (Table 4.4.1). For example, CAT activity in the gastrocnemius and SOL muscles remained unchanged with age in one study (Hollander *et al*, 2000), whereas significant age-related increases were observed in another (Gündüz *et al*, 2004). Age-related increases in GPx activity have been observed in the gastrocnemius and SOL with age (Oh-ishi *et al*, 1995, Lawler & Demaree, 2001), but other studies have

shown no age-associated alteration in these muscle groups (Bejma & Ji, 1999, Hollander *et al*, 2000). This also adds difficulty in the interpretation of the results produced in the present study.

4.4.4.1. Antioxidant enzymes

Since mitochondria are the main source of $O_2^{\cdot-}$ in skeletal muscle, they have a crucial role in aging (Lass *et al*, 1998, Cadenas & Davies, 2000, Carmeli *et al*, 2002). In aged skeletal muscle, ROS-induced alterations affect mitochondrial function, which can result in further $O_2^{\cdot-}$ production above that measured in young skeletal muscle (Section 1.8.2, Rikans & Hornbrook, 1997, Bejma & Ji, 1999, Hamilton *et al*, 2001, Miquel, 2002, Sastre *et al*, 2003, Gianni *et al*, 2004). Therefore, an age-associated increase in SOD activity is essential to accelerate $O_2^{\cdot-}$ removal from the mitochondria. In the present study, total SOD activity at 22 months was higher in the EDL only. This agrees with previous findings where an increase in total SOD activity was measured in skeletal muscle with age (Lammi-Keefe *et al*, 1984). However, SOD activity remained unchanged in SOL, GW and GR, and was reduced in PL at 22 months (Figure 4.3.12). The absence of any increase in total SOD activity with age has been measured previously (Table 4.4.1, Pansarasa *et al*, 1999, Hollander *et al*, 2000, Pansarasa *et al*, 2000, Gündüz *et al*, 2004). However, age-associated increases in SOD have been observed in the gastrocnemius (Table 4.4.1, Hollander *et al*, 2000, Gündüz *et al*, 2004). It has been shown that the $O_2^{\cdot-}$-scavenging ability of SOD, as measured by ESR, is higher in the SOL, diaphragm and deep potions of the gastrocnemius muscles compared to the plantaris and superficial portions of the gastrocnemius in 3-month-old rats (Masuda *et al*, 2003). Results from the present study agree with this finding, in so much as total SOD activity was highest in SOL at 10 and 22 months compared to other muscles (Appendix x). This muscle-specific distribution of SOD is likely to reflect fibre type composition as type I fibres have higher SOD activity than type IIa or IIb fibres.

$O_2^{\cdot-}$ is confined within the mitochondria, where it can induce modifications to the ETC and lead to further $O_2^{\cdot-}$ production (Figure 1.1, Yu, 1994, Meister, 1995b, Cardoso *et al*, 1999, Hollander *et al*, 2000, Hamilton *et al*, 2001). Therefore, since total SOD activity remained unchanged in SOL, GW and GR and was reduced in PL with age, it is likely that the mitochondria in these muscles could be a target for $O_2^{\cdot-}$-mediated damage. As total SOD activity was larger in aged EDL, $O_2^{\cdot-}$ dismutation and removal from the

mitochondria is accelerated, which reduces $O_2^{\cdot-}$-induced damage to the organelle. However, as an increase in total SOD activity accelerates H_2O_2 formation (Equation 1.11, Scott *et al*, 1987), a simultaneous increase in H_2O_2-specific antioxidant enzymes (GPx and CAT) must also occur to facilitate H_2O_2 removal (Figure 1.3, Section 1.4). Indeed, H_2O_2 levels increase with age in skeletal muscle (Bejma & Ji, 1999), therefore an age-associated increase in the activity of CAT and GPx is expected in all muscles to reduce H_2O_2-induced damage (Hammeren *et al*, 1992, Lawler *et al*, 1993, Ji *et al*, 1998, Lass *et al*, 1998, Lawler & Powers, 1998, Lawler & Demaree, 2001).

CAT activity has been shown to increase with age in skeletal muscle irrespective of changes in muscle fibre type distribution (Spiers *et al*, 2002). However, in the present study, CAT activity was higher in SOL only at 22 months, and remained unchanged in all other muscles (Figure 4.3.12). The highest CAT activity in each age group was also measured in SOL. This agrees with previous work where age-related increases in CAT were measured in SOL (Table 4.4.1, Oh-Ishi *et al*, 1995, Gündüz *et al*, 2004). In the present study, GPx activity was larger in GW at 22 months only, and remained largely unchanged in all other muscles (Figure 4.3.12). This was shown previously, where an age-related increase in GPx activity in the gastrocnemius, but not SOL, was measured between 4, 18 and 24-month-old rats (Table 4.4.1, Lawler & Demaree, 2001). Although no age-related increase in GPx was observed in SOL in the present study, activity was highest in this muscle at 10 and 22 months compared to all other muscles. This has been shown previously (Hollander *et al*, 2000, Lawler & Demaree, 2001).

Even though GPx activity remained unchanged in SOL in the present study (Figure 4.3.14), a concurrent age-associated increase in CAT activity was observed (Figure 4.3.12). This agrees with previous work performed in the same animal model as the present study (Gündüz *et al*, 2004). Therefore, it is likely that larger CAT in this muscle is capable of removing the excess H_2O_2 produced with age. The absence of an age-associated change in CAT and GPx in EDL has been observed previously (Oh-Ishi *et al*, 1995). As CAT or GPx activity remained unchanged in EDL, PL and GR, the results from the present study suggest that these muscles are ill equipped to deal with the oxidative stress associated with aged muscle, such that H_2O_2 levels are not removed as effectively as SOL. H_2O_2 can then participate in the Fenton reaction, resulting in the production of $OH^{\cdot}$ (Figure 1.3, Halliwell & Gutteridge, 1991, Yu, 1994, Lloyd *et al*, 1997, Kourie,

1999), which induces more oxidative damage to cells and tissue than any other ROS (Cadenas & Davies, 2000). In the present study, it appears that a differential response to age-associated oxidative stress occurs in predominantly slow-twitch muscle compared to a fast-twitch muscle. Therefore, an adaptation of some other component of the antioxidant defence system must occur to accommodate for age-related increases in ROS ($O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$) in skeletal muscle.

Age-associated changes in antioxidant activities could also help to explain the response observed in aged muscle to H_2O_2 incubation *in vitro* (Chapter 3). Since SOD and CAT were higher at 22 months in SOL than at 2 months and SOD was higher in aged EDL, these changes may have led to an enhanced resistance to oxidatively induced changes in force due to increased ROS scavenging and removal from the muscle bath. This appears to have delayed the reduction in force associated with a supramaximal dose of H_2O_2 *in vitro* in the aged muscle despite these muscle having higher resting levels of ROS.

4.4.4.2. Antioxidant molecules

Aging is accompanied by a progressive oxidation of GSH to GSSG, which occurs when H_2O_2 is increasingly converted to O_2 and H_2O by the glutathione system (Equation 1.16, Bejma & Ji, 1999, Miquel, 2002, Mosoni *et al*, 2004). GSH deficiency has been associated with increases in lipid peroxidation in skeletal muscle and heart (Sen *et al*, 1994), and attenuated contractile properties in rat diaphragm muscle (Morales *et al*, 1993). Therefore, age-associated reductions in GSH are likely to have important effects on skeletal muscle structure and function with age. However, in the present study, GSH concentrations were larger in SOL and GR at 22 months, and remained unchanged in all other muscle groups (Figure 4.3.14). Since GSH removes H_2O_2 and recycles vitamin C & E radicals (Figure 1.4, Niki *et al*, 1985), an increase in GSH in GR and SOL with age leads to enhanced H_2O_2 scavenging, vitamin C and E radical recycling and an overall reduction in free radical chain reactions and lipid peroxidation. Previous studies have shown that GSH concentrations are unaffected with age (Table 4.4.1, Bejma & Ji, 1999), however, in some cases this took place in conjunction with increases in GSSG (Pansarasa *et al*, 1999, 2000, Kumaran *et al*, 2004). GSSG levels in aged skeletal muscle were not assessed in the present study, however it appears likely that an increase in the production of GSH, or enhanced recycling of GSSG to GSH via GPx, occurred to maintain/increase GSH concentrations in aged skeletal muscle. Since the largest GSH concentration and

highest GPx and CAT activities were measured in SOL (Figure 4.3.13, 4.3.14 & 4.3.12), and as these antioxidants work in conjunction with one another to reduce H_2O_2 levels (Equation 1.16), it would appear that at 22 months, SOL is more resistant to age-associated ROS-induced damage than other muscles and also helps to explain the results produced in Chapter 3.

Since vitamin C scavenges a range of ROS ($OH^{\cdot}$, peroxyl radicals, $O_2^{\cdot-}$ and HOCl) and RNS ($ONOO^{-}$) (Sies & Stahl, 1995, Naidoo & Lux, 1998, Carr & Frei, 1999), and remembering that $O_2^{\cdot-}$ production increases with age, it is likely that vitamin C would become increasingly oxidized to dehydroascorbic acid as a result of increased ROS scavenging (Figure 1.4). Indeed, aging has also been shown to compromise vitamin C concentrations in skeletal muscle (Table 4.4.1, Van der Loo *et al*, 2003). Mitochondria recycle vitamin C at an externally exposed portion of mitochondrial complex III (Xia *et al*, 2002). As mitochondria undergo extensive modification with age, the ability of cells to regenerate vitamin C could be reduced partly due to ROS-induced modifications of the ETC (Section 1.2, Hamilton *et al*, 2001). However, this does not seem to be the case in the present study, as vitamin C concentrations remained largely unchanged with age, except in PL and GW, where vitamin C was higher at 1 month compared to other age groups and muscles (Figure 4.3.6). This agrees with previous work on blood samples from human subjects, where no age-related alterations in vitamin C concentrations were observed (Kasapoglu & Özben, 2001). It should be noted that rats synthesize ascorbic acid endogenously, whereas vitamin C concentrations in humans depend entirely on dietary intake (Colven & Pinnell, 1996, Griffiths & Lunec, 2001). Therefore, it is anticipated that rats should be able to maintain sufficient vitamin C concentrations by increasing cellular synthesis, and in humans by maintaining an adequate dietary intake.

4.4.5. Conclusions

Age-associated alterations in a section of the antioxidant defence system were investigated in the present study. Results support the premise that aging is associated with alterations in the antioxidant defence system, and that these may be linked to any alterations in muscle fibre type distribution.

From the results presented here, SOL possesses the most adequate defences to deal with the oxidative stress associated with aging as none of the other muscles demonstrated such dramatic changes in antioxidant levels between young, adult and aged groups. The results of this study could help to explain why the combination of age and an oxidant challenge had a more dramatic effect on P_O in EDL compared to SOL muscles, as seen in Chapter 3.

It should be noted that the antioxidant defence system is extensive in cells and encompasses a vast number of interacting systems. As only a fraction of these defences were assessed in the present study, other age-associated alterations that were not measured may have occurred to accommodate for the increase in ROS production in aged skeletal muscle.

Chapter 5.

The effects of dietary intervention on contractile function and antioxidant status in skeletal muscle

5.1. Introduction

5.1.1. Antioxidant supplementation

Significant incorporation of vitamin E into skeletal muscle mitochondrial membranes, plasma, skeletal muscle lipids and various other tissues has been observed following administration of supra-nutritional doses of vitamin E (Morrissey *et al*, 1996, Mercier *et al*, 2001, Yang *et al*, 2002, Harms *et al*, 2003, Sumien *et al*, 2003, Lo Fiego *et al*, 2004), which has been reported to increase the resistance of plasma, skeletal muscle, and other tissues to lipid peroxidation (Sumida *et al*, 1989, Kanter *et al*, 1993, Goldfarb *et al*, 1994, Mastaloudis *et al*, 2001). Short- and long-term vitamin C supplementation also significantly increases plasma and skeletal muscle vitamin C, SOD, CAT and GPx concentrations and dose-dependently improves the resistance of plasma to lipid peroxidation (Chatterjee *et al*, 1975, Rojas *et al*, 1994, Khassaf *et al*, 2003, Polidori *et al*, 2004). *In vitro* studies have demonstrated that vitamins C & E work synergistically (Section 1.4, Niki *et al*, 1982, Niki *et al*, 1995, Benzie & Strain, 1999, May, 1999, Umegaki *et al*, 2000), and *in vivo* studies report an increase in vitamin E status with vitamin C supplementation (Tanaca *et al*, 1997, Liu & Lee, 1998, Castellini *et al*, 2000, Hamilton *et al*, 2000). Antioxidant supplementation reduces ROS concentrations in rat diaphragm and peripheral skeletal muscles, as measured by reductions in mitochondrial H_2O_2 generation, reductions in protein and lipid oxidation and an overall decrease in ROS levels confirmed by reductions in ESR signals (Duchesne *et al*, 1975, Chow *et al*, 1999, Factor *et al*, 2000, Gatellier *et al*, 2000).

5.1.2. Antioxidants and muscle function

Redox modulation of skeletal muscle contraction can be influenced by modifying antioxidant defences to offset any ROS-induced effects on contractile function. One strategy that has been used previously to improve antioxidative defences in skeletal muscle is the administration of supra-nutritional doses of vitamin C and E (Section 1.4, Jayachandran *et al*, 1996, Polidori *et al*, 2004). It has been shown that ROS contribute to exercise-induced skeletal muscle damage, and studies have demonstrated that prior supplementation with dietary antioxidants (vitamin C and/or E) ameliorates muscle functional decrements, plasma creatine kinase activity and muscle soreness subsequent to eccentric muscle contraction (Bloomer *et al*, 2004, Shafat *et al*, 2004). Therefore, boosting antioxidant defences in the hind limb and diaphragm muscles with high doses of

vitamin C and E may afford these muscles a level of protection against any ROS-induced modification *in vitro*, such that muscle force may be maintained in the presence of an oxidant challenge (Section 1.7 & 3.3, Reid *et al*, 1993, Clanton *et al*, 1999).

5.1.3. Aims

The main objective of the present study was to investigate the effects of supra-nutritional doses of vitamin C and E on the oxidative stability of skeletal muscle. This was achieved by assessing the effects of an oxidant challenge *in vitro* (as simulated by exogenous H_2O_2 application) on the maximum force-producing ability of EDL, SOL and diaphragm muscles. Plasma, liver and heart tissue was also collected to determine whether vitamin supplementation had a similar effect on different tissues.

The main hypotheses were tested in this study:

1. Long-term supplementation with supra-nutritional doses of vitamin C and E would lead to an overall enhancement of antioxidant defences in vitamin-treated animals compared to controls.
2. Vitamin-treated muscles would be able to resist H_2O_2-induced reductions in force over time better than control muscles due to increased antioxidant activity.

5.2. *Materials and Methods*

5.2.1. Materials

5.2.1.1. Animals

37 adult male Wistar rats were used in this study. Animals were randomly divided into 2 groups, and were fed either a control (CON, n = 19) or vitamin diet (VIT, n = 18). Groups were further subdivided according to which muscle, hind limb (*HL*) or diaphragm (*Dia*), was tested in contractile function experiments. Therefore, CON and VIT were further spilt into CON *HL* (n = 10), VIT *HL* (n = 9), CON *Dia* (n = 9) and VIT *Dia* (n = 9). All rats survived the treatment protocol and none had large tumours, unusual weight loss or other obvious indications of poor health. Animals were sacrificed at approximately 24 weeks of age.

5.2.1.2. Diet protocol

(i) Parameters measured

Food and water intake was measured for 5 weeks before the start of the diet protocol to establish daily food and water consumptions. Body mass was recorded twice weekly. Dietary manipulation lasted for a period of 8 weeks.

(ii) Dietary manipulation

CON was fed standard laboratory rat chow (standard pelletted chow, Red Mills Ltd, Appendix ii) to which corn oil was added (Mazola pure corn oil, Bestfoods, Surrey, UK, Appendix ii), ensuring that each group received an isocaloric intake. VIT was fed rat chow mixed with dL-α-tocopherol acetate dissolved in oil (vitamin E; 250mg/rat/day; Aldrich, Dublin, Ireland), and drinking water with L-ascorbic acid added (vitamin C; 205mg/rat/day; Sigma, Dublin, Ireland). The dL-α-tocopherol acetate ester is used to prevent oxidation during storage and is hydrolysed *in vivo* generating free tocopherol (Sumien *et al*, 2001). It has been previously shown that 200mg vitamin E/kg body weight/day results in significant incorporation of tocopherol into mitochondria and homogenates of various tissues (Lass *et al*, 1999). Rats were given 95% of their average daily food and water intakes to guarantee that the full daily allowances of vitamins would be ingested. Throughout the course of the diet protocol, both CON and VIT diets were

prepared daily to ensure that the vitamins added were not significantly oxidized prior to ingestion.

5.2.2. Methods

5.2.2.1. Tissue dissection

On the last day of the diet protocol, animals were weighed and anaesthetised. A 5ml venous blood sample was taken (Section 2.2.1). Following animal sacrifice, either the hind limb muscles or diaphragm was removed (Section 2.2.1.2). EDL and SOL were also removed from *Dia* animals for antioxidant testing. In all animals, the heart was removed and weighed. A portion of the left ventricular wall and a section of the central lobe of the liver were removed, blotted on filter paper and weighed. In the EDL, SOL and diaphragm segments, tendons were tied securely leaving small loops for attachment to a force transducer.

5.2.2.2. Contractile function protocol

All CON and VIT animals were further subdivided into 2 groups to measure the effects of 8 weeks vitamin supplementation on contractile function in the diaphragm and hind limb muscles (Figure 5.2.1). CON & VIT muscles were divided into a control group, in which muscles were incubated in Krebs solution, while a treatment group was used to test contractile function of muscles in response to H_2O_2 incubation.

As with previous contractile experiments, a supramaximal concentration of H_2O_2 was used to simulate an oxidative stress. Once L_O was established, maximum tetanic force (P_O) was recorded in each muscle every 5 minutes for 30 minutes in the presence or absence of H_2O_2 (Section 2.2.2.4). Immediately following the first maximum tetanus, H_2O_2 was added to the bath in treatment groups only.

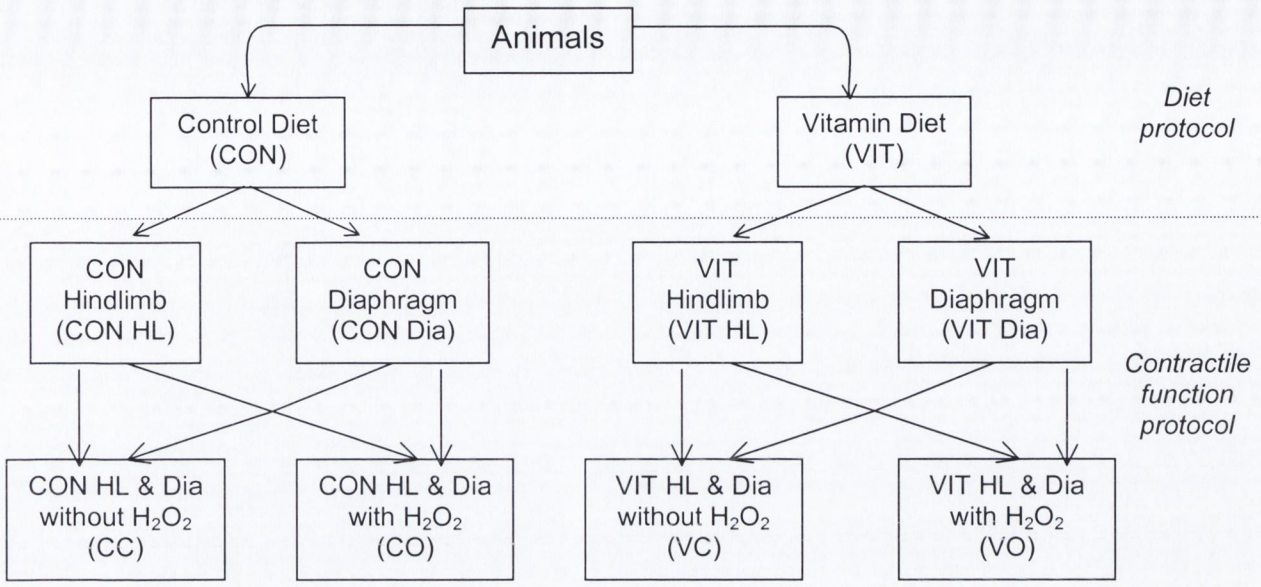


Figure 5.2.1. Flowchart representing the distribution of animals during the diet protocol, and skeletal muscles in contractile function tests.

5.2.2.3. Tissue storage

All plasma samples were frozen initially at -20°C and stored at -80°C. After completion of isometric contractile experiments, all muscles were weighed for calculation of cross-sectional area. Muscles, heart and liver samples were snap-frozen and stored at -80°C. Prior to antioxidant assessment; muscles were thawed, homogenized in Krebs solution and equalized for protein (2mg/ml) using the Bradford protein assay (Section 2.2.4.2).

5.2.2.4. Antioxidant assays

Antioxidant enzymes (SOD, CAT), antioxidant molecules (glutathione and vitamin C) and a measure of overall antioxidant power (FRAP) were assessed in this study. All assays have been described in detail previously (Section 2.2.4).

5.2.3. Statistical analysis

5.2.3.1. Diet parameters

The effect of time and dietary manipulation on food and fluid consumption and body mass was assessed using a repeated measures ANOVA. Where significance was recorded, *post-hoc* analysis was performed using a Student-Newman-Keuls test. Significance was set at $P < 0.05$.

5.2.3.2. Tissue mass

Unpaired Student t-tests were used to measure differences in tissue mass between CON and VIT. Significance was set at $P < 0.05$.

5.2.3.3. Contractile parameters

A repeated measures ANOVA was used to determine the effect of various concentrations of H_2O_2 on muscle force in diaphragm sections during a 30 min incubation protocol. A 2-way ANOVA analyzed the effects of dietary intervention on isometric characteristics (P_O, Pt, TTP, ½RT, P_t/P_O ratio and contraction and relaxation velocities). Where significance was recorded, a Student Newman Keuls *post-hoc* test was run. The effect of H_2O_2 on P_O in SOL, EDL and diaphragm from CON and VIT was analysed using a 3-way ANOVA. Where significance was recorded, *post-hoc* analysis was performed with a Tukey LSD. A significance value was set at $P < 0.05$.

5.2.3.4. Antioxidant parameters

The effect of vitamin supplementation on the concentrations and activities of antioxidants in CON and VIT tissues was tested with a 2-way-ANOVA, followed by a Student Newman Keuls *post-hoc* test when significance was recorded. Statistical analysis was divided into 2 main sets of tests:

1. A 2-way ANOVA was used for muscle groups (EDL, SOL, diaphragm and heart).
2. Unpaired t-tests were performed on the data collected from the liver and plasma.

Data was analysed in this manner because the liver and plasma had very large and small antioxidant levels, respectively, in the majority of the tests done. When included with the muscle data for statistical analysis, the power of the statistical tests in determining real differences between EDL, SOL and diaphragm groups was reduced. As the effect of the diet on antioxidant levels in muscle was the most important factor being evaluated in this study, this was the most appropriate way to analyse the data.

5.3. Results

	Week	CON Food intake (g)		VIT Food intake (g)		CON Fluid intake (ml)		VIT Fluid intake (ml)	
		Pre-diet	*Diet*	*Pre-diet*	*Diet*	*Pre-diet*	*Diet*	*Pre-diet*	*Diet*
Pre-diet	1	28.67 ± 0.47		28.05 ± 0.73		33.33 ± 1.72		35.71 ± 2.08	
	2	26.62 ± 1.05		28.00 ± 0.68		32.38 ± 1.90		36.19 ± 0.95	
	3	30.14 ± 1.31		28.76 ± 0.45		36.67 ± 2.14		39.52 ± 0.90	
	4	29.90 ± 0.65		28.71 ± 1.34		34.05 ± 2.28		38.45 ± 0.72	
	5	31.10 ± 0.44		30.88 ± 0.63		37.62 ± 0.85		38.81 ± 1.16	
Diet	6		28.05 ± 0.47		28.00 ± 0.58		35.55 ± 1.86		31.40 ± 1.07
	7		27.57 ± 1.01		28.48 ± 0.78		36.88 ± 0.92		33.81 ± 1.62
	8		27.14 ± 0.89		29.29 ± 0.62		35.00 ± 1.95		34.02 ± 2.03
	9		27.43 ± 0.90		28.71 ± 1.34		35.24 ± 1.62		36.02 ± 1.73
	10		28.52 ± 0.95		30.76 ± 1.17		40.00 ± 0.75		34.81 ± 1.66
	11		28.48 ± 1.03		30.52 ± 0.81		36.83 ± 1.78		34.86 ± 2.20
	12		29.05 ± 1.09		30.24 ± 0.72		35.60 ± 2.72		36.19 ± 1.98
	13		29.19 ± 1.14		31.10 ± 0.59		35.38 ± 1.95		35.48 ± 2.17

Table 5.3.1. Average daily food (g) and fluid (ml) consumptions per rat per week for the monitoring (pre-diet) and diet period in control groups (CON, n =19) and dietary groups (VIT, n = 18).

All results are expressed as mean ± SEM unless stated otherwise. Contractile function data (Section 5.3.2) was normalised to a percentage of the initial P_O. Specific forces ($kN.m^{-2}$) can be seen in Appendix ix.

5.3.1. Diet parameters

5.3.1.1. Food and fluid consumption

Throughout the course of this study, no significant differences were observed for food and fluid intakes between the 5-week monitoring and 8-week diet period, or between CON and VIT groups (Table 5.3.1).

5.3.1.2. Morphometric data

Morphometric data is shown below (Figure 5.3.1 & Table 5.3.2). As expected with growth, body mass increased steadily throughout the course of the monitoring and diet protocol (Figure 5.3.1). However, body mass was not different between CON and VIT at any time point indicating that the diet had no effect on the health or growth of vitamin-treated animals.

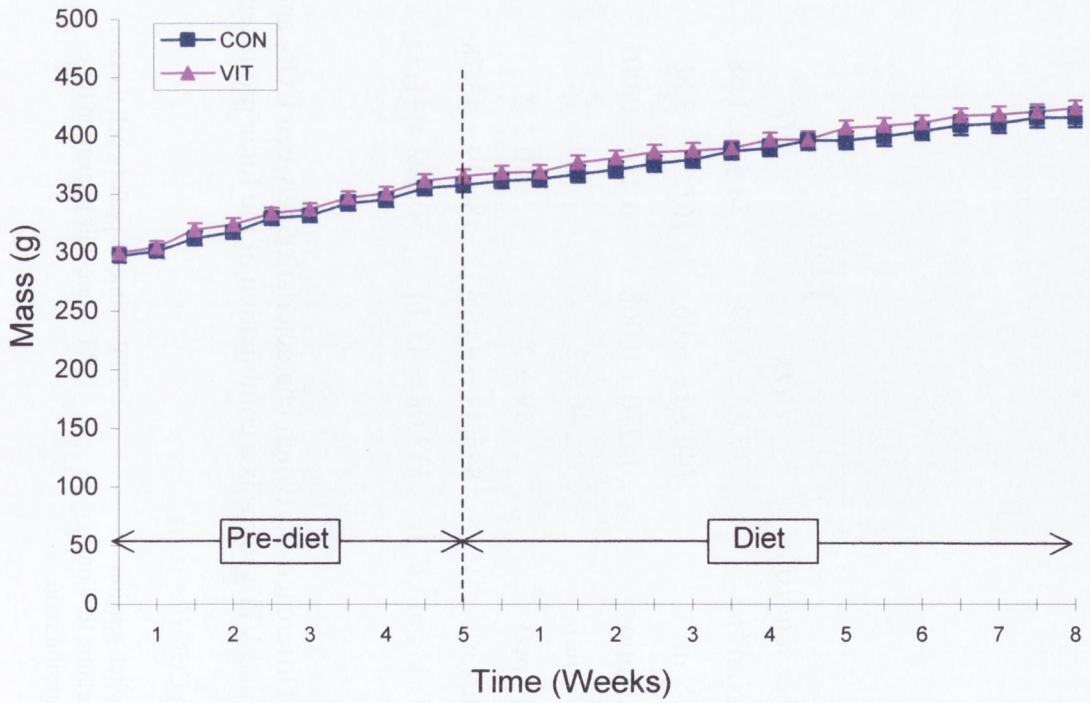


Figure 5.3.1. Change in body mass (g) measured in CON (n = 18) and VIT (n = 18) for the duration of the monitoring (pre-diet) and diet protocol.

Isometric characteristics	EDL		SOL		Diaphragm	
	CON	*VIT*	*CON*	*VIT*	*CON*	*VIT*
P_t ($kN.m^{-2}$)	37.62 ± 1.79	38.54 ± 1.58	23.06 ± 0.98	26.04 ± 1.42	68.78 ± 4.09	64.09 ± 4.12
P_O ($kN.m^{-2}$)	146.86 ± 6.96	161.15 ± 3.86	140.79 ± 7.67	142.87 ± 9.20	161.65 ± 12.76	136.89 ± 12.34
P_t/P_O ratio	0.270 ± 0.008	0.256 ± 0.007	0.165 ± 0.004	0.178 ± 0.005	0.435 ± 0.010	0.446 ± 0.016
TTP (ms)	46 ± 2	50 ± 2	129 ± 8	127 ± 9	75 ± 3	77 ± 3
½RT (ms)	39 ± 2	40 ± 2	188 ± 13	163 ± 13	74 ± 4	71 ± 4
P_t/TTP ($kN.m^{-2}.s^{-1}$)	835.43 ± 44.74	786.49 ± 43.78	193.10 ± 16.85	225.95 ± 22.37	949.72 ± 66.15	872.43 ± 72.6
$(½P_t)$/ ½RT ($kN.m^{-2}.s^{-1}$)	513.08 ± 42.11	508.92 ± 41.95	67.11 ± 6.00	95.85 ± 13.04*	486.55 ± 36.64	497.28 ± 52.42

Table 5.3.3. Differences in twitch characteristics between CON and VIT in the EDL (n = 9), SOL (n = 9) and diaphragm (n = 11) muscles. P_O data in CON and VIT groups is a combination of the force produced by control and treated muscles, prior to incubation with H_2O_2.

* Different from CON ($P < 0.01$)

P_t – isometric twitch tension
P_O – isometric tetanic tension
P_t/P_O – twitch/tension ratio

TTP – time to peak twitch tension
½RT – one-half relaxation time

P_t/TTP – contraction velocity
$(½P_t)$/ ½RT – relaxation velocity

When animals were sacrificed, no significant differences in mass were recorded between CON (n = 19) & VIT (n = 18) for EDL, SOL or heart (Table 5.3.2).

	CON	VIT
Body (g)	418.89 ± 8.49	425.50 ± 6.68
Heart (g)	1.07 ± 0.02	1.11 ± 0.03
EDL (mg)	186 ± 4	187 ± 3
SOL (mg)	163 ± 6	167 ± 5

Table 5.3.2. Difference in mass of the body, heart, EDL and SOL between CON & VIT at the time of sacrifice.

5.3.2. Isometric contractile parameters

Isometric contractile data for the EDL, SOL and diaphragm muscles are shown in Table 5.3.3. No significant differences were recorded in any muscle between CON & VIT for P_t, P_O, P_t/P_O, TTP, ½RT and contraction velocity. However, relaxation velocity was slower in SOL VIT compared to CON SOL ($P < 0.01$). Therefore, 8 weeks vitamin supplementation did not significantly affect overall skeletal muscle function in EDL, SOL and diaphragm segments.

5.3.3. Incubation protocol

Group divisions in the contractile results section have been outlined previously (Figure 5.2.1, Section 5.2.2.6). To summarize, CON and VIT muscles (*HL* & *Dia*) were further spilt into control muscles (CC & VC) and muscles exposed to an oxidant challenge, as simulated by exogenous application of a supramaximal dose of 160mM H_2O_2 (CO & VO).

5.3.3.1. EDL incubation

Baseline P_O was 146.86 ± 6.96 $kN.m^{-2}$ and 161.15 ± 3.86 $kN.m^{-2}$ in CC and VC, respectively. Although P_O declined over time in both CC and VC ($P < 0.05$), no

significant differences were observed between these groups at any time point (Figure 5.3.2).

Before application of H_2O_2, P_O was 133.17 ± 9.52 kN.m^{-2} and 141.15 ± 7.58 kN.m^{-2} in CO and VO, respectively. Over time, force also decreased in CO and VO ($P < 0.05$), but this was more significant than the decline observed in CC and VC. However, this was also time and diet dependent such that the decline in P_O over time in CO was more pronounced than in VO at all time points, except 5 mins ($P < 0.001$). No potentiation in P_O was observed in either CO or VO.

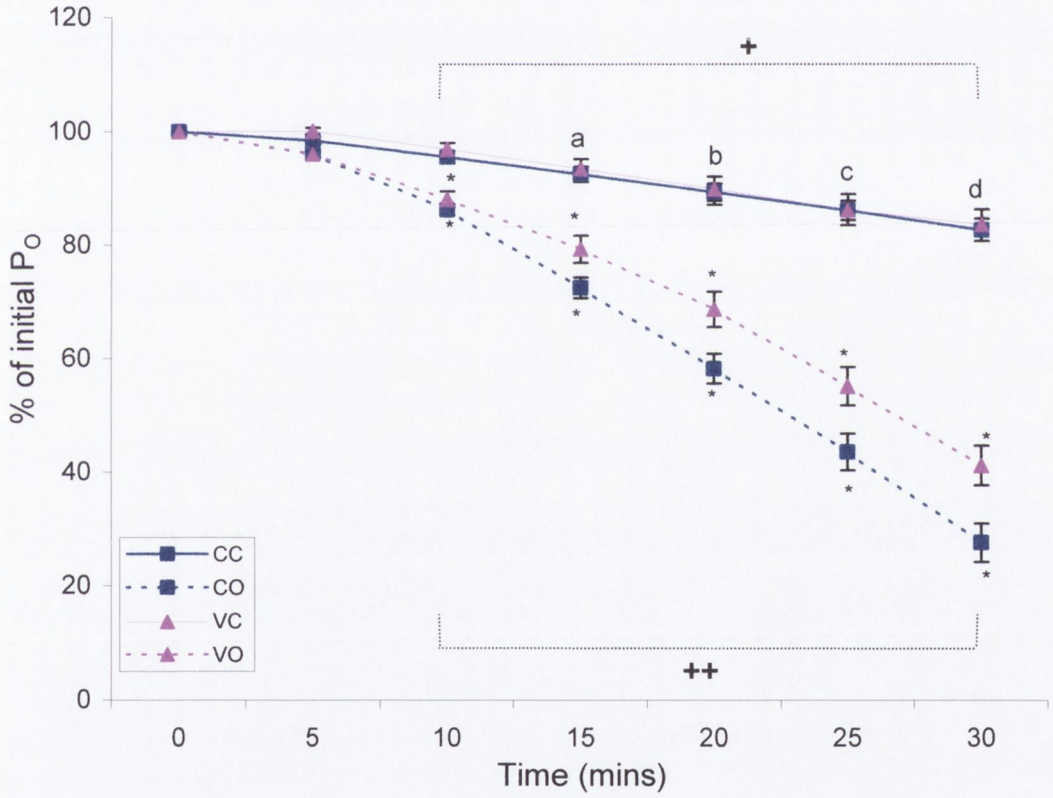


Figure 5.3.2. Force production was normalized to initial P_O in CON (n = 10) and VIT (n = 9) EDL during 30 mins incubation with H_2O_2 (CO & VO) or without (CC & VC).

+ CC and VC different from CO & VO ($P < 0.001$) * Different from preceding time points ($P < 0.05$)
++ VO different from CO ($P < 0.001$)

a Different from 0-5 mins ($P < 0.05$)
b Different from 0-10 mins ($P < 0.05$)
c Different from 0-15 mins ($P < 0.05$)
d Different from 0-20 mins ($P < 0.05$)

Therefore, results show that although vitamin supplementation had no effect on control conditions over time, the decline in P_O when muscles were exposed to a supramaximal

dose of H_2O_2 *in vitro* was delayed when compared to control muscles. This could reflect changes in antioxidant status and/or basal levels of ROS.

5.3.3.2. SOL incubation

Baseline P_O was 140.79 ± 7.67 kN.m^{-2} and 142.87 ± 9.20 kN.m^{-2} in CC and VC, respectively. An increase in P_O was observed in all groups at 5 mins ($P < 0.05$, Figure 5.3.3). Although P_O decreased in CC & VC over time ($P < 0.05$), force did not fall below initial values recorded at 0 mins. No significant differences in P_O were measured between CC and VC at any time point (Figure 5.3.3).

Prior to application of H_2O_2, P_O was 141.17 ± 9.16 kN.m^{-2} and 148.87 ± 9.41 kN.m^{-2} in CO and VO, respectively. After the initial increase in P_O at 5 mins, P_O in CO was reduced over time and was significantly lower than VO and CC at 20, 25 and 30 mins ($P < 0.001$). A potentiation of P_O in VO above VC was observed at 5 mins, and was maintained for 10 mins ($P < 0.05$). Although P_O decreased in VO over time ($P < 0.05$), force did not fall below initial values recorded at 0 mins, similar to that observed in both CC and VC.

Results demonstrate that the diet used in the present study had no effect on force production in control conditions, but slowed the reduction in P_O over time compared to control values when muscles were incubated in H_2O_2.

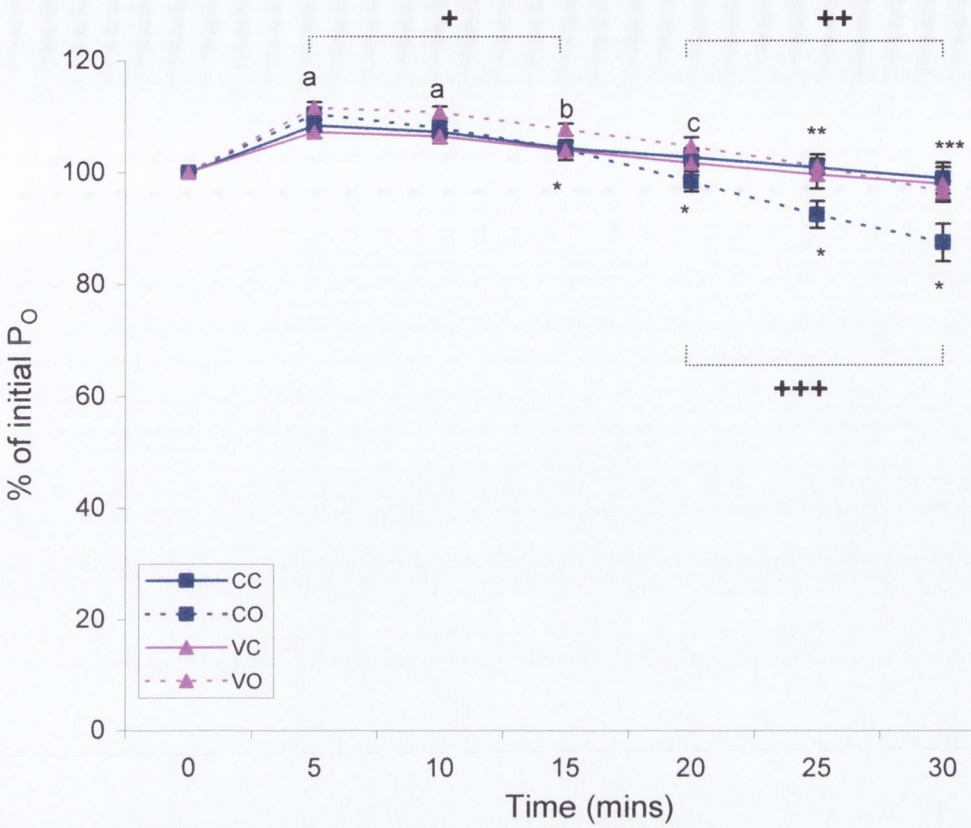


Figure 5.3.3. Force production normalized to initial P_O in CON (n = 10) and VIT (n = 9) SOL during 30 mins incubation with H_2O_2 (CO & VO) or without (CC & VC).

+ VO different from VC ($P < 0.05$)
++ VO different from CO ($P < 0.001$)
+++ CC different from CO at 20 mins ($P < 0.05$), 25 & 30 mins ($P < 0.001$)

a Different from 0 mins ($P < 0.05$)
b Different from 0 mins in VC and 0-5 mins in CC and VO ($P < 0.05$)
c Different from 5-10 mins in CC & VC, and 0-10 mins in VO ($P < 0.05$)

* Different from preceding time points in CO ($P < 0.05$)
** Different from 5-15 mins ($P < 0.05$)
*** Different from 5-25 mins ($P < 0.05$)

5.3.3.3. Diaphragm incubation

Baseline P_O was 161.65 ± 12.76 kN.m^{-2} and 136.89 ± 12.34 kN.m^{-2} in CC and VC, respectively. Although P_O significantly decreased over time ($P < 0.05$, Figure 5.3.4), no significant difference in force was observed between CC and VC at any time point.

Prior to application of H_2O_2, P_O was 168.06 ± 13.73 kN.m^{-2} and 154.09 ± 18.73 kN.m^{-2} in CO and VO, respectively. Over time, force decreased in both CO and VO ($P < 0.05$). However, this was time and diet dependent, such that the decrease in P_O over time was

significantly greater in CO compared to VO at 20, 25 and 30 mins ($P < 0.05$). No potentiation of force was observed in either CO or VO.

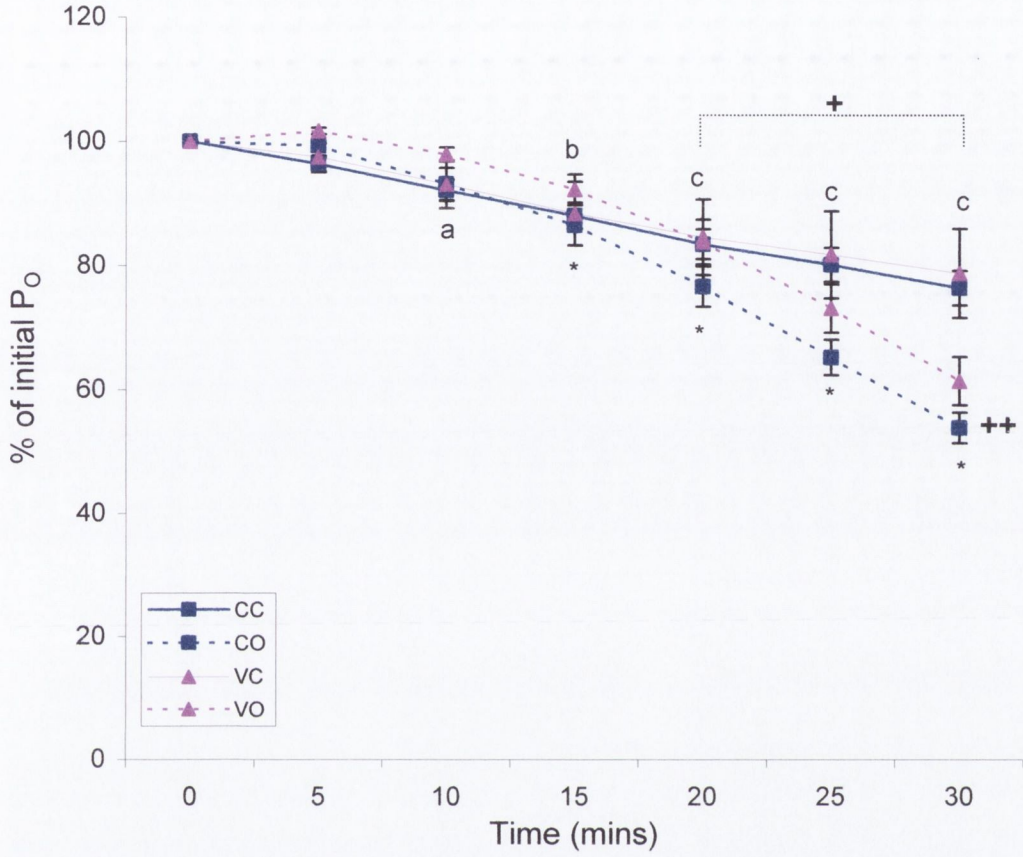


Figure 5.3.4. Force production normalized to initial P_O in CON (n = 11) and VIT (n = 11) diaphragm during 30 mins incubation with (CO & VO) or without (CC & VC) H_2O_2.

+ VO different from CO ($P < 0.05$)
++ CC different from CO ($P < 0.01$)

* Different from preceding time points in CO & VO ($P < 0.05$)

a Different from 0 mins ($P < 0.05$), except in VO
b Different from 0-5 mins in CC & VC ($P < 0.05$)
c Different from 0-10 mins in CC & VC ($P < 0.05$)

Similar to EDL and SOL, vitamin-treated diaphragm segments behaved the same as control segments under control conditions, but did not suffer the same force decrements over time in the presence of an oxidant challenge as control muscles.

5.3.4. Antioxidant assays

As previously discussed (Section 5.2.2.3), EDL and SOL muscles were removed from animals in CON & VIT *Dia* and were immediately snap-frozen, as opposed to CON &

VIT *HL* muscles, which underwent contractile function testing prior to storage. Results from the various antioxidant assays performed revealed no significant differences between EDL and SOL muscles from either the *HL* or *Dia* group. Therefore, these muscles were combined in both CON (n = 19) and VIT (n = 18). Significantly higher levels were measured in the liver compared to the other tissues for total SOD, CAT and GSH activity. Therefore, another scale was added on the opposite y-axis of these graphs, which applies to the liver only (Figure 5.3.5, 5.3.6 & 5.3.7). Differences in antioxidant levels between control tissue and vitamin-treated tissue can be seen in Appendix xi.

5.3.4.1. Total SOD activity

No significant diet-associated alteration in total SOD activity was observed in the diaphragm, EDL, heart or plasma between CON and VIT (Figure 5.3.5). However, SOD activity was 27% larger in VIT SOL compared to CON SOL ($P < 0.05$), and 55% larger in VIT liver compared to CON liver ($P < 0.001$).

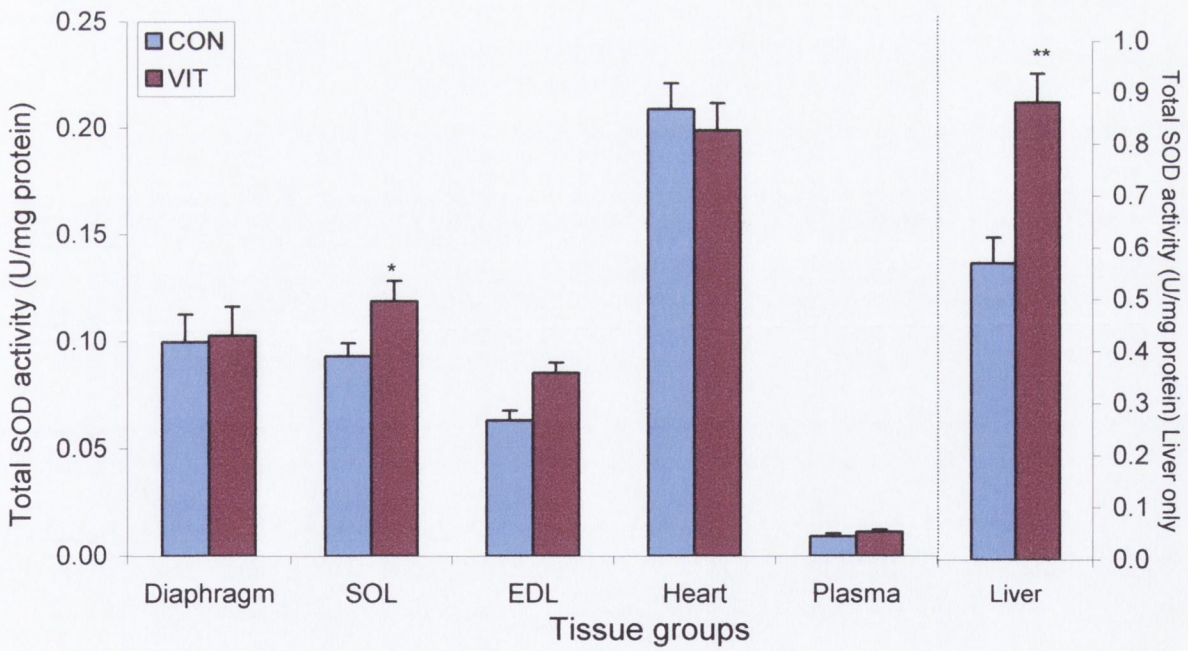


Figure 5.3.5. Total SOD activity (U/mg protein) in CON and VIT.

* Different from CON ($P < 0.05$)
** Different from CON ($P < 0.001$)

5.3.4.2. CAT activity

CAT activity was unchanged in the diaphragm, EDL or plasma between CON & VIT (Figure 5.3.6), although a 12% reduction was observed in VIT SOL compared to CON SOL ($P < 0.001$). CAT activity was 10% higher in VIT heart compared to CON heart ($P < 0.01$). Although CON and VIT livers had significantly larger levels of CAT when compared to all the other tissues (~70 times, $P < 0.001$), no difference was observed between CON and VIT groups (Figure 5.3.6).

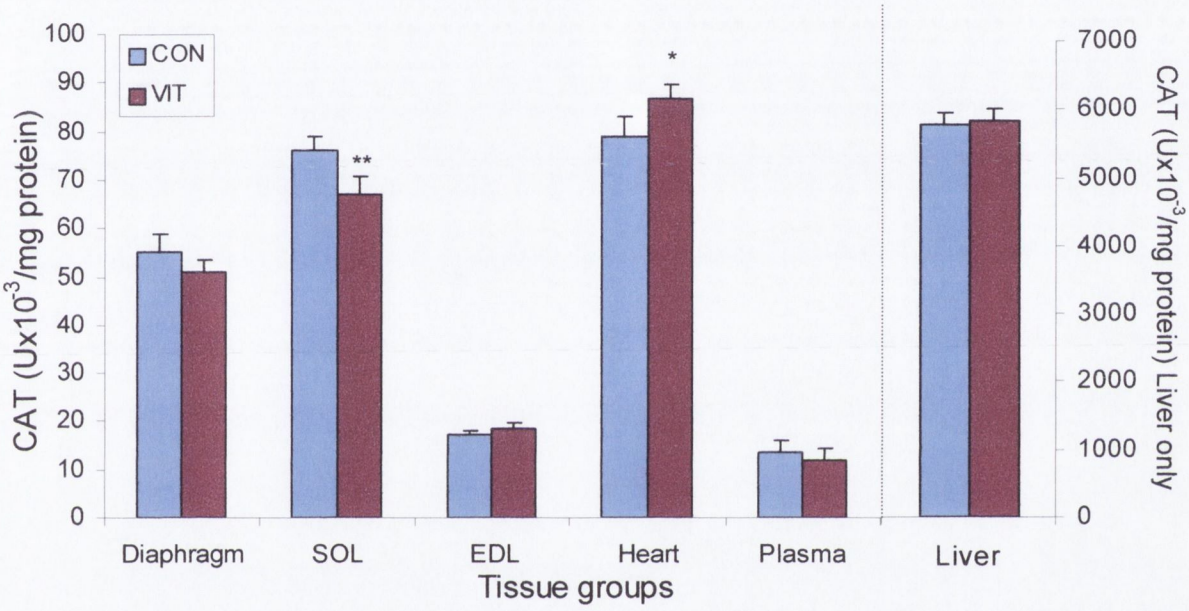


Figure 5.3.6. CAT activity ($U \times 10^{-3}$/mg protein) in CON and VIT.

* Different from CON ($P < 0.01$)
** Different from CON ($P < 0.001$)

5.3.4.3. Total glutathione concentration

Higher GSH concentrations were measured in VIT heart (32%, $P < 0.05$) and VIT plasma (350%, $P < 0.001$) compared to corresponding CON groups (Figure 5.3.7). CON and VIT livers had significantly larger GSH concentrations when compared to other tissues ($P < 0.001$) even though no diet-associated change in GSH concentration was observed.

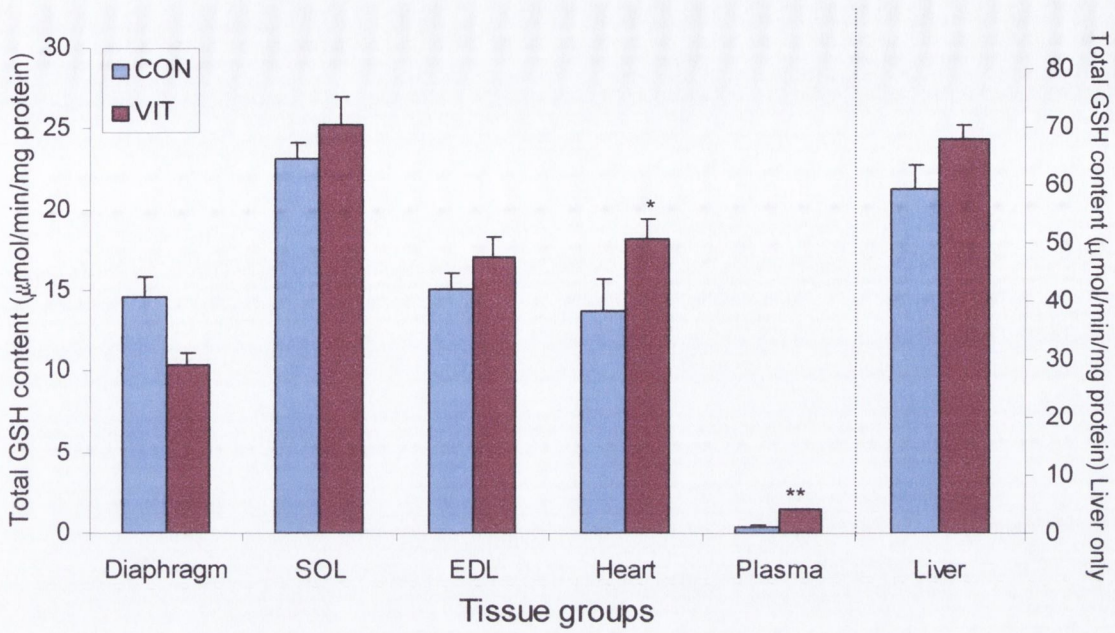


Figure 5.3.7. Total GSH concentrations (µmol/min/mg protein) in CON and VIT.

* Different from CON ($P < 0.05$)
** Different from CON ($P < 0.001$)

5.3.4.4. Vitamin C concentration

In VIT, vitamin C concentrations were larger in the liver (40%, $P < 0.001$), in the heart (20%, $P < 0.01$), in plasma (20%, $P < 0.05$) and in SOL (13%, $P < 0.01$) compared to CON groups (Figure 5.3.8). Vitamin C concentrations were highest in the liver in CON and VIT ($P < 0.001$).

5.3.4.5. FRAP assay

No significant diet-related changes in FRAP were observed in EDL, SOL, diaphragm or the liver between CON and VIT (Figure 5.3.9). However, FRAP values were 50% higher in VIT heart ($P < 0.01$) and 59% larger in VIT plasma ($P < 0.05$) compared to CON groups.

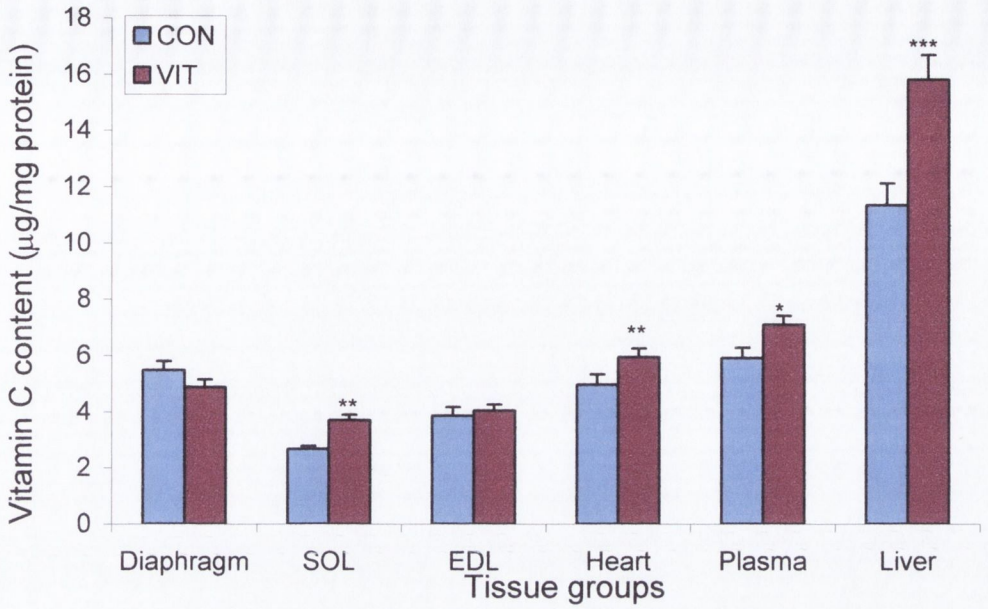


Figure 5.3.8. Vitamin C concentration (µg/mg protein) in CON and VIT.

* Different from CON ($P < 0.05$)
** Different from CON ($P < 0.01$)
*** Different from CON ($P < 0.001$)

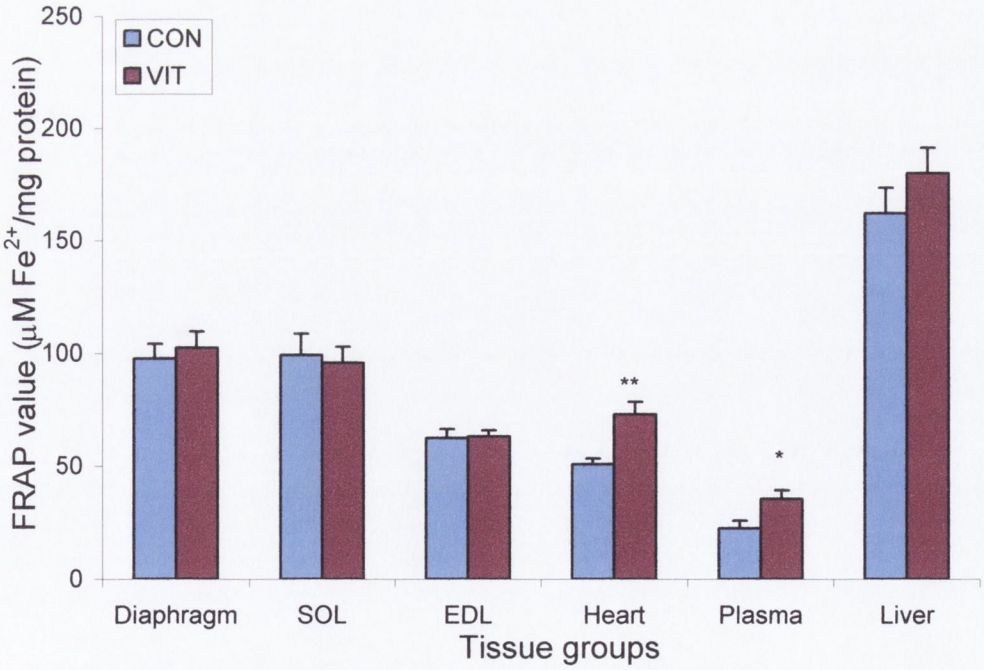


Figure 5.3.9. FRAP levels (µM Fe^{2+}/mg protein) in CON and VIT.

* Different from CON ($P < 0.05$)
** Different from CON ($P < 0.01$)

5.4. Discussion

5.1.4 Aims of the study

This study was carried out to determine if antioxidant supplementation using vitamin C and E could improve skeletal muscle antioxidant defences, such that its ability to resist an oxidative stress would be enhanced. A beneficial effect was considered to have taken place if antioxidant defences were higher in vitamin treated animals, and if a delay in the H_2O_2-induced reduction in force was observed compared to control muscles.

2 main hypotheses were tested in this study:

1. Long-term supplementation with supra-nutritional doses of vitamin C and E would lead to an overall enhancement of antioxidant defences in vitamin-treated animals compared to controls.
2. Vitamin-treated muscles would be able to resist H_2O_2-induced reductions in force over time better than control muscles due to increased antioxidant activity.

The major findings of the present study confirm that vitamin supplementation was beneficial as measured by:

1. A less prominent reduction in P_O over time in EDL, SOL and diaphragm muscles in the presence of a supramaximal dose of H_2O_2.
2. A diet-associated improvement in antioxidant status in skeletal muscle, liver, heart and plasma.

It should be noted that responses measured have been attributed to diet-related changes in antioxidant status. However, vitamin supplementation could have also lowered resting ROS levels in vitamin-treated muscle. As ROS and oxidative damage was not measured in this study, it cannot be completely ruled out that alterations in ROS did occur. However, this may only have been a contributing factor as opposed to the main cause of the results observed here.

5.4.2. Antioxidant defences

5.4.2.1. Antioxidant enzymes

Total SOD activity in liver and SOL, and CAT activity in heart was higher in VIT compared to CON in the present study, whereas plasma, EDL and diaphragm antioxidant enzyme activities remained unchanged with vitamin supplementation (Figure 5.3.5 & 5.3.6). These results agree with previous findings where a diet-related increase in Cu, Zn SOD activity in liver was observed, whereas activity levels remained unchanged in heart (Öztürk-Ürek *et al*, 2001). In the present study, an 8-week vitamin supplementation protocol had no effect on CAT activity in the liver (Figure 5.3.6). This has been observed previously (Jayachandran *et al*, 1996, Alper *et al*, 1998, Valls *et al*, 2003, Young *et al*, 2003). It has been shown that skeletal muscle SOD; CAT and GPx activities also remain unchanged following dietary intervention (Khassaf *et al*, 2003, Young *et al*, 2003).

Results from the present study suggest that vitamin-treated animals are better equipped to deal with $O_2^{\cdot-}$ and H_2O_2 production than controls, as measured by diet-associated increases in total SOD and CAT activity. In VIT animals, enhanced $O_2^{\cdot-}$ and H_2O_2 removal suggests that the potential for oxidative damage is reduced in these animals. This is likely to have had an effect on the response of skeletal muscle to an oxidant challenge, such that exogenous application of H_2O_2 was dehydrated more rapidly by a larger pool of antioxidant defences than in control muscles, thus reducing the decline in force measured in the presence of an oxidant challenge. This will be discussed further in section 5.4.4.

In the present study, total SOD and CAT activity was higher in heart and liver than in EDL, diaphragm and, to a lesser extent, SOL (Appendix xi). This has been shown previously where tissues with a high oxidative capacity (liver and heart) had significantly higher antioxidant enzyme levels than skeletal muscle (Jenkins *et al*, 1984, Jenkins, 1988, Sen, 1995, Laughlin *et al*, 1990, Alper *et al*, 1998). Differences in CAT, but not SOD, were observed among EDL, SOL and diaphragm muscles in the present study (Figure 5.3.5 & 5.3.6). This agrees with previous results where CAT activity in skeletal muscle was related to fibre type composition, whereas SOD activity was not (Ji *et al*, 1992).

5.4.2.2. Antioxidant molecules

Previous studies have demonstrated that long-term vitamin E supplementation results in an increase in α-tocopherol concentrations in plasma, skeletal muscle, liver and heart mitochondria and homogenates (Lass *et al*, 1999, Coombes *et al*, 2001, Lo Fiego *et al*, 2001, Sumien *et al*, 2003). Although diet-associated changes in vitamin E concentrations were not assessed in the present study, it is reasonable to assume that the supplementation protocol used resulted in significant incorporation of vitamin E into cellular and organelle membranes in vitamin treated animals. As vitamin E has been shown to stabilize the cell membrane against ROS-induced lipid peroxidation (Packer *et al*, 1991, Valk & Hornstra, 2000), it has been suggested that *in vivo* supplementation resulted in additional vitamin E availability at the SR membrane (Coombes *et al*, 2001). As proteins in the SR membrane have been implicated in ROS-induced reductions in force (Section 1.7.2 & 3.4.4), vitamin E incorporation into SR membranes is likely to have provided extra protection to these proteins against ROS-induced conformational changes and alterations in $[Ca^{2+}]_i$.

In the present study, vitamin C concentrations were ~40% higher in VIT liver compared to CON (Figure 5.3.8), whereas GSH remained unchanged (Figure 5.3.7). Previously, vitamin C supplementation (20mg/100g body weight for 60 days) did not result in a significant increase in vitamin C or GSH concentrations in liver (Jayachandran *et al*, 1996). However, the level of vitamin supplementation administered is likely to account for this difference, as doses were significantly larger in the present study and included vitamin E, which also has an antioxidant role and works in conjunction with vitamin C (Section 1.4). Vitamin C concentrations were also higher in VIT plasma compared to CON plasma in the present study (Figure 5.3.8). This agrees with previous findings where short- and long-term vitamin C supplementation significantly increased plasma ascorbate concentrations in humans (Polidori *et al*, 2004). Vitamin C concentrations were also significantly larger in VIT SOL compared to CON SOL, and to the author's knowledge this has not been shown before.

A significant reduction in lipid peroxides and improved resistance to lipid peroxidation *ex vivo* has been measured in plasma, heart and liver following vitamin supplementation (Jayachandran *et al*, 1996, Özürk-Ürek *et al*, 2001, Polidori *et al*, 2004). These results, in combination with those of the present study, suggest that dietary intervention increased ROS removal in vitamin treated animals due to greater levels of antioxidant enzymes

(SOD and CAT) and molecules (GSH and vitamin C) (Section 1.4). It is likely that these changes contributed to the P_O response observed in VIT muscles in the presence of an oxidant challenge.

In the present study, vitamin C concentrations in CON and VIT groups were highest in liver, followed by plasma, heart and skeletal muscles and GSH concentrations were larger in liver, followed by SOL, and then heart, EDL and diaphragm (Appendix xi). A similar vitamin C tissue-specific distribution has been reported previously in 6-month-old rats, and it has been shown that GSH concentrations are highest in liver, followed by heart; then slow-twitch muscle and finally fast-twitch muscle (Liu *et al*, 2000, Van der Loo *et al*, 2003). Tissue GSH concentration has been related to oxidative capacity, such that muscles with greater oxidative capacity have significantly higher levels of GSH than those with lower oxidative potential (Ji *et al*, 1992). Results obtained in the present study also demonstrated this relationship, where SOL had higher GSH concentrations than EDL and diaphragm (Appendix xi).

5.4.2.3. Antioxidant capacity

The FRAP assay offers an index of antioxidant defence, such that tissues with a higher FRAP value have a stronger reducing, and hence, antioxidant ability (Section 2.2.4, Benzie & Strain, 1996). As some antioxidants are not able to reduce Fe^{3+}, such as GSH, this test does not account for all antioxidants (Prior & Cao, 1999). However, a GSH assay was performed on CON and VIT tissues (Figure 5.3.7). In the present study, FRAP was higher in VIT plasma and VIT heart compared to CON groups, whereas no other diet-associated change was observed in any other tissue (Figure 5.3.9). No significant increases in the FRAP of plasma has been observed previously following acute supplementation with vitamin E and C (Choi *et al*, 2004). From these results, it would appear that a diet-related increase in FRAP, as measured in the present study, is dependent upon the duration of the supplementation protocol. The liver had the highest FRAP compared to all other tissues in CON and VIT (Appendix xi). A similar distribution has been measured previously (Runzer *et al*, 2002, Katalinic *et al*, 2005).

5.4.3. Isometric contractile parameters

In the present study, isometric contractile characteristics remained unchanged in EDL and diaphragm muscles following long-term vitamin supplementation (Table 5.3.3). However, relaxation velocity was significantly larger in VIT SOL compared to CON SOL. Vitamin supplementation lowers ROS levels in rat diaphragm and peripheral skeletal muscles (Duchesne *et al*, 1975, Chow *et al*, 1999, Factor *et al*, 2000, Gatellier *et al*, 2000). As ROS are essential for optimum contractile function in skeletal muscle (Reid *et al*, 1992), excessive removal can produce a reductive state that has been associated with a reduced contractile performance (Coombes *et al*, 2001). A reductive stress has been shown to inhibit the Ca^{2+}-ATPase pump via the reduction of regulatory sulfhydryls that must be oxidized for ATP hydrolysis and Ca^{2+} reuptake to proceed (Daiho & Kanazawa, 1994). As the CRB in SOL is already in a more reduced state compared to EDL and diaphragm muscle as a result of a higher antioxidant capacity (Figure 1.7, Laughlin *et al*, 1990, Plant *et al*, 2001), it is likely that the difference observed in twitch profiles between CON and VIT SOL only occurred as a result of a diet-induced shift in the CRB to a more reduced redox state, which is likely to have produced a reductive stress. Therefore, although a measure of the CRB was not determined in the present study, the vitamin supplementation protocol used increased overall antioxidant defences, which is likely to have shifted the CRB to a more reduced state in VIT muscles compared to CON muscles.

5.4.4. Incubation protocol

In the present study, a ~10% decay in P_O over time (30 mins) was measured under control conditions *in vitro* in EDL and diaphragm muscle, but not SOL. This result is consistent with contractile function tests performed in Chapter 3 (Figure 3.3.1 & 3.3.4) and previous studies using diaphragm sections and whole muscle (Diaz *et al*, 1998, Plant *et al*, 2001). Throughout the control incubation protocol, no significant difference in P_O was observed at any time point between CON and VIT EDL (Figure 5.3.2), SOL (Figure 5.3.3) and diaphragm muscles (Figure 5.3.4). These results show that despite the diet-related change in the twitch profile of SOL (Table 5.3.3), P_O was not significantly affected by a reductive stress, which has been previously observed in skeletal muscle following vitamin supplementation (Coombes *et al*, 2001)

When skeletal muscle proteins and lipids become oxidized by ROS, force production is reduced (Reid *et al*, 1993). This has been previously shown in this thesis (Chapter 3). ROS production is increased during exercise, which has been shown to cause muscle fatigue and skeletal muscle damage *in vitro* (Reid *et al*, 1992a & 1992b), Rajgura *et al*, 1994), whereas vitamins and ROS scavengers can protect against ROS-induced oxidative stress, thus reducing muscle fatigue and improving recovery of muscle force (Reid *et al*, 1992, Sen, 1995, Diaz *et al*, 1998, Khassaf *et al*, 2003). Exogenous application of antioxidants *in vitro* also protects against the reduction in force associated with skeletal muscle fatigue (Shindoh *et al*, 1990, Diaz *et al*, 1994, Khawli & Reid, 1994, Reid & Moody, 1994, Travaline *et al*, 1997) and vitamin supplementation has been shown to reduce oxidative stress as measured by a reduction in allantoin levels in plasma (Section 1.4, Naidoo & Lux, 1998), an improved resistance to lipid peroxidation *ex vivo* (Polidori *et al*, 2004) and a reduction in lipoperoxide levels following an exercise bout (Kanter *et al*, 1993, Schroder *et al*, 2000).

Theoretically it follows that an increase in antioxidant defences in skeletal muscle cells *in situ* should provide greater protection against a ROS-induced reduction in force *in vitro*. In the present study, a diet-related increase in antioxidant defences was observed, which is likely to have contributed to the response in P_O produced by VIT and CON muscles upon exogenous application of H_2O_2 *in vitro*, such that reductions in P_O over time were more prominent in all CON muscles compared to VIT muscles (Figure 5.3.2, 5.3.3 & 5.3.4). Although the supplementation protocol did not improve antioxidant defences in all VIT muscles to the same extent, it should be noted that other antioxidants not measured in the present study could improved with the dietary intervention used, which helps to why P_O was not as diminished over time in the presence of an oxidant challenge in VIT EDL, SOL and diaphragm muscle. These results agree with previous studies where prior supplementation with antioxidants led to an improvement in exercise tolerance during short duration leg exercise (Lands *et al*, 1999) and ameliorated muscle functional decrements subsequent to eccentric muscle contraction (Shafat *et al*, 2004). However, previous work has also shown that dietary antioxidants do not improve muscular performance (Sharman *et al*, 1971, Brady *et al*, 1979, Nielsen *et al*, 1999) and administration of high doses of antioxidants has been shown to impair muscle force production *in situ*, which has been attributed to a reductive stress (Coombes *et al*, 2001).

In the present study, it is likely that vitamin supplementation placed the CRB of skeletal muscle into an even more reduced state compared to control animals (due to an increased relaxation velocity in VIT SOL, Table 3.3.3), as a diet-associated increase in antioxidant defences leads to an increase in ROS, and hence oxidant, removal, thus reducing the CRB. This has been suggested previously (Coombes *et al*, 2001). Vitamin supplementation could have lead to a reduction of regulatory sulfhydryls on the DHPR and/or RYR, similar to that observed in the Ca^{2+}-ATPase pump in the presence of a reductive stress (Daiho & Kanazawa, 1994), which could ultimately delay any H_2O_2-induced effects on $[Ca^{2+}]_i$. Although the shift in the CRB was not enough to significantly compromise P_O at rest (Table 5.3.3), it is likely that application of an oxidant *in vitro* took longer to oxidize the CRB in VIT muscles and modify EC and contractile proteins enough to compromise force production to the same extent as in control muscles.

Corn oil contains a relatively high concentration of polyunsaturated fatty acids (PUFA) (Pan & Storlien, 1992). Due to the high levels of unsaturation in PUFA, these lipids are susceptible to ROS-induced oxidation, giving rise to the formation of lipid peroxides (Beltowski *et al*, 2000, Frenoux *et al*, 2001). Therefore, it could be argued that the diet administered to CON animals, which contained corn oil (Section 5.2.1), lead to a reduction in P_O due to an increase in lipid peroxidation as opposed to the vitamin diet enhancing contractile function. However, it has been shown that an increase in PUFA in the diet, in particular linoleic acid (the major component of corn oil), does not result in an increase in lipid peroxidation in the tissues of the rat (Valls *et al*, 2003). Also, the reductions in P_O under control conditions in CON muscles was similar to that in VIT and that measured in Chapter 3, which strongly suggests that excessive cell damage by lipid peroxidation, which would detrimentally affect contractile function, is not likely to have occurred in CON muscles in the present study.

5.4.5. Conclusions

The present study has demonstrated that long-term vitamin supplementation has a beneficial effect on antioxidant status in skeletal muscle, such that increases in antioxidant defences and less prominent reductions in force in the presence of an oxidant challenge were observed in vitamin-treated animals compared to control animals.

Chapter 6.

General Discussion

6.1. Aims of the thesis

Reactive oxygen species (ROS) are produced as a by-product of oxidative phosphorylation in mitochondria and via cellular enzyme systems (Section 1.2). ROS induce damage to cellular organelles and membranes, which can compromise cellular function (Ylä-Herttuala, 1999, Marnett, 2000, Stadtman & Levine, 2000). Antioxidant defences, consisting of enzymes (SOD, CAT and GPx) and low molecular weight molecules (GSH and vitamin C & E), prevent/reduce oxidative damage by lowering ROS concentrations (Section 1.4). In skeletal muscle, antioxidant defences are higher in muscles composed largely of type I and/or IIa fibres compared to those with a large percentage of type IIb fibres (Laughlin *et al*, 1990, Ji *et al*, 1992).

Under certain physiological states, such as exercise and aging, ROS production significantly increases and antioxidant defences can become depleted (Section 1.3, Clanton *et al*, 1999, McArdle *et al*, 2002), which produces an oxidative stress that is capable of reducing force in skeletal muscle (Barclay & Hansel, 1991, Reid *et al*, 1992a, Supinski *et al*, 1995). A strategy that has previously been used to ameliorate these force deficits is by increasing antioxidant defences (Diaz *et al*, 1994, Khawli & Reid, 1994). However, excessive ROS removal by antioxidant defences has been shown to compromise contractile function in skeletal muscle (Reid *et al*, 1993).

The continuous interaction between ROS and antioxidant defences, and the subsequent effects on skeletal muscle function, has led to the suggestion that a cellular redox balance (CRB) exists in skeletal muscle, which has a major influence over force production (Reid, 2001). Under resting conditions, antioxidant defences buffer ROS production adequately, such that the CRB is slightly reduced, which slightly limits maximum force production (Reid *et al*, 1992a & 1992b, Reid, 2001, Plant *et al*, 2001). Perturbations in the CRB via an increase in ROS production, as observed with exercise and aging, or ROS removal, due to improved antioxidant defences, can affect the redox status of the CRB, which modulates force. Throughout this thesis, a number of studies were designed to integrate this information into an investigation of oxidative stress in skeletal muscle.

As stated in Chapter 1, the main objectives of this thesis were to:

1. Determine the effect of aging and oxidative stress on skeletal muscle structure and function.
2. Assess age-associated alterations in antioxidant capacity and determine if a muscle-specific response occurred that depended on the fibre type composition of muscles.
3. Measure the effects of vitamin supplementation on skeletal muscle function in the presence of an oxidative stress, and assess if the supplementation protocol led to an improvement in antioxidant defences.

2 main sets of hypotheses were investigated. Firstly, it was hypothesized that aging in skeletal muscle would:

1. Cause a significant degree of muscle atrophy, independent of muscle type
2. Shift fibre proportions in favour of type I and/or IIa fibre types
3. Increase contraction times in both EDL and SOL muscles
4. Reduce maximum isometric tetanic force, irrespective of muscle type
5. Be more sensitive to an oxidant challenge *in vitro* due to already elevated levels of ROS
6. Alter antioxidant levels compared to younger muscles due to a combination of higher resting levels of ROS and increases in type I fibre proportion

Secondly, it was hypothesized that antioxidant supplementation would:

1. Increase tissue (skeletal muscle, liver and heart) and plasma antioxidant levels
2. Have no effect on resting isometric contractile characteristics
3. Preserve force in the presence of an oxidant challenge due to enhanced antioxidant defences

Following completion of Chapter 3 & 4, results confirmed that aging was associated with:

1. Absence of skeletal muscle atrophy
2. Absence of fibre atrophy

3. Increases in type I and/or IIa fibre proportions in EDL, SOL and PL
4. Age and/or muscle-specific alterations in antioxidant defences
5. An increase in twitch contraction duration of skeletal muscle, independent of muscle type (i.e. in both EDL and SOL)
6. A reduction in twitch force (P_t) in the SOL, but not EDL
7. A reduction in specific maximal muscle force (P_O), independent of muscle type
8. A greater reduction in P_O over time with age independent of muscle type
9. With H_2O_2 incubation, aged muscle showed a dramatically different profile of P_O over time, which was attributed to changes in antioxidant status, such that:
    i. The reduction in P_O was more dramatic in EDL compared to SOL, independent of age
    ii. A more prominent reduction in P_O was observed in young SOL compared to aged SOL
    iii. Decreases in P_O were similar in EDL independent of age

Changes in contractile parameters in Chapter 3 were attributed to the shifts in fibre type distribution in favour of type I and/or IIa fibre types, as well as alterations in antioxidant status with age observed in Chapter 4. The major findings of Chapter 5 were that vitamin supplementation was beneficial as measured by:

1. A less prominent reduction in P_O over time in vitamin-treated EDL, SOL and diaphragm in the presence of H_2O_2.
2. A diet-associated increase in antioxidant levels in skeletal muscle, liver, heart and plasma.

6.2. Antioxidant defence system

In this thesis, a section of the antioxidant defence system was measured in skeletal muscle under control conditions and under physiological states representing an oxidative stress (aging) and a condition in which antioxidant levels were expected to increase (vitamin supplementation). A comparison between these groups could help to clarify if any significant adaptation in antioxidant capacity occurred in response to aging or dietary intervention, as measured by significant deviations from control values. Also, a number

of different muscles and tissues were assessed to determine if a tissue-specific change in antioxidant status to these physiological states had occurred.

Data from this thesis has demonstrated that antioxidant defences (SOD, CAT, GPx, GSH and vitamin C) in skeletal muscle are affected with age. Age-associated changes in antioxidant capacity varied depending on the muscle being tested. For example, total SOD activity was higher in EDL with age, whereas it was reduced in PL and remained unchanged in SOL, GR and GW (Figure 4.3.11). In general, there was a hierarchy of antioxidant defence, such that SOL had higher levels of antioxidants compared to EDL and diaphragm muscle. However, age-associated shifts in fibre type distribution, in favour of type I and/or IIa fibres appeared to have had an influence on antioxidant levels with aging. Collectively, these results demonstrate that SOL had adapted to deal with the oxidative stress associated with aging, as measured by larger CAT, SOD and GSH in aged SOL compared to young and/or adult SOL, combined with the highest SOD, CAT, GPx and GSH in each age group (Chapter 4). Results also show that the other muscles tested appeared not to have adapted to age-related oxidative stress as well as SOL.

Vitamin supplementation was associated with an overall improvement in the antioxidant defence system, such that antioxidants (SOD, CAT, GSH, vitamin C) and measures of antioxidant power (FRAP) were larger in vitamin-treated skeletal muscle, organs and plasma compared to control values. Tissue-specific distributions were also evident, such that liver had significantly higher levels of antioxidants, followed by heart and SOL. These results demonstrate that supplementation has a positive effect on the antioxidant defence system in some tissues and also suggests that vitamin-treated animals are better equipped to deal with oxidative stress than controls. This was confirmed by *in vitro* functional tests where the maximum force producing ability of vitamin-treated muscles (EDL, SOL and diaphragm) was not diminished by an oxidative stress as rapidly as muscles from control animals (Chapter 5).

Collectively, the results obtained in this thesis demonstrate that the antioxidant status of skeletal muscle is modified by different physiological states and varies depending on muscle and tissue type. Again, the contribution of ROS to the results produced in this thesis should not be overlooked. Since fluctuations in antioxidant status and ROS concentrations both affect the CRB in skeletal muscle, it is likely that age and diet-

induced alterations in the antioxidants measured and changes in ROS (not assessed in this thesis) had a direct effect on skeletal muscle function (Discussed below).

6.3. Isometric contractile function

In vitro contractile function tests were performed in aged and vitamin-treated skeletal muscle to assess the effects of permanent oxidative stress and vitamin supplementation on isometric contractile parameters.

6.3.1. Twitch characteristics

In this thesis, alterations in a number of isometric contractile characteristics were measured with age, which were independent of muscle type, such that twitch profiles were prolonged and specific forces were lower in aged compared to young muscle (Table 3.3.2). Previous studies have shown that age-related alterations in contractility occur due to a combination of extensive ROS-induced conformational changes in the proteins involved in EC coupling (Delbono *et al*, 1995, Vogt, 1995, Diaz *et al*, 1998, Margreth *et al*, 1999, Winterbourn & Metodiewa, 1999), age-induced modifications to proteins involved in the cross bridge power stroke (Eddinger *et al*, 1986, Brooks & Faulkner, 1994, Thompson & Brown, 1999, Frontera *et al*, 2000, Trappe *et al*, 2003) and shifts in fibre type proportions in favour of type I and/or IIa fibres (Sugiura *et al*, 1992, Thompson, 1994, Thompson & Brown, 1999, Höök *et al*, 2001).

Indeed, in this thesis, significant increases in type I and/or IIa fibre distribution were observed in EDL (Figure 4.3.8), SOL (Figure 4.3.9) and PL (Figure 4.3.10) with age. Conversely, isometric contractile parameters were largely unaffected by long-term vitamin supplementation, where the only difference between CON and VIT was a longer relaxation velocity in SOL (Table 5.3.3). Previous work has shown that vitamin supplementation can induce a reductive stress by improving antioxidant defences and ROS removal (Coombes *et al*, 2001), which has been shown to delay Ca^{2+} reuptake to the SR (Daiho & Kanazawa, 1994). Since SOL has higher levels of antioxidants compared with EDL (Chapter 4 & 5), the CRB is already more reduced in this muscle (Figure 1.7). Therefore, vitamin supplementation may have further reduced the CRB in SOL to a point where muscle function was modified.

6.3.2. Incubation protocol

The ability to maintain P_O under control conditions over time in aged muscle was reduced compared to young muscle, independent of muscle type, such that a 30% reduction in force was observed after 30 mins in both aged EDL and SOL (Figure 3.3.1 & 3.3.4). Despite the diet-related change in the twitch profile of SOL (Table 5.3.3), P_O was not significantly affected over time, regardless of the possibility of a reductive stress that has been observed in skeletal muscle following vitamin supplementation (Coombes *et al*, 2001).

As discussed, skeletal muscle at rest maintains lower oxidant levels than are necessary for optimal contraction (Reid *et al*, 1992a & 1992b, Reid *et al*, 1993, Reid, 1996, Reid, 2001). Exogenous application of an oxidant *in vitro* (1-5mM) has previously been shown to increase force in skeletal muscle due to a shift in the CRB into a more oxidized state for force production (Figure 1.7, Andrade *et al*, 1998, Plant *et al*, 2001). However, due to alterations in ROS production and removal, it has been suggested that the CRB is likely to be oxidized in aged skeletal muscle (Figure 1.8, Bejma & Ji, 1999, Reid, 2001, Reid & Durham, 2001, Lamb & Posterino, 2003) and reduced in vitamin-treated muscle (Coombes *et al*, 2001). Therefore, incubation with a supramaximal dose of H_2O_2 produced different responses to that reported in control muscles in Chapter 3 (aging) and Chapter 5 (vitamin supplementation).

It has been suggested that exogenously applied H_2O_2 *in vitro* should shift the CRB in aged skeletal muscle into a more oxidized state more rapidly than young muscle due to the additive effect of higher endogenous ROS levels in aged skeletal muscle (Figure 1.8, Section 1.7). This would result in a more immediate reduction in force compared to that seen in young muscles. However, such a model is inconsistent with the results obtained in Chapter 3, where the decrease in P_O in the presence of H_2O_2 was less dramatic with age in SOL (Figure 3.3.5 & 3.3.6) and similar between young and aged EDL (Figure 3.3.2 & 3.3.3). These results have been attributed to differences in fibre type distribution and antioxidant status (Chapter 4). As demonstrated with age, SOL has higher CAT, SOD and GSH at 22 months compared to 2 months. These antioxidants are involved in the dismutation of $O_2^{\cdot-}$ and the dehydration of H_2O_2 to O_2 and H_2O (Equation 1.12 & 1.16). Therefore, aged SOL was able to resist H_2O_2-induced modulation to P_O more efficiently than young SOL, such that a larger reduction in P_O over time was observed in young

compared with aged SOL in response to an oxidant challenge. A similar effect could have taken place in aged EDL, where no significant difference was observed between the reductions measured in young and aged groups.

Although vitamin supplementation has been suggested to further reduce the CRB compared to control levels (Coombes *et al*, 2001), it would appear that if this had occurred in this thesis, the reductive stress produced by the supplementation protocol was not enough to significantly compromise P_O at rest (Table 5.3.3) or over time under control conditions in EDL (Figure 5.3.2), SOL (Figure 5.3.3) or diaphragm muscles (Figure 5.3.4). It also seems apparent that application of H_2O_2 *in vitro* took longer to oxidize the CRB in VIT muscles sufficiently enough to produce a decline in P_O to the same extent as control muscles. As antioxidant levels were higher in slow-twitch than fast-twitch muscles in this thesis, which has been observed previously (Jenkins, 1988, Laughlin *et al*, 1990, Ji *et al*, 1992, Lawler *et al*, 1993, Powers *et al*, 1994,), the CRB is slightly more reduced than fast-twitch muscles due to enhanced ROS scavenging/removal, such that force was maintained in slow-twitch muscles for longer in the presence of an oxidative stress compared to fast-twitch muscle (Figure 1.7, Plant *et al*, 2001). This was clearly demonstrated in Chapter 3 and 5, where EDL, a fast-twitch muscle, was more sensitive to oxidative stress than SOL, a slow-twitch muscle, which was independent of age or diet.

6.4. Age and diet-induced modifications of the CRC

6.4.1. Current CRC model

Collectively, the findings of this thesis support the concept that aging and vitamin supplementation induce sufficient perturbations to the CRB, such that contractile function at rest (aged muscle) and in the presence of an oxidant challenge (aged and vitamin-treated muscle) is significantly different compared to controls. It has been previously suggested that aged muscle at rest are at an oxidized position on the CRC (Figure 6.4.1, Reid & Durham, 2001), such that P_O is compromised compared to young muscle. This is consistent with the results observed in this thesis, where P_O was reduced in both young and aged skeletal muscle (Table 3.3.2).

According to the current CRC model (Figure 6.4.2), application of an oxidant should result in an immediate reduction in force, irrespective of the concentration of H_2O_2 applied. This was observed in aged muscle (Figure 3.3.1 & 3.3.4). However, the current CRC implies that the decline in force should be larger in aged compared to young muscle. In this thesis, similar decreases in P_O were seen in EDL, independent of age, and a greater decline in P_O over time was measured in young SOL compared to aged SOL (Chapter 3).

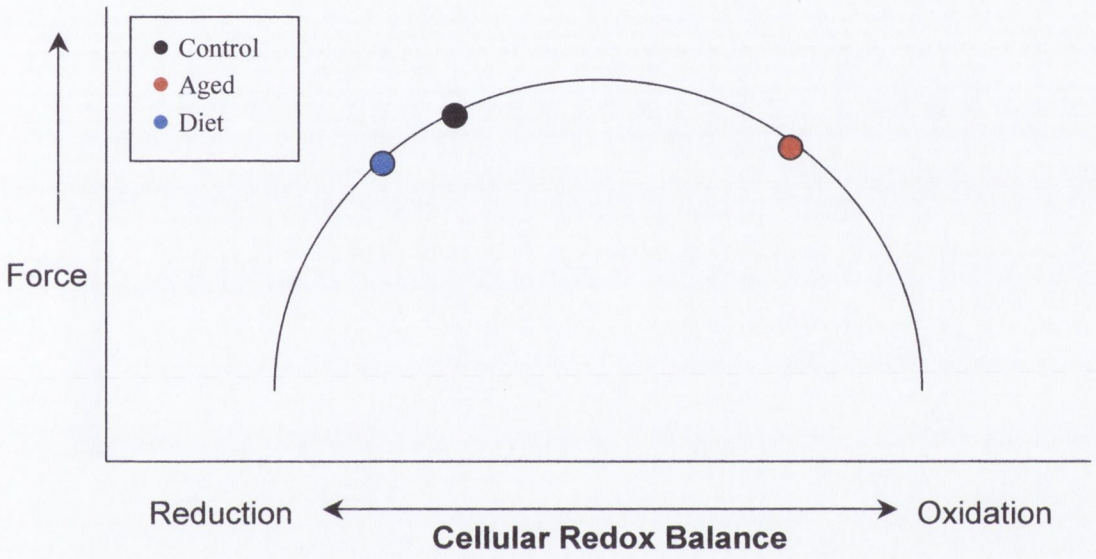


Figure 6.4.1. Model depicting the effects of age and antioxidant supplementation on the cellular redox balance and force in skeletal muscle.

It has also been previously suggested that vitamin-treated muscles, due to an increase in ROS removal via a greater antioxidant pool, have a more reduced CRB than control muscles, as measured by reductions in isometric strength and changes in twitch profiles following vitamin supplementation (Coombes *et al*, 2001). According to the current model, this position should produce a reduction in force at rest compared to control muscles (Figure 6.4.1). Although results presented in Chapter 5 show that P_O was not different between control and vitamin-treated muscles (Table 5.3.3), application of H_2O_2 did demonstrate that force did not decline as much over time in vitamin-treated muscles compared to controls (Chapter 5). This data fits the CRC model. However, it appears that the current model still needs updating to accommodate for the results presented in this thesis.

6.4.2. Updated CRC model

As aged muscle was able to resist the reduction in P_O over time induced by a supramaximal dose of H_2O_2 compared with young muscle (Chapter 3), the shape of the CRC needs to be altered. Results in Chapter 5 also demonstrate that, in the presence of an oxidant challenge, P_O in vitamin-treated muscles resisted ROS-induced reductions better than control muscle in EDL (Figure 5.3.2), SOL (Figure 5.3.3) and diaphragm (Figure 5.3.4). Therefore, it would seem that the CRC in these muscles has a plateau combined with a more gradual drop compared to control muscle (Figure 6.4.2). It was also demonstrated that alterations to the CRC are different between EDL and SOL as the reduction in force in the presence of an oxidant challenge was greater in EDL (composed largely of type II fibres, Figure 4.3.8) compared with SOL (composed largely of type I fibres, Figure 4.3.9).

From the results produced in this thesis, the ability to resist drops in P_O over time following application of a supramaximal dose of H_2O_2 has been attributed to an enhancement of the antioxidant defences. In Chapter 4, alterations in antioxidant status with age were partly credited to shifts in fibre type distribution in favour of type I and/or IIa. The change in antioxidant status measured in Chapter 5 resulted from the vitamin supplementation protocol used. Larger antioxidant defences can delay the impact of H_2O_2 on proteins involved in the contractile process by lowering its levels *in vitro* before it can cause reductions in force. However, a limitation to this interpretation is that it was not directly shown in this thesis. As discussed, these fibres have larger levels of the major antioxidant than type IIb fibres (Laughlin *et al*, 1990). This information has been summarized in Figure 6.4.2.

Therefore, from these results, a shift in the CRC can occur for a number of reasons, which reduces the severity that oxidative stress can have on P_O over time (Figure 6.4.2). Again, changes in ROS levels could also have played a role here, especially in muscles that did not show any age-related increases in antioxidant status. This could have led to a shift to the left of the CRC (Figure 6.4.2). However, since ROS were not measured at any stage throughout the course of this thesis, it is not possible to know exactly what effect they may have had on P_O in conjunction with the fibre type and antioxidant changes.

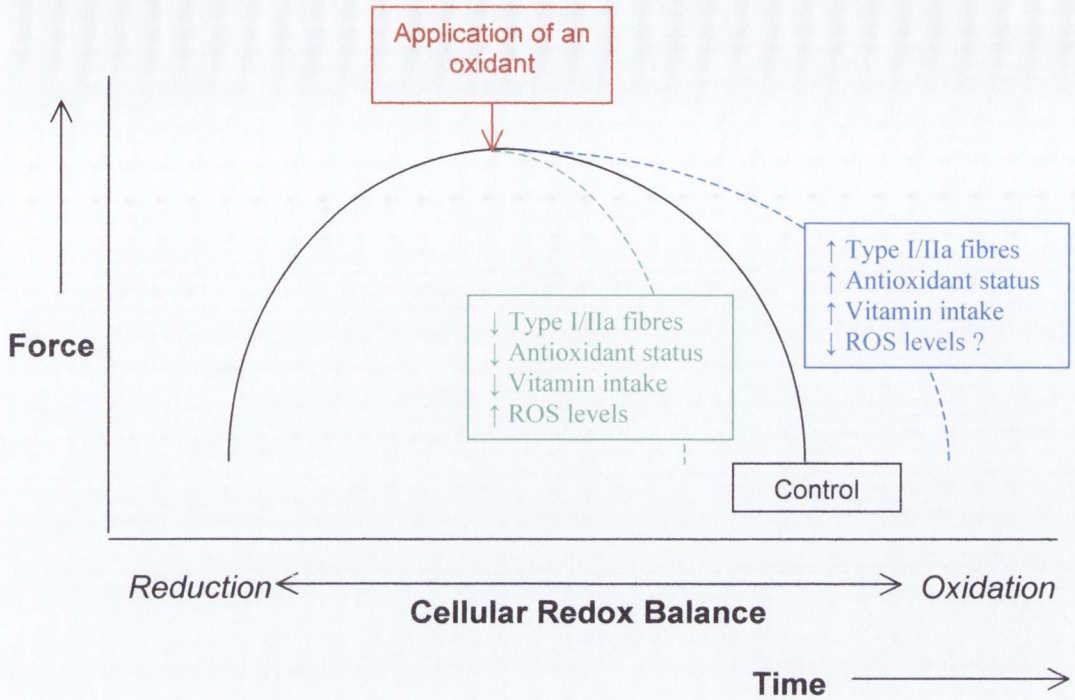


Figure 6.4.2. A modified CRC using results observed in this thesis.

Application of an oxidant (H_2O_2) induced reductions in force over time in all muscles tested throughout the course of this thesis. However, changes in fibre type distribution, antioxidant status and vitamin intake delayed the reduction in force in aged and vitamin-treated muscles compared to controls. This may have occurred due to enhanced ROS scavenging/removal both in the muscle bath and within the muscle itself although this was not assessed in this thesis.

6.4. Conclusions

From the results presented in this thesis, it can be concluded that aging has significant effects on skeletal muscle function such that force is reduced and twitch profiles are altered. Despite the absence of muscle and fibre atrophy, age-related changes in skeletal muscle composition were observed, as measured by significant shifts in muscle fibre type distribution in favour of type I and IIa fibre types in all muscles tested. More importantly, it was observed that H_2O_2-induced reductions in P_O were not accelerated in aged skeletal muscle, as was initially expected due to an additive effect of endogenously higher ROS levels.

Age-associated alterations in antioxidant status support the fact that aging is associated with a significant modification in the antioxidant defence system. SOL possessed the highest antioxidant defence levels to deal with the oxidative stress associated with aging as none of the other muscles demonstrated such dramatic changes in antioxidant levels

between young, adult and aged groups. Age-associated changes in CAT and SOD appeared to be related to shifts in the fibre type distribution in EDL, SOL and PL. These results could explain why the combination of age and an oxidant challenge caused a more dramatic fall in P_O in EDL compared to SOL over time.

Long-term vitamin supplementation was shown to have a beneficial effect on skeletal muscle, such that increases in antioxidant defences in skeletal muscle, organs and plasma, and a less dramatic reduction in force in the presence of an oxidant challenge in skeletal muscle were observed in vitamin-treated animals compared to control animals.

6.6. Future work

6.6.1. Antioxidants and age

In this thesis, aging was associated with an increase in type I fibres (Chapter 4). Since type I fibres have higher antioxidant levels than type II fibres (Laughlin *et al*, 1990), it was anticipated that antioxidant defences would be higher in any skeletal muscle showing age-related increases in type I/IIa fibre proportions. However, this was not the case. Therefore, changes in ROS production may also have influenced antioxidant levels with age. In order to clarify the exact cause of age-related alterations in antioxidant status, a direct measure of ROS levels or oxidative damage (as measured by the products of protein, lipid and DNA oxidative damage) should be taken. This will help to expand on the interpretation of the data already presented in this thesis and elucidate what other age-related changes in skeletal muscle could be contributing to the changes in antioxidants observed.

Mitochondria are a major source of ROS in skeletal muscle and, according to the mitochondrial theory of aging (Section 1.8), undergo significant structural and functional modifications with age. These changes further increase $O_2^{\cdot-}$ production due to ETC dysfunction and electron leakage. Therefore, does a concomitant increase in antioxidant levels occur with age in skeletal muscle mitochondria to prevent/reduce oxidative damage to the organelle? Analysis of antioxidant and ROS levels in mitochondrial fractions from different age and muscle groups similar to Chapter 4 could provide an insight into the ability of muscle cells to adapt to age-related oxidative stress.

Age-associated changes in total SOD, GSH and GPx were assessed in this thesis (Chapter 4). As discussed previously (Section 1.4), different isoforms of SOD and GPx exist, which are found in different cellular locations and act on different sources of ROS. An age-related increase in one isoform can occur despite a decrease in total activity/concentration, as has been shown previously in SOD (Oh-Ishi *et al*, 1995, Pansarasa *et al*, 1999 & 2000). Therefore, although a reduction in overall enzyme activity was measured in some antioxidants with age in this thesis, an increase in the activity of the individual isoforms may also have occurred. Analysis of these individual changes could give a more accurate insight regarding changes in ROS production by different cellular sources with age.

6.6.2. Contractile function

In aged muscle, force was reduced over time compared to young muscle (Chapter 3). It has been shown previously that contractile activity significantly increases ROS production *in vitro* (Reid *et al*, 1992a). This may have been higher in aged muscle compared to young muscle, thus ROS may have played a role in this drop in force over time. Therefore, resting ROS levels, and the changes contractile activity induces, should be investigated more thoroughly. Use of cytochrome C reduction and/or fluorescent probes would give an indication of ROS levels *in vitro* (Reid *et al*, 1992a & 1992b). If ROS levels were implicated, would antioxidant supplementation improve aged skeletal muscle performance under control conditions? In order to clarify this, a vitamin supplementation protocol, similar to that used in Chapter 5, could be performed to test this hypothesis.

Another important issue to develop is how the H_2O_2 incubation affected skeletal muscle. No measure of oxidative damage or oxidative stress in the muscle itself was been taken following incubation protocols. Therefore, assessment of a number of antioxidants involved in H_2O_2 dehydration (CAT, GPx & GSH) could be taken subsequent to the incubation protocol to establish whether these antioxidants lower H_2O_2 levels in the muscle bath, and ultimately reduce the impact of H_2O_2 on force.

The beneficial effect of the vitamin diet on skeletal muscle force in the presence of an oxidant challenge is without question (Chapter 5), but the precise mechanism by which these effects are achieved needs to be established. Diet-related increases in some

antioxidants were recorded, but other factors may also have been involved. Was the response produced in vitamin-treated muscles due to an increase in antioxidant defences alone or in combination with a reduction in resting ROS levels? A measure of ROS levels in skeletal muscle before and during contractile activity would clarify this. Was the ability to resist reduction in force over time in the presence of an oxidant challenge due to increased vitamin E incorporation into skeletal muscle membranes, resulting in increased cellular stability or did the vitamin diet lead to an overall reduction of sulfhydryls in proteins involved in contraction, thus taking longer for them to become oxidized?

Incorporating RYR and DHPR from vitamin-treated animals into lipid bilayers, followed by addition of sulfhydryl probes, would indicate whether reduction of these proteins was contributing to the response measured. The ability to resist lipid peroxidation *ex vivo* would give a good indication of an increase in cell stability. Ca^{2+} flux measurements, using SR vesicle preparations and patch clamp techniques, would indicate if alterations in the function of DHPR, RYR and the Ca^{2+}-ATPase pump were involved in the response measured in vitamin-treated skeletal muscle to H_2O_2 incubation, as the drop in force over time in the presence of an oxidant challenge has been partly attributed to a loss in Ca^{2+} homeostasis. Single fibre studies could also expand on whether the Ca^{2+} sensitivity of the contractile proteins was altered in these muscles fibres.

6.6.3. Vitamin diet

It is unclear whether vitamin C, vitamin E or the combination of both was responsible for the changes measured in contractile function in vitamin-treated muscle. Therefore, involving a larger variety of diet groups could help to establish which antioxidant plays the most significant role in the changes observed. Rats fed just vitamin E, just vitamin C and a combination of both could clarify which diet produces the greatest resistance to oxidative stress compared to control muscles. Also, would long- and short-term supplementation give similar results or does vitamin supplementation have to be long-term in order to see any benefits on contractile activity. As an antioxidant diet has previously been shown to reduce P_t and P_O compared to control muscle (Coombes *et al*, 2001), at which point does a reductive stress occur with supplementation? Using different vitamin doses may shed light on this point.

6.7. Clinical applications

The vitamin supplementation protocol used in this thesis was shown to have beneficial effects both on antioxidant status and on contractile performance in the presence of an oxidative stress. This may have important implications in the treatment of disease states in which oxidative stress is a cause or symptom of the disease.

Diaphragm weakness and fatigue are a common occurrence in many respiratory diseases, where a significant detrimental impact on the strength and endurance of respiratory muscles has been reported (Arora and Rochester, 1982, 1987). Considerable evidence links COPD with increased oxidative stress (Repine *et al*, 1997), where the chronically increased load imposed on the diaphragm due to a decrease in lung compliance may enhance the generation of ROS, further impairing muscle contractility (Section 1.8, Levison & Cherniack, 1968). Patients with CF are exposed to chronic oxidative stress due to an overproduction of ROS resulting from chronic activation of polymorph neutrophils and macrophages, in conjunction with impaired antioxidant status due to low dietary intake (Winklhofer-Roob, 1994, Winklhofer-Roob *et al*, 2003).

Chronic malnutrition is present in approximately 30% of patients with obstructive pulmonary disease, such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (Grievink *et al*, 1998, Linsdell & Hanrahan, 1998, Baker *et al*, 2001). In chronically malnourished animals, a significant reduction in P_t and P_O is observed in the diaphragm, and it has been shown that diaphragm strength in CF and COPD patients is compromised with increasing levels of malnutrition (Kelsen *et al*, 1985, Lewis *et al*, 1986, Lougna *et al*, 1986, Heijerman, 1993, Hart *et al*, 2004). As a result of this oxidant–antioxidant imbalance, oxidative protein modifications occur in the CF bronchial system, which further compromise lung function in these patients (Winklhofer-Roob *et al*, 1999). A high proportion of CF patients have some level of vitamin E deficiency, resulting from inefficient vitamin E absorption in the small intestine due to fat malabsorption (Winklhofer-Roob *et al*, 2003).

Therefore, increases in ROS production, combined with reduced antioxidant defences due to a low dietary intake, can drive this state of oxidative stress (Section 1.3). Antioxidant therapy could help to counteract the effects of ROS-induced damage to some degree in

these patients, partly restoring diaphragm muscle function by enhancing antioxidant defences, due to lower ROS muscle levels (Reid *et al*, 1993, Oba *et al*, 1996, Murrant *et al*, 1999).

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IX. *Appendices*

Appendix i.

List of chemical suppliers

The following is a list of the chemical suppliers that were used to buy materials from for the course of this thesis.

BD Vacutainer Systems	BD Vacutainer Systems., Preanalytical Solutions, Belliver Industrial Estate, Plymouth PL6 7BP, UK.
BioRad	BioRad Laboratories Ltd., Bio-Rad House, Marylands Ave., Hemel Hempstead, Hertfordshire HP2 7TD, UK.
Labsystems	Labsystems Oy, Research Technologies Division, P.O. Box 208 FIN-00811, Helsinki, Finland
Lennox	Lennox Laboratory Supplies, John F Kennedy Drive, Naas Road, Dublin 12, Ireland.
Mazola	Bestfoods UK Ltd., Claygate House, Esher Surrey, KT10 9PN, UK.
Tissue-Tex O.C.T.	Raymond A. Lamb Ltd., Parkview Industrial Estate, Lottbridge Drove, Eastbourne, East Sussex, BN23 6QE, UK.

Sagatal	Rhóne Mérieux Ltd., Spire Green Centre, Harlow, Essex CM19 5TS, UK.
Sigma	Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset BH12 4QH, UK.
Silkam®	B|Braun Surgical GmbH, D-34209 Melsungen, Germany.
Whatman®	Whatman International Ltd., Maldstone, England.

Appendix ii.

The constituents of the rat chow diet used in Chapter 3, 4 and 5 are outlined below. Corn oil components used in the dietary intervention study are also provided.

1.1. Rat chow constituents

1.1.1.Diet used in Chapter 4

Manufactured by Red Mills Ltd for Trinity College Dublin Bioresources Unit, 1994.

Specification for laboratory rat/mouse cubes

Crude Protein	%	17.00
Crude Oil	%	3.50
Crude Fibre	%	7.00
Crude Ash	%	8.00
Moisture	%	14.00
Digestible energy	(Mj/Kg)	11.80
Calcium	%	1.30
Phosphorous	%	0.80
Salt	%	0.70
Vitamin A	(iu/Kg)	9,000
Vitamin D3	(iu/Kg)	2,000
Vitamin E	(iu/Kg)	60
Lycine	%	0.85
Methionine	%	0.32
Threomine	%	0.64
Vitamin A	u/kg	9,000.00
Vitamin D3	iu/kg	2,000.00
Vitamin E	iu/kg	40.00
Selenium	mg/kg	0.20

Ingredients used include: Herring meal, oats, wheat, soyabean, molasses, sunflower, minerals and vitamins.

1.1.2. Diet used in Chapter 3 and 5

Manufactured by Red Mills Ltd for Trinity College Dublin Bioresources Unit, March 2001. New Diet is made without animal based proteins

Specification for laboratory rat/mouse cubes

Crude Protein	%	17.00
Crude Oil	%	3.50
Crude Fibre	%	8.50
Crude Ash	%	7.50
Moisture	%	14.00
Digestible energy	(Mj/Kg)	11.80
Calcium	%	1.20
Phosphorous	%	0.56
Salt	%	0.85
Magnesium	%	0.26
Copper	mg/kg	14.00
Iron	mg/kg	30.00
Manganese	mg/kg	100.00
Zinc	mg/kg	60.00
Vitamin A	(iu/Kg)	9,000
Vitamin K	mg/kg	6.00
Folic Acid	mg/kg	6.00
Nicotinic Acid	mg/kg	30.00
Pantothenic Acid	mg/kg	20.00
Vitamin D3	(iu/Kg)	2,000
Vitamin E	(iu/Kg)	60.00
Riboflavin	mg/kg	8.00
Cobalt	mg/kg	1.00
Iodine	mg/kg	1.50
Lycine	%	0.85
Methionine	%	0.32
Tryptophane	%	0.22
Threonine	%	0.65
Vitamin A	u/kg	9,000.00
Vitamin B12	mg/kg	0.012
Thiamin	mg/kg	5.00
Pyrodoxin	mg/kg	5.00

Biotin	mg/kg	0.030
Vitamin D3	iu/kg	2,000.00
Vitamin E	iu/kg	40.00
Selenium	mg/kg	0.10

Ingredients used include: Herring meal, oats, wheat, soyabean, molasses, sunflower, minerals and vitamins.

1.2. Mazola corn oil constituents

Listed below are the constituents of the corn oil used in the control diet in the antioxidant intervention diet study in Chapter 5.

Nutritional Information	Average values per 100ml
Energy	3409kJ/829kcal
Protein	Nil
Carbohydrate	Nil
Of which:	
sugars	
Fat	92.1g
Of which	
saturates	13.3g
monosaturates	27.5g
polyunsaturates	47.3g
Fibre	nil
Sodium	nil

Appendix iii.

Aim: The aim of this pilot study was to investigate if approximately 45 mins incubation in Krebs solution at room temperature had any effect on isometric contractile force (P_t & P_O) in diaphragm strips.

Animals: Diaphragm sections were removed from 10 male Wistar rats as described in Section 2.2.1.

Protocol: A strip from one hemidiaphragm was tested according to the protocol outlined in Section 2.2.2.4 while the other segment remained in Krebs solution for the duration of the initial experiment. The second segment was then tested to investigate whether any significant reductions in diaphragm viability had occurred, as measured by significant reductions in P_t and P_O.

Results: Results presented below show that no significant differences were observed in isometric strength between diaphragm segments following 45 mins incubation in Krebs solution (Figure 1).

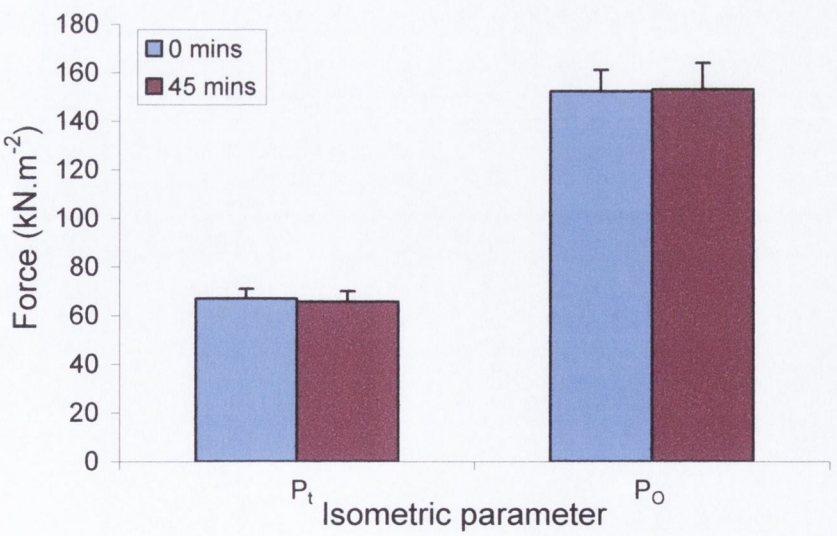

Figure 1. The effect of 45 mins incubation on P_t and P_O in diaphragm segments.

Conclusion: Diaphragm sections were unaffected by the 45 min incubation in Krebs solution.

Appendix iv

Aim: The aim of this pilot study was to establish an optimum stimulation frequency for the EDL and SOL in order to ensure that all muscle fibres were being recruited maximally.

Animals: EDL and SOL muscles from male Wistar rats were used in this study (n = 10 muscles/group) and were prepared as discussed (Section 2.2.2).

Protocol: A force-frequency curve was constructed for each muscle from records of the force exerted during periods of stimulation at increasing frequencies initially with the stimulus isolator, followed by a increasing settings on the amplifier.

Results: The force-frequency curves for EDL and SOL can be seen in Figure 1 & 2, respectively. Maximum force was produced by EDL muscles at a frequency of 70Hz and an amplifier setting of 4, whereas a similar response was measured in SOL at a frequency of 50Hz.

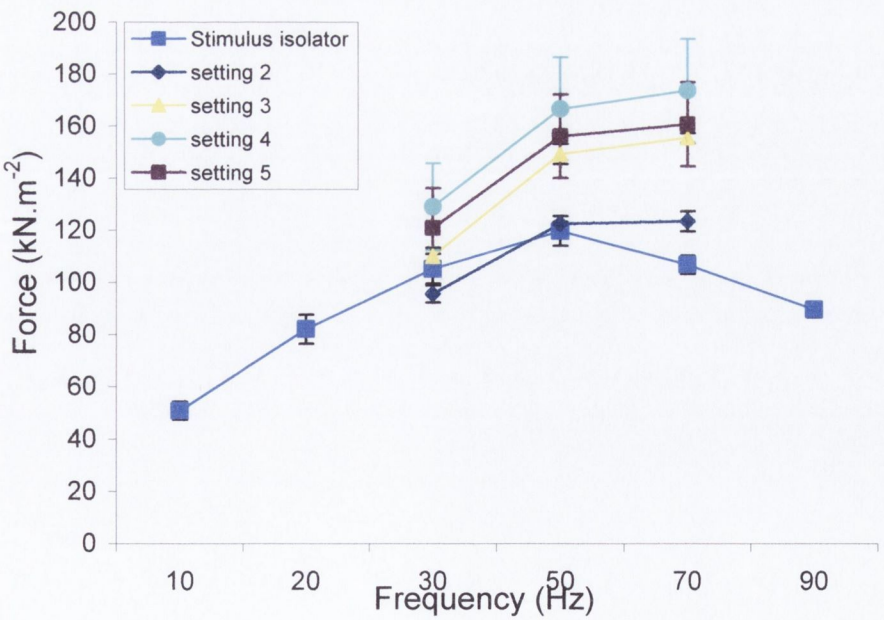

Figure 1. Force-frequency relationship for EDL.

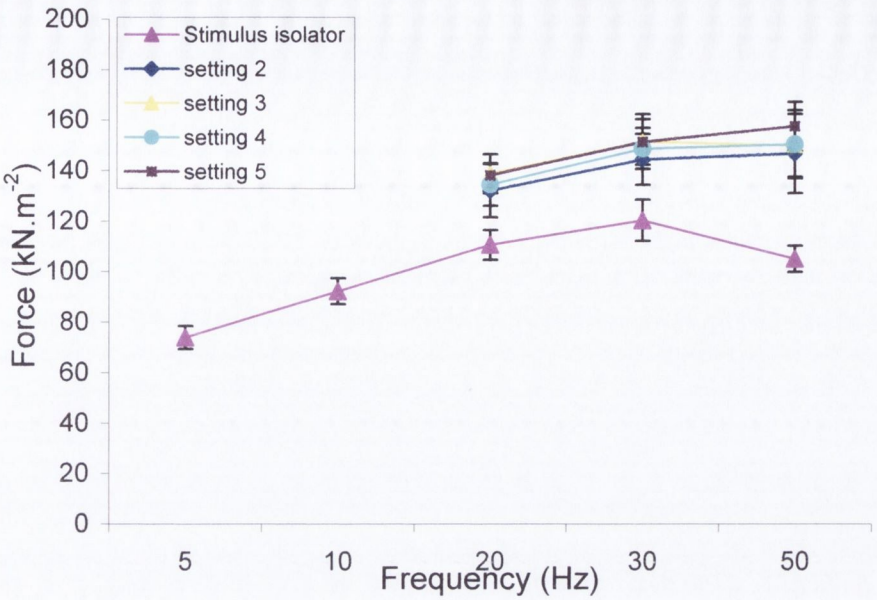

Figure 2. Force-frequency relationship for SOL.

Conclusion: The results show that in order to ensure complete recruitment of all muscle fibres, EDL must be stimulated at 70Hz and SOL at 50 Hz at an amplifier setting of 4.

Appendix v.

Aim: The aim of this pilot study was two-fold:

1. To establish the effect of various concentrations of H_2O_2 on force production in diaphragm strips
2. Produce a similar response to incubation with H_2O_2 as observed in hind limb muscle

Animals: Diaphragms from male Wistar rats were used in this pilot study (n = 11 muscle segments/group) and were prepared as discussed (Section 2.2.2).

Protocol: Concentrations of H_2O_2 tested in the pilot study ranged from 20mM to 160mM. Diaphragm sections were allocated into 1 of 6 H_2O_2 treatment groups: 0mM (control), 20mM, 40mM, 80mM, 120mM and 160mM H_2O_2. Following establishment of L_O, muscles were tetanically stimulated for 1s. Following the first tetanic contraction; H_2O_2 (30% stock solution) was added directly to the bathing medium, which was gently mixed to ensure even H_2O_2 distribution. Muscles were incubated for 30 mins and P_O was measured every 5 minutes. Each muscle section was used as its own control. P_O was expressed as a % of the initial value recorded at 0 mins in both control and treated muscle sections.

Results: The effects of H_2O_2 on P_O were dose-dependent, such that the highest concentration of H_2O_2 (160mM) caused the largest and most rapid reduction in P_O during the 30-minute incubation protocol (Figure 1).

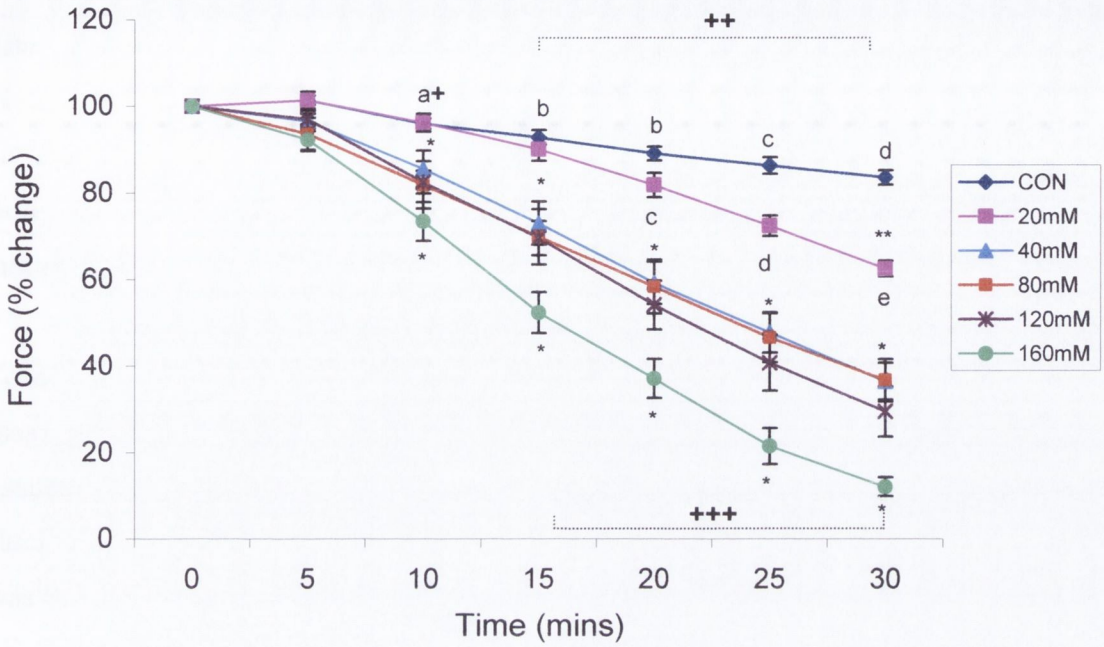

Figure 1. Dose-response relationship of H_2O_2 on P_O in diaphragm segments (n = 8-10 sections per group). Force production was normalized to the initial P_O during 30 mins incubation with H_2O_2.

* Different from all preceding time points ($P < 0.05$)

** Different from CON ($P < 0.001$)

\+ CON & 0.125mM different from 1mM ($P < 0.001$)
++ CON & 0.125mM different to all concentrations ($P < 0.001$)
+++ 1mM different from 0.25 & 0.5mM ($P < 0.001$) and 0.75mM ($P < 0.05$)

a Different from 0 mins ($P < 0.05$)
b Different from 0-5 mins ($P < 0.05$)
c Different from 0-10 mins ($P < 0.05$)
d Different from 0-15 mins ($P < 0.05$)
e Different from 0-20 mins ($P < 0.05$)

Conclusions: From this set of experiments, the response of P_O in diaphragm segments to 30 mins incubation with H_2O_2 showed a similar profile at 20mM H_2O_2 to that recorded in the hind limb muscle of 2-month-old rats in Chapter 3). Therefore, this concentration was used to test the effects of H_2O_2 on force production in the diaphragm in Chapter 5.

Appendix vi.

Aim: Two methods were used in this thesis to measure total SOD activity. The first involved a spectrophotometric assay, as used in Chapter 4 to analyse total SOD activity in rat skeletal muscle taken from animals of different ages. A microtitre chemiluminescence assay was used for analysis of total SOD activity in rat tissue samples collected from a dietary intervention study as described in Chapter 5.

Comparison of the results produced by the spectrophotometric and chemiluminescence assay revealed 10-fold differences in SOD activity (Figure 4.3.11 & 5.3.6). Since the tissues were not the same for each assay, further experimentation was required to elucidate whether this difference in activity reflected a real variation in enzyme levels or was due to an inherent property of the assay techniques used.

Protocol: Samples that were previously run on the spectrophotometric assay in Chapter 4 were analysed using the chemiluminescence assay, as described (Section 2.2.4.4). If variations in enzyme activity were due to enzyme differences between the tissues used in Chapter 4 & 5, activity levels measured using the chemiluminescence technique should duplicate those produced earlier by the spectrophotometric assay.

Results: Results produced by samples run on the chemiluminescence assay can be seen in Figure 1.

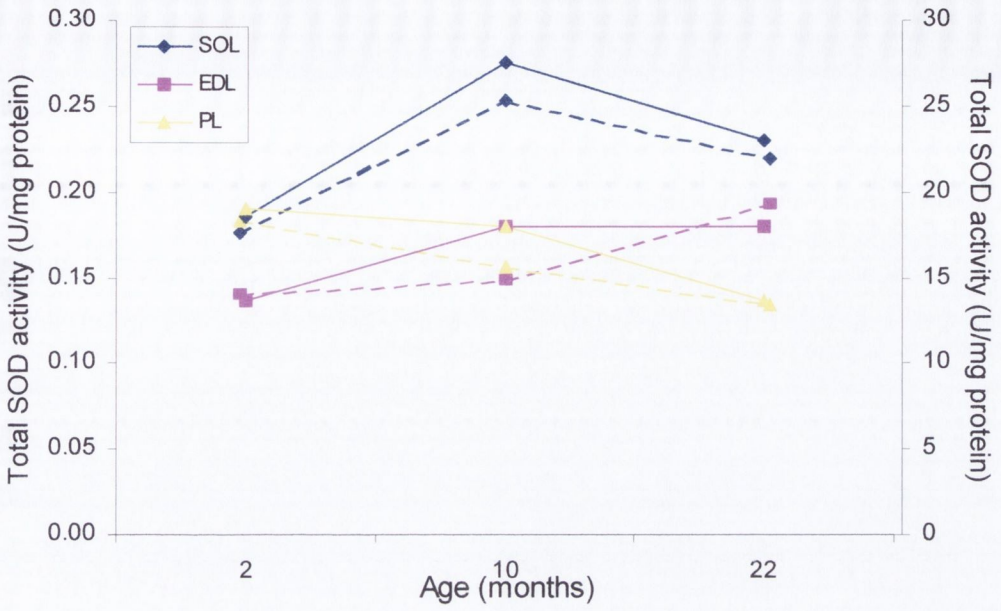

Figure 1. Total SOD activity was measured by chemiluminescence.

Solid lines represent results obtained from the chemiluminescence assay and dashed lines represent the spectrophotometric assay. The axis on the right-hand side refers to the spectrophotometric assay.

Results show that the variation in total SOD activity is attributed to an inherent property of the assay techniques as opposed to variation in enzyme levels as total SOD activity was similar to that produced in Chapter 5, as opposed to that measured earlier with the spectrophotometric technique. Although the number of samples tested was small (n = 2), muscle and age-dependent differences were observed that mirrored the response obtained by the spectrophotometric assay.

Conclusion: The differences measured in total SOD activity in Chapter 4 & 5 were attributed to an inherent property of the assay technique as opposed to a real variation in enzyme levels.

Appendix vii.

The following graphs were produced by standards and samples at different absorbencies in a range of antioxidant assays described in Chapter 4 and 5.

7.1. Protein standard

A linear relationship ($r^2 = 0.9973$) was produced by protein concentration (μg/ml) versus absorbance at 600nm (Figure 1). Sample protein concentration was determined from the equation of the line produced from the standard.

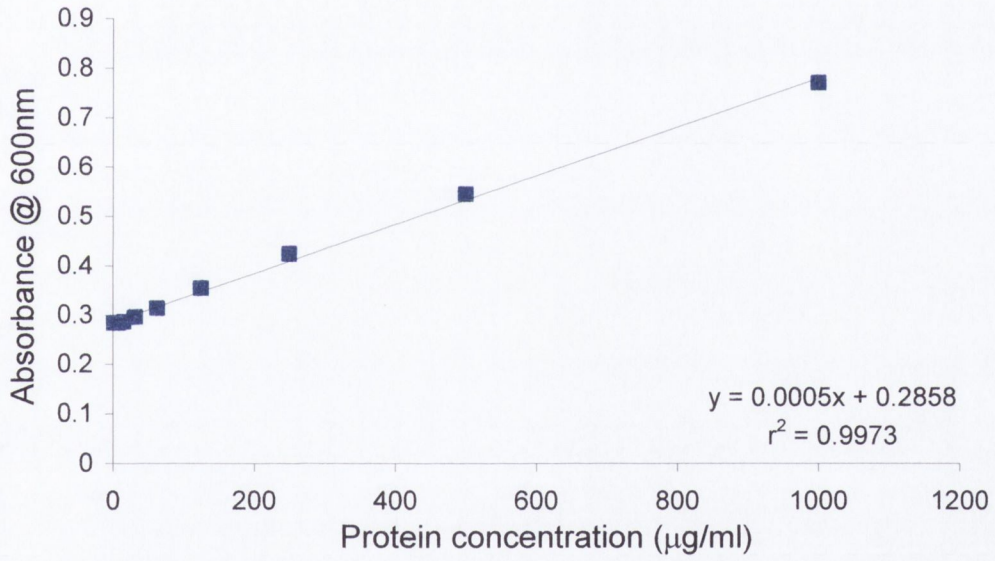

Figure 1. An example of the linear relationship produced by absorbance at 600nm versus protein concentration (μg/ml).

7.2. Total SOD activity

Figure 2 shows the relationship produced between various dilutions of sample supernatant and the change in absorbance at 560nm over time. The slopes of each line were plotted against their respective protein concentrations to produce a % inhibition curve, which was then used to determine total SOD activity.

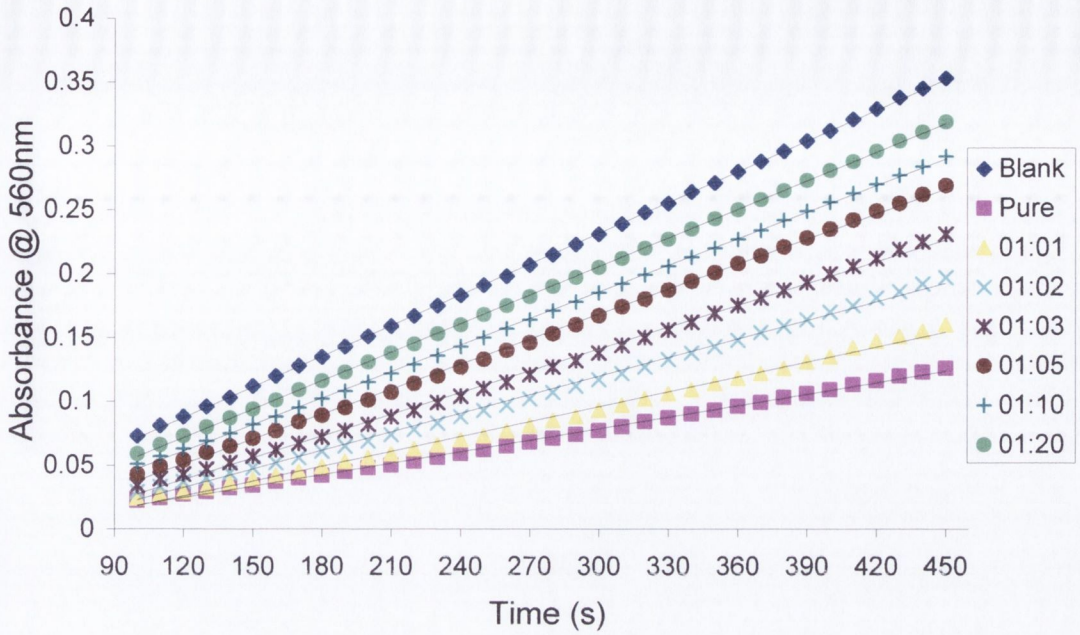

Figure 2. Example plot of the absorbance at 560nm versus time for the blank and serial diluted samples during measurement of total SOD activity.

7.3. Total glutathione concentration

Figure 3 represents the change in absorbance at 412nm over time in samples being assessed for total glutathione concentration. The slope of each line was used to calculate glutathione concentration according to the standard relationship produced in Figure 4.

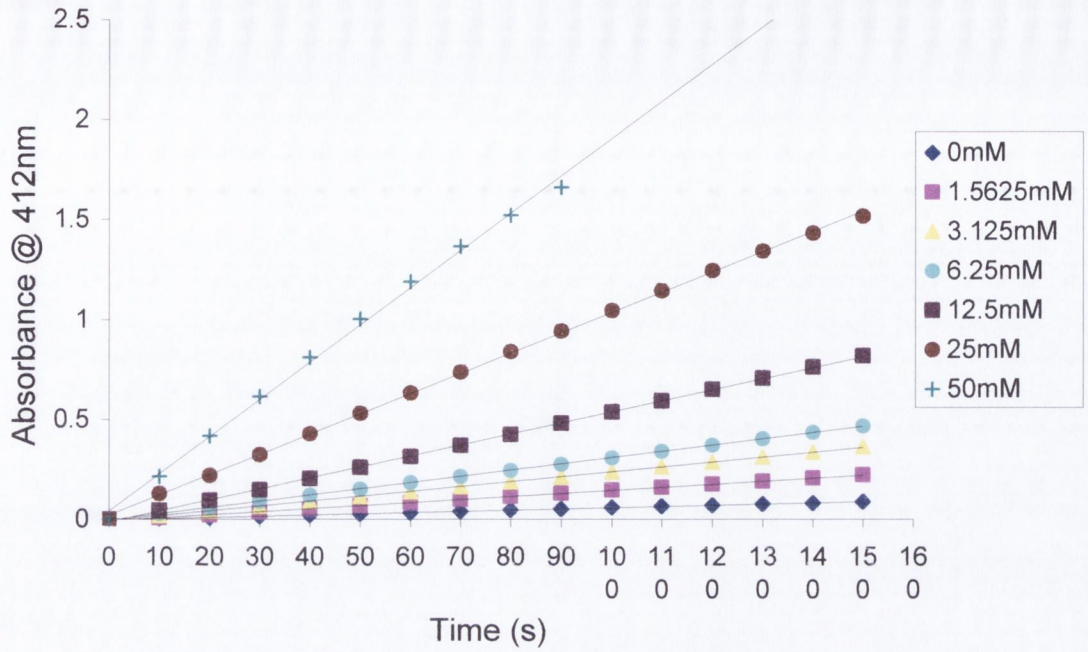

Figure 3. Example of the linear relationship of absorbance at 412nm produced by GSH standards against time.

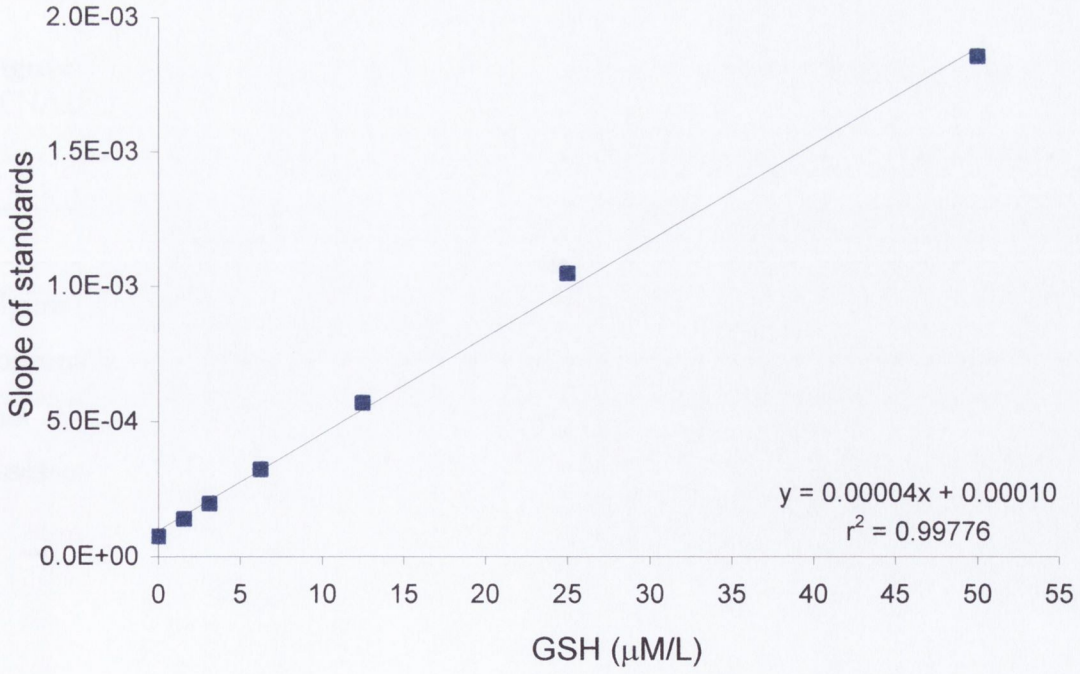

Figure 4. Linear relationship produced between the slopes measured by the absorbance of GSH standards and GSH concentration.

7.4. Total glutathione peroxidase activity

The relationship between the change in absorbance of samples over time with or without a substrate for GPx (cumene hydroperoxide) was plotted in Figure 5. The change in absorbance was calculated from the slopes of the lines produced.

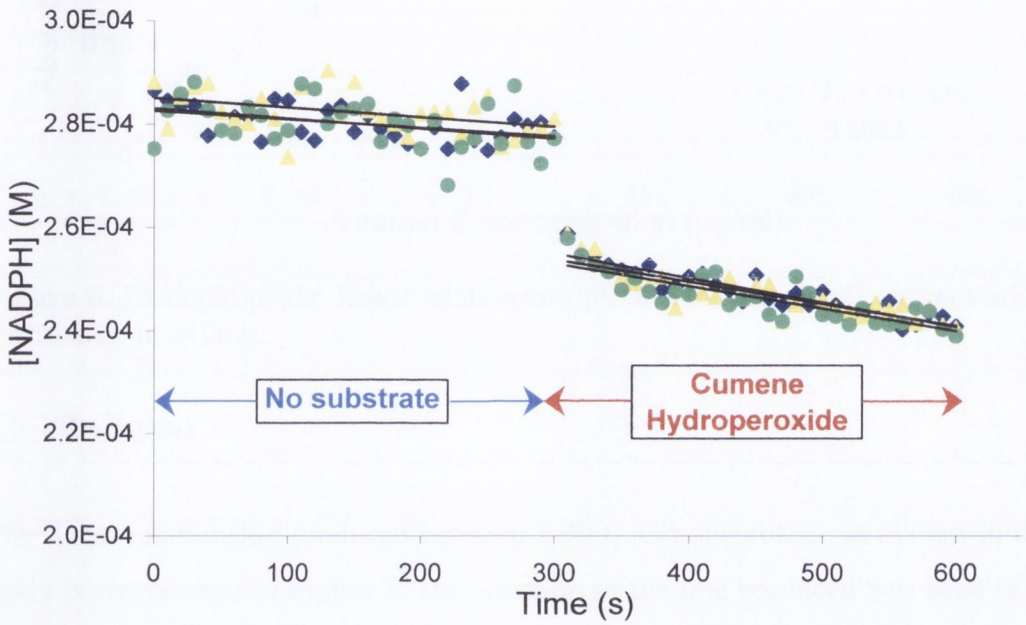

Figure 5. Example of triplicate sample absorbance versus time representing the oxidation of NADPH to $NADP^+$.

7.5. Vitamin C concentration

Figure 6 represents the linear relationship (r^2 = 0.9938) produced by vitamin C concentration (µg/ml) versus absorbance at 540nm. The equation of the line produced by this relationship was used to calculate the vitamin C concentration in all samples being assessed.

Appendix viii

The staining intensity obtained with SDH of the 3 fibre types measured in this study can be seen below in EDL, SOL and plantaris muscles. Fibre types were determined by a colour code where type IIa fibres stained darkly, type IIb fibres stained pale and type I fibres were intermediate (Schmalbruch & Kamieniecka, 1975).

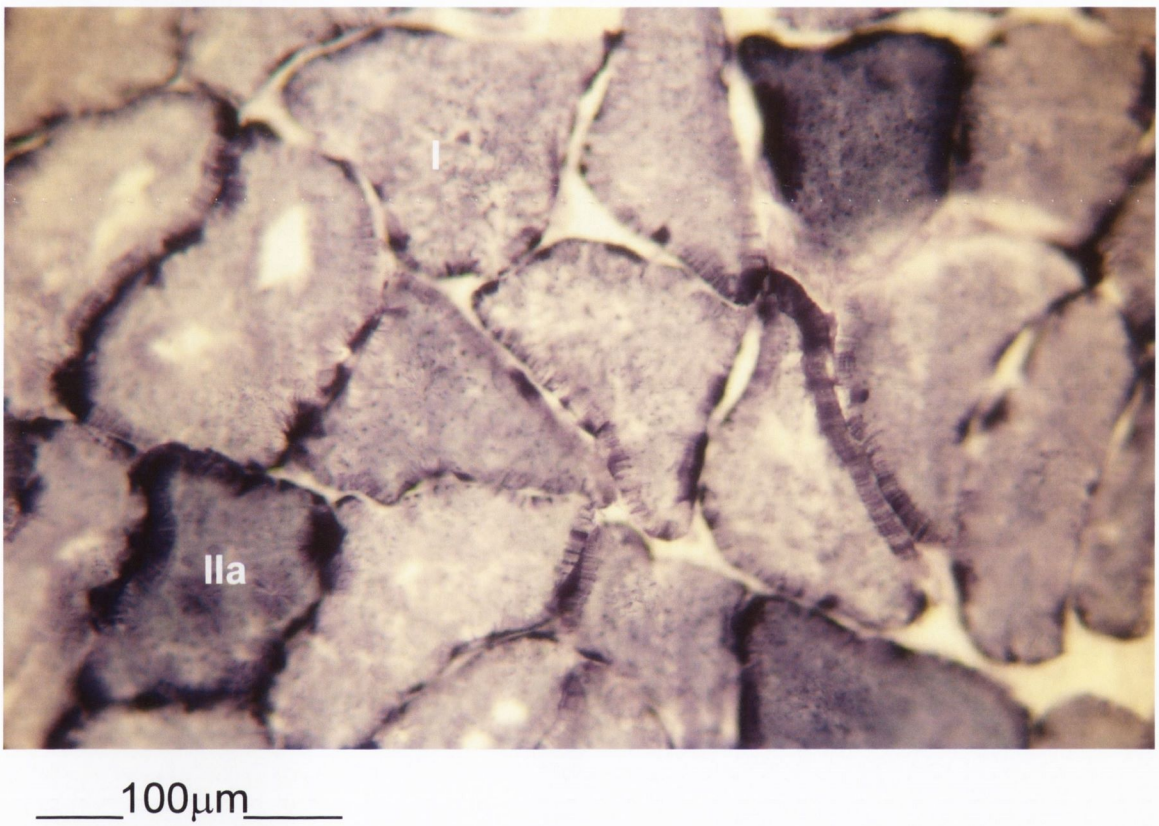

Figure 1. SDH stained section of the SOL muscle. The difference in staining intensity between type I and IIa fibre types can be clearly observed.

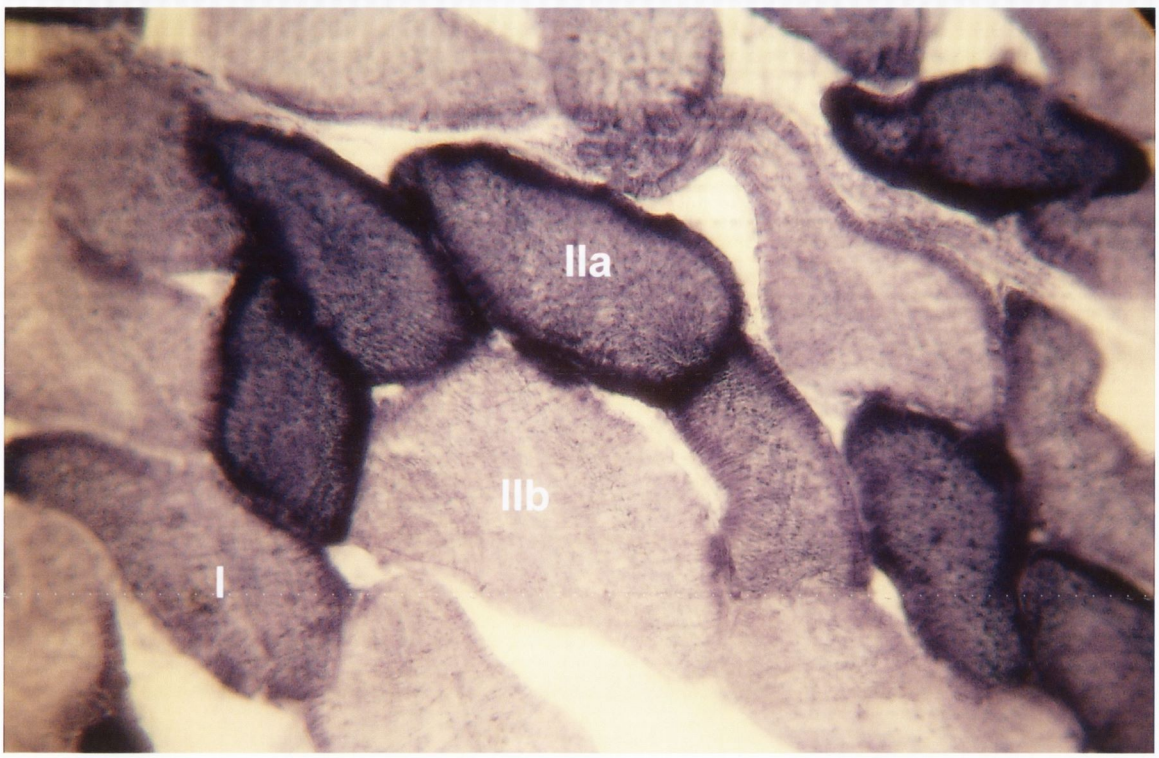

Figure 2. SDH stained section of the plantaris muscle. Type I, IIa and IIb fibre types are indicated.

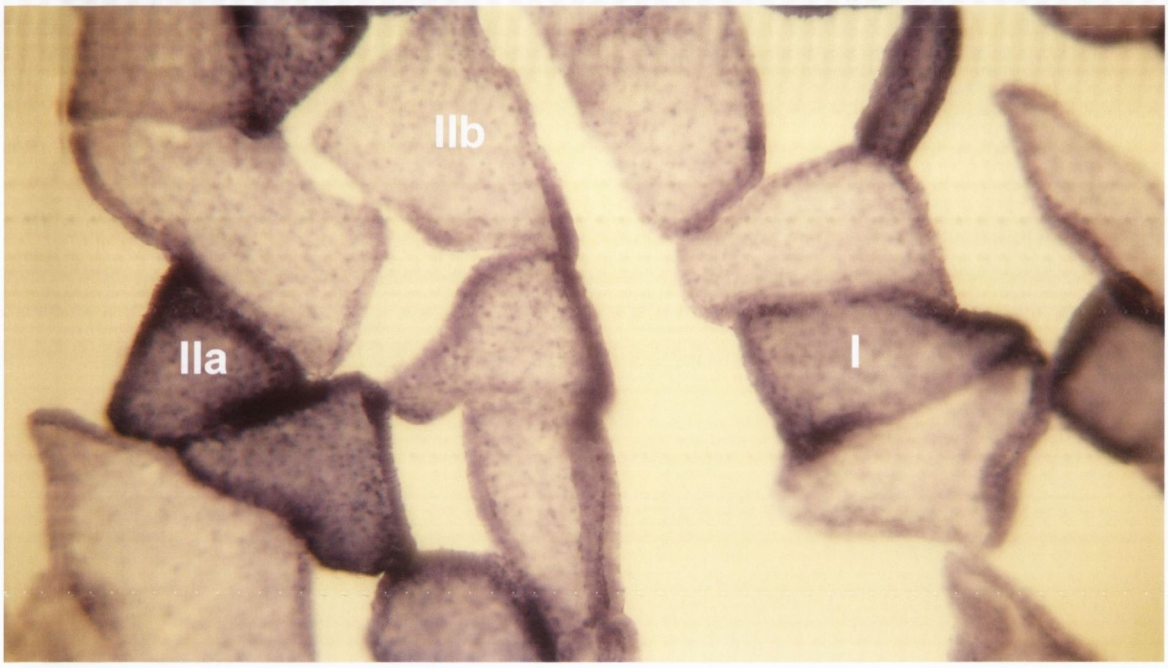

Figure 3. SDH stained section of the EDL muscle. Type I, IIa and IIb fibre types are indicated.

Appendix ix.

9.1. Aging contractile study

Specific forces ($kN.m^{-2}$) produced by EDL and SOL muscles involved in the contractile function protocol in Chapter 3 are shown in Table 1.1. and 1.2.

Time	Young		Aged	
(mins)	*YC*	*YO*	*AC*	*AO*
0	172.41 ± 4.89	158.89 ± 5.08 [a]	160.04 ± 5.60 [b]	144.49 ± 5.38 [ab]
5	174.19 ± 5.04	148.76 ± 5.19	150.54 ± 6.58	124.81 ± 9.08
10	167.76 ± 5.25 *	131.77 ± 4.94	145.64 ± 6.89	105.89 ± 10.37
15	161.12 ± 5.41 **	118.20 ± 2.63 [ad]	127.09 ± 10.27 [bd]	86.25 ± 9.99 [abd]
20	155.33 ± 5.49 **	92.02 ± 4.86	118.48 ± 10.99	63.09 ± 10.04
25	153.54 ± 5.87 ***	65.59 ± 5.36	110.11 ± 11.26	44.59 ± 9.09
30	149.47 ± 5.71 ***	39.57 ± 5.82	104.56 ± 11.64+	29.25 ± 8.30 [acd]

Table 1.1. Change in force ($kN.m^{-2}$) between young and aged EDL in the presence (H_2O_2) or absence (control) of an oxidant challenge.

a Different from corresponding C ($P < 0.001$)
b Different from corresponding young ($P < 0.001$)
c Different from corresponding young ($P < 0.05$)
d Different from all preceding time points ($P < 0.05$)

* Different from 0 mins ($P < 0.05$)
** Different from 0-5 mins ($P < 0.001$)
*** Different from 0-15 mins ($P < 0.05$)
\+ Different from 0-15 mins ($P < 0.001$)

Time (mins)	Concentrations of H_2O_2 used					
	CON	*20mM*	*40mM*	*80mM*	*120mM*	*160mM*
0	186.52 ± 14.12	163.99 ± 10.55	162.18 ± 12.43	142.83 ± 11.46	218.22 ± 13.49 *	164.07 ± 12.20
5	189.66 ± 15.15	166.14 ± 10.50	157.74 ± 13.66	134.56 ± 12.78	210.98 ± 11.34 *	152.02 ± 12.03
10	179.69 ± 14.57	157.41 ± 9.90	140.51 ± 13.87	117.56 ± 12.11	178.88 ± 10.20 **	121.61 ± 12.55 b
15	174.11 ± 14.63 ***	147.07 ± 9.02 ***	120.22 ± 13.20 b	100.60 ± 11.95 a	150.14 ± 10.14 ***	84.95 ± 8.07 c
20	167.23 ± 14.35 **	133.66 ± 8.71 ***	97.35 ± 11.56 c	+ 83.43 ± 10.65 d	115.89 ± 12.44 +	59.28 ± 6.90 c
25	162.21 ± 14.28 **b	118.41 ± 7.86 **b	78.34 ± 10.28 d	66.76 ± 9.51 c	86.55 ± 14.03 +d	41.09 ± 4.50 d
30	156.84 ± 13.58 ++d	102.39 ± 6.87 ++d	60.07 ± 8.89 e	52.22 ± 7.79 d	62.49 ± 12.60 e	23.15 ± 2.05 d

Table 1.3. Dose response of H_2O_2 on P_O in diaphragm segments.

* Different from all concentrations at that time point ($P < 0.05$)
** Different from 0.25, 0.5 and 1mM H_2O_2 at that time point ($P < 0.05$)
*** Different from 0.5 and 1mM H_2O_2 at that time point ($P < 0.05$)
\+ Different from 1mM H_2O_2 at that time point ($P < 0.05$)
++ Different from 0.25, 0.5, 0.75 and 1mM H_2O_2 at that time point ($P < 0.05$)
+++ Different from preceding time points ($P < 0.05$)

a Different from 0 mins ($P < 0.05$)s
b Different from 0-5 mins ($P < 0.05$)
c Different from 0-10 mins ($P < 0.05$)
d Different from 0-15 mins ($P < 0.05$)
e Different from 0-20 mins ($P < 0.05$)

Time (mins)	Young		Aged	
	YC	*YO*	*AC*	*AO*
0	164.98 ± 4.65	152.27 ± 5.60	141.73 ± 8.60	132.59 ± 4.82
5	186.44 ± 6.46	163.58 ± 5.91	146.59 ± 10.75	136.86 ± 7.30
10	186.04 ± 6.55	151.82 ± 4.49	135.68 ± 12.60 [a]	133.19 ± 7.76
15	185.59 ± 6.45	++ 142.94 ± 4.50 [a]	+ 126.03 ± 13.66 [b]	* 119.56 ± 8.64 [c] + *
20	184.55 ± 5.99	132.40 ± 4.01 [c]	118.15 ± 14.62 [c]	107.39 ± 9.16 [d]
25	183.95 ± 6.27	121.90 ± 4.32 [d]	109.65 ± 15.38 [d]	94.79 ± 9.38 [e]
30	182.05 ± 6.70	110.84 ± 5.29 [e]	103.13 ± 15.39 [f]	84.70 ± 10.14 [e]

Table 1.2. Change in force (kN.m^{-2}) between young and aged SOL in the presence (H_2O_2) or absence (control) of an oxidant challenge.

a Different from 5 mins ($P < 0.05$)
b Different from 0-5 mins ($P < 0.05$)
c Different from 0-10 mins ($P < 0.05$)
d Different from 0-15 mins ($P < 0.05$)
e Different from 0-20 mins ($P < 0.05$)
f Different from 0-25 mins ($P < 0.05$)

* Different from corresponding young ($P < 0.001$)
\+ Different from corresponding C ($P < 0.001$)
++ Different from 0 mins

9.2. Dose response protocol

Specific forces (kN.m^{-2}) produced by diaphragm sections in response to incubation with various H_2O_2 concentrations as described in Appendix v are shown in Table 1.3.

9.3. Diet intervention study

Specific forces (kN.m^{-2}) produced by EDL, SOL and diaphragm muscles involved in the dietary intervention study in Chapter 5 can be seen below.

Time	CON		VIT	
(mins)	*CC*	*CO*	*VC*	*VO*
0	146.86 ± 6.96	133.17 ± 9.52	161.15 ± 3.86	141.15 ± 7.58
5	144.78 ± 7.37	127.99 ± 9.48	161.38 ± 4.57	135.54 ± 7.09
10	140.60 ± 7.39	115.28 ± 8.92	156.33 ± 4.91 **	124.12 ± 6.16
15	136.15 ± 7.36 [a]	96.87 ± 7.65 +	150.80 ± 5.32	111.49 ± 5.40 +
20	131.55 ± 7.17 [c]	77.60 ± 6.30 ++	145.07 ± 5.58 [c]	96.22 ± 4.68 ++
25	126.84 ± 6.98 [d]	57.70 ± 5.23	139.13 ± 6.02 [d] *	77.09 ± 4.37 ***
30	121.80 ± 6.88 [e]	36.31 ± 4.57	135.05 ± 6.01 [e]	57.78 ± 5.01

Table 1.4. Change in force ($kN.m^{-2}$) between CON and VIT EDL in the presence (CO & VO) or absence (CC & VC) of an oxidant challenge.

* Different from corresponding CON ($P < 0.05$)
** Different from corresponding CON ($P < 0.01$)
*** Different from corresponding CON ($P < 0.001$)
\+ Different from preceding time points ($P < 0.05$)
++ Different from corresponding C ($P < 0.001$)

a Different from 0 ($P < 0.05$)
b Different from 0-5 mins ($P < 0.05$)
c Different from 0-10 mins ($P < 0.05$)
d Different from 0-15 mins ($P < 0.05$)
e Different from 0-20 mins ($P < 0.05$)

Time	CON		VIT	
(mins)	*CC*	*CO*	*VC*	*VO*
0	140.79 ± 7.67	141.17 ± 9.16	142.87 ± 9.20	148.87 ± 9.41
5	152.27 ± 7.65 ++	155.44 ± 9.12 ++	153.51 ± 10.66 ++	165.69 ± 9.73 ++
10	150.76 ± 7.68 [a]	152.41 ± 9.78	152.37 ± 10.39 [a]	164.38 ± 9.45 [a]
15	146.78 ± 7.87 [b]	147.20 ± 9.72	148.80 ± 10.29 [b]	160.08 ± 9.69 [b] +
20	144.37 ± 7.64 [c]	139.18 ± 9.42 * ++	145.74 ± 10.60 [c]	155.42 ± 9.41 [c] **
25	141.78 ± 7.92 [d]	131.39 ± 9.82 **	142.84 ± 10.74 [d]	150.58 ± 9.57 [d]
30	139.13 ± 8.24 [e]	124.80 ± 10.37 **	140.69 ± 11.12 [e]	143.79 ± 9.72 ++

Table 1.5. Change in force ($kN.m^{-2}$) between CON and VIT SOL in the presence (CO & VO) or absence (CC & VC) of an oxidant challenge.

* Different from corresponding C ($P < 0.05$)
** Different from corresponding C ($P < 0.001$)
\+ Different from corresponding CON ($P < 0.001$)
++ Different preceding time points ($P < 0.05$)

a Different from 0 mins ($P < 0.05$)
b Different from 0-5 mins ($P < 0.05$)
c Different from 0-10 mins ($P < 0.05$)
d Different from 0-15 mins ($P < 0.05$)
e Different from 0-20 mins ($P < 0.05$)

Time (mins)	CON		VIT	
	CC	*CO*	*VC*	*VO*
0	161.65 ± 12.76	168.06 ± 13.73	136.89 ± 12.34	154.09 ± 18.73
5	155.55 ± 12.41	166.08 ± 13.07	134.41 ± 13.43	156.87 ± 19.32 $^{+}$
10	148.75 ± 12.16 ^{a}	154.94 ± 12.10	128.27 ± 13.38	150.83 ± 18.54
15	141.87 ± 11.46 ^{b}	143.14 ± 11.19	121.42 ± 13.15 ^{b}	142.08 ± 18.13 ^{b}
20	134.13 ± 10.82 *	126.56 ± 10.03	115.83 ± 13.10 ^{c}	129.05 ± 17.27
25	128.47 ± 10.26 ^{d}	108.34 ± 9.38	111.39 ± 13.01 ^{c}	113.12 ± 16.07
30	122.38 ± 9.85 ^{d}	90.33 ± 8.59	107.23 ± 12.91 ^{c}	95.50 ± 14.22

Table 1.6. Change in force (kN.m^{-2}) between CON and VIT diaphragm in the presence (CO & VO) or absence (CC & VC) of an oxidant challenge.

+ Different from corresponding CON ($P < 0.05$)
++ Different from corresponding CON ($P < 0.001$)
+++ Different from corresponding C ($P < 0.05$)
* Different from preceding time points ($P < 0.05$)

a Different from 0 mins ($P < 0.05$)
b Different from 0-5 mins ($P < 0.05$)
c Different from 0-10 mins ($P < 0.05$)
d Different from 0-15 mins ($P < 0.05$)

Appendix x.

10.1. Total SOD activity

The largest SOD activity was measured in SOL at 10 & 22 months compared to all other muscles at all ages ($P < 0.01$).

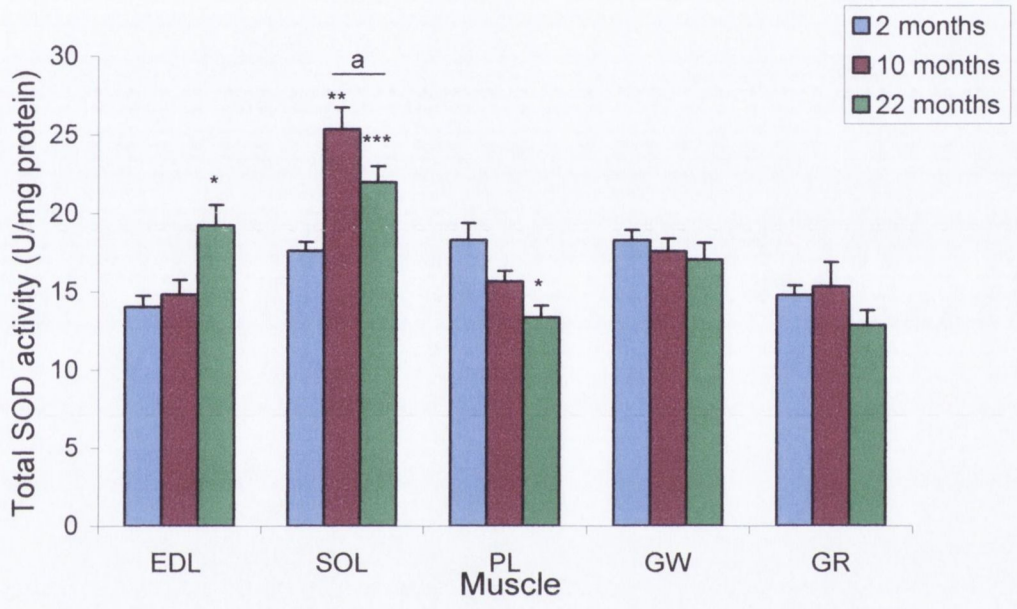

Figure 1. Effect of age and muscle type on total SOD activity (U/mg protein).

* Different from 2 months ($P < 0.05$)
** Different from 2 months ($P < 0.01$)
*** Different from 10 months ($P < 0.05$)
a Different from all other muscles at all ages ($P<0.01$)

10.2. Catalase activity

CAT activity was greater in SOL in all age groups when compared with all other muscles ($P < 0.01$, Figure 2).

10.3. Total glutathione concentration

At 22 months, total glutathione levels were larger in SOL and GR only compared with all other muscles ($P < 0.05$, Figure 3), and also compared with 10 months in SOL and GR, respectively.

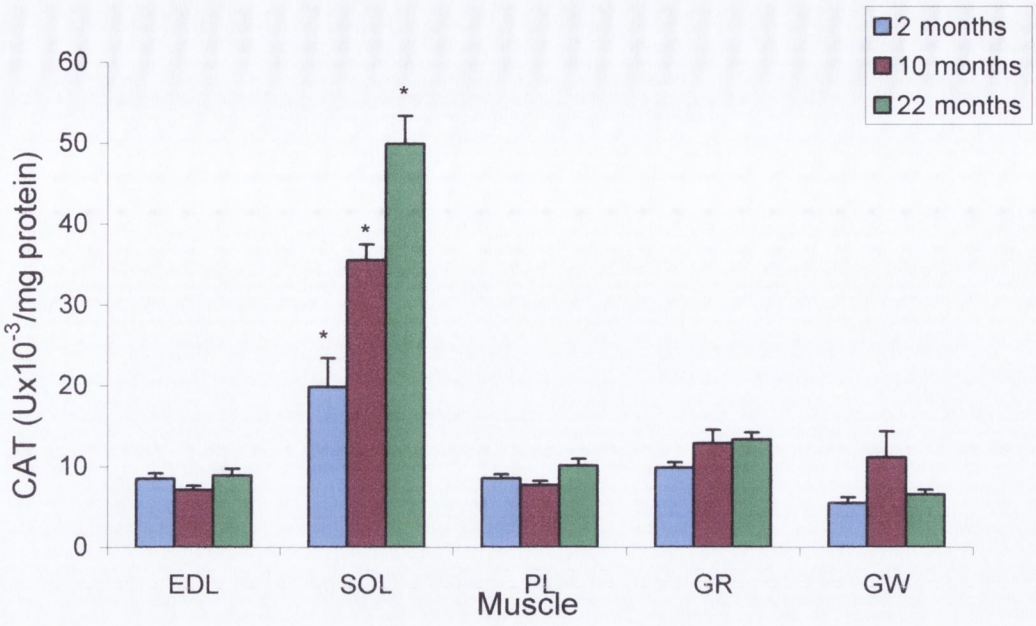

Figure 2. Effect of age and muscle type on CAT activity (U×10^{-3}/mg protein)

* Different from all age groups and all muscles ($P < 0.01$)

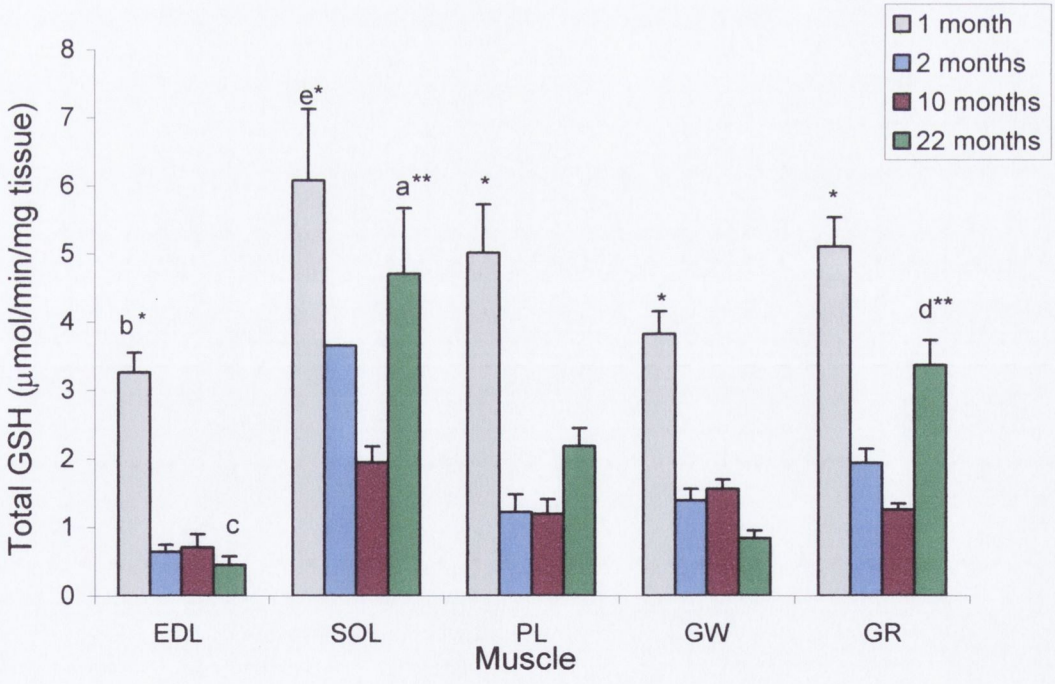

Figure 3. Effect of age and muscle type on total GSH content (µmol/min/mg tissue).

* Different from all ages ($P < 0.01$)
** Different from 10 months ($P < 0.05$)

a Different from EDL, PL & GW at 22 months ($P < 0.01$)
b Different from PL at 1 month ($P < 0.05$)
c Different from PL at 1 month ($P < 0.05$)
d Different from EDL & GW at 22 months ($P < 0.01$)
e Different from EDL & GW at 1 month ($P < 0.01$)

Note: Due to a lack of remaining homogenate, the GSH value in SOL at 2 months corresponds to 1 muscle and acts simply as a representative of total glutathione concentration.

10.4. Total glutathione peroxidase activity

At 10 and 22 months, SOL GPx activity was greater than other muscles in those age groups ($P < 0.01$, Figure 4).

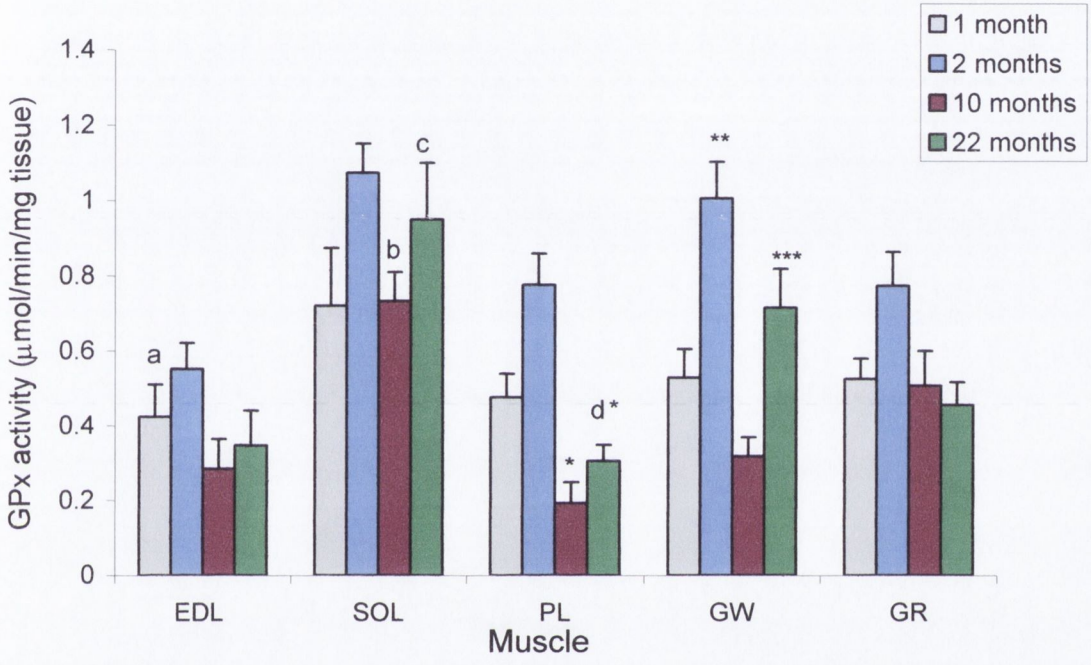

Figure 4. Effect of age and muscle type on GPx activity (µmol/min/mg tissue).

* Different from 2 months ($P < 0.05$)
** Different from 1, 10 & 22 months ($P < 0.01$)
*** Different from 10 months ($P < 0.05$)

a Different from SOL & GW at 1 month ($P < 0.01$)
b Different from EDL, PL & GW at 10 months ($P < 0.01$)
c Different from EDL, PL & GR at 22 months ($P < 0.01$)
d Different from GW at 22 months ($P < 0.05$)

10.5. Vitamin C concentration

Muscle-specific age-associated alterations for vitamin C can be seen in Figure 5.

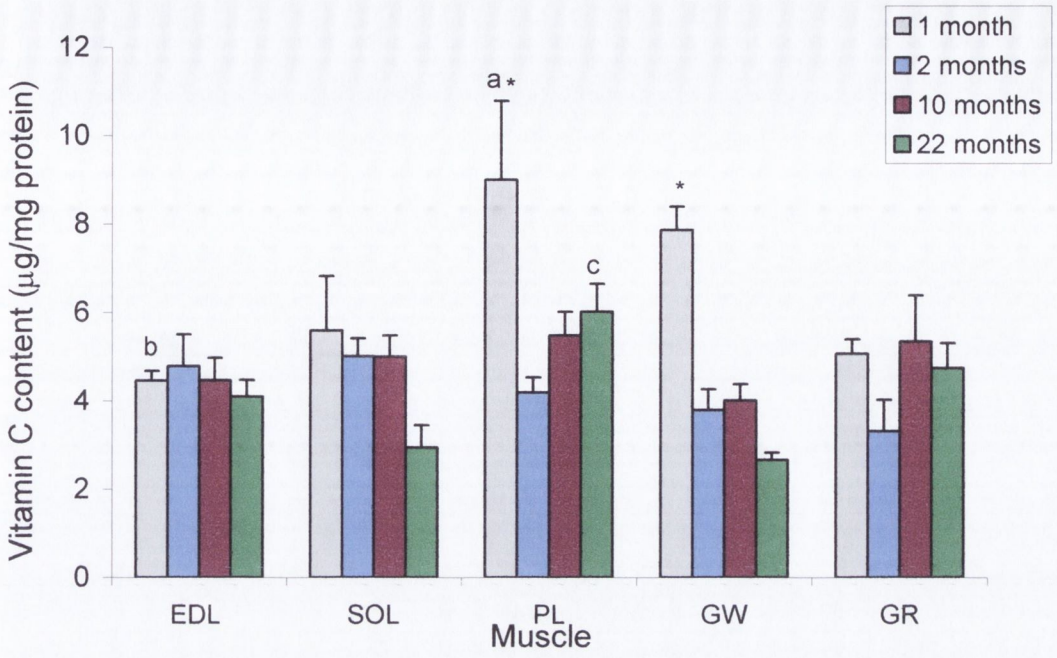

Figure 5. Effect of age and muscle type on vitamin C concentration (µg/mg protein).

* Different from all other age groups ($P < 0.01$)

a Different from EDL, SOL & GR at 1 month ($P < 0.01$)
b Different from GW at 1 month ($P < 0.05$)
c Different from SOL & GW at 22 months ($P < 0.05$)

Appendix xi.

11.1. Total SOD activity

Inter-group differences in total SOD activity can be seen in Figure 1.

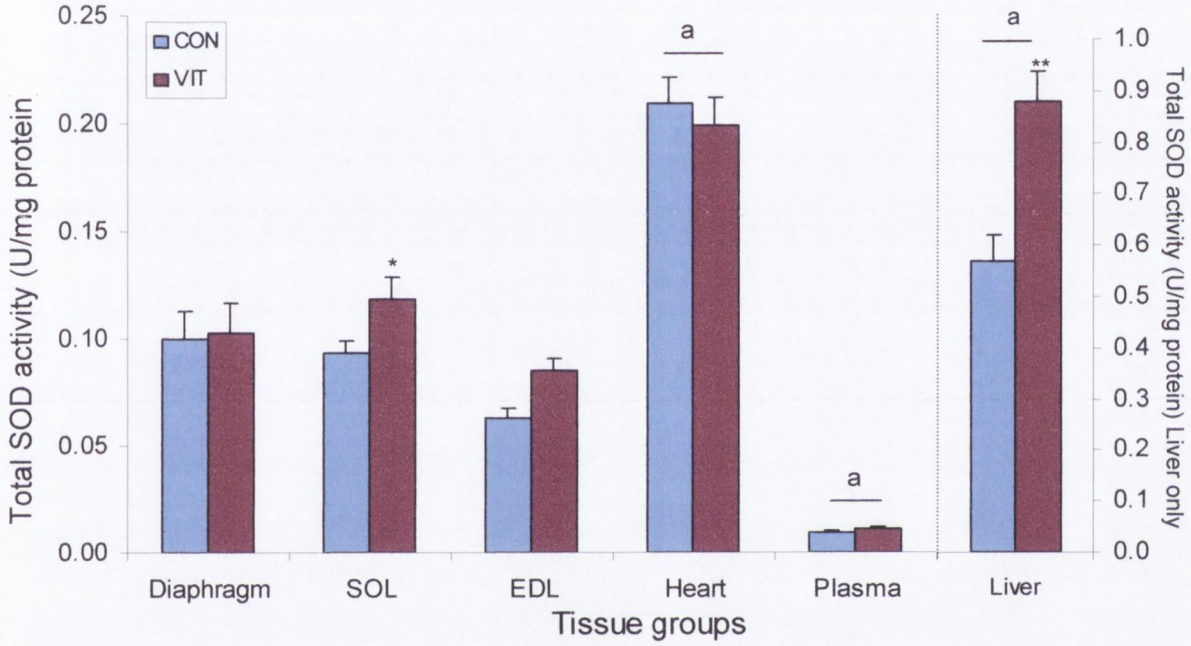

Figure 1. Total SOD activity (U/mg protein) in CON and VIT.

* Different from CON ($P < 0.05$)
** Different from CON ($P < 0.001$)
a Different from all tissues in CON & VIT ($P < 0.001$)

11.2. CAT activity

Although CON and VIT livers had significantly larger levels of CAT when compared to all the other tissues (~70 times, $P < 0.001$), no difference was observed between CON and VIT groups (Figure 2).

11.3. Total glutathione concentration

CON and VIT livers had significantly larger GSH concentrations when compared to other tissues ($P < 0.001$, Figure 3).

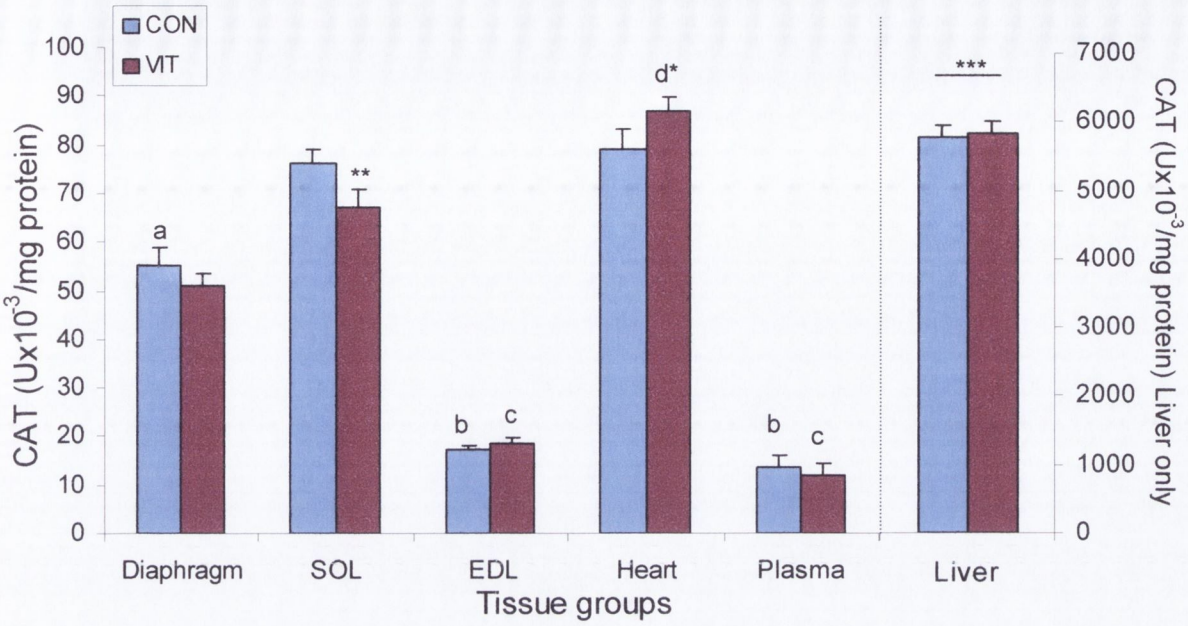

Figure 2. CAT activity (U×10^{-3}/mg protein) in CON and VIT.

* Different from CON ($P < 0.01$)
** Different from CON ($P < 0.001$)
*** Different from all tissues ($P < 0.001$)

a Different from CON heart and SOL ($P < 0.001$)
b Different from CON diaphragm, heart and SOL ($P < 0.001$)
c Different from VIT diaphragm, heart and SOL ($P < 0.001$)
d Different from VIT EDL, SOL and Dia ($P < 0.001$)

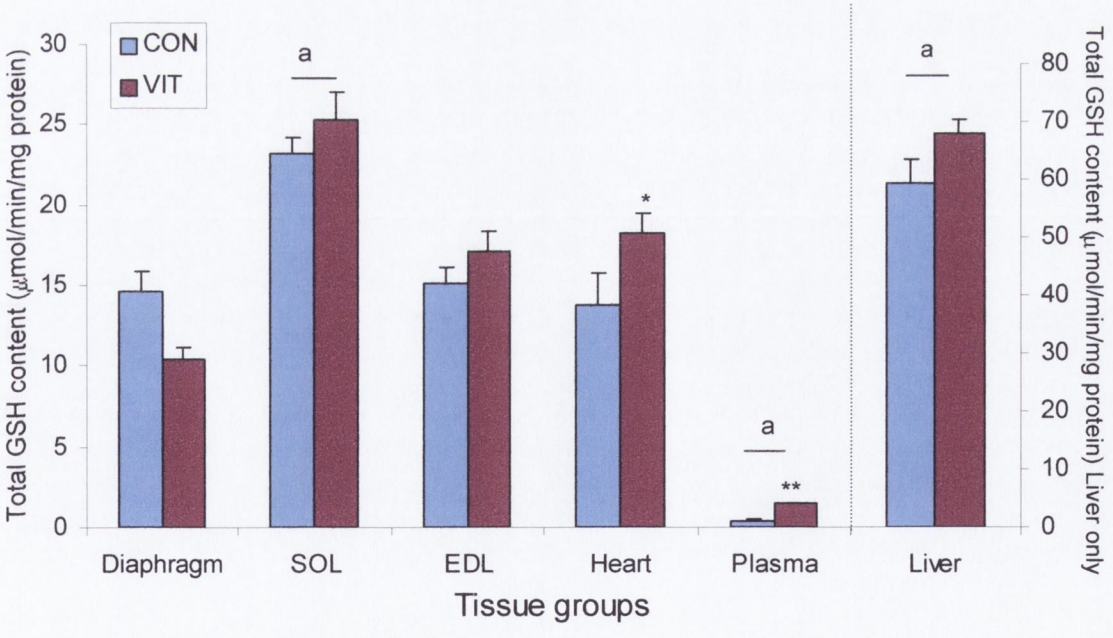

Figure 3. Total GSH concentrations (µmol/min/mg protein) in CON and VIT.

* Different from CON ($P < 0.05$)
** Different from CON ($P < 0.001$)

a Different from all other tissues ($P < 0.001$)

11.4. Vitamin C concentration

Vitamin C concentrations were highest in the liver in CON and VIT ($P < 0.001$, Figure 4).

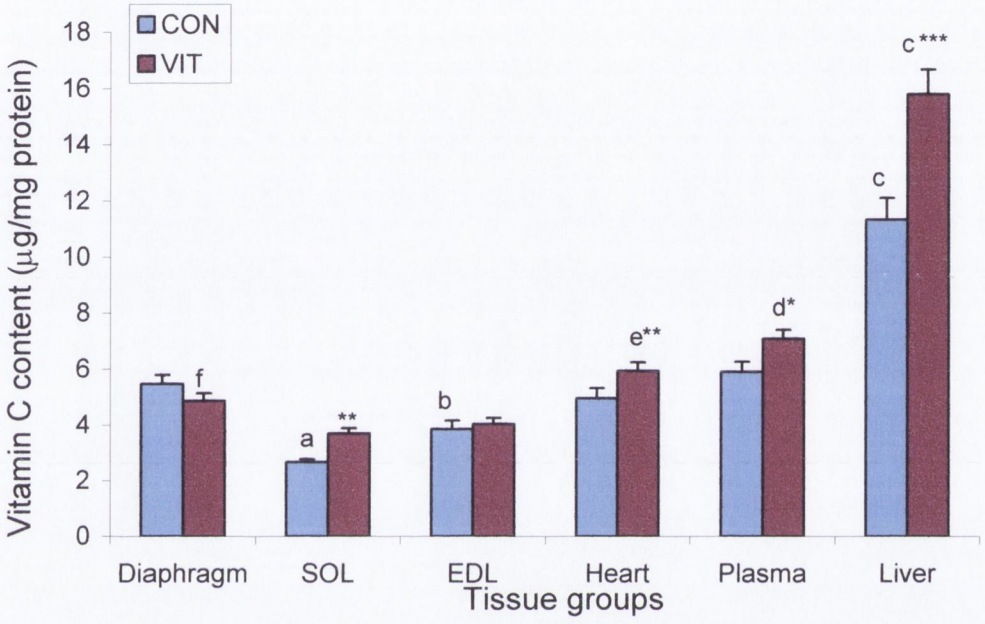

Figure 4. Vitamin C concentration (µg/mg protein) in CON and VIT.

* Different from CON ($P < 0.05$)
** Different from CON ($P < 0.01$)
*** Different from CON ($P < 0.001$)

a Different from CON diaphragm, heart & plasma ($P < 0.001$)
b Different from CON diaphragm, heart, SOL & plasma ($P < 0.001$)
c Different from all CON & VIT tissues ($P < 0.001$)
d Different from VIT heart, EDL, SOL & diaphragm ($P < 0.001$)
e Different from VIT EDL and SOL ($P < 0.001$)
f Different from VIT heart ($P < 0.05$)

11.5. FRAP assay

Inter-group differences in FRAP can be seen in Figure 5.

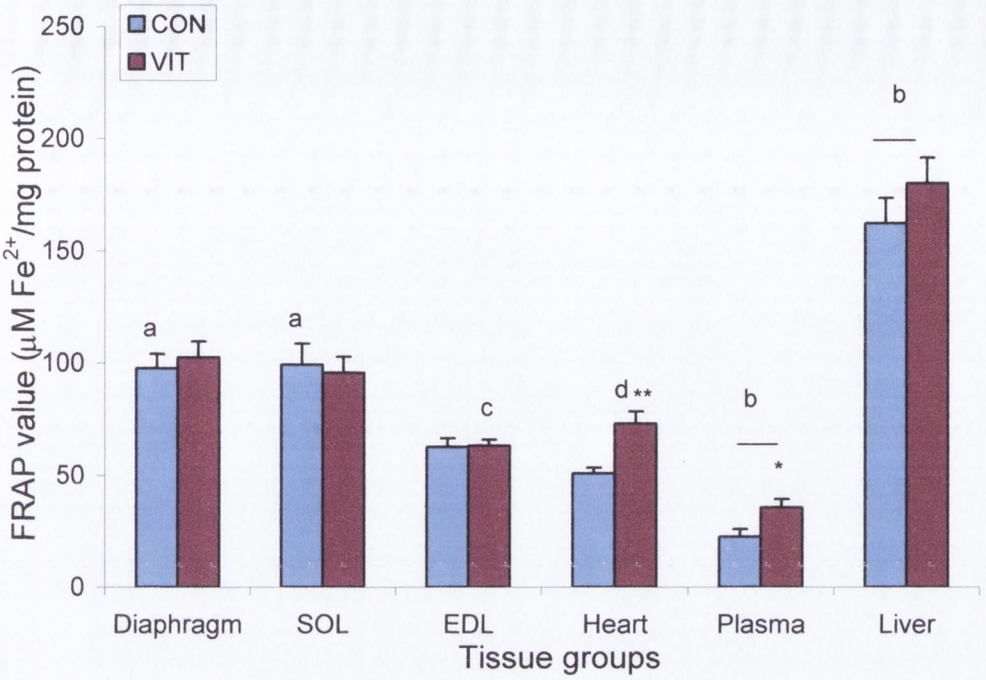

Figure 5. FRAP levels (μM Fe^{2+}/mg protein) in CON and VIT.

* Different from CON ($P < 0.05$)
** Different from CON ($P < 0.01$)

a Different from CON heart and EDL ($P < 0.001$)
b Different from all tissues ($P < 0.001$)
c Different from VIT SOL and diaphragm ($P < 0.001$)
d Different from VIT SOL and diaphragm ($P < 0.01$)