

**THE EFFECTS OF COENZYME Q₁₀ AND RESVERATROL TREATMENT ON
THE LEUKOCYTE COUNTS AND MUSCLE MORPHOLOGY OF SJL/J
MICE WITH DYSFERLINOPATHY: A QUALITATIVE MICROSCOPIC
INVESTIGATION**

By

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The Effects of Coenzyme Q₁₀ and Resveratrol Treatment on the Leukocyte Counts and Muscle Morphology of SJL/J Mice with Dysferlinopathy: A Qualitative Microscopic Investigation

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Abstract

The Dysferlinopathies are a form of Muscular Dystrophy specifically affecting muscles of the shoulder and pelvic girdles; which result from the autosomal recessive inheritance of one of a few mutant variants of the dysferlin gene which encodes the dysferlin protein. The dysferlin protein product is hypothesized to be involved in sarcolemmal vesicle fusion with a disrupted muscle fiber's plasma membrane, in effect resealing the disruption before a potentially damaging immune response is able to take place. Dysferlinopathic muscle presents extensively with inflammatory characteristics.

Mouse models are widely used in the study of Dysferlinopathy, as the mouse dysferlin gene possesses an overall amino acid sequence homology to the human gene in excess of ninety percent, and is also inherited in an autosomal recessive pattern. The SJL/J mouse model was the model of choice in the current study as it demonstrates characteristics that resemble the most common human condition due to the presentation of a dysferlin deficiency as well as a changed inflammatory profile in the muscles. Implementation of this animal model involved the

treatment of SJL/J mice over a 90 day period with the anti-oxidant supplements Resveratrol and Coenzyme Q₁₀ separately and in combination, to achieve immune modulation.

Modulation of inflammation could, in this disease process, assist in delaying the degeneration processes of muscle fibers with inherently defective healing mechanisms, possibly affording the response to degenerating fibers an extended survival time before complete muscle homeostasis is lost. The anti-oxidants were administered in order to suppress inflammation and thus prevent the immune response to degenerating muscle from causing the additional and untimely death of muscle cells. The leukocyte counts as well as muscle fiber morphology of two muscle complexes were investigated at conclusion of the 90 day experimental period.

Differential white blood cell counts did not provide a clear answer as to which therapeutic regime would be the best remedy for Dysferlinopathy sufferers; but muscle biopsy data revealed that Resveratrol does indeed display anti-inflammatory properties at the tissue level and that Coenzyme Q₁₀ does afford a degree of membrane protection to the muscle fibers at an ultrastructural level.

From this study it can be concluded that effective therapy could be achieved through the administration of either high levels of Coenzyme Q₁₀ (120mg/kg/day) or combination Resveratrol/Coenzyme Q₁₀ (60:40mg/kg/day); as findings supported beneficial effects on muscle groups of both significantly- and insignificantly-disease-affected muscle complexes. However, in the more significantly-affected muscle group, combination treatment seemed to be favourable to a greater extent than treatment with high levels of Coenzyme Q₁₀ in exclusivity, due to the co-operative effect of combination treatment on muscle health at the tissue level. Accordingly, combination Resveratrol/Coenzyme Q₁₀ supplement administration is recommended through the findings of this study for the maintenance of comprehensive muscle health in sufferers of Dysferlinopathy.

For future studies, extensive research focused on immunohistochemistry and proteomics of dysferlin gene and protein expression within Pectoralis and Gluteus complex muscles may lead to a more comprehensive understanding of the ultrastructural and morphological findings in both affected and unaffected animal models utilised in this study.

Declaration

I, Wendy Jeannette van der Spuy, hereby declare that this research dissertation is my own work and has not been presented for any degree at another University

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Foreword

First and foremost, I must express thanks to God for granting me the patience and perseverance to achieve anything which I put my mind to. Without Him nothing that means anything would be possible!

To my family – Daddy, the one who instilled in me that there are no boundaries to that which can be accomplished. Even in his absence, the memory of him remains a mighty force within me! Mommy, a pillar of strength and motivation in my life, without whom I would not have been the person I am today. Her belief in me, though unvoiced, is heartfelt. Frederick and Zoë, my dear brother and sister whose support means more to me than words can convey.

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Abbreviations, Symbols and Chemical Formulae

%	Percentage
®	Registered trademark
°C	Degrees centigrade
2p13	Chromosome position of the dysferlin gene
A/J	Substrain of inbred A mouse strain originating from The Jackson Laboratory
<i>Ad libitum</i>	Without restraint
ANOVA	Analysis of variance
BALB/c	Inbred strain of mouse
C2 Domain	Protein structural domain involved in targeting proteins to cell membranes
Ca(II)	Calcium ion
CD4	Cluster of Differentiation 4
cDNA	Complementary deoxyribonucleic acid
CK	Creatine Kinase
cm ²	Centimetres squared
CoQ ₁₀	Coenzyme Q ₁₀ (Ubiquinone)
COX1	Cyclo-oxygenase-1
C-Terminal	Transmembrane domain anchoring dysferlin to the plasma membrane
ddH ₂ O	Double distilled water
DGC	Dystrophin Glycoprotein Complex

DNA	Deoxyribonucleic acid
Dysf ^{-/-}	Inbred dysferlin-knockout mouse strain
e.g.	<i>Exempli gratia</i> (for example)
<i>et al.</i>	<i>Et alia</i> (and others)
etc.	<i>Et cetera</i> (and so forth)
Fer-1	Nematode <i>Caenorhabditis elegans</i> spermatogenesis factor
Fer1L4	Fer-1-like 4 pseudogene
g	Gram
GIT	Gastrointestinal tract
HEPA	High Efficiency Particulate Air filter
i.e.	<i>Id est</i> (that is)
IRRAD	Irradiated mouse food
IVC	Individual Ventilated Cage for mice
kDa	KiloDalton
LDL	Low Density Lipoprotein
LGMD2B	Limb-Girdle Muscular Dystrophy Type 2B
LLC	Limited Liability Company
LR-White	Low viscosity acrylic embedding medium
M	Molar
Mg/kg/day	Milligrams per kilogram per day
MM	Miyoshi Myopathy
mRNA	Messenger ribonucleic acid

NCSS	Number Cruncher Statistical System
nm	Nanometer (1×10^{-9} meter)
OsO ₄	Osmium tetroxide
OVA	Ovalbumin
pH	Logarithmic scale for the measurement of the acidity or alkalinity of an aqueous solution
p-Value	Probability value
RuO ₄	Ruthenium tetroxide
SEM	Scanning Electron Microscopy
SJL	Swiss Jim Lambert inbred strain of mouse
SJL/J	Substrain of inbred SJL mouse strain originating from The Jackson Laboratory
SWR/J	Substrain of inbred SWR mouse strain originating from The Jackson Laboratory
T-cells	Thymus-derived lymphocytes
TEM	Transmission Electron Microscopy
UPBRC	University of Pretoria Biomedical Research Centre
USA	United States of America
Vitamin C	Ascorbic acid
Vitamin E	α -Tocopherol
X	Times (in reference to magnification)
μ l	Microlitre (1×10^{-6} litre)
μ m	Micrometer (1×10^{-6} meter)

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Chapter 1: Introduction

Dysferlinopathy is a form of Muscular Dystrophy inherited in an autosomal recessive hereditary pattern which affects muscles of the shoulder and pelvic girdles. The myopathy is the result of inheritance of one of a few mutation variants of the dysferlin gene which encodes the dysferlin protein. The dysferlin protein product is hypothesized to be involved in sarcolemmal vesicle fusion with a disrupted plasma membrane, in effect resealing the disruption before an immune response is able to take place.

With defective protein function, due to the gene mutation, the sarcolemmal vesicles accumulate beneath a plasma membrane disruption but are unable to fuse with it and reseal the membrane. Due to this fact, cells of the immune system are given enough time to initiate a response toward the damage, and the muscle fiber will most likely degenerate and become necrotic after which it will undergo myophagocytosis. It follows that as more damage is incurred to muscle, without the prospect of repair, the myopathy takes on an active state and the condition of skeletal muscle will thus deteriorate, resulting in the Muscular Dystrophy. Dysferlinopathy is also characterized by a high level of muscle fiber regeneration in response to the heightened levels of degeneration, as compensation. Muscle is thus in a constant state of alteration, with weakening thereof also being prominent.

Dysferlinopathic muscle is additionally known to present with a large degree of inflammatory characteristics, with much leukocyte infiltration perivascularly as well as between and within muscle fibers themselves. The animal model presenting with characteristics most like the commonest human condition is the SJL/J mouse model; presenting with a dysferlin deficiency as well as inflammatory changes in muscle. The mouse dysferlin gene possesses a >90% percent overall amino acid sequence homology to the human gene, and is also inherited in an autosomal recessive pattern.

It is believed that modulation of inflammation could serve to prolong the life of muscle fibers with inherently defective healing mechanisms, possibly affording the overall response to degenerating fibers an extended existence before complete muscle homeostasis is lost. Immune modulation would thus involve the use of drugs or supplements in the suppression of inflammation in order to prevent the immune response toward degenerating muscle from causing additional and untimely death of muscle cells.

Two immune modulators of interest are Coenzyme Q₁₀ and Resveratrol. Coenzyme Q₁₀ is an endogenous antioxidant which protects cells and tissues from the damaging effects of free

radicals on proteins (thus DNA) and lipids (thus membranes) as well as other cellular components. High concentrations thereof are present in skeletal muscle under normal circumstances; but levels do decline with age as there are increased requirements for the antioxidant and decreased production of it by the body. Just as in an aged state, the levels of Coenzyme Q₁₀ could rapidly decline with the loss of muscle homeostasis in Dysferlinopathy as the requirement therefore is increased but the production thereof remains at normal levels. Due to the lipid solubility of the antioxidant, it is recommended as a membrane protective supplement, which is of great importance in a disease such as Dysferlinopathy.

Resveratrol is an antioxidant and anti-inflammatory compound found largely in the skins of red grapes. The compound is known to enhance nitric oxide production, which through certain mechanisms assists in the suppression of the damaging effects of leukocytes such as macrophages and neutrophils. Resveratrol has additionally been shown to increase motor function and modulate longevity pathways through improving the aerobic capacity of mitochondria.

We thus hypothesize that administration of Coenzyme Q₁₀ and Resveratrol separately and in combination will provide membrane-protective and inflammatory-protective effects on muscle fibers of animals of the SJL/J mouse model. The following research objectives will thus govern this study:

1. Implementation of the SJL/J dysferlin-null mouse model
2. Histological investigation and determination with light microscopy, through blood smears, of the morphology and differential leukocyte counts respectively
3. Histological investigation with light, transmission- and scanning- electron microscopy, of muscle morphology of tissue biopsy samples collected

Chapter 2: Literature Review

2.1. Introduction

Muscular Dystrophy is defined as a set of hereditary or sporadic progressive muscle disorders. It is caused by a defect in one or more genes that control various muscle functions; and generally affects multiple systems (Emery 2002) (depending on the type of muscular dystrophy) including the heart, gastrointestinal tract (GIT), nervous and endocrine systems, skin, eyes as well as other organs.

Muscular Dystrophies are characterised by defects in muscle proteins, progressive skeletal muscle weakness, irreversible skeletal muscle wasting, death of muscle cells and tissue, and finally the replacement of muscle by scar tissue and fat termed pseudohypertrophy (Emery 2002).

Finally, Muscular Dystrophies are classified according to the pattern of inheritance, age at the onset of symptoms, and the part of the body primarily affected. There are nine primary forms of Muscular Dystrophy (Angelini 2002, HMC 2008), divided further into groups of inheritance patterns as follows – X-linked (dominant and recessive), and Autosomal (dominant and recessive) forms of disease.

The topic of this research project is Dysferlinopathy, namely Limb-Girdle Muscular Dystrophy type 2B (LGMD2B) and Miyoshi Myopathy (MM). A myriad of studies have been conducted on the muscular and inflammatory changes of Dysferlinopathy sufferers (humans and animal models). The differential white blood cell counts have never been quantified in animals suffering from this disease, nor have the effects of immune modulators on the muscle tissue itself been studied on the SJL animal model for the disease.

2.2. The Dysferlinopathies

The Dysferlinopathies display an autosomal recessive inheritance pattern and are divided into LGMD2B and MM. LGMD2B (Angelini 2002) shows onset in the second decade of life. It affects the muscles of the shoulder and pelvic girdles, displays an inflammatory response, and can affect the cardiopulmonary system. This form of Limb-Girdle Muscular Dystrophy (LGMD) is slowly progressive compared to LGMD type 1 which has an autosomal dominant inheritance pattern. Patients with LGMD2B generally have a loss of ambulation (ability to walk) by the fourth decade of life. MM (Angelini 2002, HMC 2008) shows onset in adulthood and affects mostly the distal muscles i.e. Gastrocnemius and Soleus muscles. It is similar to LGMD2B in many ways, but has a slower disease progression.

These Dysferlinopathies are both caused by a mutation in the dysferlin gene, mapped to chromosome 2p13 (Angelini 2002). Despite sharing a mutation of the same gene, the Dysferlinopathies are not allelic variants of one another (Angelini 2002). In fact there have been members of the same family (Angelini 2002) found to have the exact same mutation; but presenting, inexplicably, with either LGMD2B or MM. In search of an answer to this clinical heterogeneity, which may indeed be attributed to additional epigenetic factors resulting in variable dysferlin expression, the myoferlin gene is under study as a possible modifier (Davis *et al.* 2000) in Dysferlinopathy; as myoferlin is a protein homologue to dysferlin (Angelini 2002).

2.2.1. Diagnosis of Dysferlinopathy

Muscular Dystrophies are diagnosed on a basis of elevated serum Creatine Kinase (CK) levels (Galassi *et al.* 1987, Davis *et al.* 2000, Bansal *et al.* 2003, Bansal & Campbell 2004, Kirschner & Bonnemann 2004). In Dysferlinopathy, levels are elevated even early in life despite normal muscle function (Davis *et al.* 2000, Kirschner & Bonnemann 2004). Elevated CK levels are suggestive of membrane damage, and in the case of Dysferlinopathies this is proposed to be due to inadequate muscle membrane repair (see 2.2.3.2 later).

Also essential to diagnosis is the taking of a muscle biopsy. The microscopic changes (MDAUSA 2008) indicative of dystrophy are a variation in muscle fiber size, degeneration of muscle myofibers, necrosis or destruction of myofibers, regeneration of muscle (in response to degeneration (Bansal *et al.* 2003)), and fibrosis replacing the necrotic tissue.

The defining factor in the diagnosis of Dysferlinopathies is immunostaining for the dysferlin protein. There is a partial or complete loss of functional dysferlin due to nonsense,

missense, and/or frame-shift mutations of the dysferlin gene (Davis *et al.* 2000, Bansal & Campbell 2004, Comerlato *et al.* 2005).

2.2.2. The Dysferlin Protein

Dysferlin is a large (230kDa) membrane-based protein, encoded by the dysferlin gene (55 exons) which is mapped (Angelini 2002) to chromosome 2p13. Dysferlin is separate from the dystrophin-associated proteins (see 2.2.3.1 later) defects of which are responsible for the majority of other muscular dystrophies. The dysferlin protein localizes at the plasma membrane or sarcolemma and in cytoplasmic vesicles (Bansal & Campbell 2004) in normal skeletal muscle immunostaining; but in LGMD2B and MM skeletal muscle there is no dysferlin staining, as the functional protein is absent in Dysferlinopathies.

In structure (Bansal & Campbell 2004), the dysferlin protein includes a single transmembrane domain at its C-terminal and a large intracellular domain with six C2 domains at the region of its N-terminal. It is termed a type II transmembrane protein. The dysferlin protein has been shown to be similar to the nematode *Caenorhabditis elegans* spermatogenesis factor *Fer-1*, which is proposed to be responsible for vesicle fusion of spermatids with plasma membrane (Weiler *et al.* 1999, Bansal & Campbell 2004). Without the presence of this *Fer-1* protein nematodes are rendered infertile; yet no evidence of infertility has been supported in human or mouse males with LGMD2B or MM (Liu *et al.* 1998). Of importance here is that the major transcript of dysferlin mRNA is expressed strongly in skeletal and cardiac muscle (Liu *et al.* 1998).

Dysferlin is also found to be similar to other members of the Ferlin family to which it belongs; these members are otoferlin (Bansal & Campbell 2004), Fer1L4 (Bansal & Campbell 2004) and myoferlin (Davis *et al.* 2000, Bansal & Campbell 2004). Myoferlin is the most like dysferlin – it is also thought to be a type II transmembrane protein, and contains six C2 domains. Immunohistochemical analysis suggests that myoferlin is also present on the sarcolemma (like dysferlin); but unlike dysferlin, it is additionally present in the nucleus of muscle fibers (Bansal & Campbell 2004). As aforementioned, myoferlin is suggested to be a possible modifier gene of the phenotypic variance seen in Dysferlinopathies (Davis *et al.* 2000, Bansal & Campbell 2004).

2.2.3. Dysferlin Function

Dysferlin is presumably involved in calcium-dependent vesicle fusion with the plasma membrane (Bansal & Campbell 2004); this presumption is made on the basis of dysferlin's similarities to the synaptogamins, which are essentially calcium sensors (Bansal & Campbell 2004) that regulate the process of fast exocytosis (Davis *et al.* 2002). Proteins which are directly implicated in membrane fusion, such as the synaptogamins, contain at least two C2 domains. The first C2 domain of synaptogamin binds calcium and anionic phospholipids. The second C2 domain of synaptogamin aids in protein-protein interaction and also binds phospholipids (Davis *et al.* 2002).

Dysferlin is thus hypothesized to facilitate in vesicle trafficking and fusion to the plasma membrane due to the presence of six C2 domains with similarity to synaptogamins which are involved in this manner to membrane repair in other cell types (Bansal *et al.* 2003). This hypothesis is strengthened by the finding that dysferlin occurs both during muscle development and membrane repair (Davis *et al.* 2002).

Dysferlin localizes to where damage to the sarcolemma is induced; and the first C2 domain of dysferlin binds to phospholipids in a calcium-dependent manner – calcium being essential for resealing of sarcolemmal disruptions (Bansal *et al.* 2003). Evidence exists, revealing that muscle membrane repair is impaired in the absence of dysferlin; and this is thought to be the key factor in the pathogenesis of Dysferlinopathies (Kirschner & Bonnemant 2004, Glover & Brown 2007).

2.2.3.1. Dysferlin and the Dystrophin Glycoprotein Complex (DGC)

In the majority of Muscular Dystrophy types the function of the DGC is found to be compromised due to the mutation of a gene which encodes one of its many associated proteins (Angelini 2002). Much research emphasises the importance of the DGC in conferring structural integrity or stability to the sarcolemma, by mediating a link between the cytoskeleton and the extracellular matrix. Loss of any component (sarcoglycans, dystrophin, and others) causes secondary instability in the whole complex, rendering the muscular membrane susceptible to mechanical damage (Glover & Brown 2007). Dysferlin, like dystrophin, localizes to the sarcolemma; but does not associate with or form a direct constituency of the DGC (Angelini 2002). Thus mutation of dysferlin should not alter the integrity of the sarcolemmal structure.

This is indeed supported by studies of the Dysferlinopathies; revealing that the DGC is functional and stable as the sarcolemma itself is structurally stable. There is also no change in

the expression of components of the DGC in Dysferlinopathies, thus the sarcolemmal structure is indeed unaffected in these Muscular Dystrophies or myopathies (Bansal *et al.* 2003). Because the sarcolemmal structure is not affected by a deficiency of dysferlin, there is another mechanism of muscle degeneration in play. For this reason, it is proposed that dysferlin has a role in the sarcolemmal repair process (Bansal *et al.* 2003).

2.2.3.2. Membrane Repair Model

Skeletal muscle is susceptible to plasma membrane injuries due to force generated by stretching and contraction. There is thus a need for the maintenance of structural integrity of the plasma membrane as defects in repair thereof would cause muscle degeneration which may lead to Muscular Dystrophy (Bansal & Campbell 2004).

Mechanical stress is proposed to cause muscle fibre necrosis via one of two mechanisms. Firstly, the sarcolemma could be more susceptible to injury, as in the DGC-linked Muscular Dystrophies where the plasma membrane structure is disrupted or inherently unstable. Secondly, the sarcolemma repair mechanism of the muscle fiber could be defective. This second mechanism is proposed for dysferlin-linked muscular dystrophies, in which it is suggested that damaged membrane is unable to repair itself adequately (Bansal *et al.* 2003).

This proposal is supported in studies by Bansal & Campbell (Bansal & Campbell 2004) among others. Their research shows that in normal muscle, dysferlin facilitates vesicle docking and fusion with plasma membrane by interacting with other dysferlin molecules and proteins at the plasma membrane. Vesicle fusion actively causes addition of membrane to the damaged sarcolemma (in the presence of high Ca(II) levels) and results in the patching and resealing of disrupted membrane. However, in dysferlin deficient muscle vesicles accumulate under membrane disruption sites but are unable to fuse with the membrane (Bansal & Campbell 2004). Also, the muscle fibers are found to be defective in Ca(II)-dependent resealing of the sarcolemma (Bansal *et al.* 2003). With vesicle docking and fusion not possible, disrupted membrane is unable to reseal and the damaged muscle fibre degenerates (Bansal & Campbell 2004).

2.3. Muscle Regeneration & Degeneration

Muscle regeneration is a response to muscle degeneration (Bansal *et al.* 2003). The process of muscle regeneration is characterised by a series of events. These are: necrosis of muscle tissue, revascularisation, inflammatory cell infiltration (especially neutrophils and macrophages), phagocytosis of necrotic muscle tissue, proliferation of mononuclear muscle precursors (satellite cells), and then fusion of these precursors to form myotubes or multinucleated young muscle cells (Carlson 1973, Mitchell CA *et al.* 1992).

Minor myofiber injury can be repaired locally by restoration of the sarcolemmal membrane in order to limit cellular breakdown and avoid focal necrosis. Minor damage occurs regularly in response to modifications in muscle loading and other mechanical stress (Grounds 1998). When skeletal muscle incurs non-lethal injury it regenerates by means of proliferation and fusion of muscle precursor cells (Mitchell CA *et al.* 1995). In instances of sublethal damage to myofibers where only inflammation takes place and there is no need for necrosis and regeneration, macrophages are sufficient to repair muscle fibers, and there is no need for satellite cells to undergo replication (Grounds 1998).

If however the incurred injury is lethal to the muscle fibers, degeneration takes place and necrosis is the end result. Regardless of whether damage is lethal to muscle fibers or not, the infiltration of leukocytes, particularly macrophages, is essential for removal of cellular debris. The absence of leukocytes at the injury site does not impede proliferation and fusion of muscle precursor cells (Robertson *et al.* 1992), but the formation of new muscle is severely impeded by the persistence of muscle debris (Mitchell CA *et al.* 1995).

2.3.1. Satellite Cells

Adult muscle fibers contain small uninucleated satellite cells, a small population of morphologically undifferentiated cells situated between the sarcolemma and endomysium of uninjured muscle fibers (Kelly *et al.* 1984, Leeson *et al.* 1988). Satellite cells are mitotically quiescent in adult skeletal muscle, but are activated – possibly through the inflammatory response and release of growth factors – in response to stress stimuli which include denervation, stretching, exercise or trauma (Grounds 1998, Seale & Rudnicki 2000, Smythe *et al.* 2008). Thus satellite cells represent a reserve of embryonic myoblasts, better termed endogenous myogenic stem cells because they actually constitute a myogenic cell lineage distinct from embryonic lineages (DeAngelis *et al.* 1999, Seale & Rudnicki 2000), which can proliferate and play a major role in the repair and regeneration of skeletal muscle (Leeson *et al.*

1988, Seale & Rudnicki 2000). Gross damage is alternatively repaired by the formation of fibroconnective scar tissue (Leeson *et al.* 1988).

During postnatal muscle growth, satellite cells fuse with their adjacent growing myofiber, resulting in an increase in the number of myonuclei. However, not all satellite cells fuse during muscle growth, and those that remain in adult skeletal muscle may have an important role in the repair and regeneration that does occur in mature skeletal muscle (Kelly *et al.* 1984). In fact, they mediate postnatal growth of muscles and are the primary means of formation of adult muscle (Schultz 1996, Seale & Rudnicki 2000).

Muscle injury activates satellite cells to proliferate and generate a myoblast pool, which subsequently differentiate and fuse to each other or to injured myofibers to promote repair and/or regeneration (Snow 1978, Conboy *et al.* 2003, Collins *et al.* 2007). It would seem that satellite cells demonstrate an inherent capacity for self-renewal, in that the numbers of quiescent satellite cells in adult muscle remain relatively constant in number through multiple cycles of degeneration and regeneration (Gibson & Schultz 1983, Seale & Rudnicki 2000). However, the number of satellite cells is reduced in aged muscle, supporting the known finding that skeletal muscle in the aged manifests a progressive failure to maintain itself (Collins *et al.* 2007).

Interestingly, human satellite cells have been shown to release factors which attract monocytes/macrophages through an endothelial layer *in vitro* (Chazaud *et al.* 2003). Their chemoattractive properties are highest immediately following their activation to proliferate and then decline as the muscle fibers exit the cell cycle and begin to differentiate. In the context of injury, this indicates that activated satellite cells could signal for the speedy invasion of macrophages immediately after injury (Tidball 2005).

2.3.2. Leukocytes

It is well established that macrophages and neutrophils are required for phagocytosis of necrotic fibers at early stages of injury and muscle fiber degeneration. A range of studies have suggested that neutrophils reach peak infiltration levels within 24 hours of muscle injury. At a later time-point macrophages reach the damage site to remove cellular debris and possibly even limit the damaging potential of neutrophils (St Pierre & Tidball 1994, Tidball 2005, Brunelli & Rovere-Querini 2008).

Neutrophil-mediated skeletal muscle injury seems to be largely dependent on the production of free radicals (Rando 2001, Hodgetts *et al.* 2006); additionally, cytosolic protein release from muscle seems to indicate that the target of injury of neutrophils is the muscle

membrane (Tidball 2005). Reactive nitrogen and oxygen species have also been reported as contributors of skeletal muscle injury and necrosis associated with Muscular Dystrophies (Pizza *et al.* 1998, Hodgetts *et al.* 2006). Interestingly, *in vitro* studies have reported a significant protective role of nitric oxide (NO) against neutrophil-mediated cellular injury (Rando 2001, Hodgetts *et al.* 2006). Furthermore, strategies blocking neutrophil function have been shown to reduce muscle damage (Mitchell CA *et al.* 1995, Hodgetts *et al.* 2006, Toumi *et al.* 2006); and it has been shown that neutrophil-mediated damage is not a requirement for muscle growth. However, whether neutrophils play a role in muscle repair independent of growth or phagocytosis is as yet unknown (Tidball 2005).

Macrophages are reported to play a beneficial role in muscle healing as it has been found that depletion of macrophages in fact impairs muscle regeneration (Lescaudron *et al.* 1999, Summan *et al.* 2006, Arnold *et al.* 2007, Brunelli & Rovere-Querini 2008, Smythe *et al.* 2008). It would seem that muscle-derived NO and initial muscle damage (which protects from excessive leukocyte adhesion) promotes increased NO production by macrophages (Tidball 2005) and this mechanism could in fact contribute to the downregulation of the damaging action of neutrophils. The initial macrophage requirement is for the phagocytosis of necrotic muscle fibers. Thereafter, however, they actively promote muscle fiber regeneration through the release of certain mitogenic growth factors that possibly contribute to the recruitment of myogenic precursors in muscle tissue (Seale & Rudnicki 2000, Arnold *et al.* 2007, Brunelli & Rovere-Querini 2008).

This discovery has led to the identification of two distinct macrophage populations: classically activated (type I) macrophages with pro-inflammatory functions; and alternatively activated (type II) macrophages with wound healing and immunoregulatory functions (Mantovani *et al.* 2007, Brunelli & Rovere-Querini 2008). In effect, type I macrophages have been shown to be associated with muscle necrosis and type II macrophages to be associated with regenerative myofibers (St Pierre & Tidball 1994, Brunelli & Rovere-Querini 2008). Macrophages additionally have an apparent plasticity; in that type I macrophages are able to acquire a type II phenotype when appropriate factors are present within the muscle tissue (Arnold *et al.* 2007, Brunelli & Rovere-Querini 2008).

If there is a defect in this necessary transition, then necrosis and inflammatory responses will exceed the regenerative capacity of muscle tissue. In expansion of this statement, it is logical that in the case of chronic muscle defect or injury as experienced in Muscular Dystrophies, the depletion of this macrophage transition will also cause necrosis to exceed regenerative capacity, and in so doing, result in the scarring and fat accumulation observed in

Muscular Dystrophies (figure 2.1). Furthermore, Nagaraju and colleagues (Nagaraju *et al.* 2008) have demonstrated that activated macrophages which heavily infiltrate dysferlin deficient muscle show evidence of increased phagocytic activity. It is thus plausible that mild dysferlinopathic myofiber damage stimulates an inflammatory cascade that initiates, exacerbates, and possibly perpetuates the underlying myofiber damage in affected muscles (Brunelli & Rovere-Querini 2008).

Furthermore, strong evidence exists that inflammatory cells i.e. neutrophils and macrophages contribute to necrosis of healthy muscle cells when an excessive inflammatory response is present. It is proposed that such excessive inflammatory responses directly damage myofibers in myopathic conditions such as Muscular Dystrophies (Hodgetts *et al.* 2006).

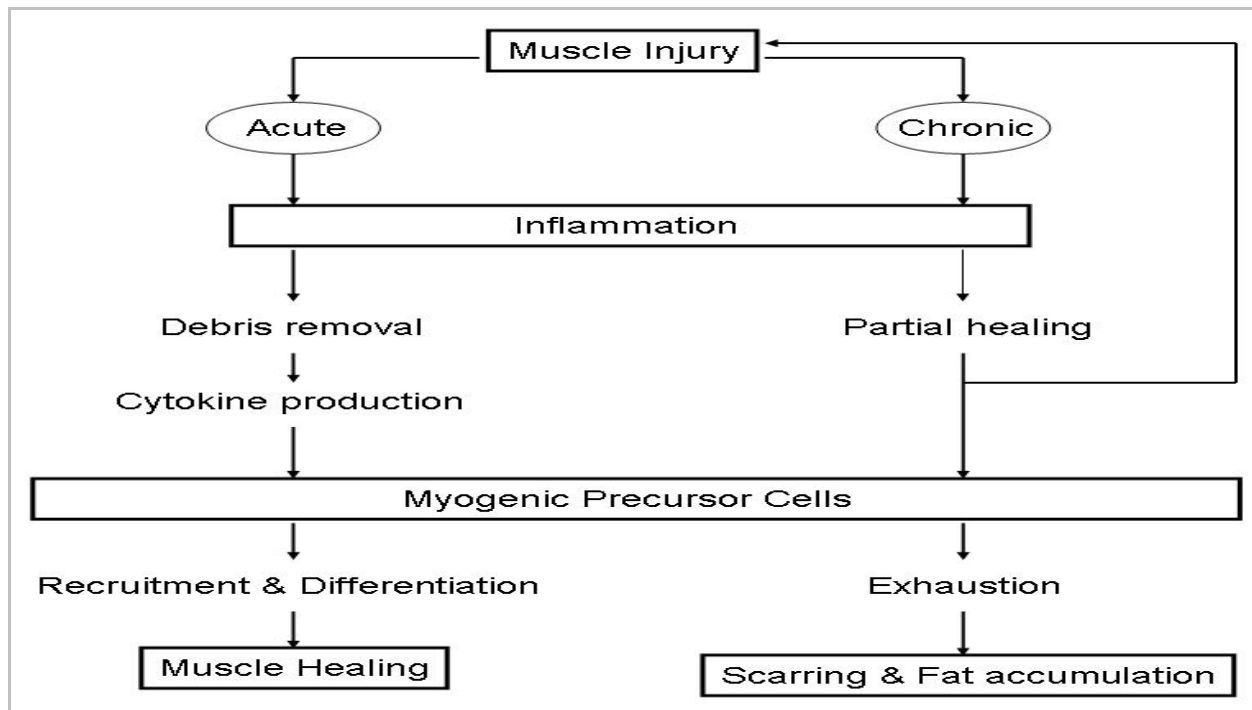


Fig.2.1: Schematic representation of the macrophage-stimulated events which follow acute or chronic muscle injury (adapted from Brunelli & Rovere-Querini 2008).

2.4. The SJL Mouse Model

The SJL mouse is a naturally occurring model for LGMD2B and MM. These mice develop a spontaneously occurring myopathy, inherited as an autosomal recessive trait, resulting in muscle weakness (Bittner *et al.* 1999, Vafiadaki *et al.* 2001). SJL mice show (Bittner *et al.* 1999, Suzuki *et al.* 2005) a deficiency in dysferlin and inflammatory changes in muscle. The mouse dysferlin cDNA has been cloned and analysis thereof revealed a >90% percent overall amino acid sequence homology between the human and mouse dysferlin genes (Vafiadaki *et al.* 2001). The SJL mice do however present with a milder phenotype than human patients with LGMD2B and MM; thought to be due to the in-frame deletion of 171 base pairs of the protein in mice, rather than the variety of stop codon or frame-shift mutations seen in human patients with Dysferlinopathy (Suzuki *et al.* 2005). The SJL phenotype is most likened to that of human patients with a missense mutation of dysferlin (Bittner *et al.* 1999).

The spontaneous myopathy (Suzuki *et al.* 2005) of SJL mice begins at four to six weeks of age and is nearly complete by eight months of age. In a study by Suzuki and colleagues (Suzuki *et al.* 2005), Quadriceps Femoris muscles were dissected from mice to investigate muscle pathology and the progression of myopathy. They reported that in two month old SJL mice, there were slight or no muscle changes compared to two month old control mice. On the other hand, nine month old SJL mice had an increased variety of muscle fiber diameters, degenerating and regenerating fibers, as well as an increased inflammatory response. They then defined the two month time-point as the early stage of Dysferlinopathy, and the nine month time-point as the progressive stage of Dysferlinopathy.

In another study by Bansal and co-workers (Bansal *et al.* 2003) muscle regeneration was studied in SJL mice in various stages of disease. Muscle regeneration is a response to muscle degeneration in muscular dystrophy. Muscle undergoing regeneration is easily identified by the presence of centrally nucleated muscle fibers and large variability in fiber size. Their findings were that about ten percent of all fibers in dysferlin-null mice were centrally nucleated by two months of age, twenty percent by four months, forty-eight percent by eight months and sixty-five percent by ten months of age. Centrally nucleated muscle fibers in control or wild-type mice never exceeded two percent at any age.

In the study of inflammation in SJL mice muscle, it is found that most of the muscle-infiltrating cells are macrophages and CD4⁺ T-cells (Kostek *et al.* 2002, Suzuki *et al.* 2005). Macrophages are found within or near injured muscle fibers (Selcen *et al.* 2001), consistent with their role in responding to muscle fiber damage (Tidball & Wehling-Hendricks 2005).

Morphological blood counts (differential or otherwise) of these animals have not, as yet, been documented in research papers.

Also of interest is the observation that SJL/J muscles display a superior regeneration, when compared to the BALB/c mouse model, due to the faster and more extensive inflammatory response in these animals. This, combined with a greater capacity for myotube formation as well as the presence of intrinsic muscle factors appears to play a central role in the regeneration of damaged muscle fibers (Mitchell CA *et al.* 1992, Smythe *et al.* 2008).

2.5. Immune Modulation

Immune modulation involves the use of drugs to suppress inflammation in order to prevent a patient's immune response to degenerating muscle from causing additional death of muscle cells (Jain Foundation Inc. 2008). According to researchers of the Jain Foundation (Jain Foundation Inc. 2008), this strategy is used partially to manage other Muscular Dystrophies, but is still in development for dysferlin deficiency. No immune modulators are specified in this regard.

Inflammation is a characteristic observation in dysferlin deficient muscle in humans and mice (Diers *et al.* 2007) although a clear explanation is still missing. According to Diers and colleagues (Diers *et al.* 2007), due to clinical and histological features of inflammation, LGMD2B has been mistaken for an inflammatory myopathy in some patients especially when the CK activity is found to be extremely high. For some unknown reasons, the inflammatory response in LGMD2B seems to be more prominent in less affected muscle groups, such as the Gastrocnemius and Soleus muscles of the calves (Diers *et al.* 2007).

Ongoing studies of the mechanism by which anti-inflammatory drugs or supplements are able to affect the severity of Muscular Dystrophies have shown that some of the observed effects of treatment may occur through direct action on muscle fibers, through preventing exercise-induced loss of strength and fiber damage as well as decreasing fibrosis (Tidball 2005).

2.5.1. Coenzyme Q₁₀ (CoQ₁₀)

CoQ₁₀ or Ubiquinone (structure depicted in figure 2.2) is a vitamin-like substance which is synthesized naturally by the human body and found in the membranes of endoplasmic reticulum, peroxisomes, lysosomes, vesicles as well as the inner mitochondrial membrane (Ernster & Dallner 1995) where it is used by the mitochondria for respiration or metabolism to produce energy for growth and maintenance of the cell. CoQ₁₀ is well known as an endogenous antioxidant which protects cells and tissues from free radicals capable of damaging proteins (thus DNA) and lipids (thus membranes) as well as other cellular components (NIH 2008).

CoQ₁₀ plasma levels are used as a measure of oxidative stress (in which antioxidant levels are largely reduced). The highest tissue concentrations of CoQ₁₀ are present in the heart (cardiac muscle), liver, kidneys and pancreas, as well as in skeletal muscle tissue (Eniva Corporation 2006). The Eniva Corporation (Eniva Corporation 2006) reports that muscle tissue is extremely vulnerable to CoQ₁₀ concentrations as this tissue cannot perform without the energy-producing machinery of the cells intact.

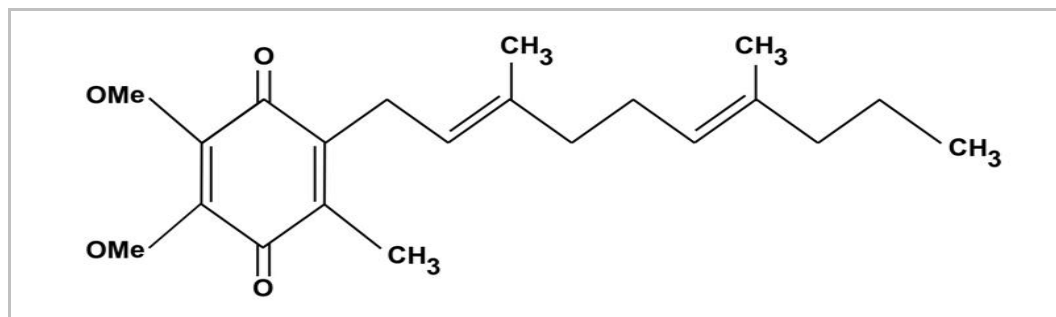


Fig.2.2: Chemical structure of Coenzyme Q₁₀ (2,3-dimethyl-6-decaprenyl-benzoquinone)

The CoQ₁₀ compound is known to have immune-stimulatory potential and has been used as an adjuvant therapy in various cancers. Stimulation of the immune system by this compound has also been observed in animal and human studies unrelated to cancer. It is best absorbed as lipid preparations rather than as a purified compound (NIH 2008). The fact that it is a highly lipid soluble compound makes it a recommended membrane protective supplement.

Tissue levels of CoQ₁₀ obviously decline with advancing age as there are increased requirements for the antioxidant and a decreased production of it by the body, usually due to an insufficient intake of its chemical precursors (NIH 2008). CoQ₁₀ levels are reported to be low in Muscular Dystrophies; and preliminary studies by various authors in Muscular Dystrophy patients taking CoQ₁₀ supplements describe improvements in exercise capacity, heart function, and overall quality of life. Folkers and Simonsen (Folkers & Simonsen 1995) support that CoQ₁₀ is vital to muscle cells in that it is involved in cellular energy production and thus growth control. Additionally, the fact that it displays membrane protective properties warrants its supplementary consideration for the upkeep of patients with Muscular Dystrophy (Folkers & Simonsen 1995). Additional research is needed in this regard as scientific evidence is as yet unclear for the use of CoQ₁₀ in treatment of Muscular Dystrophies (MedlinePlus 2007).

The fact that CoQ₁₀ is membrane protective should decrease the need for an inflammatory response in SJL mice, and it would be interesting to observe whether a decrease in inflammation will actually slow down the myopathy of muscle tissue. Additionally, the membrane protective properties of CoQ₁₀ should, theoretically, decrease the workload of the inherently insufficient membrane repair mechanism of dysferlinopathic muscle.

2.5.2. Resveratrol

Resveratrol is a polyphenolic compound (classified according to its chemical structure, depicted in figure 2.3) found largely in the skins of red grapes (McElderry 1999, Mitchell T 2008). It is classified as a phytoalexin, which is a class of antibiotic compounds produced naturally as part of a plants defence system against bacterial and other infection (McElderry 1999, Breen *et al.* 2008, Naderali 2009) as well as environmental stressors (Das KD & Maulik 2006) including UV radiation (Roy & Lundy 2005). Resveratrol has been examined for antioxidant (McElderry 1999, Roy & Lundy 2005, Mitchell T 2008), anti-inflammatory (Roy & Lundy 2005, Mitchell T 2008), blood-sugar-lowering, anti-cancer and phytoestrogen properties (McElderry 1999), as well as anti-lipid, anti-aging, and vasoprotective effects (Breen *et al.* 2008, Naderali 2009), together with its anti-atherogenic effects in atherosclerosis (Roy & Lundy 2005).

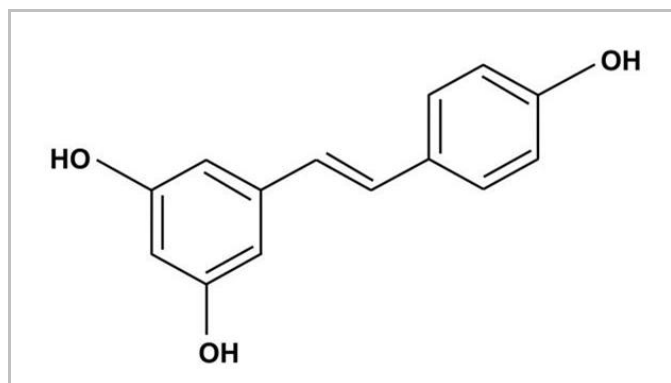


Fig.2.3: Chemical structure of Resveratrol (3,5,4'-trihydroxy-trans-stilbene)

Anti-inflammatory characteristics are exerted by Resveratrol due to its ability to act against Linoleic acid (Mitchell T 2008), as well as inhibit cyclo-oxygenase-1 (COX1) (McElderry 1999). Linoleic acid is converted to Arachidonic acid which is then converted to pro-inflammatory substances by COX1. The promotion of inflammatory processes may stimulate cancer cell growth, amongst other things (Mitchell T 2008).

Scientific studies demonstrate beneficial cardiovascular effects. Resveratrol is able to function as an *in vivo* antioxidant (Das DK *et al.* 1999, Palsamy & Subramanian 2008) and markedly reduces the risk of developing coronary heart disease; this property is likely due to its modulation of lipid metabolism and prevention of oxidation of the low density lipoproteins (LDL) (Frankel *et al.* 1993, Palsamy & Subramanian 2008), as well as inhibition of eicosanoid production and platelet aggregation (Pace-Asciak *et al.* 1995, Palsamy & Subramanian 2008). Resveratrol contains highly hydrophilic and lipophilic properties, and is thus thought to provide

more effective protection than other well-known antioxidants such as vitamins C and E (McElderry 1999). Additionally, reduced platelet aggregation has been demonstrated (McElderry 1999, Roy & Lundy 2005), as well as inhibition of inflammatory enzymes (Roy & Lundy 2005). Furthermore, promotion of vasodilation is demonstrated (Roy & Lundy 2005, Mitchell T 2008) as Resveratrol enhances NO production, inhibits the proliferation of cells in blood vessels that narrow the arteries, and prevents blood cells from adhering to one another through inhibition of thrombin and other “stickiness”-promoting factors (Mitchell T 2008).

Resveratrol can additionally inhibit several important enzymes involved in carcinogenesis (Fontecave *et al.* 1998, Chun *et al.* 1999, Palsamy & Subramanian 2008). It has been shown to act as an antioxidant by inhibiting free radical formation and also to have anti-mutagenic properties in tumour initiation. An appealing characteristic of the compound is its minimal toxicity to blood-forming cells (McElderry 1999). Very impressive is its exhibition of little or no toxic effects in healthy cells, as well as its high efficacy against multiple sites (Mitchell T 2008). Additionally, Resveratrol has been reported to elicit numerous cellular responses including cell cycle arrest, differentiation, and apoptosis (Joe *et al.* 2002, Palsamy & Subramanian 2008). It also displays anti-leukaemic, antiviral and neuroprotective properties (Virgili & Contestabile 2000, Bhat *et al.* 2001, Gao *et al.* 2002, Palsamy & Subramanian 2008). The *in vivo* effectiveness of Resveratrol is however limited by its poor systemic bioavailability. This is due to the rapid metabolism of the compound in the GIT and liver into conjugated forms, namely glucuronate and sulfonate. The strongest evidence for anti-cancer action thus exists for tumours which Resveratrol can come into direct contact with.

Studies on spinal cord injury have also produced some beneficial results. Resveratrol was demonstrated to reverse the signs of inflammatory response to spinal cord injury on a level comparable to Prednisone, a steroid used to prevent inflammation; but with better energy compensation and protection against free radicals, when injected immediately after injury. Besides helping to ameliorate spinal cord injury through free radical blockade, Resveratrol actually inhibits specific enzymes that change the way individual cells respond to the injury (Mitchell T 2008).

There is also a suggested upregulation of gene expression regulating mitochondrial activity through Resveratrol treatment as well as a proven increase in aerobic capacity. The increase in mitochondrial oxidative phosphorylation and aerobic capacity are associated with increased longevity, therefore improving animal survival time (Dasgupta & Milbrandt 2007, Naderali 2009). Mitochondria are the principal energy sources of the cell that convert nutrients into energy through cellular respiration (Wallace 2005, Lagouge *et al.* 2006). Compromised

mitochondrial function has been linked to numerous diseases, including those of the metabolic and cardiovascular systems (Petersen *et al.* 2003, Lagouge *et al.* 2006).

With impaired mitochondrial function, fatty acids are directed toward storage as opposed to oxidation. This may contribute to the lipid accumulation within muscle cells, which has been linked to insulin resistance in obesity and Diabetes Mellitus type 2 (Virkamaki *et al.* 2001, Patti *et al.* 2003, Petersen *et al.* 2004, Lagouge *et al.* 2006). In line with this, Resveratrol significantly improves both muscle oxidative capacity and sensitivity to insulin in high-calorie fed mice (Lagouge *et al.* 2006). In a study by Baur and colleagues (Baur *et al.* 2006) Resveratrol was also shown to modulate known longevity pathways and improve general health; including survival, motor function, insulin sensitivity, organ pathology, and mitochondrial number.

Resveratrol's anti-inflammatory effects and improvement of motor function are of the most interest to this study. We wish to perceive the effects of the compound against the inflammatory processes distinctive of Dysferlinopathy as well as to observe a slowing in negative motor function as the disease progresses.

Chapter 3: Implementation of the Animal Model

3.1. Introduction

There are several rodent strains (Ho *et al.* 2004) available for the study of Dysferlinopathy. The mouse dysferlin gene possesses an overall amino acid sequence homology in excess of ninety percent when compared to that of humans (Vafiadaki *et al.* 2001). Commonly used animals include the A/J (late age of onset, slow progression), *Dysf*^{-/-} (intermediate age of onset, fast progression), as well as the SJL (early age of onset, fast progression) mouse models (Ho *et al.* 2004). The SJL mouse model was chosen for this study due to the fact that the mutation of the dysferlin gene in this animal model is most like the commonest mutation seen in the human disease, i.e. a missense mutation of dysferlin (Bittner *et al.* 1999).

SJL/J mice are an inbred strain of Swiss mice with albino appearance, bred since 1955. The animals are characterised by extreme aggression in males, and are susceptible to experimental autoimmune encephalomyelitis for which they are used in Multiple Sclerosis research. These animals develop a spontaneous myopathy resulting from a splice-site mutation in the dysferlin gene (JAX Mice Database 2009). They thus display decreased levels of dysferlin protein and characteristic inflammatory changes in muscle (Bittner *et al.* 1999, Suzuki *et al.* 2005), making the animals a good model for Limb-Girdle Muscular Dystrophy type 2B (LGMD2B) and Miyoshi Myopathy (MM) research. The spontaneous myopathy which they undergo is characterised by a progressive loss of muscle mass and strength corresponding with an increase in muscle pathology (JAX Mice Database 2009). Suzuki and co-workers (Suzuki *et al.* 2005) have defined the early stage of Dysferlinopathy in SJL mice as occurring at the time-point of two months of age, and nine months of age was defined as the progressive stage of Dysferlinopathy. SJL/J muscle weakness is detected as early as three weeks of age, with the greatest muscle pathology after six months of age. The animals are immunocompetent and are known to have elevated levels of circulating T-cells (JAX Mice Database 2009).

SWR/J mice are an inbred strain of Swiss mice with albino appearance, bred since 1926. These animals are used widely as a general purpose strain; but are highly susceptible to experimental allergic encephalomyelitis. Additionally, these animals are not immunocompromised (JAX Mice Database 2009).

The variable of extreme male aggression warranted the use of females in the present study, and the termination dates were selected to coincide with the lapse of age time-points known for greatest muscle pathology.

3.2. Materials and Methods

3.2.1. Implementation of the Animal Models

SJL/J dysferlin-null mice as well as SWR/J mice with normal dysferlin were used in this study and housed at the University of Pretoria's Biomedical Research Centre (UPBRC). An import permit was obtained from the Department of Veterinary Services, Ministry of Agriculture, and the conditions stipulated therein were strictly adhered to. The animals – SWR/J as negative control mice without Muscular Dystrophy and SJL/J as dysferlin-null mice for experimentation – were imported from The Jackson Laboratory (Bay Harbor, USA) and allowed a 40 day acclimatisation period before commencement of the study. All experimental procedures, to follow, were carried out in strict accordance with the requirements of the University of Pretoria's Animal Use and Care Committee. Ethical clearance was obtained from the UPBRC.

60 Female mice consisting of 50 eight week old SJL/J and 10 nine week old SWR/J mice (each an average weight of 20g) were maintained and housed at the laboratory animal facility of the UPBRC at Onderstepoort (University of Pretoria, Pretoria, South Africa). The animals were maintained in the facility's barrier unit in Tecniplast IVC cages, Eurostandard type II L with individual air supply, equipped with a pre-filter and HEPA filter system. The barrier unit was free of all major pathogens as serology, bacteriology and parasitology tests performed were all negative. Agrebe basic cotton sheet laboratory animal cloth bedding of 55cm² for boxes Type II/III LI was used and changed once weekly. The main air supply consisted of a 50% Fresh Air Primary filter and a Secondary Bag filter with 10-15 air changes per hour. A constant temperature of 23-24°C, a relative humidity of 40-60%, and a 12 hour dark-light cycle were maintained. The animals were provided JL Rat and Mouse 6F-IRRAD food (PMI Nutrition International, LLC) and water *ad libitum*. As a decrease and slowing in the mobility of the animals was observed with disease progression, it was decided upon to supplement the animals' diet by addition of Cerelac, sterile baby cereal on the floor of each cage, from day 50 of the 90 day trial.

At study commencement, the animals were 14 and 15 weeks old respectively for SJL/J and SWR/J mice. Animals were divided into seven experimental groups as displayed in table 3.1, which depicts the strain of animals in each of the groups as well as the treatment and dosages administered to them. Six randomly selected SJL/J animals were terminated for Baseline (Group G) results at this time. The remainder of the animals were entered into a 90 day experimental period, treatment being administered as follows: Control groups (Groups A and B) received a placebo in the same volume as the animals who received the substances tested in

the study. Experimental groups (Groups C-F) received volumes of Resveratrol and Coenzyme Q₁₀ (CoQ₁₀) in dosages displayed in table 3.1. All dosing was performed orally, using a 1000µl syringe with a mouse oral gavage needle nr.20g, once daily. The study was effectively concluded when the animals had reached approximately 27- and 28-weeks of age respectively for SJL/J and SWR/J mice.

Termination procedures were thus performed for Group G at 14 weeks of age, for Groups B-F at 27 weeks of age and for Group A at 28 weeks of age. The animals were terminated by heart puncture under anaesthesia. During the termination process, blood smears of each individual animal were made. After termination, muscle biopsies were taken of the Pectoralis and Gluteus complexes for light microscopy as well as transmission- and scanning-electron microscopy.

Table 3.1: Subdivision of experimental animals into groups, indicating the treatment dosages administered to each animal within respective groups, as well as the age of the mice at which termination was performed.

Experimental Group	Strain	Treatment	Treatment Dosage (mg/kg/day)	Age at Termination (weeks)
Group A	SWR/J	None (Negative Control)	-	28
Group B	SJL/J	None (Positive Control)	-	27
Group C	SJL/J	Resveratrol	60	27
Group D	SJL/J	Low-CoQ ₁₀	40	27
Group E	SJL/J	High-CoQ ₁₀	120	27
Group F	SJL/J	Resveratrol/CoQ ₁₀ (Combination)	60/40	27
Group G	SJL/J	None (Baseline)	-	14

3.2.2. Histological Investigation of Blood

3.2.2.1. Blood Smears

Blood smears were made onto glass slides in triplicate on the day of termination, with blood drawn from each animal. All blood smears were allowed to air-dry before slide storage. The blood smears were subsequently stained using Wright's stain (quick-diff). The staining procedure is as follows: The slide is dipped into

1. Methanol five times to fix the blood smear to the slide
2. Eosin fifteen times to stain the acidic cells
3. Methylene Blue twenty-five times to stain the basic cells

Slides were then rinsed in distilled water to remove residual dye, after which they were allowed to air-dry. The best of the three slides for each animal was selected for counting. These slides were then mounted with cover-slips, using Entellan as mounting fluid, as oil submission microscope lenses were utilised. Thus a single blood smear was evaluated via light microscopy using a Nikon Optiphot Transmitted Light Microscope for each of five animals within the seven groups under study, in order to compare leukocyte morphology between groups and determine the differential leukocyte counts of the seven groups of animals. The morphological differences of leukocytes i.e. differentiation between identification characteristics of respective leukocytes, as well as the statistical differences between differential leukocyte counts in the different experimental groups will be reported on.

3.2.2.2. Statistical Analysis

The purpose of the evaluation was to quantify each cell of the leukocyte species; namely lymphocytes, monocytes, neutrophils, basophils and eosinophils; counting up to 100 leukocytes in a minimum of three longitudinal zones per slide.

The NCSS statistical program was employed to perform statistical analyses of the various leukocyte counts between the seven groups under study. Each leukocyte species was considered separately between the experimental groups via a parametric one-way ANOVA or non-parametric Kruskal-Wallis one-way ANOVA, depending on whether the necessary assumptions for the parametric test were met or not. These assumptions include the existence of normally distributed data, where equal variance can be ignored based upon equal sample size, provided that all observation is randomly and independently conducted.

3.2.3. Histological Investigation of Muscle Biopsy Samples

Muscle biopsies were taken of the Pectoralis and Gluteus complexes for light, transmission- (TEM) and scanning- (SEM) electron microscopy. All muscles were dissected from the left side of the animal, unless this was unavailable due to destruction of these muscle samples by the first dissector (taking bilateral muscle samples of Quadriceps Femoris and Gastrocnemius for a concurrent study). For purposes of repeatability, a random selection of three animals from each of the groups was made for this process before termination.

3.2.3.1. Light Microscopy

Light Microscopy samples were placed into a 2.5% Formaldehyde fixative for at least one hour. Tissue samples were rinsed three times for 15 minutes each with 0.075M Sodium Phosphate buffer, after which series dehydration was performed in 30%, 50%, 70%, 90% and three times absolute ethanol for periods of 10 minutes each. Once dehydrated, tissue samples were infiltrated with LR-White resin for one hour, changed and subsequently infiltrated overnight for two days. Finally, tissue samples were individually embedded in LR-White resin, placed into gelatine capsule moulds and allowed to dry in an oven at 60°C for approximately 39 hours in order to properly polymerise the samples for microtoming. Samples were cut at an approximate thickness of 1µm and placed onto glass slides. All light microscopic slides were stained using Toluidine Blue at a pH of approximately 11.5 for 30 seconds per slide, dried and mounted with cover-slips. Light microscopic analysis followed using a Nikon Optiphot Transmitted Light Microscope.

The following morphological alterations in dysferlinopathic muscle have been noted in literature (Selcen *et al.* 2001, Fanin & Angelini 2002, Bansal *et al.* 2003, Ho *et al.* 2004, Cennachi *et al.* 2005), and were thus scrutinised. Concerning fiber characteristics: fiber size variability, which is indicative of muscle undergoing regeneration; longitudinal fiber splitting, with fibers enclosed within the same endomysial sheath; fiber lobulation; degeneration and/or necrosis of muscle fibers, necrotic fibers being of opaque or hyaline appearance, undergoing myophagocytosis; fiber regeneration, demarcated by central or internal nuclei; fiber hypertrophy or enlargement, fibers usually round in cross-section; and finally fiber atrophy or shrinkage, where fibers are also round in cross-section. Fatty replacement of muscle fibers is found in the advanced stages of myopathy.

With regards to connective tissue: proliferation thereof as well as endomysial and perimysial thickening were scrutinised. The replacement of necrotic tissue with fibroconnective

scar tissue was also analysed, especially endomysial fibrosis which is an excessive production of connective tissue often seen in Dystrophies. It must be noted that a variable number of leukocytes are found in the interstitium, with or without the presence of fibrosis.

Finally, where infiltration is concerned: inflammatory infiltrate, which is a response secondary to necrosis, initiates the clearing of cellular debris as well as subsequent regeneration or scar formation, depending on the duration and severity of infiltrate. Perivascular infiltrate of leukocytes was also expected.

3.2.3.2. Electron Microscopy

Electron Microscopy samples were placed into a 2.5% Formaldehyde/Glutaraldehyde fixative for at least one hour. Tissue samples were rinsed three times for 15 minutes each with 0.075M Sodium Phosphate buffer, after which the samples were placed in a 1% Osmium Tetraoxide (OsO_4) secondary fixative for one hour. After a second round of rinsing three times for 15 minutes each in 0.075M Sodium Phosphate buffer, series dehydration was performed in 30%, 50%, 70%, 90% and three times absolute ethanol for a period of 10 minutes each. Once dehydrated, SEM tissue samples were dried in a critical-point drier and TEM tissue samples were infiltrated with a Quetol Epoxy resin in a 1:1 ratio with 100% ethanol for one hour, then changed and infiltrated in Quetol Epoxy resin exclusively for one day overnight. Samples were finally embedded with Quetol Epoxy resin in rubber moulds and allowed to dry in an oven at 60°C for approximately 39 hours to polymerise for ultramicrotoming.

TEM samples were cut at a thickness of approximately 70-100nm and placed onto copper grids. These were contrast stained using Uranyl Acetate for 10 minutes and Reynolds Lead Citrate for five minutes, rinsing the samples 16 times in triplicate with ddH_2O and dabbing on filter paper to dry after each staining step. Transmission electron microscopic analysis followed using a JEOL Transmission Electron Microscope (JEM 2100F).

The following morphological observations have been made in literature for TEM samples (Selcen *et al.* 2001, Ho *et al.* 2004) and were thus scrutinised: Defects and discontinuity of the sarcolemma as well as vesicle accumulation in this region; sub-sarcolemmal vacuoles and macromolecular synthesis; alterations as well as thickening and focal duplication of the basal lamina; and finally the presence of papillary projections.

SEM samples were mounted whole onto copper stubs and coated with Ruthenium Tetraoxide (RuO_4) for 30 minutes. Scanning electron microscopic analysis followed using a ZEISS ULTRA plus FEG Scanning Electron Microscope.

No literature was available in which SEM was employed. Since connective tissue between fibers is shown to be thickened in extensive light microscopic research, it was decided upon to photograph longitudinally arranged muscle fibers at 5,000X magnification, measure the connective tissue between them and subsequently compare the measurements between groups through the performance of statistical analysis thereon. Thus, for each of the muscle types analysed, three mice from each experimental group were assessed with the aid of SEM analysis. The connective tissue thickness between the muscle fibers was measured at random in nine areas of an ultramicrograph for each individual mouse, with the aid of the iTEM Olympus Soft Imaging Solutions® software. The NCSS statistical program was employed to perform statistical analysis on these measurements. The experimental groups were compared by means of either a parametric one-way ANOVA or non-parametric Kruskal-Wallis one-way ANOVA, depending on whether or not the necessary assumptions for the parametric test were met.

Chapter 4: Morphological Classification and Differential Counts of Leukocytes

4.1. Introduction

Leukocytes are involved in the cellular/innate and humoral/adaptive defences of an animal against foreign antigens. Leukocytes are functional only to a small extent in the bloodstream, their greatest activity being exhibited in the connective tissues (Leeson *et al.* 1988). They are capable of amoeboid movement, enabling them to travel out of the circulatory system in order to elicit an immune response in the tissues where such response is needed.

Leukocytes of two main types exist, namely granulocytes and agranulocytes. The granulocytes have a cytoplasm containing numerous characteristic granular bodies and possess nuclei which exhibit considerable variation in shape. Neutrophils, basophils and eosinophils are classified as granulocytes. The agranulocytes are characterised by a clear, homogenous, slightly basophilic cytoplasm. The nuclei of these leukocytes are spherical to reniform in shape. Lymphocytes and monocytes are classified as agranulocytes (Leeson *et al.* 1988).

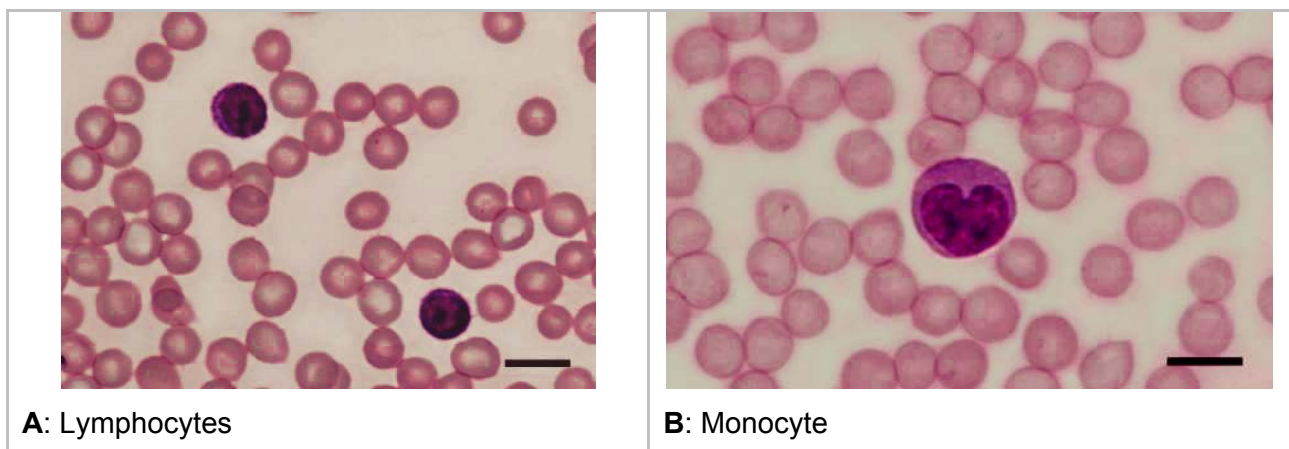
Functionally, neutrophils constitute the first line of defense against invading organisms. They actively phagocytose small particles and their granules are lysosomal in character, containing hydrolytic enzymes. Eosinophils possess granules that are lysosomal in nature and are thought to phagocytose antibody-antigen complexes formed as part of the allergic response. These granules can also reduce inflammation by inactivating histamine and leukotrienes produced by basophils. Basophil granules contain heparin (an anticoagulant) as well as histamine and serotonin. These granules are capable of producing leukotrienes, which slow the contraction of smooth muscles. The basophil, like mast cells, releases its granular content in response to the action of certain antigens. Monocytes are actively phagocytic cells. In tissues, they interact with lymphocytes of the immune defense system. Lymphocytes play a major role in the initiation of the immune responses (Leeson *et al.* 1988).

The antioxidant and sequentially anti-inflammatory properties of Resveratrol and Coenzyme Q₁₀ (CoQ₁₀) are of interest in the present study as the aim of this chapter is to determine whether or not their administration will affect the levels of inflammatory leukocytes at the haematopoietic level.

4.2. Morphological Classification of Leukocytes in Mice

Lymphocytes are small cells with very little cytoplasm, the nucleus taking up almost the entire cell (figure 4.1A). Monocytes are slightly larger cells than lymphocytes and contain greater amounts of cytoplasm (figures 4.1B and 4.1C). Mostly, the amount of cytoplasm and the size of the cells are a dead giveaway in their identification. Some lymphocytes and monocytes (figure 4.1D) can however overlap in terms of size, with small monocytes sometimes being in the same size range as larger lymphocytes. The manner in which to distinguish between them is then as follows: lymphocyte nuclei are denser and thus darker-staining than monocyte nuclei as chromatin is not as tightly wound in monocytes as in lymphocytes.

Granular leukocytes differ in the staining of their cytoplasmic granules. Neutrophil granules stain more greyish; basophil granules stain a deep blue/purple; and eosinophil granules stain pink/orange. Sometimes the difference between neutrophil and eosinophil cytoplasm is not very clear. The most reliable characteristic which distinguishes between the cells is thus the shape of the nucleus. Neutrophils have a multi-lobed nucleus with thin threads of nuclear material joining the lobes (figure 4.1E). Basophils possess a large bi-lobed (sometimes split) nucleus (figure 4.1F) which takes up most of the cell. The nucleus is sometimes however not clearly visible due to the dark staining of the cytoplasm, but it would be difficult to confuse these cells for any other. Eosinophil nuclei present in one of two forms in mice; they are either in the shape of a figure eight (figure 4.1G) or in the shape of a donut (figure 4.1H).



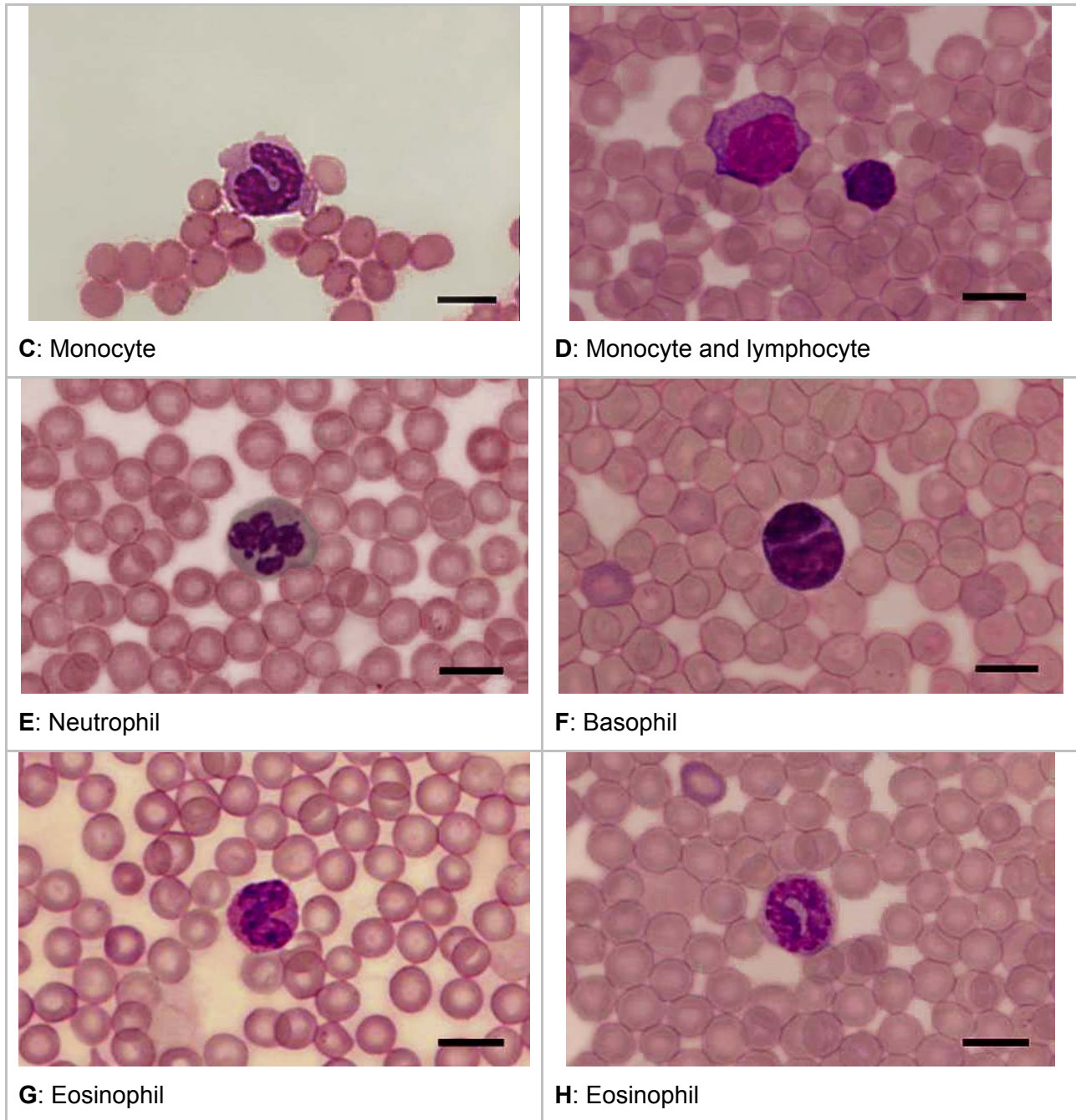


Fig.4.1: Morphological differences between leukocyte species in mice. All leukocytes were photographed at a magnification of 40X. **A:** Two lymphocytes with dense nuclei and little cytoplasm; **B:** Monocyte with classic kidney-shaped nucleus; **C:** Monocyte with extended pseudopodia; **D:** Monocyte and lymphocyte; **E:** Neutrophil with multi-lobed nucleus; **F:** Basophil with split bi-lobed nucleus; **G:** Eosinophil with figure-8 nuclear shape; **H:** Eosinophil with donut-shaped nucleus. (Scale bar = 10µm)

4.3. Differential Leukocyte Counts

The mean overall leukocyte counts per experimental group are shown in figure 4.2. Firstly it must be noted that both monocytes and macrophages circulating in the bloodstream were counted as monocytes. From the graph, it can be seen that the Combination treated group had the highest lymphocyte count. This group also had the lowest monocyte, neutrophil and eosinophil counts, as well as the lowest non-zero basophil count. It is thus believed that this group will display the least inflammation at the tissue level. The Baseline group had the highest neutrophil, eosinophil and basophil counts. The High-CoQ₁₀ treated group showed the lowest lymphocyte counts, as well as the lowest (zero) basophil count, in conjunction with the Positive Control group. It is noteworthy that the High-CoQ₁₀ treated group and the Positive Control group, with basophil counts of zero had the highest monocyte counts overall. Furthermore, the High-CoQ₁₀ treated group had the smallest lymphocyte to monocyte ratio of the experimental groups, in conjunction with the Negative Control group.

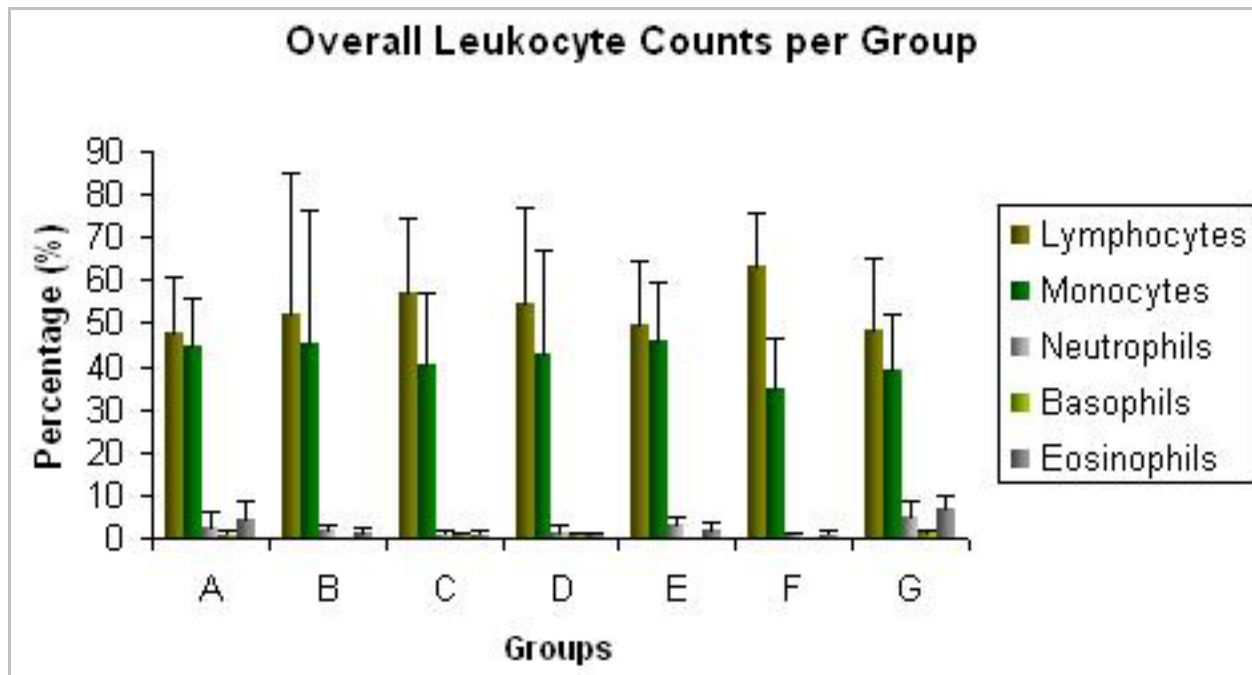


Fig.4.2: Mean leukocyte counts of five animals assessed per group A-G. Group A: Negative Control SWR/J mice at 28 weeks of age. Groups B: Positive Control; C: Resveratrol treated; D: Low-CoQ₁₀ treated; E: High-CoQ₁₀ treated; F: Combination Resveratrol/CoQ₁₀ treated SJL/J mice at 27 weeks of age. Group G: Baseline untreated SJL/J mice at 14 weeks of age. Standard deviations are indicated, it must be noted that these are relevant both above and below the maximum value represented in the bar graphs though only indicated above.

The statistical analysis of each of the seven groups monocyte, lymphocyte and neutrophil counts was performed through a one-way ANOVA, as all the necessary assumptions for these parametric tests were met. Analysis of eosinophil and basophil counts were conducted with the aid of the non-parametric Kruskal-Wallis one-way ANOVA, as the data considered did not assume a normal distribution. Table 4.1 expresses the numerical results of these statistical tests run for leukocyte species as well as the outcomes thereof, with statistical significance set at 95%, i.e. with P-values of less than 0.05.

Monocyte, lymphocyte and basophil counts displayed no significant differences ($P > 0.05$) between any of the groups assessed. Eosinophil counts were significantly higher ($P < 0.05$) in the Baseline group than all other experimental groups assessed except for the Negative Control SWR/J mice. Neutrophil counts of the Baseline termination group were significantly higher ($P < 0.05$) only when compared to the Combination treated group.

Table 4.1: Statistical comparisons performed on the various leukocyte species, derived from the blood counts of the assessed SJL/J and SWR/J mice under various experimental conditions

Leukocyte	Test	P-value*	Inter-group Comparison
Monocytes	Parametric	0.961	No significant differences
Lymphocytes	Parametric	0.857	No significant differences
Eosinophils	Non-parametric	0.0104	Group G significantly higher than Groups B-F
Neutrophils	Parametric	0.0454	Group G significantly higher than Group F
Basophils	Non-parametric	0.0654	No significant differences

* Significance was set at a level of 0.05

Granulocytes circulate in the blood unless recruited to act as effector cells at sites of infection and/or inflammation; whereas agranulocytes continuously circulate between the tissues and the bloodstream (Janeway *et al.* 2005).

Monocytes complete their differentiation in the tissues, where they are referred to as macrophages. Macrophages and neutrophils are of the innate immune system, they are essential for the control of common infections, and provide the first line of defence against many common microorganisms. Cells of the innate immune system play a crucial role in the initiation

and direction of adaptive immune responses, as well as subsequent removal of pathogens that have been targeted by an adaptive immune response (Janeway *et al.* 2005).

Monocytes continuously mature into macrophages upon migration into the tissues and are most abundant in the connective tissues but continuously recirculate using the bloodstream as transport medium. Previous histological study has revealed that macrophages are the dominant interstitial inflammatory cells in SJL/J mice at any age (Nemoto *et al.* 2007). Neutrophils are short-lived (only a few days) and produced in increased numbers during an immune response, when they leave the blood to migrate to sites of infection or inflammation.

Neutrophils are linked to early inflammatory responses and often sensitised in self-antigen recognition characteristic to autoimmune disease (Thomas *et al.* 2005), a known experimentally induced state of the SJL mouse strain (Vafiadaki *et al.* 2001). Neutrophils play a crucial role early in myofiber necrosis in dystrophic myofibers (Hodgetts *et al.* 2006). The fact that the neutrophil counts were highest in the Baseline group is entirely expected when it is reasoned that neutrophils are highest in early innate immune responses to injury, as they are considered the first line of defence. Other leukocytes are recruited to the site of infection or injury later on in an immune response, and the neutrophil numbers drop as can be seen in Groups B-F as the disease progresses.

Since neutrophil-mediated muscle injury is largely dependent on the production of free radicals (Rando 2001, Hodgetts *et al.* 2006) it is conceivable that the additive effects of the antioxidants in the Combination treated group, possessing the lowest neutrophil counts, would later display the least muscle inflammation and/or damage, especially since this group additionally has the lowest eosinophil and monocyte counts overall. At this point it is important to note that clinical significance is often seen at levels where mathematical significance does not yet exist.

Lymphocytes are cells of adaptive immunity, which, once differentiated are able to provide a more versatile means of defense than cells of innate immunity. This provides an increased protection against reinfection with the same pathogen. Later in an inflammatory response, lymphocytes are also recruited to the sites of inflammation (Janeway *et al.* 2005). It is normal for lymphocytes to be abundant in circulation, as a large population of these are memory cells and thus key in prevention of reinfection down the line.

Eosinophils are thought to be important primarily in defence against parasitic infections. Very small numbers of eosinophils are normally present in the circulation; most are in fact found in the tissues, especially connective tissues. Few eosinophils are produced in the absence of infection or other immune stimulation; but upon stimulation, there is an increased production of

these cells in the bone marrow and their release into the circulation. Eosinophil activation and degranulation is strictly regulated. Their synthesis of chemical mediators amplifies the immune response by activating epithelial cells and recruiting and activating more eosinophils and other leukocytes. In local allergic reactions, eosinophils accumulate in large numbers in tissues. Their continued presence, however, is characteristic of chronic allergic inflammation and is thought to be a major contributor to tissue damage (Janeway *et al.* 2005).

Eosinophil granules have previously been suggested to reduce inflammation caused by other leukocytes (Leeson *et al.* 1988). At onset of Dysferlinopathy between four and six weeks of age, the increase in eosinophil counts could very likely be a compensation mechanism to decrease initial inflammation in the muscular tissues of the dysferlinopathic mice. However, in excess they cause tissue damage and counteract this proposition.

We presume that in this disease state, eosinophil numbers are very likely higher in tissue than in the bloodstream. With this assumption follows the reasoning that eosinophils may play a definitive role in the initial pathogenesis of Dysferlinopathy. If they cause the main tissue damage at onset of the disease, and dysferlinopathic muscle is unable to repair the damage, it is logical that eosinophil counts will be down-regulated by the immune system to lower-than-normal numbers, as seen in SJL/J animal Groups B-F as compared to the Negative Control SWR/J animals of Group A, to prevent further tissue damage which the inherently defective repair mechanism is unable to keep up with.

Basophils, like eosinophils, are present at the sites of inflammatory reactions. They are normally present in very low numbers in circulation and seem to have a similar role to that of eosinophils in the defence against pathogens, as they are also recruited to sites of allergic reactions. They release substances that affect vascular permeability and are believed to protect mucosal surfaces against pathogens, as well as playing a definite role in orchestrating allergic responses (Janeway *et al.* 2005).

4.4. Concluding Remarks

Although the anti-inflammatory properties of Coenzyme Q₁₀ and Resveratrol are not evident at the haematopoietic level, it must be noted that clinical significance is often seen at levels where mathematical significance does not yet exist, and it is thus hoped that it will be prominent at the tissue level. Further microscopic investigation of dissected muscle tissue will follow to ascertain these results.

In the present study, eosinophils are proposed to play a definitive role in the pathogenesis of Dysferlinopathy. In small numbers, eosinophils may well serve an anti-inflammatory purpose; but in the heightened numbers experienced in the Baseline group, it is proposed that they cause tissue damage. If they are the cells responsible for major tissue damage, secondary to initial damage by neutrophils, then a compensation mechanism inherent in muscle may well down-regulate their numbers to lower-than-normal levels in order to prevent further massive damage as dysferlinopathic muscle is indeed deficient in its repair mechanism.

Neutrophils are proposed to be largely responsible for the inflammation at onset of myopathy, as these are the primary cells of an inflammatory response; additionally they play a crucial role early in myofiber necrosis of Dystrophic muscle. It is conceivable that the effects of antioxidants, especially that of the Combination treated group, possessing the lowest neutrophil counts, would later display the least muscle inflammation and/or damage, especially since this group additionally has the lowest eosinophil and monocyte counts overall.

Chapter 5: Microscopic Investigation of Pectoralis Complex Muscle Samples

5.1. Introduction

The Pectoral muscles are situated at the upper anterior part of the chest, in front of the axilla (Gray 2001) and are, in effect, the muscle group that connects the front walls of the chest with the bones of the upper forelimb and shoulder (Encyclopaedia Britannica 2009). In animals, the pectoral or shoulder girdle is the anterior skeletal girdle, offering support to the pectoral or forelimb. The Pectoralis complex is a prominent ventral muscle group of the chest originating along the sternum and converging on the humerus in mice (Kardong 2006). This muscle complex is not commonly studied in cases of Dysferlinopathy. It is of interest as a less- or insignificantly-affected muscle group as it may provide data indicating how the disease affects muscles not directly responsible for the weakness associated with Dysferlinopathy.

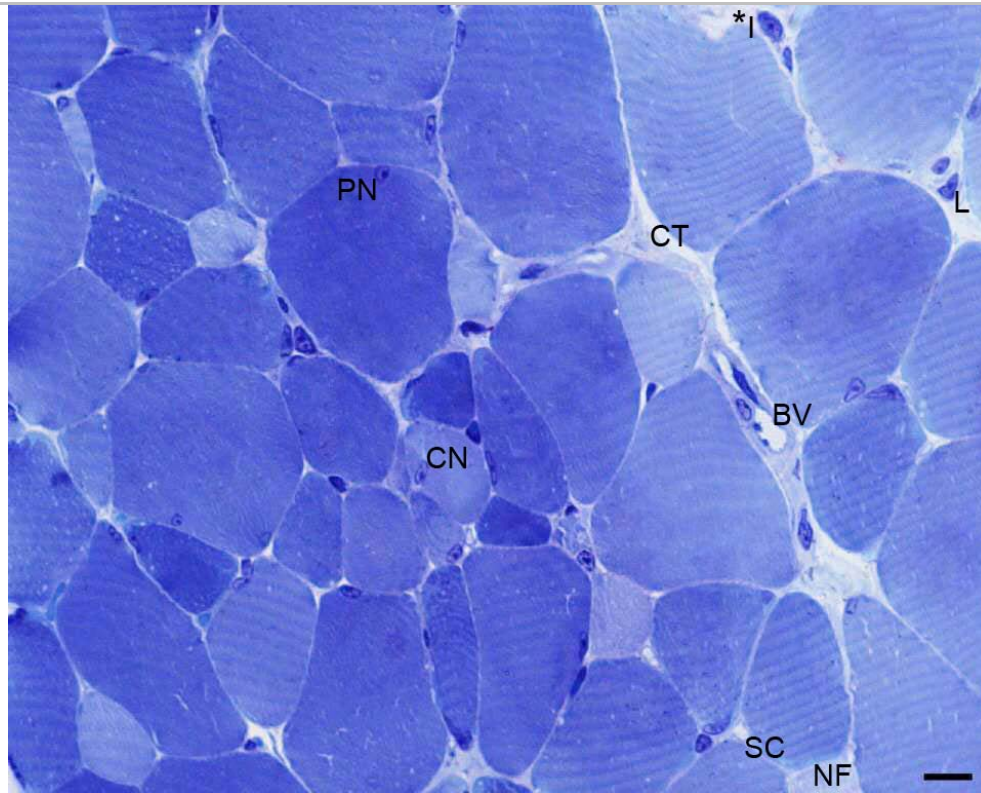
Photomicrographs were taken using light microscopy as well as transmission- and scanning- electron microscopy. Each of these subsets were analysed for various characteristics or the lack thereof, as set out in this chapter.

For the purposes of light microscopic analysis micrographs were taken at 40X magnification and performance of morphological analysis on fiber and infiltrative characteristics as well as the presence of myogenic stem cells was subsequently undertaken. Transmission electron ultramicrographs were taken at a magnification of approximately 60,000X and morphological analysis was concentrated on the sarcolemma and vesicle accumulation and fusion therewith as well as duplication and thickening of the basal lamina overlying the membrane. Finally, with regards to scanning electron microscopy, ultramicrographs were taken at a magnification of 5,000X. Statistical analysis was then performed on calibrated measurements of the connective tissue thickness between longitudinally arranged muscle fibers.

5.2. Light Microscopic Investigation

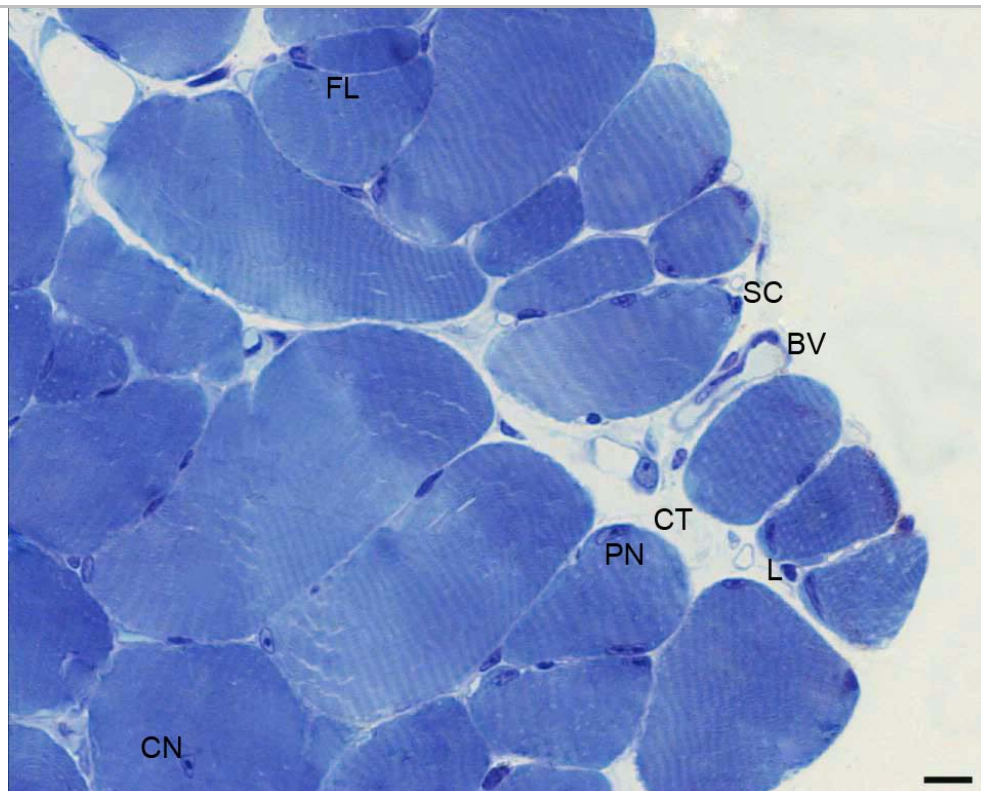
Light microscopic photographic images were taken of Pectoralis complex muscle samples of each of three animals from the seven groups under study. Of these, the image most representative of the group was selected for insertion in figure 5.1 (A-G). The characteristics most representative of Dysferlinopathy were scrutinised throughout the sample and are labelled as such in the figures. Figure 5.2 offers a summary of changes assessed within the full sample; these include various fiber characteristics, infiltrative and proliferative changes as well as the presence of satellite cells, set out in detail in the relevant section.

With regards to fiber characteristics in the light microscopic investigation, the following is of note: Fiber size variability, especially in large degrees, is suggestive of muscle fibers undergoing regenerative changes. Peripheral nuclei are characteristic of mature muscle fibers, whereas central or internal nuclei are indicative of regeneration. Necrotic fibers are distinguishable from other myofibers in that they are of opaque or glassy appearance. Fiber splitting within pictures is marked as the early stages of splitting, whereas fiber lobulation was chosen to mark the later stages of splitting where a fiber's endomysial sheath appears to enclose wholly more than one myofiber. Leukocyte infiltration takes three forms: inflammatory and regenerative infiltration which are indistinguishable morphologically, and postnecrotic infiltration which is responsible for the removal of cellular debris once necrosis of a fiber has taken place. Finally, the presence of satellite cells (and the numbers thereof) is indicative of the regenerative ability of muscle tissue. Regardless of the presence of multiple characteristics, only one of each is marked in each of the micrographs. The micrographs serve to present the morphological differences between Pectoralis complex muscles in groups A-G, with reference to characteristics mentioned above.



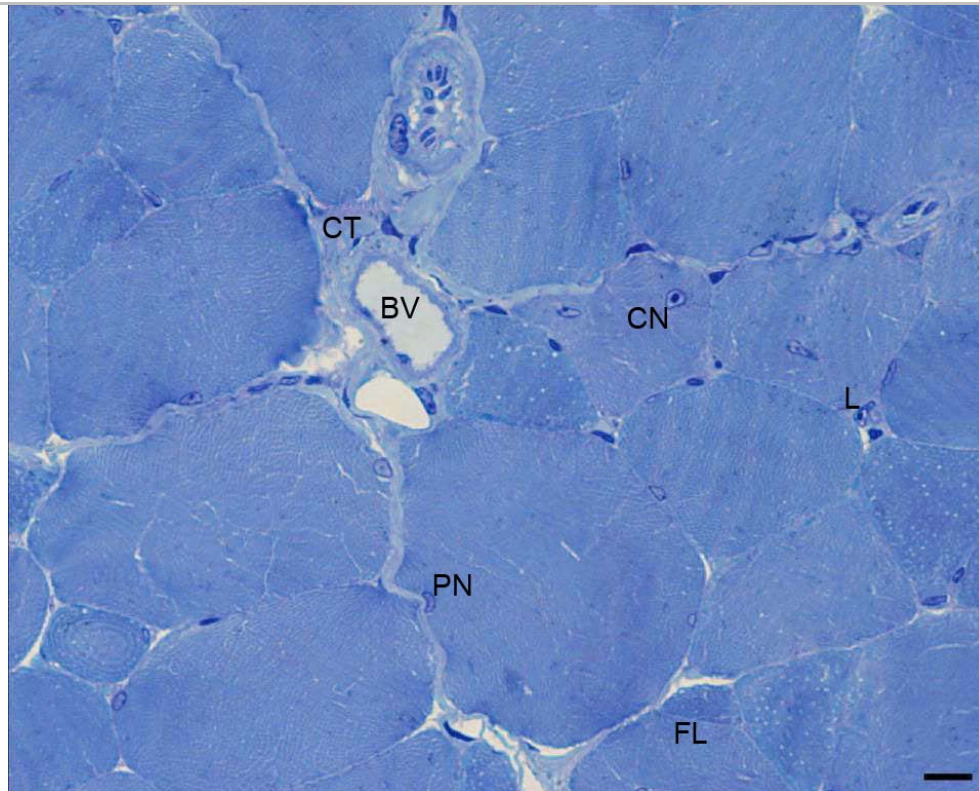
A: Negative Control

SC: Satellite Cell
L: Leukocyte
***I:** Infiltrate
PN: Peripheral Nucleus
CN: Central Nucleus
NF: Necrotic Fiber
CT: Connective Tissue
BV: Blood Vessel



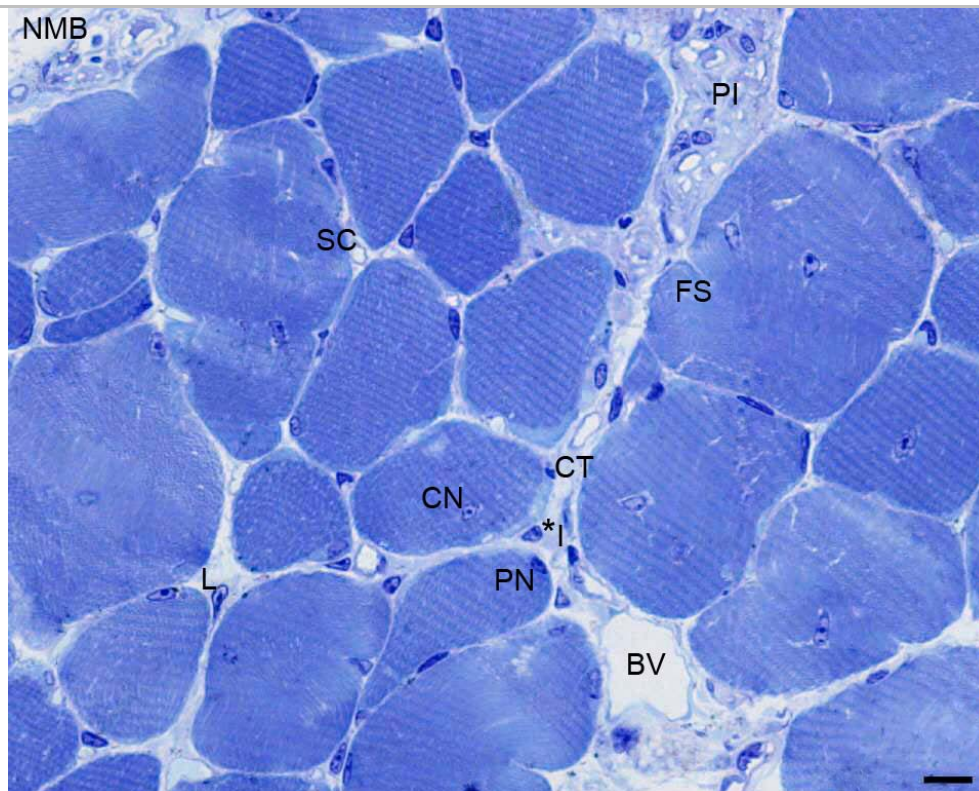
B: Positive Control

SC: Satellite Cell
L: Leukocyte
PN: Peripheral Nucleus
CN: Central Nucleus
FL: Fiber Lobulation
CT: Connective Tissue
BV: Blood Vessel



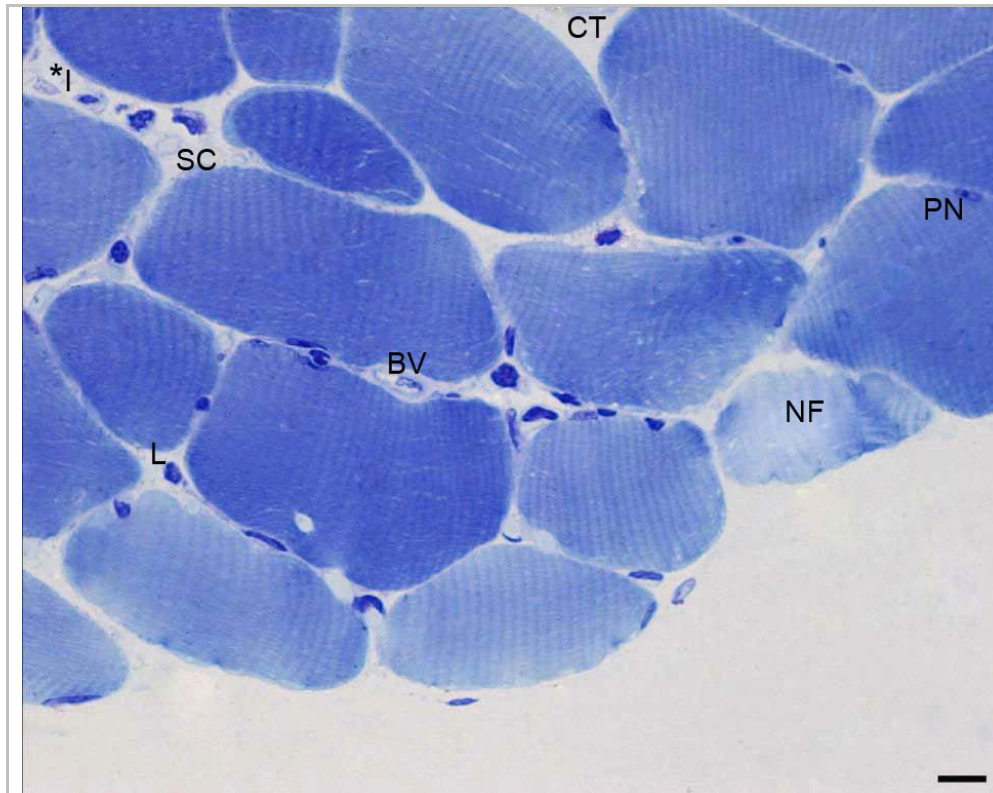
C: Resveratrol

L: Leukocyte
PN: Peripheral Nucleus
CN: Central Nucleus
FL: Fiber Lobulation
CT: Connective Tissue
BV: Blood Vessel



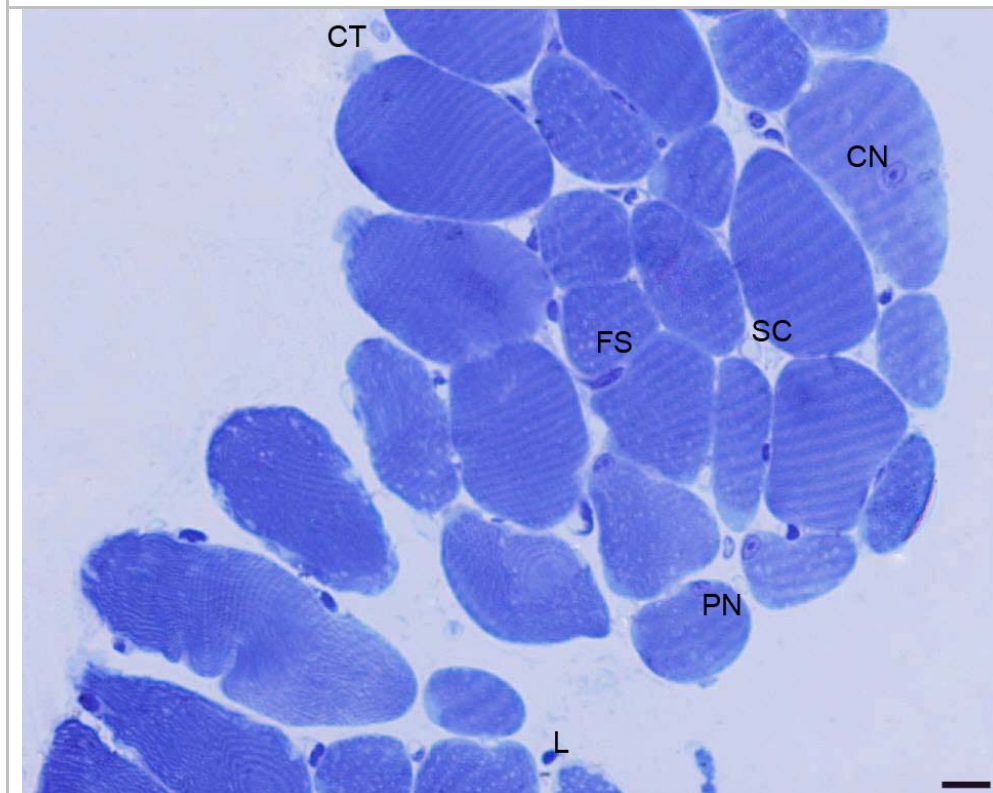
D: Low-CoQ₁₀

SC: Satellite Cell
L: Leukocyte
***I:** Infiltrate
PN: Peripheral Nucleus
CN: Central Nucleus
CT: Connective Tissue
FS: Fiber Splitting
PI: Postnecrotic Infiltrate
BV: Blood Vessel
NMB: Neuromuscular Bundle



E: High-CoQ₁₀

SC: Satellite Cell
L: Leukocyte
***I:** Infiltrate
PN: Peripheral Nucleus
CT: Connective Tissue
NF: Necrotic Fiber
BV: Blood Vessel



F: Combination Resveratrol/CoQ₁₀

SC: Satellite Cell
L: Leukocyte
PN: Peripheral Nucleus
CN: Central Nucleus
FS: Fiber Splitting
CT: Connective Tissue

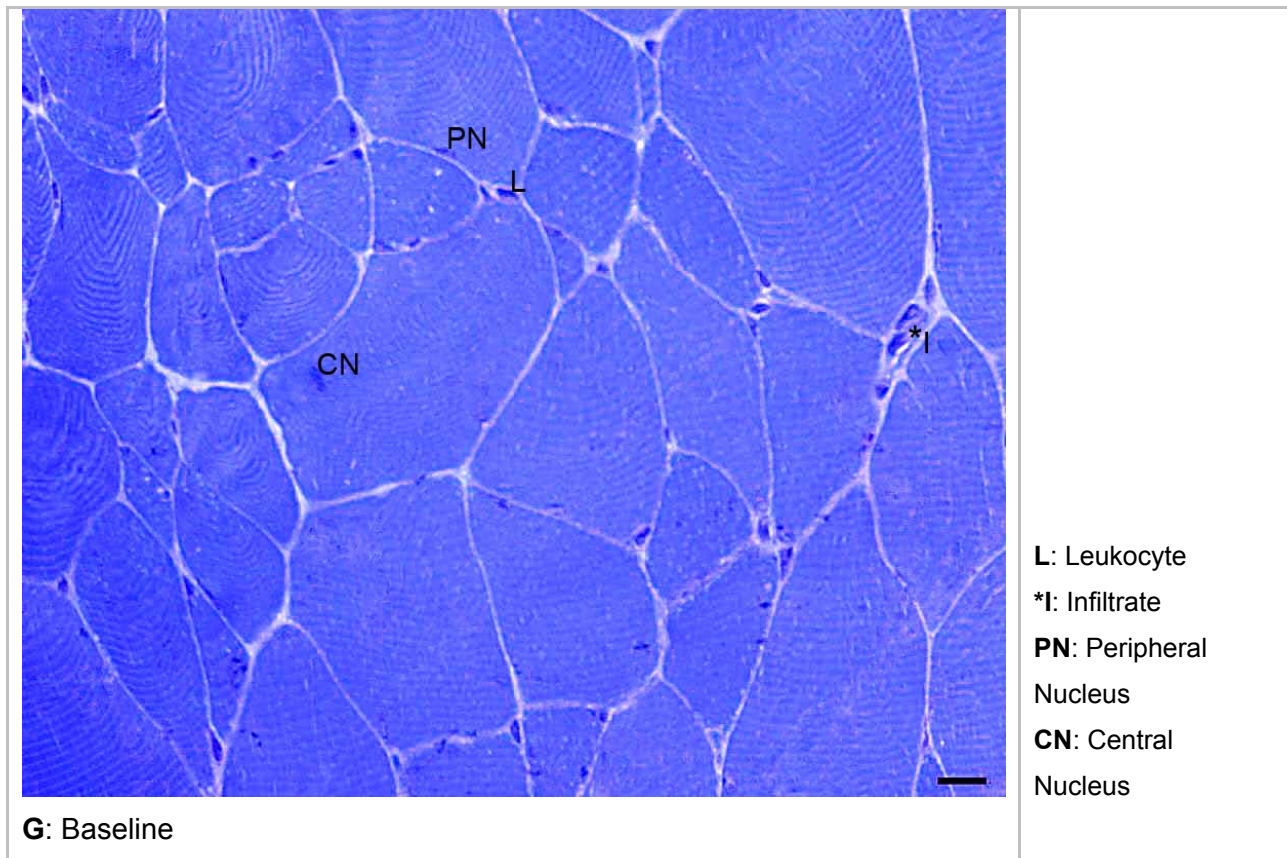


Fig.5.1: Depicting morphological differences between Pectoralis complex muscles in groups A-G. All muscle sections were photographed under 40X magnification. Labels A-G are group specific, thus: **A:** Negative Control SWR/J mice at 28 weeks of age. **B:** Positive Control; **C:** Resveratrol treated; **D:** Low-CoQ₁₀ treated; **E:** High-CoQ₁₀ treated; **F:** Combination Resveratrol/CoQ₁₀ treated SJL/J mice at 27 weeks of age. **G:** Baseline untreated SJL/J mice at 14 weeks of age. (Scale bar = 10µm)

Light microscopic changes within the Pectoralis muscle complex were scored in each of the seven groups to offer a simplified summary thereof. For each characteristic scored, zero was allocated where no changes were visible and four allocated to the maximal presence of each variable examined. Scoring for each category was then totalled and these values adjusted to generate the graph in figure 5.2. Examination was performed on a qualitative basis through scrutiny of the presence of each of the following observations under the three listed categories:

- Fiber Characteristics – Size variability, splitting and lobulation, degeneration and/or necrosis, and regeneration (central nuclei)
- Infiltration/Proliferation – Leukocyte (inflammatory, post-necrotic, and regenerative), perivascular, fibrotic or connective tissue, and adipocytic
- Satellite Cells

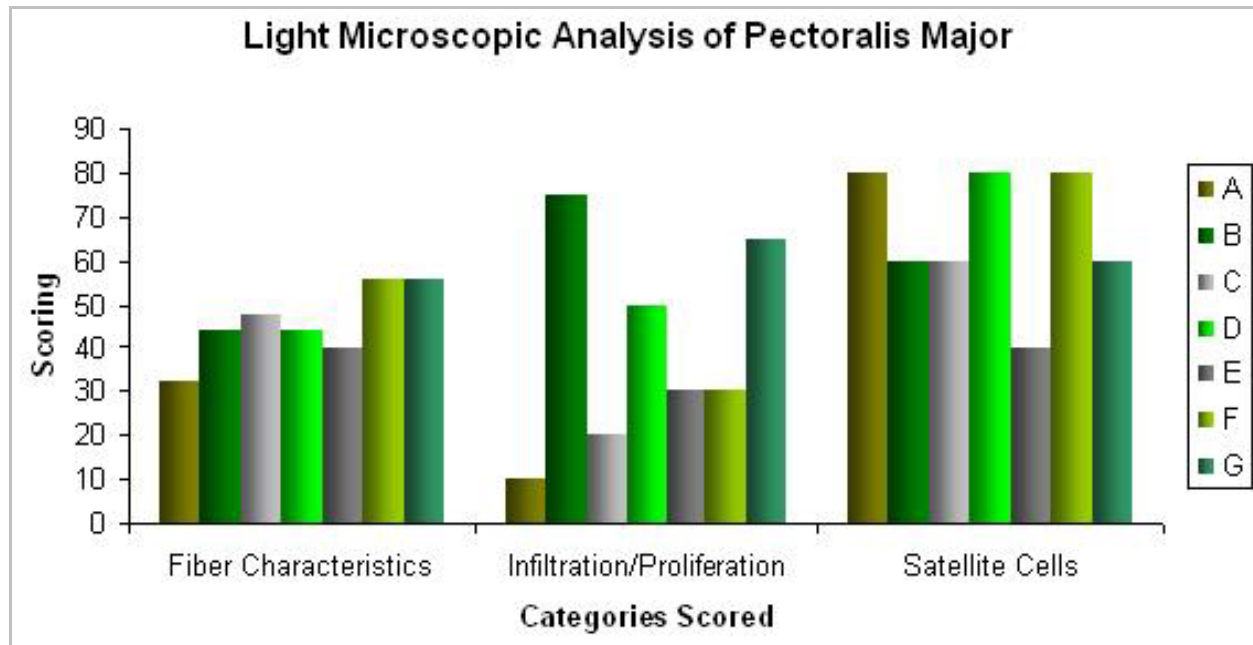


Fig.5.2: Qualitative summary of morphological light microscopic changes in the Pectoralis complex muscle per group A-G. Group A: Negative Control SWR/J mice at 28 weeks of age. Groups B: Positive Control; C: Resveratrol treated; D: Low-CoQ₁₀ treated; E: High-CoQ₁₀ treated; F: Combination Resveratrol/CoQ₁₀ treated SJL/J mice at 27 weeks of age. Group G: Baseline untreated SJL/J mice at 14 weeks of age.

From this scoring it is evident that changes in fiber characteristics are not significantly altered in the Pectoralis complex muscles of SJL/J mice when compared to SWR/J mice. Note that changes in fiber characteristics include negative as well as positive changes and give only an overview of characteristics under scrutiny. In the category of infiltration or proliferation, it can be seen that the Positive Control as well as Low-CoQ₁₀ treated and Baseline SJL/J mice display the most inflammatory infiltrate and/or connective tissue proliferation. When assessing the presence of satellite cells, it is evident that Negative Control, Low-CoQ₁₀ and Combination treated groups display the most potential for regeneration of muscle tissue.

It is important now to discuss in detail the fiber characteristics of the various groups under study. The Negative Control, Resveratrol, High-CoQ₁₀ and Combination treated as well as Baseline groups were comparable in their levels of fiber size variability, of which only little was evident; whereas the Positive Control and Low-CoQ₁₀ treated groups displayed the highest degree of fiber size variability, though at moderate levels. With regards to fiber splitting and lobulation: the Negative Control and Low-CoQ₁₀ treated groups exhibited almost none; Resveratrol and High-CoQ₁₀ treated groups showed little; the Baseline group moderate; and the

Positive Control and Combination treated groups displayed the most fiber splitting and lobulation, at maximal levels. The groups showing the least evidence of fiber degeneration and necrosis were the Negative Control, Resveratrol, Low-CoQ₁₀, High-CoQ₁₀ and Combination treated groups; with the Positive Controls displaying moderate amounts thereof; and the Baseline group exhibiting the most damage in this regard. Finally, with regards to fiber regeneration; the Negative and Positive Controls' as well as the High-CoQ₁₀ treated group showed the least regenerative characteristics; the Baseline showing moderate regeneration; and the Resveratrol, Low-CoQ₁₀ and Combination treated groups displaying the most regenerating fibers.

Satellite cells, being indicative of the regenerative ability of mature muscle tissue, are not necessarily related to the actual regeneration seen at cellular level. In this regard, the High-CoQ₁₀ treated group showed a minimal presence of satellite cells; the Positive Control, Resveratrol treated and Baseline groups showed a moderate presence of satellite cells; and finally, the Negative Control, Low-CoQ₁₀ and Combination treated groups displayed maximal satellite cell presence.

In order to correlate the presence of satellite cells with actual levels of regeneration, multiple characteristics within a group must be related. The Negative Control group (figure 5.1A), although presenting with the highest levels of satellite cells did not exhibit more than a minimal level of fiber regeneration, as this is clearly not necessary. In correlation with this are the minimal levels of fiber size variability (with only the area demonstrating any evidence of size variability displayed in figure 5.1A) as well as minimal degeneration and necrosis; thus the Negative Control group is indeed the picture of muscle health. The Positive Control group (figure 5.1B), although having moderate satellite cell numbers did not appear to be very efficient in its regeneration of fibers, and degeneration clearly outweighed regeneration. Though there was a moderate level of fiber size variability evident; the maximal presence of fiber splitting and lobulation indicates that damage does indeed exceed the capability of the Positive Control group's muscle to regenerate itself.

The Resveratrol treated group (figure 5.1C) showed a high level of fiber regeneration and a moderate presence of satellite cells, indicating that Resveratrol may enhance non-myogenic fiber regeneration. In conjunction with this is the fact that minimal degeneration and necrosis as well as fiber splitting and lobulation were evident. Finally, the Resveratrol treated group displayed levels of fiber size variability correlative to the Negative Control group. The Low-CoQ₁₀ treated group (figure 5.1D) exhibited maximal satellite cell presence as well as maximal fiber regeneration in conjunction with minimal degeneration. Fiber size variability was however at

similar levels to that of the Positive Control group, but fiber splitting and lobulation was comparable to that in the Negative Control group with very little evident. The High-CoQ₁₀ treated group (figure 5.1E) presented with the least satellite cells overall, but also minimal regeneration as well as degeneration of fibers. This could indeed be indicative of the efficacy of High-CoQ₁₀ treatment as membrane protective agent in otherwise damage-susceptible muscle. Fiber size variability was very low and there was also little fiber splitting and lobulation evident, comparable to the Resveratrol treated group. The Combination treated group (figure 5.1F) showed maximal satellite cell presence and fiber regeneration in conjunction with minimal degeneration. Fiber size variability was additionally minimal; though fiber splitting and lobulation was present at high levels, correlative to the Positive Control group.

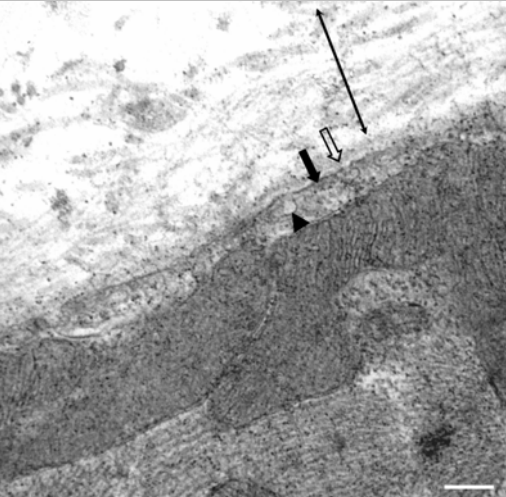
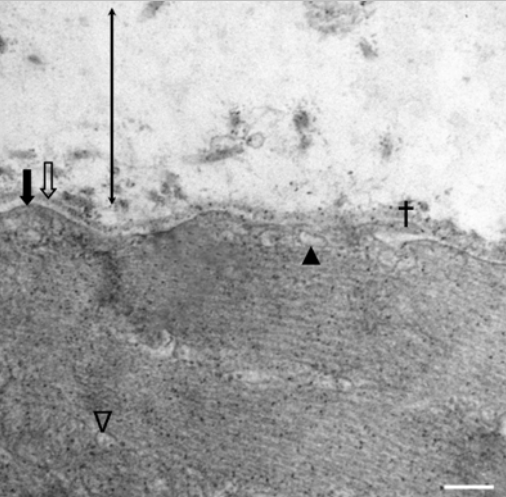
The Baseline group (figure 5.1G) exhibited moderate satellite cell presence as well as moderate regeneration; though little fiber size variability was evident. Degeneration was at maximal levels compared to all other groups; though fiber splitting and lobulation were present at only moderate levels. This indicates that initially, muscle injury or damage definitely exceeds healing, and without treatment of some sort, this picture does not improve at a later stage of the disease as in the Positive Control group.

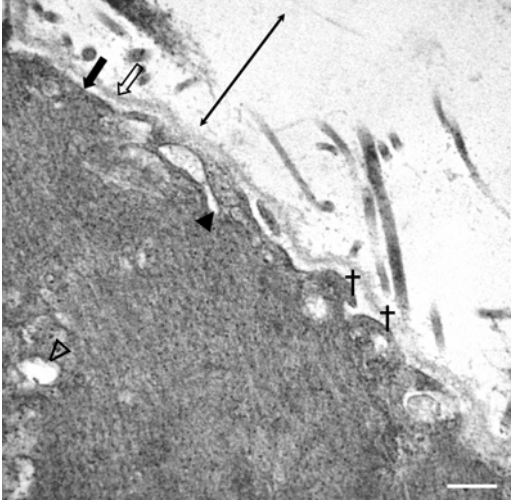
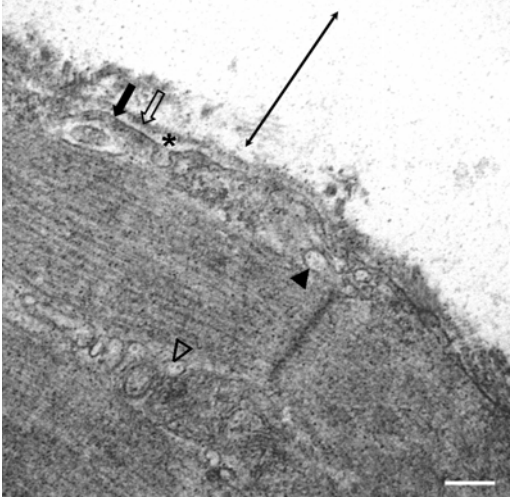
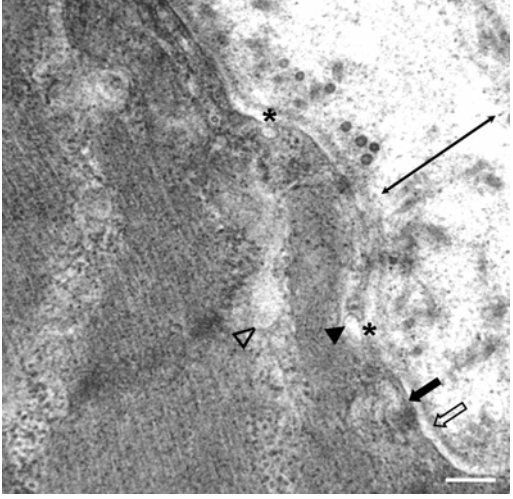
5.3. Transmission Electron Microscopic Investigation

Transmission electron microscopic ultramicrographs were taken of Pectoralis complex muscle samples of each animal displaying the worst changes (determined from light microscopic investigation) within the seven groups under study. Sarcolemmal vesicles were of the most interest as the fusion of these is indicative of membrane repair at the incurrence of damage. Of these, the image most representative of the group was selected for insertion in figure 5.3 (A-H).

With regards to TEM analysis, the following is of note where the plasma membrane is the area of focus. Membrane integrity is the first indication of a seemingly healthy muscle fiber, whether mature or regenerating. However, more important is the presence of sarcolemmal vesicles. If continuity or discontinuity is evident, the process of fusion or lack thereof with these vesicles to the membrane is indicative of repair of the inconsistency or the inability of the membrane repair mechanism to function optimally if at all. Papillary projections as well as alterations of the overlying basal lamina are highly indicative of an advanced state of Dysferlinopathy.

Sub-sarcolemmal vacuoles seem to be indicative of some loss of homeostasis in a muscle fiber. These, with the presence of sarcolemmal vesicle fusion to the membrane indicate repair of a muscle fiber which may have incurred some form of injury. The presence of these vacuoles without the presence of sarcolemmal vesicle fusion is indicative of injury or damage of a muscle fiber without the prospect of repair. Degeneration of a fiber such as this will then follow at some stage. Once the fiber is necrotic, these sub-sarcolemmal vacuoles, together with other cell contents are phagocytosed as they are expelled from the now membraneless muscle fiber. The ultramicrographs serve to compare morphological differences between Pectoralis complex muscle membranes in groups A-G, with reference to the above-mentioned characteristics.

 A: Negative Control	→ Sarcolemma ⇒ Basal Lamina ▲ Sarcolemmal Vesicles Δ Sub-sarcolemmal Vacuoles * Vesicle Fusion Sites ↔ Connective Tissue between Fibers
 B: Positive Control	→ Sarcolemma ⇒ Basal Lamina ▲ Sarcolemmal Vesicles ↔ Connective Tissue between Fibers
 C: Resveratrol	→ Sarcolemma ⇒ Basal Lamina ▲ Sarcolemmal Vesicles Δ Sub-sarcolemmal Vacuoles † Papillary Projections ↔ Connective Tissue between Fibers

 D: Low-CoQ₁₀	➡ Sarcolemma ⇒ Basal Lamina ▲ Sarcolemmal Vesicles - Δ Sub-sarcolemmal Vacuoles † Papillary Projections ↔ Connective Tissue between Fibers
 E: High-CoQ₁₀	➡ Sarcolemma ⇒ Basal Lamina ▲ Sarcolemmal Vesicles - Δ Sub-sarcolemmal Vacuoles * Vesicle Fusion Sites ↔ Connective Tissue between Fibers
 F: Combination Resveratrol/CoQ₁₀	➡ Sarcolemma ⇒ Basal Lamina ▲ Sarcolemmal Vesicles - Δ Sub-sarcolemmal Vacuoles * Vesicle Fusion Sites ↔ Connective Tissue between Fibers

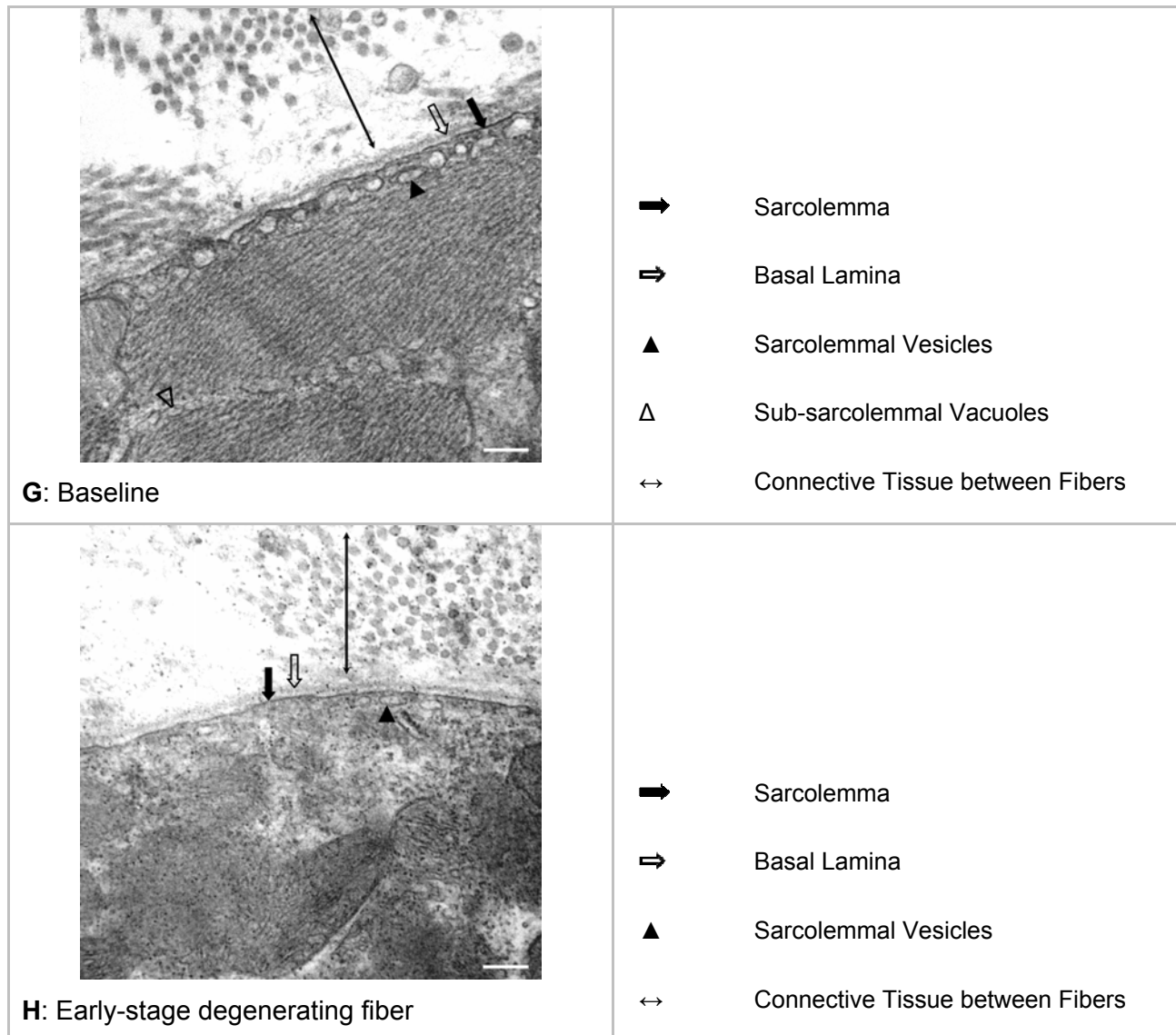


Fig.5.3: Depicting morphological differences between Pectoralis complex muscle membranes in groups A-G. All muscle sections were photographed at a magnification of approximately 60,000X. **A:** Negative Control SWR/J mice at 28 weeks of age. **B:** Positive Control; **C:** Resveratrol treated; **D:** Low-CoQ₁₀ treated; **E:** High-CoQ₁₀ treated; **F:** Combination Resveratrol/CoQ₁₀ treated SJL/J mice at 27 weeks of age. **G:** Baseline untreated SJL/J mice at 14 weeks of age. **H:** Depicts a degenerating muscle fiber in a fairly early stage with an as yet undisrupted membrane. (Scale bar = 200nm)

The Negative Control group (figure 5.3A) displayed entirely intact membranes, the ultramicrograph selected for insertion shows two areas of sarcolemmal vesicle fusion with the membrane, there were very few free sarcolemmal vesicles in biopsies taken of this group. Additionally, there was no leukocyte infiltration in the Negative Control group and also very little collagen surrounding fibers. The Negative Controls are thus the epitome of rapid and effective repair of damaged membranes without recruitment of inflammatory cells. The Positive Control group (figure 5.3B) presented with some sarcolemmal vesicle accumulation, some membrane discontinuity and defects, and the basal lamina was pronouncedly thickened. There was much inflammatory infiltration within this group and also a lot more connective tissue between muscle fibers, largely in the form of collagen bundle aggregation.

The Resveratrol treated group (figure 5.3C) exhibited sarcolemmal vesicle accumulation as well as a presence of sub-sarcolemmal vacuoles. The basal lamina was only minimally thickened, and this group possessed some areas of papillary projection. Collagen fibers and connective tissue were not in excess in this group. The Low-CoQ₁₀ treated group (figure 5.3D) demonstrated the presence of large sarcolemmal vesicles as well as large sub-sarcolemmal vacuoles in places. There was some thickening of the basal lamina and very prominent papillary projections were discernible in this group. Furthermore, many collagen bundles were present between muscle fibers. The High-CoQ₁₀ treated group (figure 5.3E) showed areas of sarcolemmal vesicle accumulation as well as some sites of vesicle fusion with the membrane. Sub-sarcolemmal vacuoles were present and some basal lamina thickening was noted. There were very few collagen fibers between muscle fibers of this group. The Combination treated group (figure 5.3F) exhibited minimal sarcolemmal vesicle accumulation and some sites of vesicle fusion with the membrane. Some sub-sarcolemmal vacuoles were present, and a large amount of collagen bundles were noted between muscle fibers. Nevertheless, the basal lamina appeared much better than that of the other groups of SJL/J mice.

The Baseline group (figure 5.3G) displayed pronounced sarcolemmal vesicle accumulation as well as a presence of sub-sarcolemmal vacuoles. The basal lamina was thickened in places but the amount of collagen present was comparable to that of the Negative Control group.

Finally, looking at an early-stage degenerating fiber (figure 5.3H), sarcolemmal vesicles were found accumulating near the membrane surface and muscle fibers beneath the still intact membrane were observed in the process of degeneration. There was as yet not much alteration of the basal lamina. The presence of many bundles of collagen fibers was however distinct.

5.4. Scanning Electron Microscopic Investigation

In the scanning electron microscopic investigation relevant to this study, photomicrographs were taken of longitudinally arranged muscle fibers at 5,000X magnification in order for analysis to be performed in determination of the significance of connective tissue thickening between fibers of the seven groups under investigation. All measurements were calibrated in the iTEM Olympus Soft Imaging Solutions® software to measure in micrometer increments. Nine measurements were taken at random for each of three animals within the seven groups under study (demonstrated in figure 5.4) and a graphical representation of the mean measurements for each Group A-G is depicted in figure 5.5.

The statistical program, NCSS, was employed in the performance of statistical analysis upon these derived thicknesses. The experimental groups were compared by means of either a one-way ANOVA or Kruskal-Wallis one-way ANOVA, depending on whether or not the necessary assumptions for the parametric test were met. Analysis was conducted at a significance level of 95% ($P=0.05$).

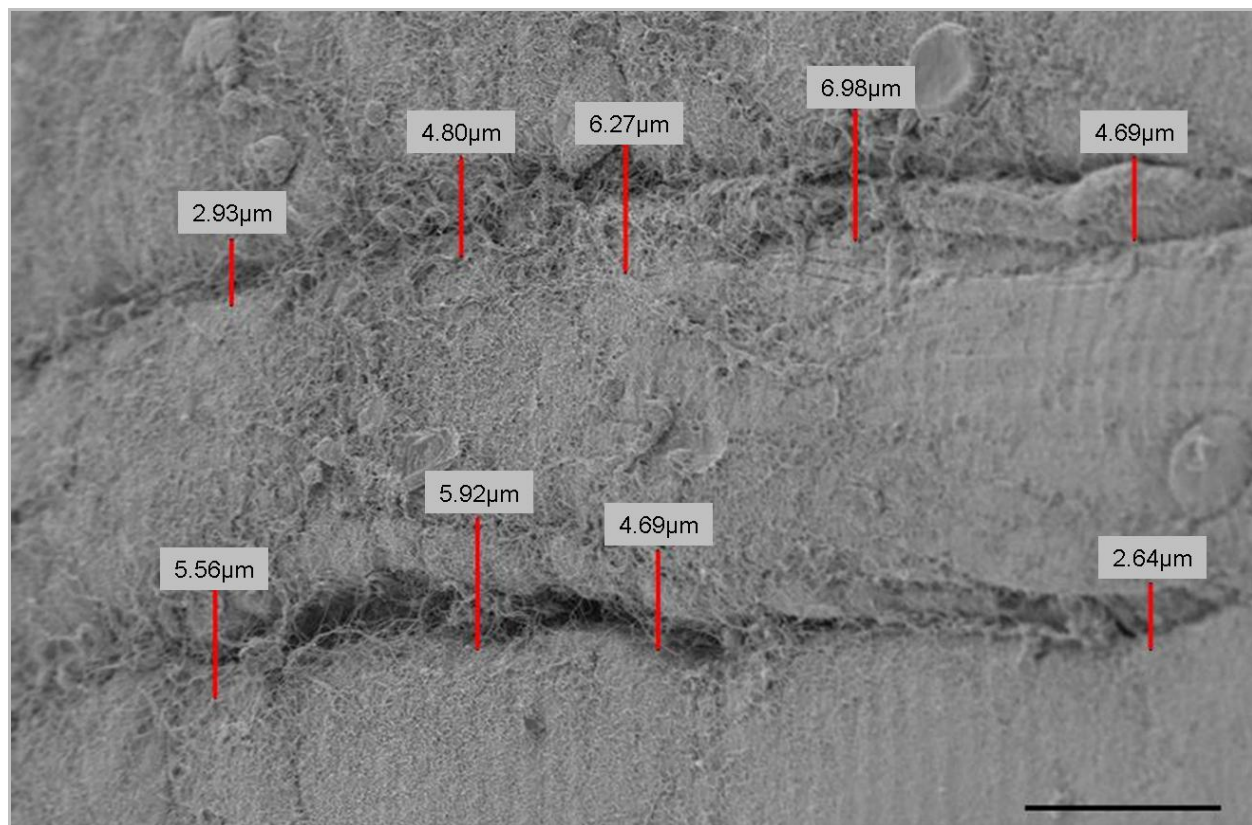


Fig.5.4: Depicting an example of connective tissue thickness measurements taken across the width between longitudinally arranged muscle fibers. (Scale bar = 10µm)

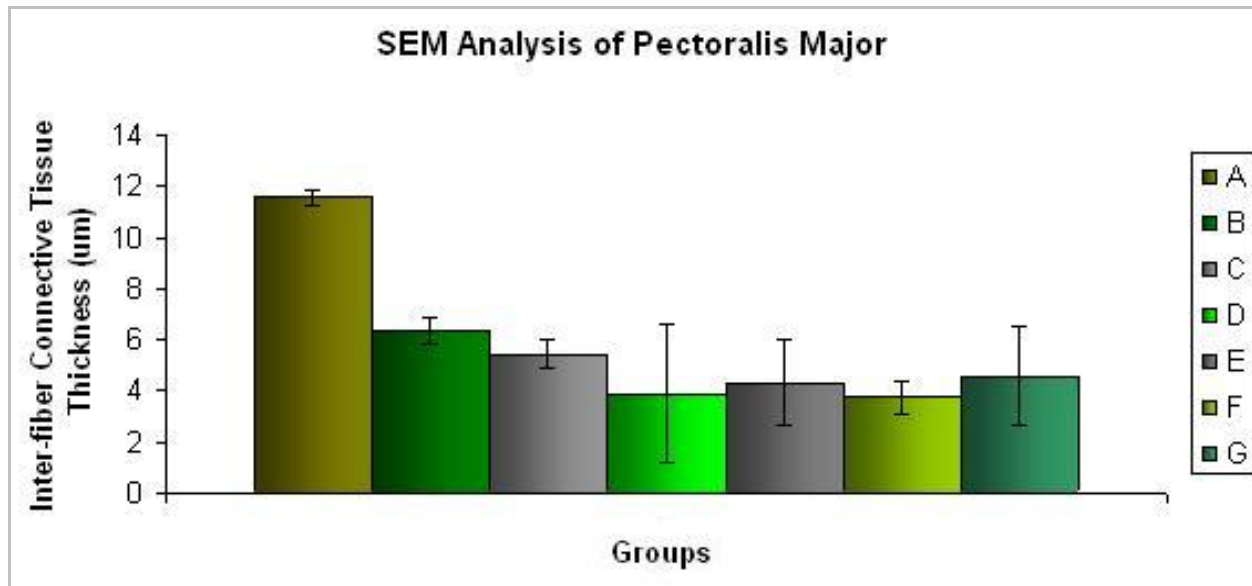


Fig.5.5: Mean connective tissue thickness measurements, with indicated standard deviations, of the Pectoralis complex muscles assessed in animals of groups A-G. Group A: Negative Control SWR/J mice at 28 weeks of age. Groups B: Positive Control; C: Resveratrol treated; D: Low-CoQ₁₀ treated; E: High-CoQ₁₀ treated; F: Combination Resveratrol/CoQ₁₀ treated SJL/J mice at 27 weeks of age. Group G: Baseline untreated SJL/J mice at 14 weeks of age.

Due to the collective data possessing a non-normal distribution, the Kruskal-Wallis one-way ANOVA was utilized for statistical assessment of the seven experimental groups. The requirement of equal variance was ignored due to the criteria of equal sample sizes being met. One or more of the groups were found to differ significantly from each other in terms of their inter-fiber connective tissue thickness ($P < 0.00001$), which was classified with the subsequent use of the Tukey-Kramers pos hoc test. The results thereof are presented in table 5.1.

Table 5.1: Significance of statistical comparisons performed on the connective tissue thickness measurements of Pectoralis complex muscle SEM micrographs of the assessed SJL/J and SWR/J mice under various experimental conditions

Experimental Group	Statistical Significance		
	Thinner Than	Indifferent To	Thicker Than
Group A Negative Control	-	-	B,C,D,E,F,G
Group B Positive Control	A	-	C,D,E,F,G
Group C Resveratrol	A,B	D,E,G	F
Group D Low-CoQ ₁₀	A,B,C	E,F	-
Group E High-CoQ ₁₀	A,B	C,D,F,G	-
Group F Combination Resveratrol/CoQ ₁₀	A,B,C	D,E,G	-
Group G Baseline	A,B	C,D,E,F	-

The pos hoc test run showed that the Low-CoQ₁₀ and Combination treated groups possessed a significantly thinner inter-fiber connective tissue thickness in comparison to the Negative and Positive Control groups as well as the Resveratrol treated group, although they were not statistically different to each other. The Baseline and High-CoQ₁₀ treated groups showed a significantly thinner inter-fiber connective tissue thickness in comparison to the Negative and Positive Control groups, although not statistically different to each other.

The Resveratrol group was statistically thicker than the Combination group, while being statistically thinner than the Negative and Positive Control groups in terms of its connective tissue thickness. The Positive Control had a significantly thicker inter-fiber connective tissue thickness when compared to all the other groups except the Negative Control. The Negative Control group's inter-fiber connective tissue was significantly thicker than all other groups (Groups B-G).

5.5. Integrative Discussion

The Negative Control group was the picture of health and optimal repair with intact membranes and rapid vesicle fusion at instances of damage, in that no degenerating characteristics were given time to take place. All degenerative as well as regenerative (a response to degeneration) changes were at an absolute minimum; though satellite cell numbers (thus regenerative capabilities) were at a maximum within this group, affording the muscles the potential to fully regenerate and develop to maturity should irreparable injury occur. Along with this, no infiltration of leukocytes was observed at the ultrastructural level and very little collagen was visible between fibers. The fact that the SEM connective tissue thickness measurements contrast the TEM collagen presence between fibers perhaps suggests that there were larger spaces between muscle fibers rather than actual connective tissue material being measured between fibers. This group is the standard of comparison for muscle in a healthy state.

The Positive Control group showed minimal to moderate regenerative changes and capabilities and moderate to maximal degenerative changes overall. Membrane discontinuities and defects as well as vesicle accumulation and marked basal lamina thickening were evident. In addition, much infiltration was present and collagen fibers were in definite excess. Connective tissue thickness was thicker in this group than in all experimental groups as well as in the Baseline group. This group is thus the standard of comparison for muscle in a moderate stage of Dysferlinopathy.

The Baseline group presented with minimum to moderate regenerative changes and capabilities, and moderate to maximal degenerative changes, as with the Positive Control group. Vesicle accumulation and vacuole presence was pronounced at the ultrastructural level and the basal lamina was observed to be thickened in places. Very little collagen was present, comparable to the Negative Control and High-CoQ₁₀ groups; but connective tissue measurements showed this to be indifferent to the four experimental groups. This group thus provides the comparison criteria for muscle in an early stage of Dysferlinopathy at an ultrastructural level.

When comparing the four experimental groups; the Resveratrol treated group displayed overall moderate regenerative changes and capabilities and minimal degenerative changes. Though collagen was not excessively present, SEM results showed that connective tissue was thicker than the Combination treated group though indifferent to the other experimental groups as well as the Baseline group. Vesicle accumulation and vacuoles were present; the basal lamina only marginally thickened and papillary projections were evident. Resveratrol treatment does seem to function as an anti-inflammatory agent at the muscular level, and appears to

marginally improve the condition of muscle from the age of Baseline animals (14 weeks) to the age of Positive Control animals (27 weeks).

The Low-CoQ₁₀ treated group manifested with minimal to moderate degenerative changes and maximal regenerative changes and capabilities. Though many collagen bundles were present in ultrastructure, connective tissue thickness seemed to be indifferent to the other experimental groups. Large vesicles and vacuoles were however present in muscle samples of this group, with some basal lamina thickening and prominent papillary projections evident. At low concentrations, CoQ₁₀ does not seem to have a significant membrane protective effect on muscle fibers; though regenerative characteristics of muscle fibers do seem to be heightened when compared to 14 week old animals as well as Positive Control animals at 27 weeks of age.

High-CoQ₁₀ treatment kept both regenerative and degenerative changes at a minimum; however, unlike the Negative Controls, satellite cell numbers and thus regenerative capabilities were also at a minimum. Though some basal lamina thickening was evident, vesicle fusion with the membrane does bring this experimental group closer to a healthy state than the two prior experimental groups under discussion. The presence of minimal collagen at an ultrastructural level, once again comparable to the Negative Control group, is also a positive sign in terms of the condition of this group's muscle. SEM results however show that connective tissue thickness is statistically insignificant to all other experimental groups as well as the Baseline group. It would seem that a high concentration dosage of CoQ₁₀ is more effective than lower levels in terms of membrane protective ability, and furthermore brings the state of muscle health closest to that seen in the Negative Control group after the initial baseline damage before commencement of treatment.

The Combination treated group exhibited overall moderate regenerative and degenerative changes; however, regenerative capabilities were higher in this group in all but the Low-CoQ₁₀ treated experimental group. There was also evidence of vesicle fusion with and thus repair of the sarcolemma in this group, as in the High-CoQ₁₀ treated group. Some vacuoles were present in the sub-sarcolemmal regions, and though much collagen was present ultrastructurally, connective tissue thickness was indifferent to the other experimental groups. Basal lamina ultrastructure in this group was in the best condition of all SJL/J animals under investigation. Thus, Combination Resveratrol/CoQ₁₀ treatment seems to offer the best of both worlds; displaying the anti-inflammatory effects of Resveratrol and providing similar levels of membrane protection offered by high levels of CoQ₁₀ treatment though only administered in low levels in this Combination treated group. It can thus be deduced that the two supplements function optimally in combination.

5.6. Concluding Remarks

The anti-inflammatory properties of Resveratrol do indeed become evident at the tissue level though not having a significant effect on the overall health of muscle fibers. The membrane protective abilities of Coenzyme Q₁₀ are unmistakeable at high concentrations and indeed seem to afford an added muscle healing capability when compared to the Baseline and Positive Control groups in that the condition of muscle between these two age groups is dramatically improved by High-Coenzyme Q₁₀ treatment. In combination, Resveratrol and low levels of Coenzyme Q₁₀ appear to achieve an optimal cooperative result, being anti-inflammatory and membrane protective to such an extent that muscle health is also improved between 14 and 27 weeks of age, unlike when the two supplements are administered exclusively in these concentrations.

From the findings in the current chapter, it can be concluded that either High-Coenzyme Q₁₀ or Combination Resveratrol/Coenzyme Q₁₀ treatment options prove to be preferential in the management of Dysferlinopathy, as their action brings muscle health closest to the state observed in the non-dysferlinopathic Negative Controls.

Chapter 6: Microscopic Investigation of Gluteus Complex Muscle Samples

6.1. Introduction

The Gluteus muscles are superficial muscles in the gluteal region (Gray 2001) of the pelvic girdle which are responsible for much of the rotational ability of the hip (Encyclopaedia Britannica 2009). In animals, the hip or pelvic girdle is the posterior skeletal girdle, supporting the pelvic or hindlimb. The Gluteus complex is a group of dorsal muscles of the hip, extending from the ilium to the femur, functioning in the extension of the hindlimb (Kardong 2006). This muscle is not commonly studied in research related to Dysferlinopathy. The Gluteus complex is however directly associated with the pelvic girdle, and is of interest as a muscle believed to be largely involved in the weakness experienced in Dysferlinopathy.

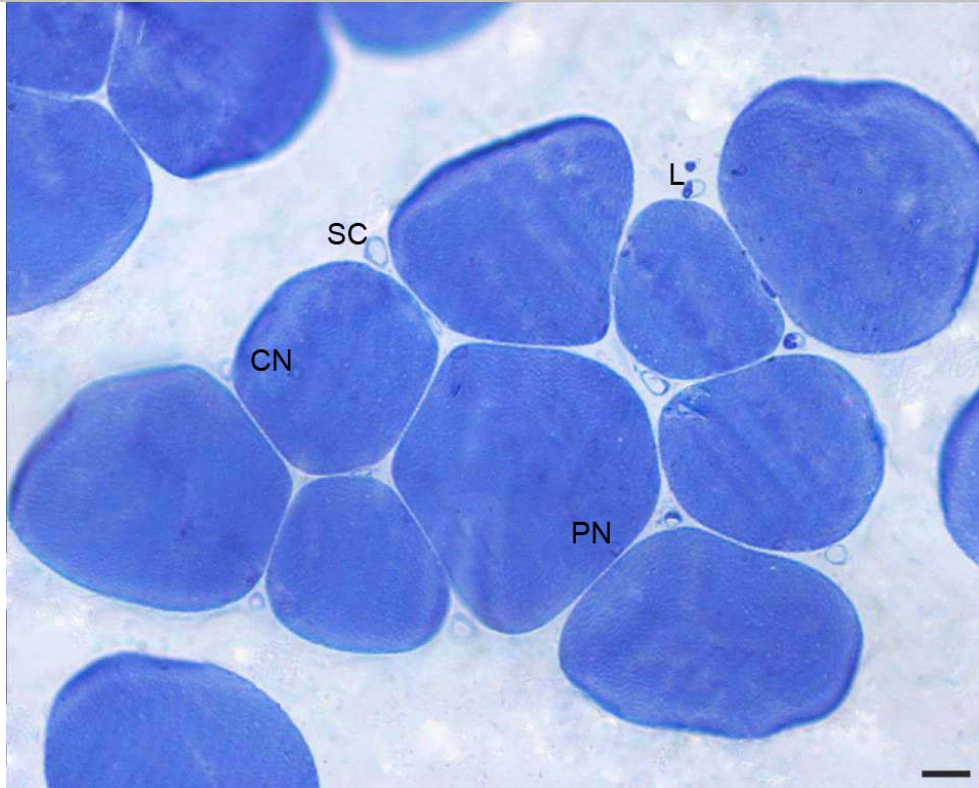
Photomicrographs were taken using light microscopy as well as transmission- and scanning- electron microscopy and each of these subsets were analysed for various characteristics or the lack thereof, as set out in this chapter.

For the purposes of light microscopic analysis micrographs were taken at 40X magnification and morphological analysis was subsequently performed on fiber and infiltrative characteristics as well as the presence of myogenic stem cells. Transmission electron ultramicrographs were taken at a magnification of approximately 60,000X and morphological analysis concentrated upon the sarcolemma and vesicle accumulation and fusion therewith was undertaken. Finally, with regards to scanning electron microscopy, ultramicrographs were taken at a magnification of 5,000X, after which statistical analysis was performed on measurements of the connective tissue thickness between longitudinally arranged muscle fibers.

6.2. Light Microscopic Investigation

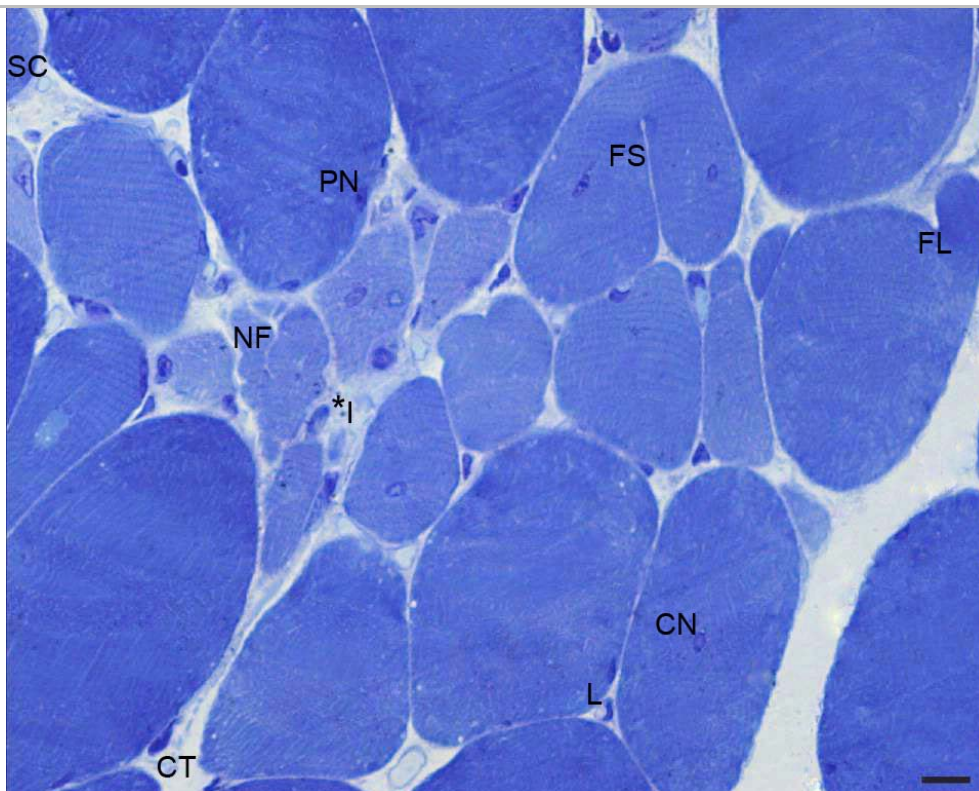
Light microscopic photographic images were taken of Gluteus complex muscle samples of each of three animals from the seven groups under study. Of these, the image most representative of the group was selected for insertion in figure 6.1 (A-G). The characteristics most representative of Dysferlinopathy were scrutinised throughout the sample and are labelled as such in the figures. Figure 6.2 offers a summary of changes assessed within the entire sample; which include various fiber characteristics, infiltrative and proliferative changes as well as the presence of satellite cells, which are set out in detail in the relevant section.

With regards to fiber characteristics in the light microscopic investigation, the following is of note: Fiber size variability, especially in large degrees, is suggestive of muscle fibers undergoing changes associated with regeneration. Peripheral nuclei are characteristic of mature muscle fibers, whereas central or internal nuclei are indicative of regeneration. Necrotic fibers are distinguishable from other myofibers in that they are of opaque or glassy appearance. Fiber splitting within pictures is marked as the early stages of splitting, whereas fiber lobulation was chosen to mark the later stages of splitting where a fiber's endomysial sheath appears to wholly enclose more than one myofiber, as well as fibers where splitting seems to fold rather than clearly split the specific fiber. Leukocyte infiltration takes three forms: inflammatory and regenerative infiltration which are indistinguishable morphologically, and postnecrotic infiltration which is responsible for the removal of cellular debris once necrosis of a fiber has taken place. Finally, the presence of satellite cells (and the numbers thereof) is indicative of the regenerative ability of muscle tissue. Regardless of the presence of multiple characteristics, only one of each is marked in each of the micrographs. The micrographs serve to present the morphological differences between Gluteus complex muscles in groups A-G, with reference to characteristics mentioned above.



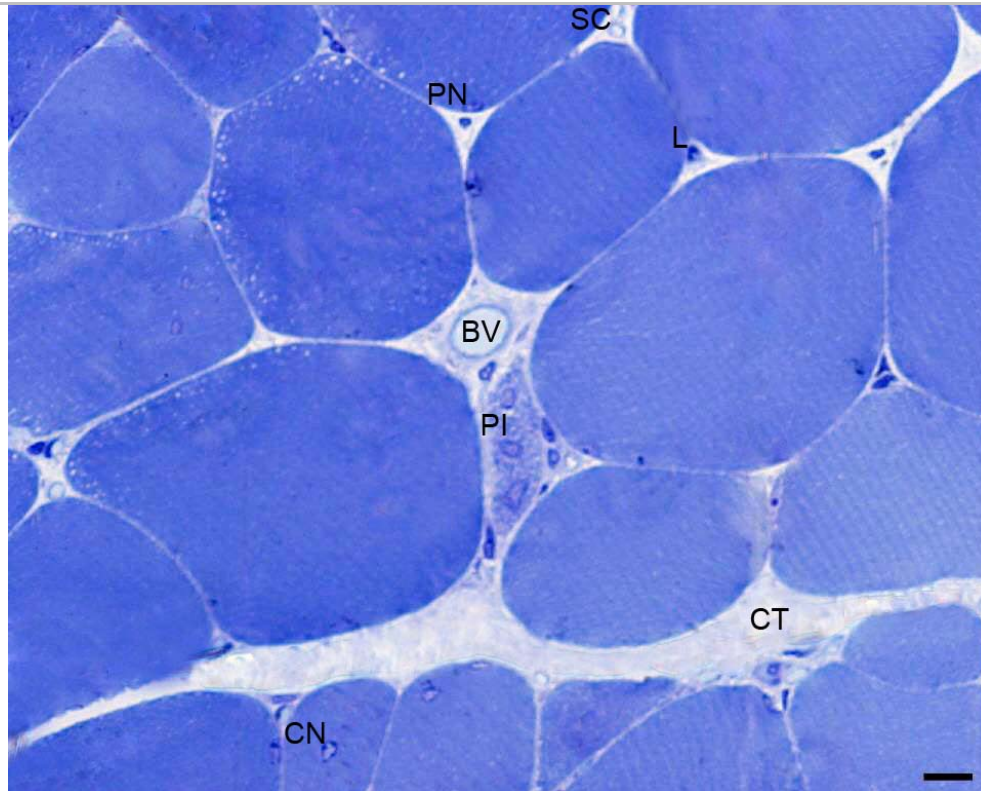
A: Negative Control

SC: Satellite Cell
L: Leukocyte
PN: Peripheral Nucleus
CN: Central Nucleus



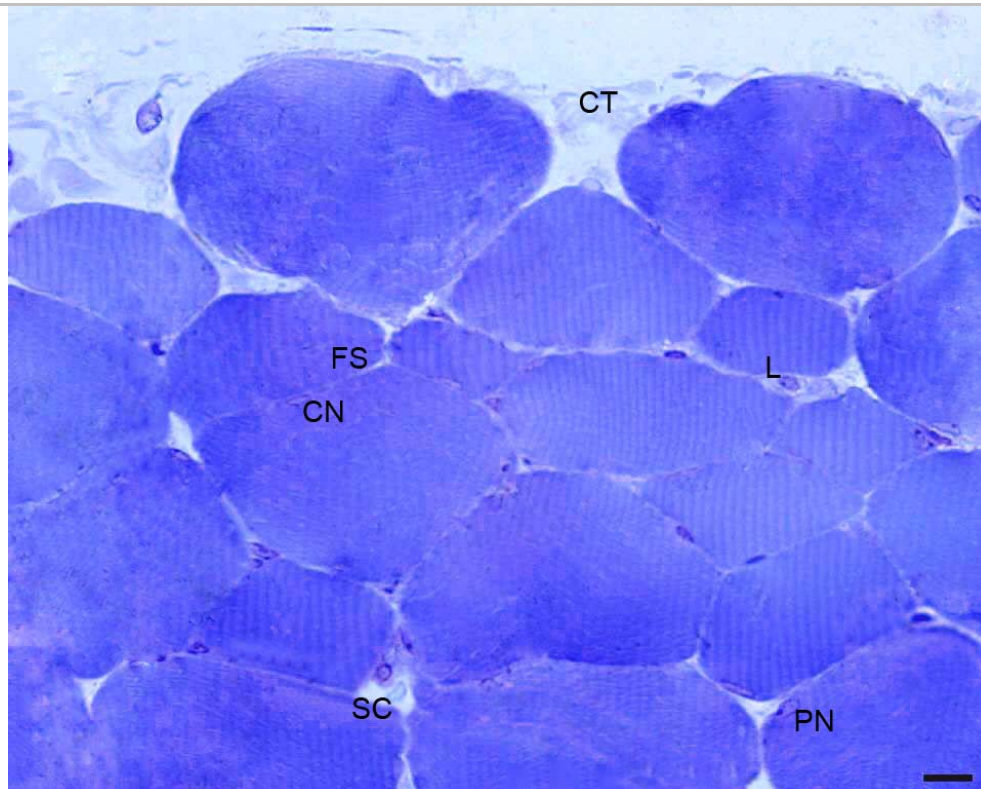
B: Positive Control

SC: Satellite Cell
L: Leukocyte
***I:** Infiltrate
PN: Peripheral Nucleus
CN: Central Nucleus
FS: Fiber Splitting
FL: Fiber Lobulation
NF: Necrotic Fiber
CT: Connective Tissue



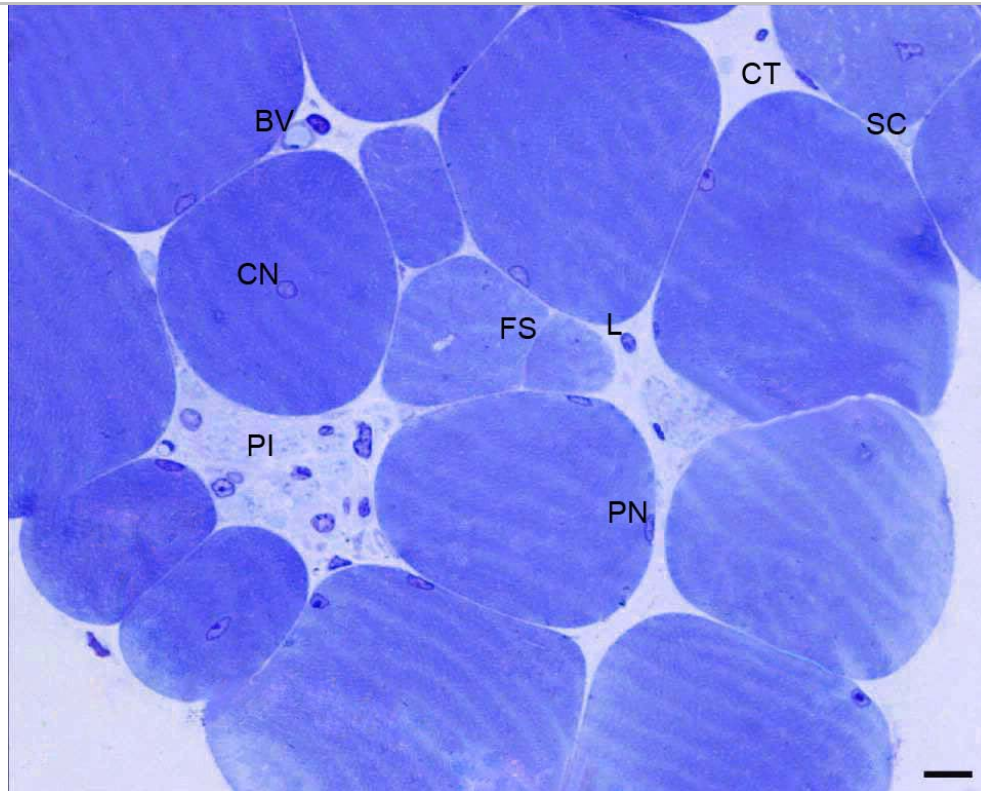
C: Resveratrol

SC: Satellite Cell
L: Leukocyte
PN: Peripheral Nucleus
CN: Central Nucleus
PI: Postnecrotic Infiltrate
CT: Connective Tissue
BV: Blood Vessel



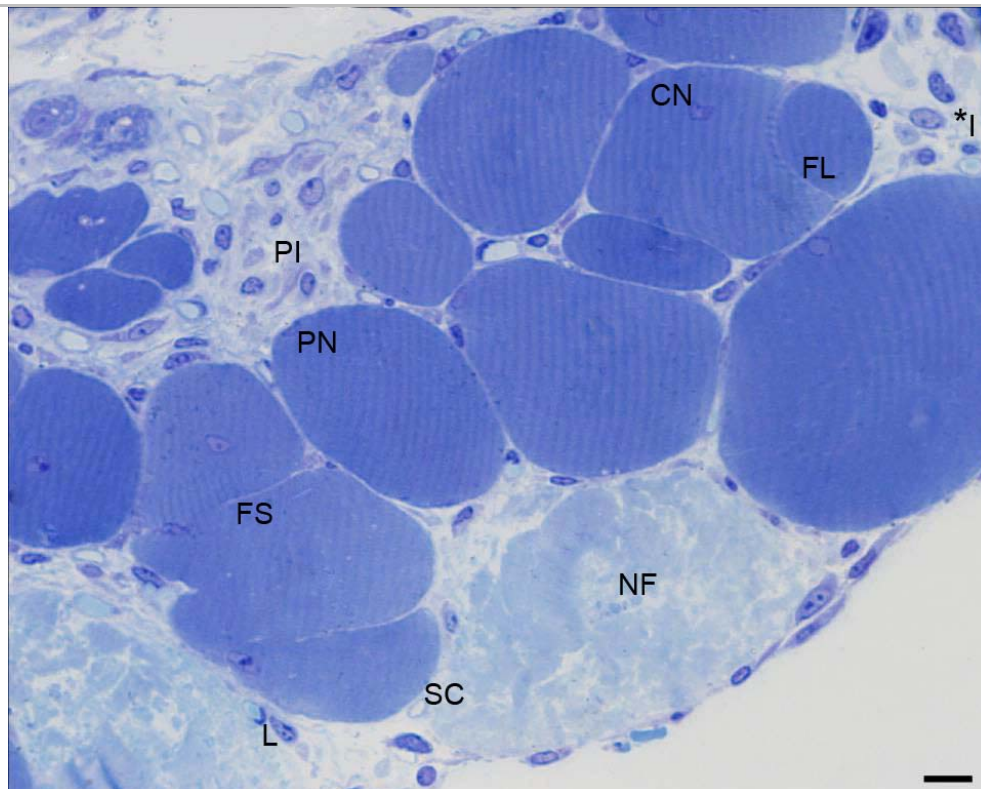
D: Low-CoQ₁₀

SC: Satellite Cell
L: Leukocyte
PN: Peripheral Nucleus
CN: Central Nucleus
FS: Fiber Splitting
CT: Connective Tissue



E: High-CoQ₁₀

SC: Satellite Cell
L: Leukocyte
PN: Peripheral Nucleus
CN: Central Nucleus
FS: Fiber Splitting
PI: Postnecrotic Infiltrate
CT: Connective Tissue
BV: Blood Vessel



F: Combination Resveratrol/CoQ₁₀

SC: Satellite Cell
L: Leukocyte
***I:** Infiltrate
PN: Peripheral Nucleus
CN: Central Nucleus
FS: Fiber Splitting
FL: Fiber Lobulation
NF: Necrotic Fiber
PI: Postnecrotic Infiltrate

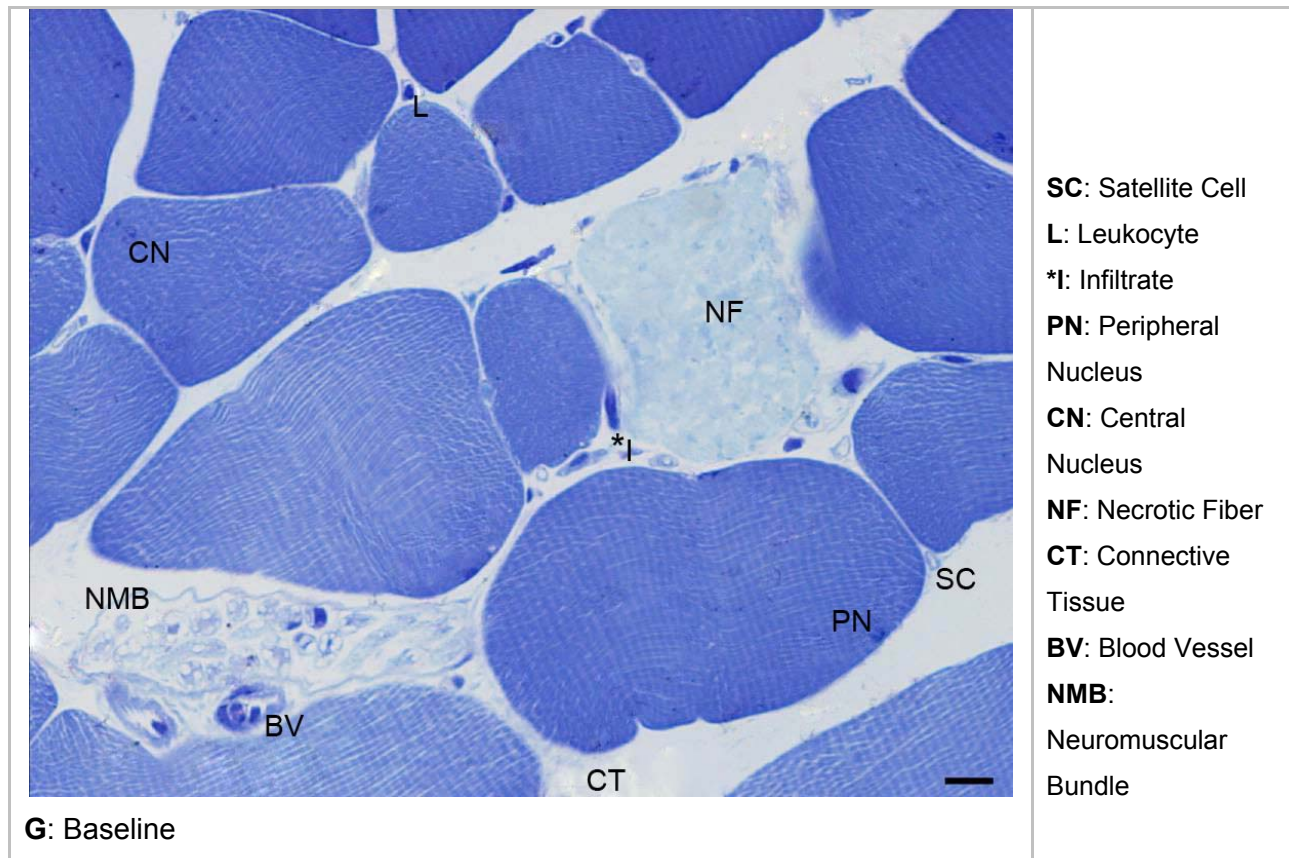


Fig.6.1: Depicting morphological differences between Gluteus complex muscles in groups A-G. All muscle sections were photographed under 40X magnification. Labels A-G are group specific, thus, **A:** Negative Control SWR/J mice at 28 weeks of age. **B:** Positive Control; **C:** Resveratrol treated; **D:** Low-CoQ₁₀ treated; **E:** High-CoQ₁₀ treated; **F:** Combination Resveratrol/CoQ₁₀ treated SJL/J mice at 27 weeks of age. **G:** Baseline untreated SJL/J mice at 14 weeks of age. (Scale bar = 10µm)

Scoring of light microscopic changes in the Gluteus complex muscles in each of the seven groups was performed, with a score of zero allocated for no changes and a maximum score of four allocated to much change, i.e. the maximal presence of each variable examined. Scoring for each category was then totalled and these values adjusted to generate the graph in figure 6.2. Examination was performed on a qualitative basis through the scrutiny of the presence of each of the following observations under the three listed categories:

- Fiber Characteristics – Size variability, splitting and lobulation, degeneration and/or necrosis, and regeneration (central nuclei)
- Infiltration/Proliferation – Leukocyte (inflammatory, post-necrotic, and regenerative), perivascular, fibrotic or connective tissue, and adipocytic
- Satellite Cells

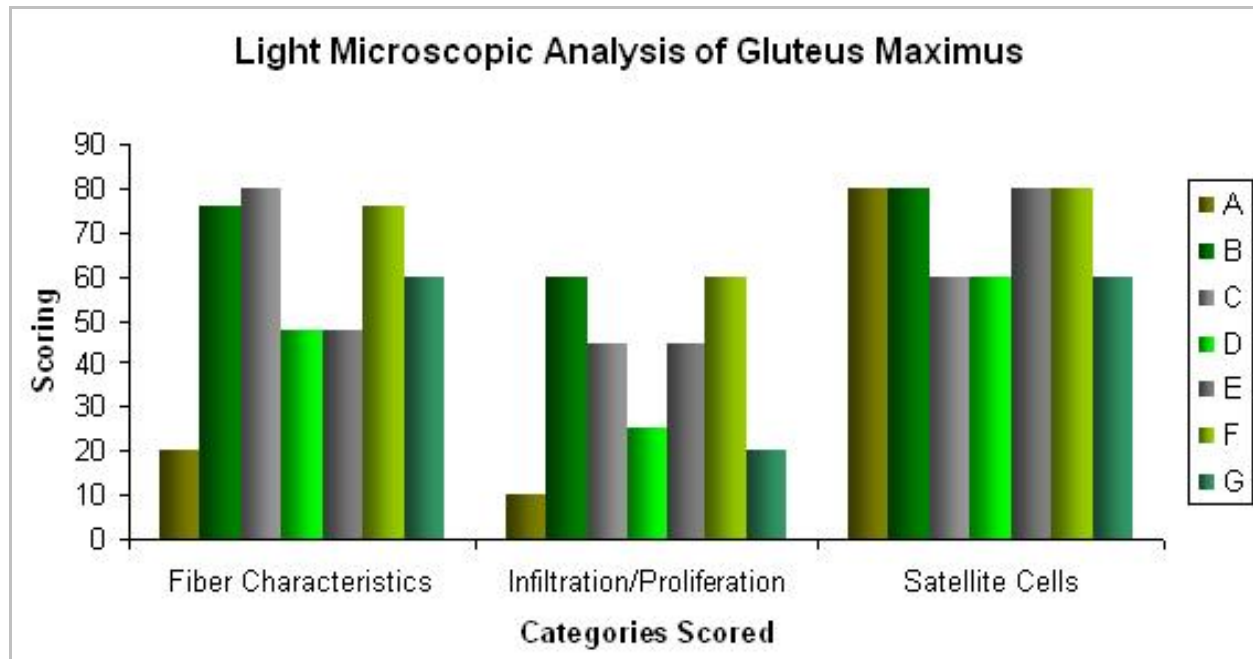


Fig.6.2: Qualitative summary of morphological light microscopic changes in the Gluteus complex muscles per group A-G. Group A: Negative Control SWR/J mice at 28 weeks of age. Groups B: Positive Control; C: Resveratrol treated; D: Low-CoQ₁₀ treated; E: High-CoQ₁₀ treated; F: Combination Resveratrol/CoQ₁₀ treated SJL/J mice at 27 weeks of age. Group G: Baseline untreated SJL/J mice at 14 weeks of age.

From the above scoring it is evident that fiber characteristics (both negative and positive changes) are significantly different when comparing the Negative Control SWR/J mice to all SJL/J mice (Groups B-G). Inflammatory infiltrate and/or connective tissue proliferation are significantly higher than the Negative Controls only in the Positive Controls, Resveratrol, High-CoQ₁₀ and Combination Resveratrol/CoQ₁₀ treated animals. The presence of satellite cells indicates that the animals with the highest muscle regenerative capacity are the ones in the Negative and Positive Controls' as well as those in the High-CoQ₁₀ and Combination treated groups.

Fiber size characteristics viewed in the light microscopic analysis would serve to deduce the following: The Negative Control group displayed almost no fiber size variability; the Low-CoQ₁₀, High-CoQ₁₀ treated and Baseline groups exhibited little size variability; and the Positive Control, Resveratrol and Combination treated groups showed the most fiber size variability at maximal levels. With regards to fiber splitting and lobulation: the Negative Controls showed almost none; the Low-CoQ₁₀ and High-CoQ₁₀ treated as well as the Baseline group displayed minimal levels thereof; the Positive Controls moderate levels; and the Resveratrol and Combination groups exhibited the most splitting and lobulation of myofibers. The Negative

Control group evidenced the least necrotic and degenerative fibers; the High-CoQ₁₀ treated and Baseline groups showed little; Low-CoQ₁₀ treated moderate; and the Positive Control, Resveratrol and Combination treated groups' exhibited the most fiber necrosis and degeneration. Finally, the Negative Control and Baseline groups displayed the least fiber regeneration; the Low-CoQ₁₀ and Combination treated groups exhibited moderate regeneration; and the Positive Control, Resveratrol and High-CoQ₁₀ treated groups showed evidence of the most fiber regeneration.

Satellite cells, being indicative of the regenerative ability of mature muscle tissue, are not necessarily related to the actual regeneration seen at cellular level. In regard herewith, the Resveratrol and Low-CoQ₁₀ treated as well as the Baseline groups exhibited a moderate satellite cell presence; and the Negative and Positive Controls', High-CoQ₁₀ and Combination treated groups presented with maximal levels of satellite cells.

In order to correlate the presence of satellite cells with actual levels of regeneration, once again, multiple characteristics within a group must be related. The Negative Control group (figure 6.1A) presented with satellite cells at a maximum, fiber regeneration as well as degeneration and necrosis were at minimum levels, and finally fiber size variability as well as splitting and lobulation were present at absolute minimum levels with almost none present. Thus, once again, the Negative Control group was the epitome of healthy muscle. The Positive Controls (figure 6.1B) also displayed a maximal presence of satellite cells; however fiber regenerative characteristics, including size variability and visible regeneration with central nuclei, as well as degeneration and necrosis were at maximal levels. Fiber splitting was additionally present in moderate levels. The fact that fiber regeneration and degeneration were in such close correlation to one another indicates that the Gluteus complex muscles are able to keep up in their attempt to repair damage caused by the disease state. This mechanism is however known to be flawed in Dysferlinopathy and some adipocytic replacement of muscle fibers was present only within samples of this group, though in minuscule amounts.

The Resveratrol treated animals (figure 6.1C) only displayed moderate levels of satellite cell presence. Fiber regeneration and degeneration were evident at levels comparable to that in the Positive Controls. Additionally, fiber size variability as well as splitting and lobulation within this group was distinguished at maximal levels, higher than that of the Positive Control group. Thus in muscles of the Gluteus complex, it would seem that the Resveratrol group displays the worst evidence of Dysferlinopathy, even higher than in the Positive Control group. The Low-CoQ₁₀ treated group (figure 6.1D) displayed satellite cells at moderate levels, comparable to the Resveratrol treated group. Fiber size variability as well as splitting and lobulation were present

only at minimal levels within this group, comparable to both the High-CoQ₁₀ treated and Baseline groups. Fiber degeneration and necrosis as well as fiber regeneration exhibited at moderate levels. It would thus seem that both damage and healing were almost at a state of equilibrium in this group, and the muscles themselves seemed to be in a fairly healthy state. The High-CoQ₁₀ treated group (figure 6.1E) exhibited maximal satellite cell numbers, in conjunction with both Control groups and the Combination treated group. Fiber regeneration was at maximal levels, far outweighing degenerative and necrotic characteristics which were at minimal levels. Additionally, fiber size variability as well as splitting and lobulation were only present at minimal levels. Thus far, this group exhibits the most evidence of being in a healthy state, when compared to the other treated groups. The Combination treated animals (figure 6.1F) presented with satellite cells at maximal levels. Fiber regeneration is evident at moderate levels, and degeneration and necrosis outweighed this at maximal levels. Fiber size variability as well as splitting and lobulation were both present at maximal levels, in correlation with animals in the Resveratrol treated group which was by far the group displaying the most unhealthy Gluteus complex muscles.

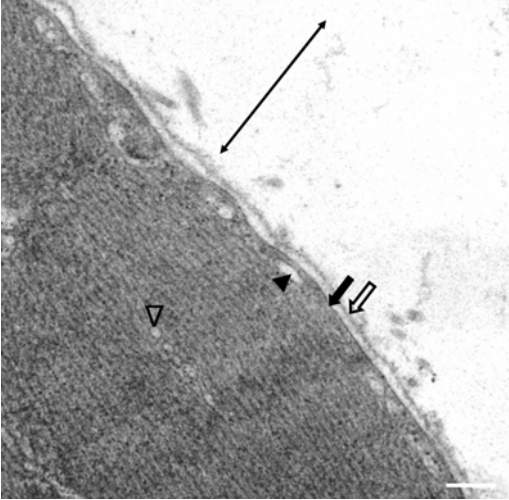
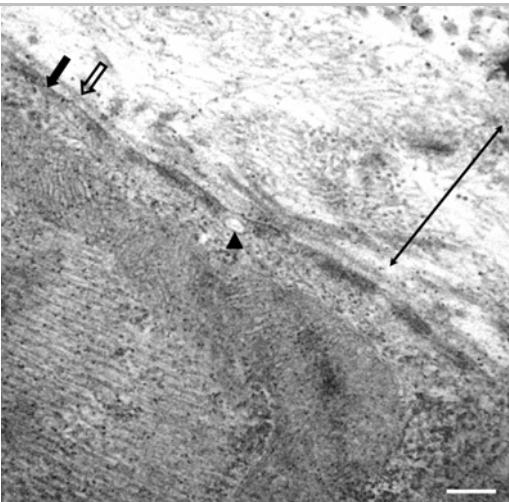
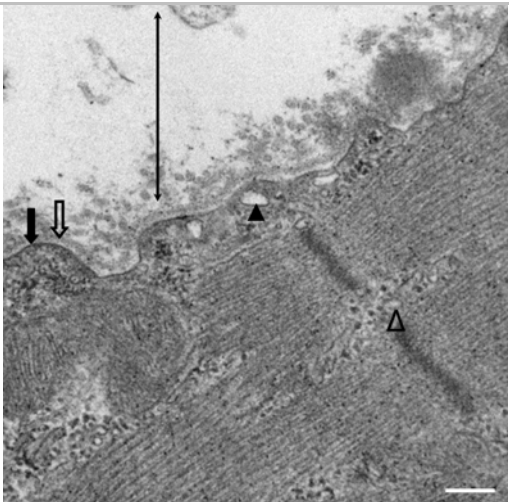
Finally, the Baseline group (figure 6.1G) only showed moderate satellite cell levels. Fiber degeneration and necrosis, splitting and lobulation as well as fiber regeneration and size variability were present at minimal levels most comparable to the High-CoQ₁₀ treated and Negative Control groups overall.

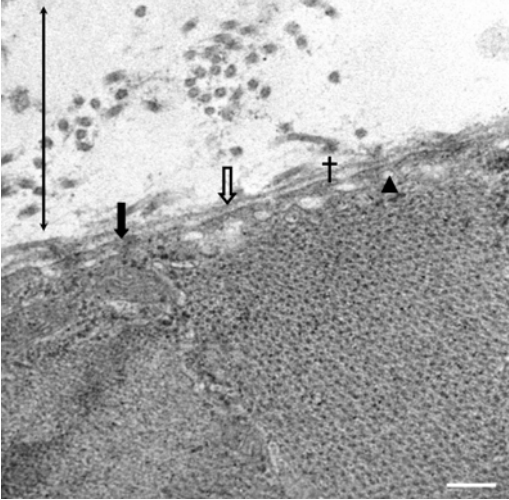
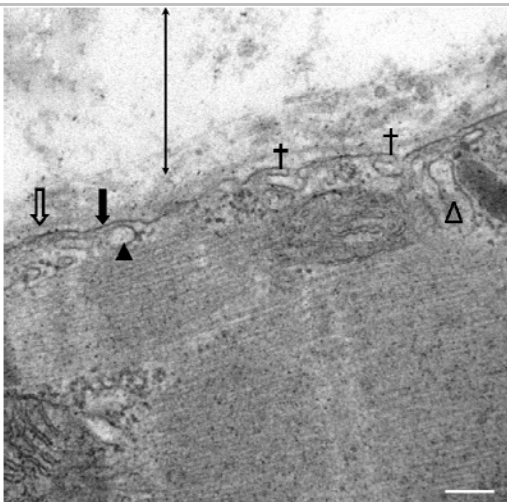
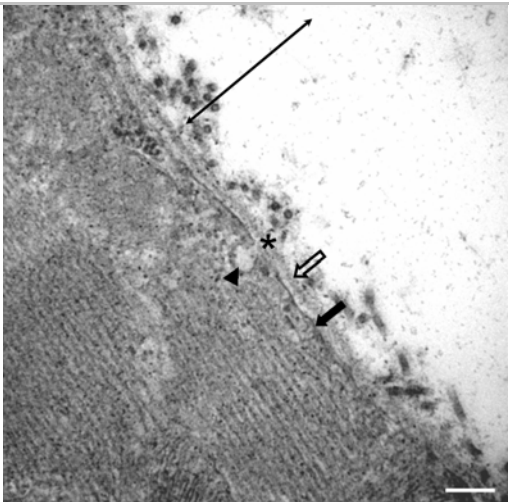
6.3. Transmission Electron Microscopic Investigation

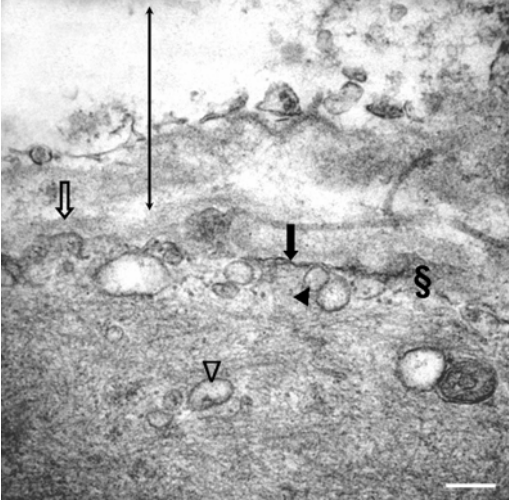
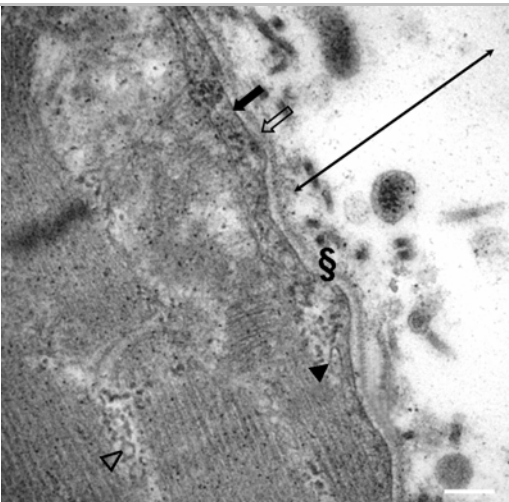
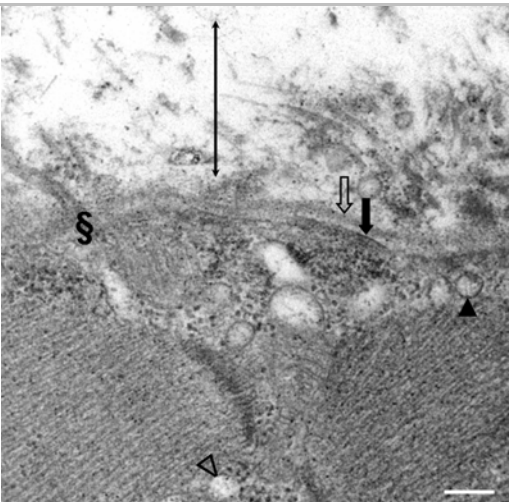
Transmission electron microscopic ultramicrographs were taken of Gluteus complex muscle samples of each animal displaying the worst changes (determined via light microscopic investigation) within the seven groups under study. Sarcolemmal vesicles were of the most interest as the fusion of these is indicative of membrane repair at the incurrence of damage. Of these, the image most representative of the group was selected for insertion in figure 6.3 (A-J).

With regards to TEM analysis, the following is of note where the plasma membrane is the area of focus. Membrane integrity is the first indication of a seemingly healthy muscle fiber, whether mature or regenerating. However, more important is the presence of sarcolemmal vesicles. If continuity or discontinuity is evident, the process of fusion or lack thereof with these vesicles to the membrane is indicative of repair of the inconsistency or the inability of the membrane repair mechanism to function optimally if at all. Papillary projections of the sarcolemma as well as alterations of the overlying basal lamina are indicative of an advanced state of Dysferlinopathy.

Sub-sarcolemmal vacuoles seem to be indicative of some loss of homeostasis in a muscle fiber. These, with the presence of sarcolemmal vesicle fusion with the membrane indicate repair of a muscle fiber which may have incurred some form of injury. The presence of these vacuoles without the presence of sarcolemmal vesicle fusion is indicative of injury or damage of a muscle fiber without the prospect of repair. Degeneration of a fiber such as this will then follow at some stage. Once the fiber is necrotic, these sub-sarcolemmal vacuoles, together with other cell contents are phagocytosed as they are expelled from the now membraneless muscle fiber. The ultramicrographs serve to compare morphological differences between Gluteus complex muscle membranes in groups A-G, with reference to the above-mentioned characteristics.

 A: Negative Control	→ Sarcolemma ⇨ Basal Lamina ▲ Sarcolemmal Vesicles △ Sub-sarcolemmal Vacuoles ↔ Connective Tissue between Fibers
 B: Positive Control	→ Sarcolemma ⇨ Basal Lamina ▲ Sarcolemmal Vesicles ↔ Connective Tissue between Fibers
 C: Resveratrol	→ Sarcolemma ⇨ Basal Lamina ▲ Sarcolemmal Vesicles △ Sub-sarcolemmal Vacuoles ↔ Connective Tissue between Fibers

 D: Low-CoQ₁₀	→ Sarcolemma ⇨ Basal Lamina ▲ Sarcolemmal Vesicles † Papillary Projections ↔ Connective Tissue between Fibers
 E: High-CoQ₁₀	→ Sarcolemma ⇨ Basal Lamina ▲ Sarcolemmal Vesicles † Papillary Projections ↔ Connective Tissue between Fibers
 F: Combination Resveratrol/CoQ₁₀	→ Sarcolemma ⇨ Basal Lamina ▲ Sarcolemmal Vesicles * Vesicle Fusion Sites ↔ Connective Tissue between Fibers

 G: Baseline	→ Sarcolemma ⇒ Basal Lamina ▲ Sarcolemmal Vesicles △ Sub-sarcolemmal Vacuoles § Sarcolemmal Disruption Site ↔ Connective Tissue between Fibers
 H: Fiber with sarcolemmal disruption	→ Sarcolemma ⇒ Basal Lamina ▲ Sarcolemmal Vesicles △ Sub-sarcolemmal Vacuoles § Sarcolemmal Disruption Site ↔ Connective Tissue between Fibers
 I: Degenerating fiber	→ Sarcolemma ⇒ Basal Lamina ▲ Sarcolemmal Vesicles △ Sub-sarcolemmal Vacuoles § Sarcolemmal Disruption Site ↔ Connective Tissue between Fibers

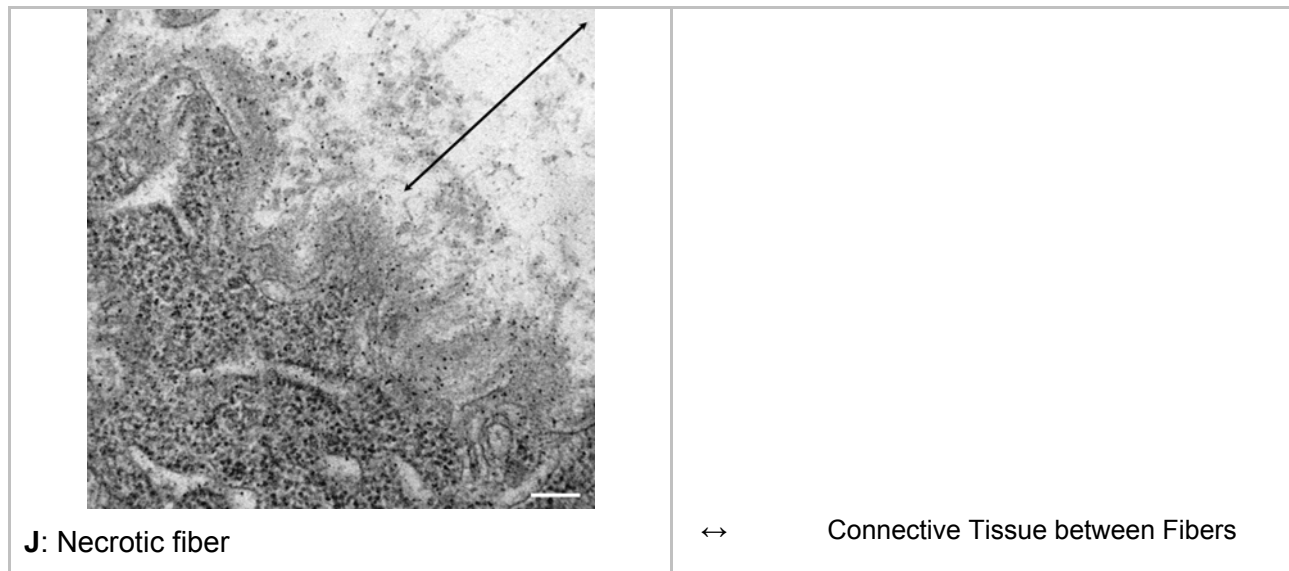


Fig.6.3: Depicting morphological differences between Gluteus complex muscle membranes in groups A-G. All muscle sections were photographed at a magnification of approximately 60,000X. **A:** Negative Control SWR/J mice at 28 weeks of age. **B:** Positive Control; **C:** Resveratrol treated; **D:** Low-CoQ₁₀ treated; **E:** High-CoQ₁₀ treated; **F:** Combination Resveratrol/CoQ₁₀ treated SJL/J mice at 27 weeks of age. **G:** Baseline untreated SJL/J mice at 14 weeks of age. **H:** Depicts a fiber with a membrane disruption; **I:** a degenerating fiber where the membrane is already disrupted; and **J:** a necrotic fiber which was already in the process of post-necrotic debris removal in adjacent areas. (Scale bar = 200nm)

The Negative Control group's (figure 6.3A) membranes were principally normal; however, some sarcolemmal vesicles were present almost throughout. There was a minimal presence of small sub-sarcolemmal vacuoles and the basal lamina was normal in its entirety. The Gluteus complex muscle is a prominent one and much stretching will necessitate the need for an efficient repair mechanism, thus it is conceivable that the evident presence of large numbers of sarcolemmal vesicles in the vicinity allow for optimal repair of damaged sites in as short a time period as possible. The Positive Control group (figure 6.3B) displayed some membrane defects as well as thickening of the basal lamina. Sarcolemmal vesicles were present but not as numerous as in the Negative Control group. Inflammatory infiltrate was definitely pronounced within the Positive Control group and it appeared that the sarcolemma itself was also thickened, in addition to basal lamina thickening observed within this group. This group definitely exhibited the largest amount of collagen fibers and bundles between muscle fibers when compared to all other groups.

The Resveratrol treated group (figure 6.3C) exhibited a larger presence of sarcolemmal vesicles than the Positive Control group as well as sarcolemmal defects in places. The basal lamina was also thickened and alterations thereof were noted. There were considerable areas of

necrosis and inflammatory infiltrate within this group. The Low-CoQ₁₀ treated group (figure 6.3D) presented with many sarcolemmal vesicles and some areas of vesicle fusion with the membrane, though there were small areas of un-repaired membrane discontinuity also. The basal lamina was thickened in places and a small number of papillary projections were observed. The state of inflammatory infiltration was marked; however, not much collagen proliferation was present. In the High-CoQ₁₀ treated group (figure 6.3E), sarcolemmal vesicles were once again noted, some of which were very large. Thickening of the basal lamina and the presence of papillary projections were also distinguishable. The Combination treated group (figure 6.3F) did not display with many sarcolemmal vesicles, the ultramicrograph selected for insertion shows distinct fusion of a vesicle with the membrane indicating the ability to repair membrane damage effectively. Some basal lamina thickening was evident, but not to a large degree. The state of inflammatory infiltration was not at all severe within the animals of this group.

The Baseline group (figure 6.3G) exhibited a presence of very large sarcolemmal vesicles, there was much membrane disruption noted throughout muscle samples of this group and the basal lamina was observed as indistinct and/or immensely thickened in places.

The ultramicrograph depicting a disrupted membrane (figure 6.3H) presents with sarcolemmal vesicles as well as sub-sarcolemmal vacuoles. The area of sarcolemmal injury is distinct and duplication of the basal lamina was visible in places. The degenerating fiber (figure 6.3I) displayed sarcolemmal vesicles and sub-sarcolemmal vacuoles being lost from the fiber through areas of membrane disruption, the basal lamina was thickened and indistinct in areas. Finally, with regards to a necrotic fiber (figure 6.3J) a multitude of vacuoles were present and a sarcolemma no longer held the cell contents in place, the basal lamina was additionally completely indistinguishable. Phagocytosis of cellular contents was taking place adjacent to this ultramicrograph.

6.4. Scanning Electron Microscopic Investigation

For the scanning electron microscopic investigation of muscle samples, photomicrographs were taken of longitudinally arranged muscle fibers at 5,000X magnification. The connective tissue thickness between Gluteus complex muscle fibers was assessed statistically between the seven experimental groups of this study by measuring the connective tissue thicknesses nine times at random between muscle fibers of three mice from each experimental group, using the iTEM Olympus Soft Imaging Solutions® software which was calibrated to measure in micrometers. Measurements are demonstrated in figure 6.4 and a graphical representation of the mean measurements for each Group A-G is depicted in figure 6.5.

The NCSS statistical program was then utilized to perform statistical analysis on these derived measurements of thickness. The experimental groups were compared by means of either a one-way ANOVA or Kruskal-Wallis one-way ANOVA, depending on whether or not the necessary assumptions for the parametric test were met. Analysis was conducted at a significance level of 95% ($P=0.05$).

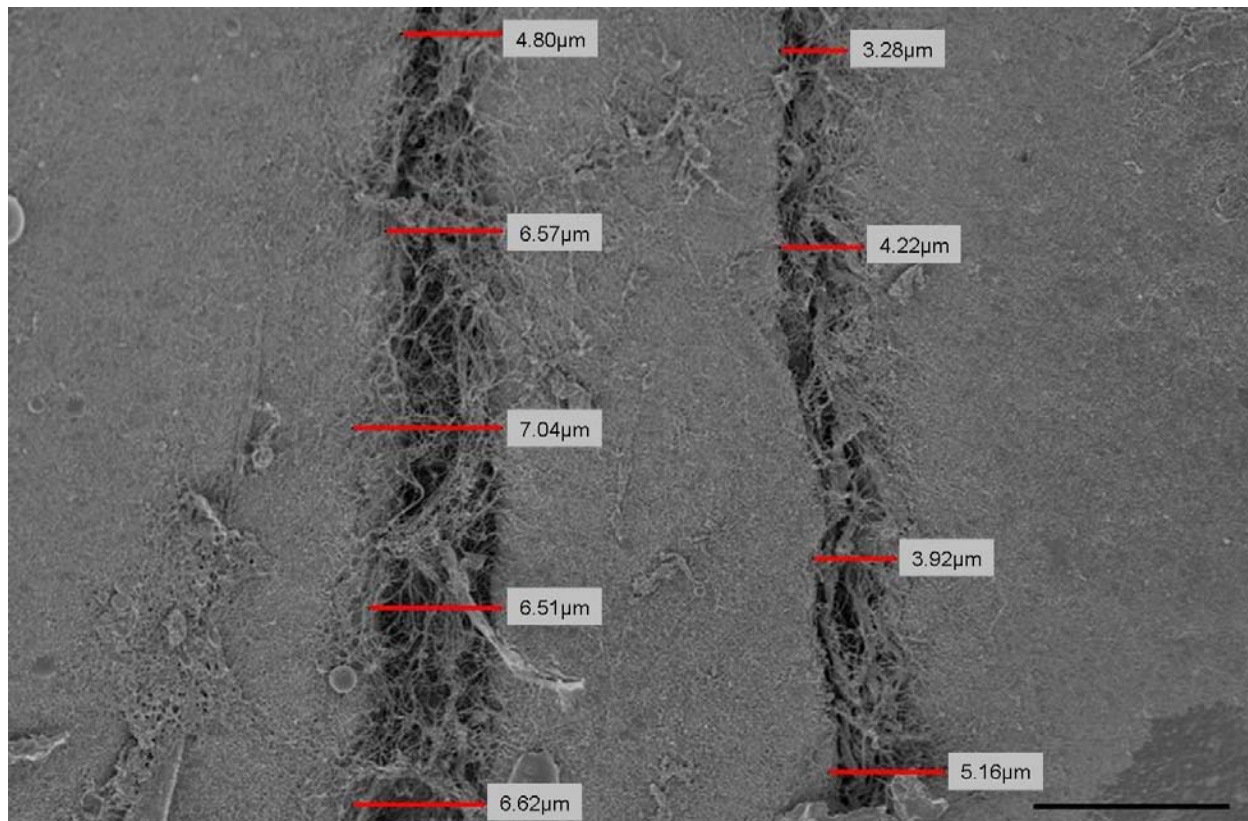


Fig.6.4: Depicting an example of connective tissue thickness measurements taken across the width between longitudinally arranged muscle fibers. (Scale bar = 10µm)

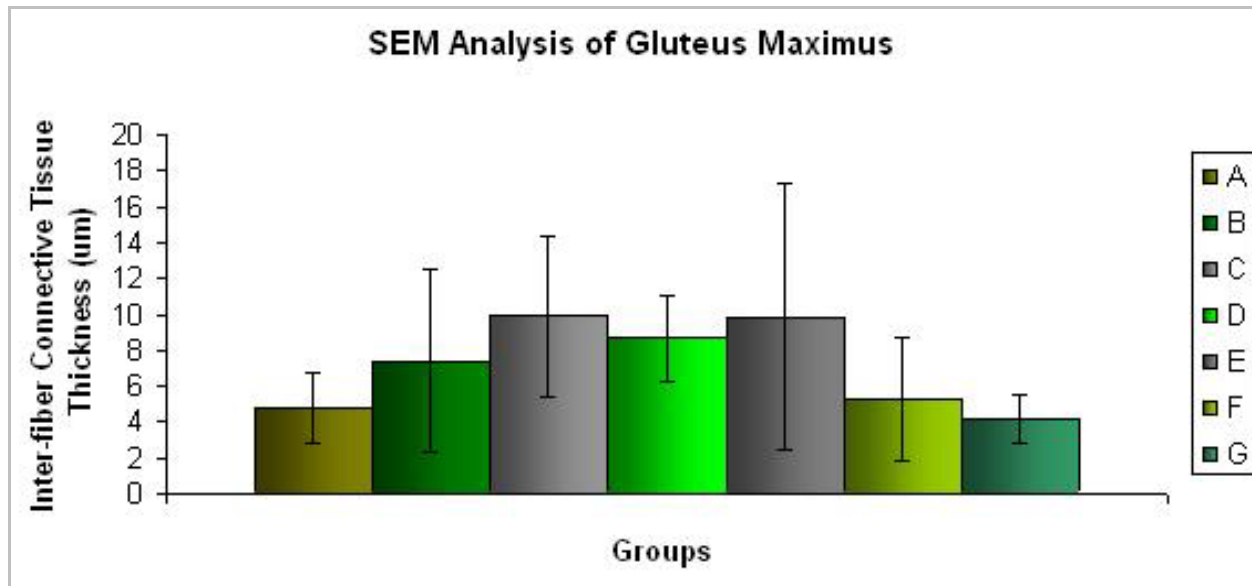


Fig.6.5: Mean connective tissue thickness measurements, with indicated standard deviations, of the Gluteus complex muscles assessed in animals of groups A-G. Group A: Negative Control SWR/J mice at 28 weeks of age. Groups B: Positive Control; C: Resveratrol treated; D: Low-CoQ₁₀ treated; E: High-CoQ₁₀ treated; F: Combination Resveratrol/CoQ₁₀ treated SJL/J mice at 27 weeks of age. Group G: Baseline untreated SJL/J mice at 14 weeks of age.

Due to the data collected possessing a non-normal distribution the Kruskal-Wallis one-way ANOVA was employed for statistical assessment of the seven experimental groups. The requirement of equal variance was ignored due to equal sample size. One or more of the groups were found to differ from each other in terms of their inter-fiber connective tissue thickness ($P < 0.00001$). This difference was then located with the subsequent use of the Tukey-Kramers pos hoc test. The results pertaining to this analysis are represented in table 6.1.

Table 6.1: Significance of statistical comparisons performed on the connective tissue thickness measurements of Gluteus complex muscle SEM micrographs of the assessed SJL/J and SWR/J mice under various experimental conditions

Experimental Group	Statistical Significance		
	Thinner Than	Indifferent To	Thicker Than
Group A Negative Control	C,D,E	B,F,G	-
Group B Positive Control	-	A,C,D,E,F	G
Group C Resveratrol	-	B,D,E	A,F,G
Group D Low-CoQ ₁₀	-	B,C,E	A,F,G
Group E High-CoQ ₁₀	-	B,C,D	A,F,G
Group F Combination Resveratrol/CoQ ₁₀	C,D,E	A,B,G	-
Group G Baseline	B,C,D,E	A,F	-

The pos hoc test run showed that the Baseline group possessed a significantly thinner inter-fiber connective tissue thickness in comparison to the Positive Control, Resveratrol as well as the Low-CoQ₁₀ and High-CoQ₁₀ treated groups. The Negative Control and Combination groups were shown to have a significantly thinner inter-fiber connective tissue thickness when compared to the Resveratrol, Low-CoQ₁₀ and High-CoQ₁₀ treated groups, but were not significantly different to each other.

The Positive Control group was only significantly thicker when compared to the Baseline group, but statistically indifferent to all other experimental groups. The inter-fiber connective tissue thickness of the Resveratrol, Low-CoQ₁₀ and High-CoQ₁₀ treated groups were all significantly thicker than the Negative Control, Combination treated and Baseline groups but not significantly different to each other.

6.5. Integrative Discussion

The Negative Control group was again the picture of healthy muscle, as expected. Degenerative changes were at an absolute minimum, regenerative changes were also at a minimum, and regenerative capabilities i.e. satellite cell numbers were at maximal levels. Sarcolemmal integrity as well as the basal lamina were normal though some sarcolemmal vesicles and sub-sarcolemmal vacuoles were present, suggesting the ability to promptly repair a site of injury should it occur in this massively utilised muscle complex. The presence of collagen bundles was insignificant at an ultrastructural level; though connective tissue thickness measurements revealed an indifference to the Positive Control, Combination treated and the Baseline groups despite the fact that these groups were visualised to possess more collagen than the Negative Controls. This group is the model to which treated groups will be compared in order to determine the status of muscular wellbeing.

The Positive Control group possessed moderate to maximal degenerative changes, and presented with maximal regenerative changes and capabilities. Sarcolemmal defects were however pronounced, as were basal lamina thickening and vesicle accumulation. Inflammatory infiltrate within muscle samples of this group was marked, and collagen bundles were observed in excess, indeed in the highest levels when compared to all other groups under investigation. Connective tissue thickness measurements did not share this visual observation, only being significantly thicker than that of the Baseline group and proving indifferent to all other groups analysed. This group is the standard of moderate to advanced Dysferlinopathy to which treated groups shall be compared.

The Baseline group possessed minimal regenerative and degenerative changes, as well as moderate regenerative capabilities, closer to that observed in the Negative Control group than the Positive Control group. Ultrastructurally however, a different representation was apparent, large vesicles and areas of membrane disruption were observed along with an indistinct and/or very thick basal lamina in places. Measurements of connective tissue thickness showed the Baseline group to be indifferent to the Negative Control and Combination treated groups. It would seem that in a more largely utilised muscle complex, the 14 week old SJL/J mouse possesses a greater ability to regenerate than in a less prominent muscle complex such as the Pectoralis complex (discussed in chapter 5). This group provides the comparison standard for groups administered treatment, allowing the determination of enhancement in muscle wellbeing from 14 to 27 weeks of age.

When comparing the four experimental groups, the following was observed; Resveratrol treatment showed a maximal balance between regenerative and degenerative changes, with

only moderate regenerative capabilities being evident. Vesicles and membrane defects were visible ultrastructurally, the basal lamina possessed areas of thickening and structural alteration. Much inflammatory infiltrate was apparent, and collagen fibers were marked but not in excess. Connective tissue thickness measurements proved to be thicker than the Negative Control, Combination treated and Baseline groups; but were statistically indifferent to the remaining groups. It appears that the exclusive anti-inflammatory role of Resveratrol is ineffective in a prominently utilised muscle complex.

The Low-CoQ₁₀ treated group showed minimal to moderate degenerative as well as regenerative changes, and moderate regenerative capabilities. Many sarcolemmal vesicles, with areas of fusion with and thus repair of the sarcolemma was evident. Some discontinuity of the membrane was however also evident. Additionally, basal lamina thickening and papillary projections were observed. Inflammatory infiltration was marked; but collagen fibers were not present in large amounts. Connective tissue thickness measurements were established to be statistically thicker than the Negative Control, Combination treated and Baseline groups (as with the Resveratrol treated group) and were indifferent to the remaining groups. Low-CoQ₁₀ treatment does seem to be marginally membrane protective in the case of Gluteus complex muscles, but is not successful in protecting the fibers to a large extent as some discontinuity is visualised, with the overall state of muscle similar to that of the Baseline group.

High-CoQ₁₀ treatment was shown to present muscle fibers with minimal degenerative changes and moderate to maximal regenerative changes and capabilities. Though large vesicles, thickening of the basal lamina and some papillary projections were evident, collagen fiber presence was not at all striking. Connective tissue thickness measurements proved inter-fiber connective tissue to be thicker than the Negative Control, Combination treated and Baseline groups (as with the Resveratrol and Low-CoQ₁₀ treated groups) and indifferent to the remaining groups. This treatment option once again seems to be more effective in improving the condition of muscle fibers from 14 to 27 weeks of age than the above two treatment options. Muscle fibers are closer to the state of health observed in the Negative Control group than to either 14 or 27 week old untreated SJL/J mice of the Baseline or Positive Control groups.

The Combination treated group displayed moderate to maximal regenerative changes and capabilities as well as maximal degenerative changes. Ultrastructurally, few vesicles were evident with some fusion thereof with the sarcolemma observed and only a small degree of basal lamina thickening present. Inflammatory infiltrate was additionally minimal and the presence of collagen fibers was almost insignificant. Ultrastructurally, the Combination treatment once again appears to display integrated beneficence of both treatment options.

6.6. Concluding Remarks

In a more prominently active muscle group such as the Gluteus complex, Resveratrol administered exclusively does not display a highly efficient anti-inflammatory capability; nor does an exclusive dose of Low-Coenzyme Q₁₀ appear to have a significant effect on the overall wellbeing of muscle fibers, though its membrane protective capabilities are more apparent in this muscle group than in the Pectoralis complex. High-Coenzyme Q₁₀ treatment once again exhibits a largely successful membrane protective capacity. Treatment with a Combination of Resveratrol and low levels of Coenzyme Q₁₀ does seem to be highly efficient in suppressing inflammation as well as protecting the sarcolemma from damage, thus achieving an optimal co-operative result of muscle wellbeing.

The current chapter's findings suggest, in conclusion, that the Combination Resveratrol/Coenzyme Q₁₀ treatment proves to be more effective in its preservation of muscle health in Dysferlinopathy, with High-Coenzyme Q₁₀ treatment following in close succession. Combination treatment is in this case more effective than any exclusive treatment option in its collective membrane protective as well as anti-inflammatory properties; thus providing the most holistically positive physical condition in animals with this disease state.

Chapter 7: Concluding Discussion

Immune modulators have long been under study in the maintenance of muscle health in patients with Muscular Dystrophies, though not as yet studied in Dysferlinopathies specifically. Treatment with such supplements would ideally suppress inflammation in order to prevent an immune response toward degenerating muscle from causing additional muscle fiber death. The promise of treatment with immune modulators thus lies in the fact that suppression of inflammation could prolong the life of muscles in a disease state known to present with prominent inflammatory characteristics. Modulating the inflammatory response of muscle tissues to the disease condition could thus provide a mechanism by which to prolong the life of muscle fibers with inherently defective healing apparatus.

Resveratrol is an antioxidant and furthermore a vastly studied anti-inflammatory compound found largely in the skins of red grapes. This compound is known to enhance nitric oxide production, which through certain mechanisms supports the suppression of damaging effects leukocytes such as macrophages and neutrophils may have on cells and tissues. Neutrophils are the primary cells of an inflammatory response and are proposed to be largely responsible for the inflammation at onset of the dysferlinopathic disease process.

Coenzyme Q₁₀ is an endogenous antioxidant which protects cells and tissues from the harmful effects of free radicals on proteins and membranes as well as other cellular components. Under normal circumstances, high concentrations thereof are present in skeletal muscle; but levels decline with age as there are increased requirements for the antioxidant and decreased production of it by the body. As with the aged state, the levels of Coenzyme Q₁₀ may well rapidly decline with the loss of muscle homeostasis in Dysferlinopathy as the body's requirement is increased but the antioxidant's production remains at normal levels. Due to its lipid solubility Coenzyme Q₁₀ is recommended as a membrane protective supplement, which was a factor of great interest to the current study.

A most successful tool in the study of disease and possible therapy thereof is an animal model. In the case of Dysferlinopathy, the SJL/J mouse model is widely used as it presents with characteristics most like the commonest human condition; characterized by dysferlin deficiency as well as inflammatory changes in muscle. The mouse dysferlin gene possesses an overall amino acid sequence homology to the human gene in excess of ninety percent, and is also inherited in an autosomal recessive pattern, making it the optimal means of studying therapeutic options to Dysferlinopathy.

Once implemented, blood and tissue samples were taken from each group of animals at completion of the study. Negative Control animals at 28 weeks of age with healthy muscle were compared to dysferlinopathic Baseline animals at an untreated 14 weeks of age and Positive Controls at an untreated 27 weeks of age, as well as to four experimental groups administered Resveratrol (60mg/kg/day), Low (40mg/kg/day) and High (120mg/kg/day) concentrations of Coenzyme Q₁₀, and Combination Resveratrol/Coenzyme Q₁₀ (60:40mg/kg/day) for a period of 90 days (from 14 to 27 weeks of age). From these, we wished to determine which therapeutic option would prove most beneficial to animal wellbeing and thus an option for human therapy in Dysferlinopathy.

We proposed early on that treatment with the Combination of Resveratrol and Coenzyme Q₁₀, since possessing the lowest neutrophil counts overall, would display very little muscle inflammation and/or damage of the sarcolemma at the tissue level. The Combination treated group also exhibited the lowest monocyte and eosinophil counts overall; eosinophils being thought to cause tissue damage secondary to initial neutrophil damage of tissues, and thus also playing a definitive role in dysferlinopathic pathogenesis. This proposition of the effectiveness of Combination Resveratrol/Coenzyme Q₁₀ treatment proved to be true at the tissue level in both the Pectoralis and Gluteus complex muscles.

Resveratrol and Low-Coenzyme Q₁₀ treatment in exclusivity were unable to afford much protection to muscle fibers at the tissue level, both in terms of inflammation suppression and membrane protective capabilities. High-Coenzyme Q₁₀ treatment offered convincing protection against membrane damage and improved muscle fiber wellbeing dramatically from a Baseline age of 14 weeks to the age of Positive Control animals at 27 weeks. The Combination Resveratrol/Coenzyme Q₁₀ treatment provided not only membrane protective effects likened to that observed in the High-Coenzyme Q₁₀ treated group, but also provided the anti-inflammatory effects of Resveratrol which failed to materialise at sufficient levels in exclusive Resveratrol treatment.

Thus in conclusion of the study, the effectiveness of treatment with either High-Coenzyme Q₁₀ or Combination Resveratrol/Coenzyme Q₁₀ were supported in the findings of both insignificantly-affected muscle groups i.e. Pectoralis complex and more significantly-affected muscle groups i.e. Gluteus complex. However, in the Gluteus complex muscle group, Combination treatment seemed to rise slightly above High-Coenzyme Q₁₀ treatment due to its co-operatively holistic effect on muscle health at the tissue level. Accordingly, Combination Resveratrol/Coenzyme Q₁₀ (60:40mg/kg/day) is the therapeutic supplement regime recommended through the findings of this study for the maintenance of comprehensive muscle health in sufferers of Dysferlinopathy.

The main focus of this thesis was ultrastructure and morphology. More extensive research focused on immunohistochemistry of the presence of the dysferlin gene and protein within these muscle samples could lead to a more comprehensive understanding of the morphological findings of the specific study. Additionally, proteomics which is the global analysis of gene expression and complex mixtures of proteins for the main purpose of qualitative, quantitative as well as functional analysis of all proteins within a specific sample. Thus, proteomics studies could be of importance in the comparison of protein maps of the Pectoralis and Gluteus muscle complexes in both affected and unaffected animal models.

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Appendix A

Van der Spuy WJ & Pretorius E. Is the use of resveratrol in the treatment and prevention of obesity premature? *Nutrition Research Reviews* 2009; 22:111-7

Is the use of resveratrol in the treatment and prevention of obesity premature?

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Obesity is a multi-faceted disease, predisposing sufferers to numerous co-morbidities such as epithelial dysfunction and insulin resistance which ultimately result in CVD. Visceral adipose tissue in particular is associated with inflammation due to the release of pro-inflammatory cytokines by adipocytes. Inflammation seems to be rather central in causing damage to endothelial cells as well as exerting negative effects on glucose metabolism, ultimately leading to insulin resistance. Resveratrol is a naturally occurring phenolic substance which has been found to display anti-inflammatory, vasoprotective and insulin-sensitising effects, among others. The popularity of resveratrol use is escalating in the treatment of various ailments including obesity in adults. The use of the substance in childhood obesity is, however, a worrying factor, as no studies have as yet been performed on adolescent animals and there is evidence of kidney toxicity of resveratrol and its metabolites at intake levels below those currently approved as safe. Another cause for concern is the uncertainty surrounding long-term, low-dose administration of the substance in humans. The supplement should thus not be recommended for use in the prevention and treatment of obesity until conclusive research is established on the safety of long-term usage of resveratrol in both children and adults.

Obesity: Inflammation: Cardiovascular disease: Resveratrol

Introduction

The amount of obese individuals in the world today is steadily reaching epidemic proportions. Importantly, obesity is not only an adult issue, but children are also increasingly affected. This increase in frequency of sufferers of obesity is mainly attributed to high-energy diets and low activity levels. Because of increased knowledge regarding the effects of obesity, many products and quick-fix solutions are currently on the market. Many of these products are alternative medicines or vitamins, typically seen as harmless and able to improve health. Particularly, products that are known to have antioxidant value claim to address obesity. The effects of some of these products on children and adults have not been adequately tested, and particularly in a condition like obesity, which is a health concern that is multi-faceted, could potentially have very negative outcomes.

Obesity is not a health complication on its own, but brings with it significant associated co-morbidities. These include CVD and heart attacks, type 2 diabetes mellitus, non-alcoholic fatty liver disease, insulin resistance^(1,2), hypertension, dyslipidaemia, impaired glucose homeostasis, sleep apnoea, accelerated skeletal and pubertal development (ageing processes), as well as cancer, orthopaedic disorders

and psychological consequences^(3–5). Although many of these conditions only develop later in life, obese individuals are already setting the scene for their future health concerns when they are young.

The adipose tissue of visceral obesity in particular has been found to release pro-inflammatory cytokines. Inflammation seems to be central to the multitude of obesity-related complications that shorten the lifespan of suffering individuals. Inflammation leads to endothelial dysfunction which ultimately results in CVD, either through atherogenesis or through more direct mechanisms. Inflammation also has a negative effect on glucose metabolism, leading to insulin resistance which is problematic either through resultant type 2 diabetes mellitus (impaired glucose homeostasis), or through causal relationships with endothelial dysfunction (insulin stimulates NO synthesis) which then eventually leads to CVD yet again. Insulin resistance is complicating in that it not only creates grounds for vascular effect impairment but also for metabolic impairment. Thus, obesity literally causes a cascade of interrelated health complications that ultimately shorten the lifespan of suffering individuals. For a schematic diagram of the inter-relationship of the obesity-related co-morbidities discussed here, see Fig. 1.

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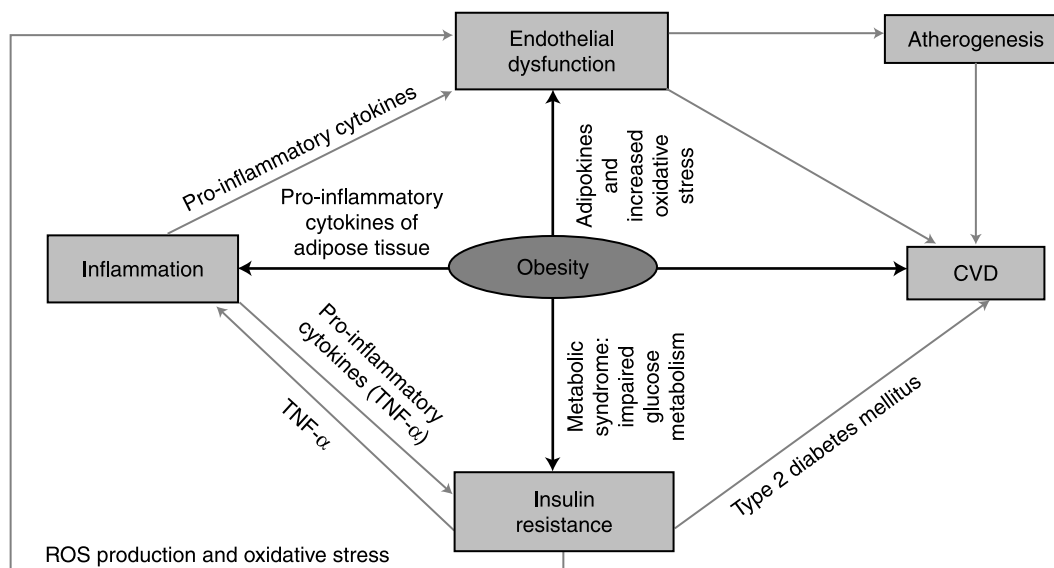


Fig. 1. Schematic diagram of the inter-relationship of most prominent co-morbidities of obesity. ROS, reactive oxygen species.

The link between obesity and endothelial dysfunction and insulin resistance is strengthened by the finding that endothelium-dependent vasodilation is impaired in proportion to insulin resistance and various indices of adiposity under baseline conditions^(1,6).

One of the antioxidants/anti-inflammatories that is now seen as being advantageous in the treatment of obesity is resveratrol, and, although it is typically used by adults, there are no restrictions against its use by children. Resveratrol is a naturally occurring phenolic substance, present in a variety of plants as a defence mechanism against bacterial and other infections^(5,7) as well as environmental stressors⁽⁸⁾. A wide range of beneficial effects have been attributed to resveratrol, including anti-inflammatory, anti-cancer, anti-lipid, anti-ageing, as well as vasoprotective effects^(5,7). The substance has been reported to elicit numerous cellular responses including cell cycle arrest, differentiation and apoptosis^(9,10). It also displays anti-leukaemic, antiviral and neuroprotective properties^(10–13). Resveratrol is able to function as an *in vivo* antioxidant^(10,14) and markedly reduces the risk of developing CHD; this property is probably due to its modulation of lipid metabolism and prevention of oxidation of the LDL^(10,15), as well as inhibition of eicosanoid production and platelet aggregation^(10,16). Additionally, resveratrol has been shown to inhibit several important enzymes involved in carcinogenesis^(10,17,18).

Complications of obesity

Obesity (Fig. 1) is characterised by an excess amount of adipose tissue. It has become recognised as an independent factor of cardiovascular risk, predisposing the obese individual to type 2 diabetes mellitus, hypertension, reduced endothelial function, as well as inducing insulin resistance^(3,5). Noteworthy is the fact that obesity is a component of the metabolic syndrome, of which insulin resistance is the hallmark. Metabolic risk factors associated with obesity are

serum TAG elevations, low levels of HDL, elevated blood pressure, insulin resistance due to elevated glucose levels, as well as prothrombotic and pro-inflammatory states^(1,19).

Inflammation

Obesity is known to be accompanied by an inflammatory response (Fig. 1) in various tissues. Adipose tissue is being implicated, more and more, as a key regulator of inflammation through secretion of pro-inflammatory cytokines (i.e. TNF- α) which seem to play a chief role in affecting both endothelial function and glucose metabolism^(1,20). The negative influence on endothelial physiology may lead to the formation of atherosclerotic plaques⁽¹⁾. Endothelial dysfunction and dysfunctional glucose metabolism (ultimately insulin resistance) seem to head the two main cascades which link obesity to CVD. In fact, growing evidence is pointing to a causative relationship between inflammation and insulin resistance, as TNF- α has been established to mediate insulin resistance as a result of obesity in a multitude of rodent obesity models^(1,21).

Resveratrol inhibits monocyte chemo-attractant protein secretion and gene transcription via down-regulation of TNF-induced transcription. It also reverses secretion and expression of atherogenic adipokines, actually reducing the size, density and mean area of atherosclerotic plaques, as well as the thickness of the vessel intima layer of atherosclerotic lesions^(5,22). Treatment with resveratrol, in addition to relieving glycerol-induced renal injury by suppressing the inflammatory process and by inhibiting lipid peroxidation^(5,23), also significantly attenuates renal dysfunction and oxidative stress in diabetic rats^(5,24); therefore, assuming a significant anti-inflammatory role (Fig. 2) for resveratrol is plausible⁽⁵⁾.

However, evidence has arisen showing an increase in both lipid peroxidation and DNA and protein carbonyl damage markers with resveratrol treatment in the kidneys, suggesting the possibility of kidney toxicity of resveratrol

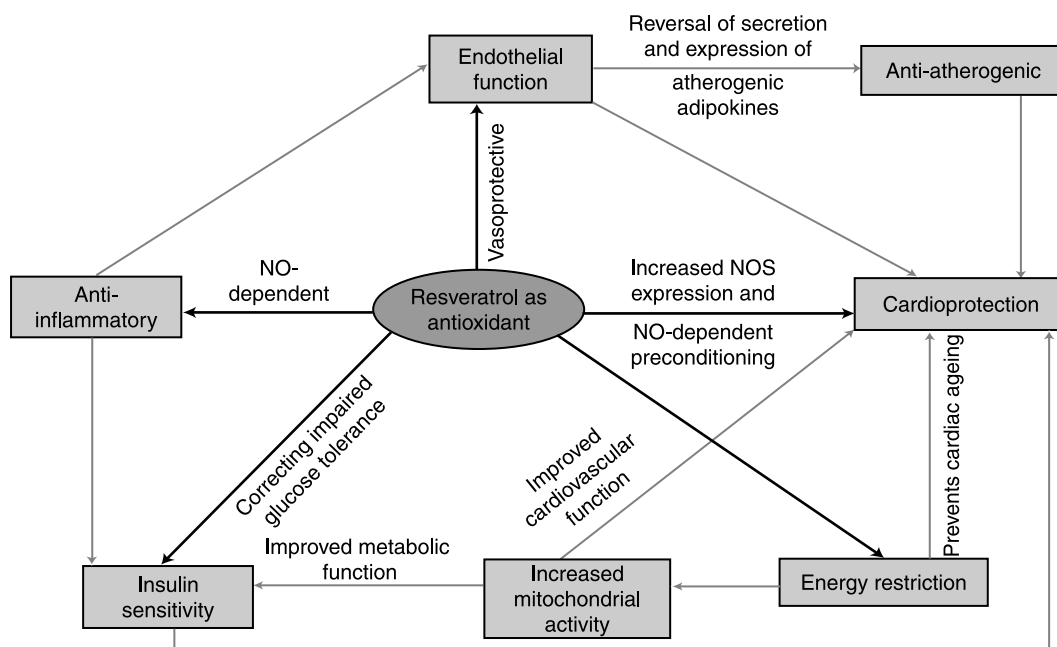


Fig. 2. Schematic diagram of benefits of resveratrol in prominent co-morbidities of obesity.

and its metabolites. Resveratrol forms extensive conjugated metabolites including glucuronides, sulfates and methylated products^(25–27). Pharmacokinetic studies on acute resveratrol doses indicate significant distribution thereof to the kidneys^(27,28), with rapid excretion in urine^(25,27,29). These metabolites are capable of crossing glomerular membranes and accumulating in the kidney tubules where they may act as pro-oxidants or activate oxidative stress, as seen for various polyphenolic conjugates^(27,30). Wong *et al.*⁽²⁷⁾ hypothesised that this may have led to the increase in lipid and protein damage observed in the kidneys of the resveratrol-treated mice in the study which they undertook. However, further *in vivo* studies on the biological activity of resveratrol metabolites are needed to investigate the chronic effects of low-dose resveratrol on the kidney.

Given the growing popularity of resveratrol as a supplement, the possibility of kidney damage is a cause for concern and warrants further investigation, especially before the supplement is recommended for usage in obesity treatment.

Reduced endothelial function

Vascular endothelial cells maintain cardiovascular homeostasis. Importantly, in addition to the physical barrier which they create, endothelial cells secrete a number of mediators that regulate platelet aggregation, coagulation, fibrinolysis and vessel tone⁽¹⁾. There is strong evidence indicating an interaction between endothelium and the secretory proteins of adipocytes (adipokines), suggesting that the ability of adipokines to directly affect vascular homeostasis may be of importance in the developmental mechanism of CVD (Fig. 1) in obese patients^(1,31).

Endothelium is a contributor to the regulation of blood pressure and flow, by releasing NO as well as other

compounds which contribute to both vasodilation and vasoconstriction⁽¹⁾. NO is the major contributor to endothelium-dependent relaxation in conduit arteries^(1,32). Obesity has been shown to increase endothelium-dependent vasoconstriction in the absence of endothelial NO^(5,33).

It is the increased oxidative stress in accumulated fat which plays a major role in the induction of endothelial dysfunction. Endothelium thus loses its physiological properties which include the tendency to promote vasodilation, fibrinolysis, and its anti-aggregation properties⁽¹⁾. NO has numerous important effects on the vasculature; these include the maintenance of basal tone by relaxing smooth muscle cells, inhibition of platelet adhesion, activation, secretion and aggregation, as well as the promotion of platelet disaggregation^(1,34). Additionally, NO is a potent inhibitor of mechanisms ultimately leading to neointimal proliferation and atherosclerosis, through preventing endothelial leucocyte adhesion and smooth muscle cell migration and proliferation⁽¹⁾.

Resveratrol has the ability to increase NO synthesis, which in turn functions as an *in vivo* antioxidant in the ischaemic–reperfused heart, brain or kidney, and lowers oxidative stress^(8,35).

Cardiovascular effects

Resveratrol's protective mechanism (Fig. 2) includes its role as an intracellular antioxidant, anti-inflammatory agent, its ability to induce NO synthase (NOS) expression, as well as its ability to induce angiogenesis^(8,12). NO was thus identified to have a direct role in vasorelaxation when increased NOS activity was found in cultured pulmonary artery endothelial cells treated with resveratrol⁽³⁶⁾, suggesting cardioprotection by resveratrol through affecting the expression of NOS⁽⁸⁾.

Much evidence suggests that resveratrol-mediated cardioprotection is achieved by preconditioning^(8,35). Preconditioning cardioprotection is achieved by subjecting the heart to a therapeutic amount of stress, so as to disturb normal cardiovascular homeostasis and re-establish a modified homeostatic condition with increased cardiac defences that can withstand subsequent stress insult⁽⁸⁾. Resveratrol's pharmacological action for preconditioning of the heart takes place in an NO-dependent manner. The anti-inflammatory action itself of resveratrol seems to take place through an NO-dependent mechanism⁽⁸⁾.

Use of resveratrol improves post-ischaemic cardiac function regardless of the presence of glucose intake, and reduces the incidence of ventricular fibrillation and infarct size. Moreover, resveratrol increases GLUT-4 expression while reducing endothelin expression and cardiac apoptosis in ischaemic-reperfused hearts, also regardless of the absence of glucose intake, suggesting a direct protective effect on the heart^(5,8,37,38). GLUT-4 translocation is not involved in resveratrol's action, but the mechanism of action appears to lead to stimulation of the intrinsic activity of the GLUT-4 glucose transporters themselves⁽⁷⁾.

Insulin resistance

Insulin resistance (Fig. 1) is one of the pathophysiological mechanisms leading to diabetes mellitus^(10,39) and the state of resistance is attained through a decreased sensitivity or responsiveness of peripheral tissues to the metabolic action of insulin. This, together with the other components of the metabolic syndrome, is associated with altered endothelial function, which ultimately leads to CVD⁽¹⁾. Insulin resistance occurs due to high glucose levels, and can contribute to reactive oxygen species production^(1,40) (oxidative stress then plays a role in endothelial dysfunction). The insulin-resistant state is additionally characterised by an increase in pro-inflammatory cytokines such as TNF- α , which may also contribute to hepatocellular injury^(10,41). The process leading to insulin resistance leads to long-term damage, dysfunction, and failure of various organs particularly the eyes, kidneys, nerves, heart and blood vessels^(10,42).

Resveratrol is capable of scavenging some intracellular reactive oxygen species⁽¹²⁾. Oral treatment with resveratrol significantly decreases the levels of glycosylated Hb (blood glucose indicator), suggesting that it may in fact prevent oxidative damage caused by the glycation reaction in diabetic conditions. Thus, resveratrol seems to be beneficial in preventing the pathogenesis of diabetic complications caused by impaired glucose metabolism⁽¹⁰⁾. Impaired glucose tolerance serves as a marker for insulin-resistant states and predicts vascular complications^(10,43). With resveratrol treatment, the impaired glucose tolerance observed in various groups of diabetic animals was corrected to near normal, strengthening the data on resveratrol's insulin-stimulatory (Fig. 2) effects^(7,10).

Data from a study by Breen *et al.*⁽⁷⁾ indicate that resveratrol increases glucose uptake without stimulating the translocation of glucose transporters (i.e. GLUT-4) in skeletal muscle tissue. They demonstrated that resveratrol

stimulates glucose uptake in skeletal muscle cells in a dose- and time-dependent fashion, independent of insulin.

Energy restriction

The purpose of energy restriction is to retard several aspects of ageing; these include age-related mortality, tumorigenesis, as well as physiological decline and the establishment of age-related transcriptional profiles^(44,45). It is proposed that energy restriction confers health benefits by provoking an organismal defence response through mild stress^(46,47).

Animal studies have reported effects of energy restriction (Fig. 2) for resveratrol^(2,5,38,45), ultimately resulting in weight reduction, through prevention of abdominal obesity and TAG accumulation in hepatic cells, and improvement of lifespan of animals fed a high-energy diet. This weight-reduction effect of resveratrol is speculated to be in part attributed to its effects on adipocytes^(2,5,38). Studies have reported a decrease in adipocyte size and decreased cell viability of adipocytes (both maturing and mature) through increasing apoptosis in these cells⁽⁵⁾.

Resveratrol mimics the effects of energy restriction to prevent cardiac ageing at both the transcriptional and functional levels. Additionally, mice on an energy-restricted diet or supplemented with resveratrol have improved insulin sensitivity (Fig. 2), as both these lower blood glucose levels without lowering plasma insulin⁽⁴⁵⁾.

There is also a suggested up-regulation of gene expression regulating mitochondrial activity (Fig. 2) through resveratrol treatment as well as a proven increase in aerobic capacity. The increases in mitochondrial oxidative phosphorylation and aerobic capacity are associated with increased longevity, therefore improving animal survival time^(5,48).

Mitochondria are the principal energy sources of the cell that convert nutrients into energy through cellular respiration^(49,50). Compromised mitochondrial function has been linked to numerous diseases, including those of the metabolic and cardiovascular systems^(50,51). With impaired mitochondrial function, fatty acids are directed toward storage as opposed to oxidation. This may contribute to the lipid accumulation within muscle cells, which has been linked to insulin resistance in obesity and type 2 diabetes mellitus^(50,52–54). In line with this, resveratrol significantly improves both muscle oxidative capacity and sensitivity to insulin in high-energy-fed mice⁽⁵⁰⁾.

Baur *et al.*⁽²⁾ hypothesised that resveratrol might shift the physiology of middle-aged mice on a high-energy diet towards that of mice on a standard diet and provide the associated health benefits of such a diet without having to reduce energy intake. After 6 months of treatment, they reported that there was a clear trend towards increased survival and insulin sensitivity. Also, it was found that resveratrol-treated mice on a high-energy diet displayed decreased organ pathology (liver, pancreas) when compared with untreated mice on a high-energy diet. The ability of resveratrol to improve motor function was also noted, indicating that the effects of resveratrol are not confined to the liver. Improvements in the morphology of the aortic elastic lamina were also apparent in resveratrol-treated mice. The livers of resveratrol-treated animals showed

a considerable increase in the number of mitochondria when compared with untreated high-energy-fed mice, and were not significantly different from mice on a standard diet. The data collected by this group demonstrate quite clearly that resveratrol can alleviate the negative impact of a high-energy diet on the overall health and lifespan of middle-aged mice.

The findings of this group⁽²⁾ showed that resveratrol does indeed shift the physiology of mice consuming high-energy diets towards that of mice on a standard diet. Resveratrol was also shown to modulate known longevity pathways and improve general health, including survival, motor function, insulin sensitivity, organ pathology, and mitochondrial number. These changes occurred, notably, without significant reduction in body weight.

It is, however, important to note that all experimental treatments have thus far been administered to middle-aged animal models; therefore, it has not yet been ascertained what the effects of resveratrol treatment would be on young animals. Although the present review focuses on the general effects of resveratrol on obesity, it cannot be ignored that children may prove to be more prone than adults to its potentially harmful effects. Thus, before any recommendation is made for the use of resveratrol in the treatment or prevention of obesity, studies will have to specifically test the product on adolescent animals, as well as compare differences in toxicity data with those of experiments performed on adult animals.

Multi-faceted effects of resveratrol

Resveratrol seems to present with two faces. It protects cells by potentiating a survival signal, but selectively kills cancer cells. It behaves as an antioxidant, yet can induce redox signalling. It is an anti-proliferative agent for cancer, inducing apoptosis in tumour cells and sensitising cancer cells by inhibiting cell survival signal transduction and anti-apoptotic pathways. In contrast, it also triggers a survival signal in ischaemic tissue by inducing anti-apoptotic genes and blocking apoptosis in the ischaemic heart. At low doses, resveratrol stimulates angiogenesis, but at higher doses it blocks the angiogenic response. At low concentrations, resveratrol scavenges reactive oxygen species, but at higher concentrations, it behaves like a pro-oxidant. Low concentrations of resveratrol are quite sufficient for cardiovascular preconditioning, whereas higher concentrations and chronic use thereof may, in fact, exert toxic effects in the heart and other organs⁽⁸⁾.

In general, however, adverse effects of resveratrol have not been documented in humans, and a recommended dosage of 5–10 mg/d is suggested to be safe after a review of toxicological literature for resveratrol by the National Institute of Environmental Health Sciences⁽⁵⁵⁾. However, the elevation in oxidative damage observed in the kidneys of mice in the study by Wong *et al.*⁽²⁷⁾ over an extended period took place at levels well below that for which toxicity has previously been reported^(2,56,57). This raises concern regarding long-term, low-dose administration of resveratrol⁽²⁷⁾. Thus, it would not be considered wise to use resveratrol at this time for the prevention or treatment of obesity, especially in children, as long-term toxicity could in

fact cause more detrimental harm to the systems of suffering individuals than currently anticipated.

Conclusion

Obesity is a growing problem worldwide, and treatment potentials are constantly under scrutiny. There is a prevalence of inflammation in obese patients, and this brings with it a cascade of problems including endothelial dysfunction and insulin resistance, ultimately resulting in cardiovascular complications. Resveratrol is a phenolic substance in which much interest has been placed. The substance is anti-inflammatory, vasoprotective, and increases glucose uptake, thus improving insulin sensitivity. These effects are the ones most applicable to obesity prevention and treatment. However, the use of resveratrol over the long term even in low doses has not yet been conclusively ascertained. Additionally, there are reports of kidney toxicity of resveratrol and its metabolites even at concentration levels currently approved by the National Institute of Environmental Health Sciences. It is thus wise not to recommend the use of resveratrol in the treatment of obesity, especially childhood obesity, as all experimental data available are from studies utilising middle-aged animal models, unless and until the safety of resveratrol and its metabolites have been scrutinised to a definitive level.

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Appendix B

Van der Spuy WJ, Potgieter M, Pretorius E, Vieira WA. Differential leukocyte counts of SJL/J mice with Dysferlinopathy treated with resveratrol and coenzyme Q₁₀. *Scandinavian Journal of Lab Animal Science*

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>>> Jann Hau <JHAU@sund.ku.dk> 09/11/09 10:06 AM >>>

Dear Dr Pretorius

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Thank you for sending this interesting manuscript to us.

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Jann

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DIFFERENTIAL LEUKOCYTE COUNTS OF SJL/J MICE WITH DYSFERLINOPATHY
TREATED WITH RESVERATROL AND COENZYME Q10

Thank you!
Resia

**DIFFERENTIAL LEUKOCYTE COUNTS OF SJL/J MICE WITH DYSFERLINOPATHY
TREATED WITH RESVERATROL AND COENZYME Q₁₀**

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Running Head:

Leukocyte counts of animals with dysferlinopathy

Keywords: SJL/J mice; dysferlinopathy animal model; Resveratrol; Coenzyme Q₁₀; blood cell counts

Summary

Dysferlinopathies include Limb-Girdle muscular dystrophy type 2B and Miyoshi myopathy, which exhibit an autosomal recessive inheritance pattern of the dysferlin gene and characteristic inflammatory infiltrate in muscle. A study of prospective treatment options was conducted on SJL/J mice, a natural model for dysferlinopathy. The animals are immunocompetent but have elevated levels of circulating T-cells. A baseline termination of SJL/J mice was made at 14 weeks, and differential leukocyte counts determined for these animals through microscopy. After administration of Resveratrol and Coenzyme Q₁₀ exclusively and in combination to the four treatment groups for approximately three months, the remaining six groups (negative and positive controls as well as four treatment groups) were terminated and differential leukocyte counts once again determined. Eosinophil counts were significantly higher in the baseline termination group than all other experimental groups assessed except for the negative control SWR/J mice, possessing normal muscle and used in research as a general purpose strain. Eosinophil granules are suggested to reduce inflammation caused by other leukocytes. At onset of dysferlinopathy between four and six weeks of age, the increase in eosinophil counts could very likely be a compensation mechanism to decrease initial inflammation in the muscular tissues of the dysferlinopathic mice. Neutrophil counts of the baseline termination group were significantly higher only when compared to the Resveratrol/Coenzyme Q₁₀ combination group. Neutrophils are linked to early inflammatory responses and often sensitised in self-antigen recognition characteristic to autoimmune disease, a known complication in the SJL strain. Thus the higher neutrophil count in the six week old mice is probably related to inflammation at diseases onset, but may also be indicative of autoimmunity; whereas the eosinophil counts may possibly play a more definitive role in the pathogenesis of dysferlinopathy. Further morphological studies will serve to clarify the roles of these leukocytes in dysferlinopathy.

Introduction

The dysferlinopathies are a set of myopathies in which a mutation in the dysferlin gene is inherited in an autosomal recessive pattern. They include Limb-Girdle Muscular Dystrophy Type 2B (LGMD2B) and Miyoshi Myopathy (MM). The dysferlinopathies mostly affect muscles of the shoulder and pelvic girdles (*Angelini, 2002*); LGMD2B first exhibits weakness in the proximal muscles and MM exhibits weakness in the distal muscles. These myopathies display a prominent inflammatory response (*Angelini, 2002; Diers et al., 2007*).

The SJL/J mouse model is a naturally occurring model for dysferlinopathy, displaying much the same characteristics as the human disease, and the dysferlin mutation is also inherited in an autosomal recessive pattern. These mice present with a prominent inflammatory response in affected muscles (*Bittner et al., 1999; Vafiadaki et al., 2000; Suzuki et al., 2005; Diers et al., 2007*). There is a more-than-ninety percent overall amino acid sequence homology between the human and mouse dysferlin genes (*Vafiadaki et al., 2000*). As a negative control, the SWR/J mouse with normal muscle is recommended as a general purpose strain.

Immune modulation involves the use of drugs to suppress inflammation in order to prevent a patient's immune response to degenerating muscle from causing additional death of muscle cells (*Jain Foundation Inc., 2008*). Coenzyme Q₁₀ (CoQ₁₀) a well-known endogenous antioxidant which protects cells and tissues from free radicals capable of damaging proteins and lipids as well as other cellular components (*Eniva Corporation, 2006*). CoQ₁₀ levels are reported to be low in muscular dystrophies. It is vital to the cellular energy production and thus growth control of muscle cells and the fact that it displays membrane protective properties warrants its supplementary consideration for the upkeep of patients with muscular dystrophy (*Folkers & Simonsen, 1995*). Resveratrol is a polyphenolic compound found largely in the skins of red grapes (*McElderry, 1999; Mitchell, 2008*). Resveratrol has been examined for its antioxidant (*McElderry, 1999; Roy & Lundy, 2005; Mitchell, 2008*) and anti-inflammatory properties (*Roy & Lundy, 2005; Mitchell, 2008*).

The antioxidant and sequentially anti-inflammatory properties of these two products are of interest to the present study as it is our aim to determine whether or not their administration will affect the levels of inflammatory leukocytes at the haematopoietic level.

Leukocytes are involved in the cellular and humoral defences of an animal against foreign materials. Leukocytes are functional only to a small extent in the bloodstream, their greatest activity being exhibited in the tissues (*Leeson et al., 1988*). They are capable of amyloid

movement, enabling them to travel out of the circulatory system in order to elicit an immune response in the tissues where such response is needed.

Leukocytes of two main types exist, namely granulocytes and agranulocytes. The granulocytes have a cytoplasm containing numerous characteristic granular bodies and possess nuclei which exhibit considerable variation in shape. Neutrophils, basophils and eosinophils are classified as granulocytes. The agranulocytes are characterised by a clear, homogenous, slightly basophilic cytoplasm. The nuclei of these leukocytes are spherical to reniform in shape. Lymphocytes and monocytes are classified as agranulocytes (Leeson *et al.*, 1988).

Materials & Methods

Animals

A total of 60 animals with a mean weight of 20g were used in this study. An import permit was obtained from the Department of Veterinary Services, Ministry of Agriculture, and the conditions stipulated therein were strictly adhered to. The Inbred animals consisting of 50 eight-week old SJL/J (dysferlinopathic muscle) and 10 nine-week old SWR/J (normal muscle) mice were obtained from Jackson Laboratory (Bar Harbor, USA) and allowed a 40-day acclimatisation period before commencement of the study.

Animals were housed at the laboratory animal facility of the UPBRC at Onderstepoort (University of Pretoria, Pretoria, South Africa). The animals were maintained in the facility's barrier unit in Tecniplast IVC cages, Eurostandard type II L with individual air supply, equipped with a pre-filter and HEPA filter system. The barrier unit was free of all major pathogens as serology, bacteriology and parasitology tests performed were all negative. Agrebe basic, 55cm², cotton sheet laboratory animal cloth bedding for boxes Type II/III LI were used as bedding and changed once weekly. The main air supply consisted of a 50% Fresh Air Primary filter and a Secondary Bag filter with 10-15 air changes per hour. A constant temperature of 23-24°C, a relative humidity of 40-60%, and a 12-hour dark-light cycle were maintained. The animals were provided JL Rat and Mouse 6F-IRRAD food (PMI Nutrition International, LLC) and water *ad libitum*. As a decrease and slowing in the mobility of the animals was observed with disease progression, it was decided upon to supplement the animals' diet by addition of Cerelac, sterile baby cereal on the floor of each cage, from day 50 of the 90-day trial.

At study commencement, the animals were 14- and 15-weeks old respectively for SJL/J and SWR/J mice. Six SJL/J animals were terminated for base-line results at this time. The remainder of the animals were entered into a 90-day experimental period, after which the study was

concluded once the animals had reached approximately 27- and 28-weeks of age respectively for SJL/J and SWR/J mice. All experimental procedures were carried out in strict accordance with the requirements of the University of Pretoria's Animal Use and Care Committee. Ethical clearance was obtained from the University of Pretoria's Biomedical Research Center (UPBRC).

Administration of Test Agents

The animals that entered the study on day one of the 90-day trial were divided and caged communally in group order (as laid out in table 1). Control groups received a placebo in the same volume as the animals who received the substances tested in the study. Experimental groups received volumes of resveratrol and Coenzyme Q₁₀ in dosages displayed in table 1. All dosing was performed orally, using a 1000 µl syringe with a mouse oral gavage needle nr.20g, once daily.

Blood Smears

Three blood smears were made for each of the animals and allowed to air-dry. All slides were later stained with Wright's stain. A single blood smear was evaluated via light microscopy (Nikon Optiphot Transmitted Light Microscope) for each of five animals within the seven groups under study. The purpose of the evaluation was to quantify each cell of the leukocyte species; namely lymphocytes, monocytes, neutrophils, basophils and eosinophils; counting up to one hundred leukocytes per slide in a total of three zones.

Statistical Analysis

The NCSS statistical program was employed to perform statistical analyses of the various leukocyte counts between the seven groups under study. Each leukocyte species was considered separately between the experimental groups via a one-way ANOVA or Kruskal-Wallis one-way ANOVA, depending upon whether the necessary assumptions for the parametric test were met or not.

Results

Animals were divided into seven experimental groups as displayed in Table 1, which importantly depicts the strain of animals in each of the groups as well as the treatment and dosage administered to them. Blood smears were made for animals in Group G at 14-weeks of age, for Groups B-F at 27-weeks of age and for Group A at 28-weeks of age.

Table 1: Subdivision of experimental animals into groups, indicating the treatment dosages administered to each animal within respective groups, as well as the age of the mice at which blood smears were made for differential leukocyte counts.

Group	Strain	Treatment	Dosage (mg/kg/day)	Age at Termination (in weeks)
A	SWR/J (Negative Control)	None	-	28
B	SJL/J (Positive Control)	None	-	27
C	SJL/J	Resveratrol	60	27
D	SJL/J	Low CoQ ₁₀	40	27
E	SJL/J	High CoQ ₁₀	120	27
F	SJL/J	Resveratrol/CoQ ₁₀	60/40	27
G	SJL/J (Baseline)	None	-	14

The overall leukocyte counts per experimental group are shown in Figure 1. Table 1, above, offers expansion of the abbreviations for groups set out on the x-axis in Figure 1. Firstly it must be noted that both monocytes and macrophages circulating in the bloodstream were counted as monocytes. From the graph, it can be seen that the combination group F has the highest lymphocyte count. This group also has the lowest monocyte, neutrophil and eosinophil counts, as well as the lowest non-zero basophil count. It is thus believed that this group will display the least inflammation at the tissue level. The baseline group G has the highest neutrophil, eosinophil and basophil counts. The high CoQ₁₀ group E shows the lowest lymphocyte counts, as well as the lowest (zero) basophil count, in conjunction with the positive control group B. It is noteworthy that the high CoQ₁₀ group E and the positive control group B, with basophil counts of zero have the highest monocyte counts overall. Further, the high CoQ₁₀ group E has the smallest lymphocyte to monocyte ratio of the experimental groups, in conjunction with the negative controls group A.

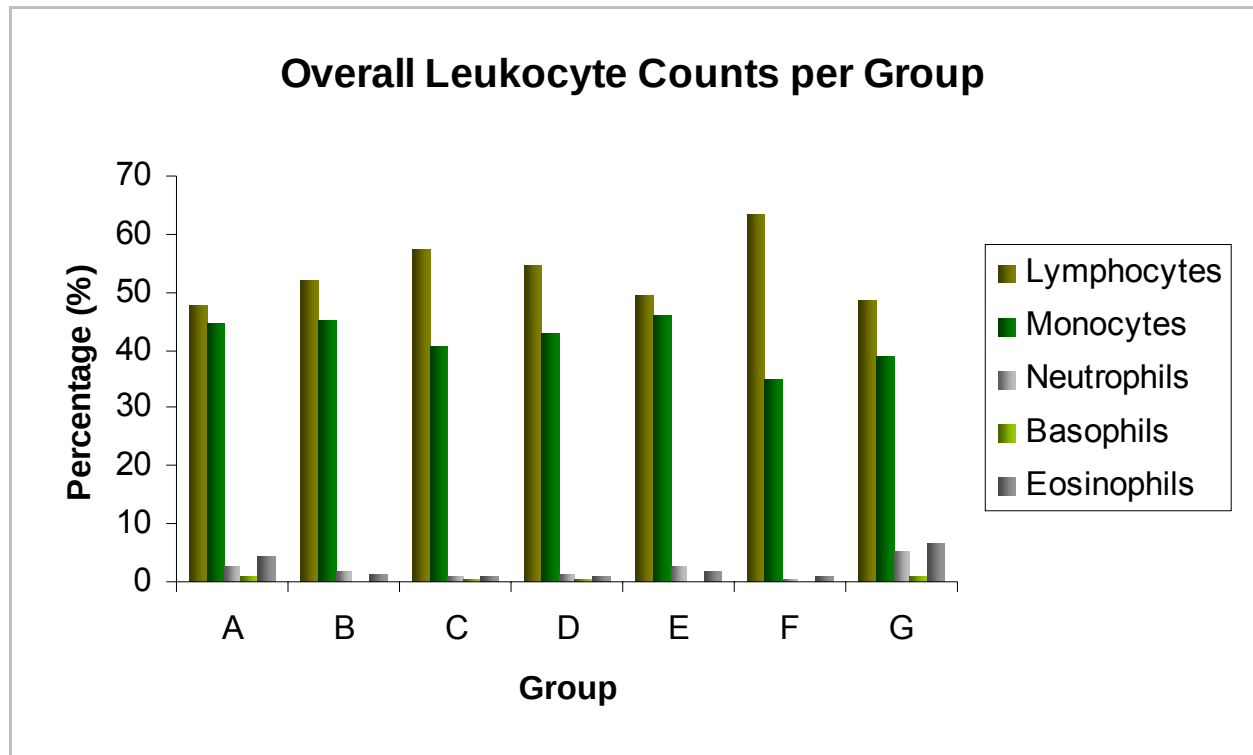


Figure 1: Mean leukocyte counts of five animals assessed per group A-G.

The analysis of each of the seven groups' monocyte, lymphocyte and neutrophil counts was performed through a one-way ANOVA, as all the necessary assumptions for these parametric tests were met. Analysis of eosinophil and basophil counts were conducted with the aid of the non-parametric Kruskal-Wallis one-way ANOVA, as the data considered did not assume a normal distribution. Table 2 expresses the values of these statistical tests run for leukocyte species as well as the outcomes thereof.

Table 2: Statistical comparisons performed on the various leukocyte species, derived from the blood of the assessed SJL/J and SWR/J mice under various experimental conditions

Leukocyte	Test	P value*	Inter-group Comparison
Monocytes	Parametric	0.961	No significant differences
Lymphocytes	Parametric	0.857	No significant differences
Eosinophils	Non-parametric	0.0104	Group G significantly higher than Groups B-F

Neutrophils	Parametric	0.0454	Group G significantly higher than Group F
Basophils	Non-parametric	0.0654	No significant differences

* Significance was set at a level of 0.05

Monocyte, lymphocyte and basophil counts displayed no significant differences ($P>0.05$) between any of the groups assessed. Eosinophil counts were significantly higher ($P<0.05$) in the baseline group G than all other experimental groups assessed except for the negative control SWR/J mice of group A. Neutrophil counts of the baseline termination group were significantly higher ($P<0.05$) only when compared to the combination group F.

Discussion

Granulocytes circulate in the blood unless recruited to act as effector cells at sites of infection and/or inflammation; whereas agranulocytes continuously circulate between the tissues and the bloodstream (*Janeway et al., 2005*).

Monocytes complete their differentiation in the tissues, where they are referred to as macrophages. Macrophages and neutrophils are of the innate immune system, they are essential for the control of common infections, and provide the first line of defence against many common microorganisms. Cells of the innate immune system play a crucial role in the initiation and direction of adaptive immune responses, as well as subsequent removal of pathogens that have been targeted by an adaptive immune response (*Janeway et al., 2005*).

Monocytes mature continuously into macrophages upon migration into the tissues and are most abundant in the connective tissues but continuously recirculate using the bloodstream as transport medium. Neutrophils are short-lived (only a few days) and produced in increased numbers during an immune response, when they leave the blood to migrate to sites of infection or inflammation. Neutrophils are linked to early inflammatory responses and often sensitised in self-antigen recognition characteristic to autoimmune disease (*Thomas et al., 2005*), a known complication in the SJL strain (*Vafiadaki et al., 2000*). The fact that the neutrophil counts were highest in the baseline group G is entirely expected when it is reasoned that neutrophils are highest in early innate immune responses to injury, as they are considered the first line of defence. Other leukocytes are recruited to the site of infection or injury later on in an immune response, and the neutrophil numbers drop as can be seen in groups B-F as the disease progresses.

Lymphocytes are of adaptive immunity, once differentiated, they are able to provide a more versatile means of defence than cells of innate immunity. This provides an increased protection against reinfection with the same pathogen. Later on in an inflammatory response, lymphocytes are also recruited to the sites of inflammation (*Janeway et al., 2005*). It is normal for lymphocytes to be abundant in circulation, as a large population of these are memory cells and thus key in prevention of reinfection down the line.

Eosinophils are thought to be important primarily in defence against parasitic infections. Very small numbers of eosinophils are normally present in the circulation; most are in fact found in the tissues, especially connective tissues. Few eosinophils are produced in the absence of infection or other immune stimulation; but upon stimulation, there is an increased production of these cells in the bone marrow and their release into the circulation. Eosinophil activation and degranulation is strictly regulated. Their synthesis of chemical mediators amplifies the immune response by activating epithelial cells and recruiting and activating more eosinophils and other leukocytes. In local allergic reactions, eosinophils accumulate in large numbers in tissues. Their continued presence, however, is characteristic of chronic allergic inflammation and is thought to be a major contributor to tissue damage (*Janeway et al., 2005*).

Eosinophil granules have previously been suggested to reduce inflammation caused by other leukocytes (*Leeson et al., 1988*). At onset of dysferlinopathy between four and six weeks of age, the increase in eosinophil counts could very likely be a compensation mechanism to decrease initial inflammation in the muscular tissues of the dysferlinopathic mice. However, in excess they cause tissue damage and counteract this proposition.

We presume that in this disease state, eosinophil numbers are very likely higher in tissue than in the bloodstream. With this assumption follows the reasoning that eosinophils may play a definitive role in the initial pathogenesis of dysferlinopathy. If they cause the main tissue damage at onset of the disease, and dysferlinopathic muscle is unable to repair the damage, it is logical that eosinophil counts will be down-regulated by the immune system to lower-than-normal numbers, as seen in groups B-F as compared to group A, to prevent further tissue damage which the inherently defective repair mechanism is unable to keep up with.

Basophils, like eosinophils, are present at the sites of inflammatory reactions. They are normally present in very low numbers in circulation and seem to have a similar role to that of eosinophils in the defence against pathogens, as they are also recruited to sites of allergic reactions.

They release substances that affect vascular permeability and are believed to protect mucosal surfaces against pathogens, as well as playing a definite role in orchestrating allergic responses (*Janeway et al., 2005*).

Conclusion

Although the anti-inflammatory properties of Coenzyme Q₁₀ and Resveratrol are not evident at the blood level, it is hoped that it will be prominent at the tissue level. Further microscopic investigation of dissected muscle tissue will follow to ascertain these results.

In the present study, eosinophils are proposed to play a definitive role in the pathogenesis of dysferlinopathy. In small numbers, eosinophils may well serve an anti-inflammatory purpose; but in the heightened numbers experienced in the baseline group G, it is proposed that they cause tissue damage. If they are the cells responsible for major tissue damage, secondary to initial damage by neutrophils, then a compensation mechanism inherent in muscle may well down-regulate their numbers to lower than normal levels in order to prevent further massive damage as dysferlinopathic muscle is indeed deficient in its repair mechanism.

Neutrophils are proposed to be largely responsible for the inflammation at onset of myopathy, as these are the primary cells of an inflammatory response. Neutrophils are additionally sensitised in the self-antigen recognition characteristic of autoimmune disease. Though this is a known complication in SJL mice, certain factors must be present to induce the experimental autoimmune encephalomyelitis (EAE) and these are not believed to have been present in our experimental procedures.

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