Chapter 3

A 90 day antioxidant supplementation trail in the SJL/J mouse model for dysferlinopathy

3.1 INTRODUCTION

An ideal animal model for a disease has been described as one in which all animals of a strain develops the disease spontaneously (Weller *et al.*, 1997). The natural induction process of the disease can be studied and an optimally reproducible environment exists in which to test treatments. Many of these models have been painstakingly developed; others have been discovered by chance (Weller *et al*., 1997). Considering that the SJL mouse has been widely used as a model for different human diseases, and for experimental research on muscle regeneration and transplantation for more than 40 years, it was not without irony, a decade ago, that the SJL mouse was found to have a genetically determined degenerative muscle disease of its own (Bittner *et al*., 1999). The spontaneous onset and 100% incidence of myopathy in the SJL/J mouse rendered this strain a useful model for idiopathic myopathy (Weller *et al*., 1997) and now also for dysferlinopathy.

3.2 ANIMAL MODELS IN SCIENTIFIC PRACTICE

A major focus of modern biological research is to elucidate the molecular and cellular mechanisms that underlie human health and disease. Over the last few decades, both naturally occurring and genetically engineered animal models of human diseases have been employed for evaluating new therapeutic options and for elucidating pathological pathways (Doran *et al*., 2007). Doran and co-workers, 2007, stated that biological studies of animal models in conjunction with findings from clinical investigations can be extremely helpful for the initial evaluation of alternative treatment strategies. Animal models further offer a valuable tool, as they provide crucial information on genetic manipulations via transgenic or gene knockout technology as well as in cell transplantation studies in the development of therapeutic approaches for addressing human disorders. A variety of animal species are currently being used for detailed cell biological, physiological and biochemical analysis of numerous human diseases. These models are also applied in essential toxicological investigations for the development and testing of potential side effects of treatment regimes (Doran *et al*., 2007).

A good animal model should ideally exhibit specific characteristics. These should include (Doran *et al*., 2007):

i. resemblance of the etiology of the human disease in complexity and severity, onset, and progression,

ii. development of most or all of the multifactorial aspects usually observed in end-stage human pathology,

iii. imitation of basic mechanisms of the human pathology and metabolism that are important for disease progression or drug treatment,

iv. demonstration of similar primary genetic abnormalities in the case of inherited disorders, *eg.* have a genetic mutation causing absence of the same protein absent in the human condition,

v. illustration of comparable susceptibility to infectious or pharmacological agents in the case of infectious or pharmacogenetic diseases,

vi. being suitable for cell based treatment strategies and genetic manipulations, and

vii. being of adequate size to facilitate physiological and surgical procedures or to yield sufficient amounts of biological samples for extended biochemical analyses.

It is also important to keep the limitations of animal experiments in mind. The extrapolation of animal data to the human situation is sometimes very difficult due to species-specific differences. Small rodents commonly used in biological research may exhibit fundamental physiological differences as compared to humans. This includes differences in development, cellular differentiation, potential for tissue regeneration, mechanisms of cell regeneration, immune responses, susceptibility to infections, and patterns of detoxification of xenobiotics, metabolism and bioenergetics (Doran *et al*. 2007). Variations in basic biological mechanisms such as the maintenance of ion homeostasis, cell signalling, hormonal regulation or metabolic integration may exist between cellular systems in animal models *versus* human patients. Differences in compensatory mechanisms or redundant pathways between different species may complicate the interpretation of results from animal experiments (Doran *et al*., 2007).

Another important consideration, of which the importance might often be overlooked in studies testing substances other than anticancer drugs, is the dose calculation. In the development and testing of therapeutic agents, whether pharmaceutical or nutraceutical in nature, it is essential to appropriately translate the drug dosage from one animal species to another (Reagan-Shaw *et al*.,

2008). Confusion and concern may emanate from incorrect dose translations across species. Direct translation of animal doses to human doses may result in misleading interpretations (Reagan-Shaw *et al*., 2008; Halliwell, 2009). The importance of determining the appropriate dose of anticancer agents has been emphasized by the fact that individuals might have varying abilities to metabolize and eliminate drugs. The same dose of anticancer agents might show different pharmacokinetics and pharmacodynamics in different individuals (Kouno *et al*., 2003). For drugs that produce therapeutic effects at doses far less than those that cause toxicity, the incentive for precise dosing is far less than for drugs with a narrower therapeutic index (Hahn, 2005). The narrow therapeutic index of most antineoplastic agents has provided great impetus to deliver doses as precisely as possible (Hahn, 2005). Halliwell, 2009, reasoned that one explanation for the lack of demonstrating the benefits of antioxidant therapy in humans might be the utilization of wrong doses. One of the practices embedded in dosing of antitumor drugs is dosing by body surface area (BSA). More commonly, milligrams per square meter (mg/m^2). Body surface area is reported to be equivalent to the two-dimensional surface area of the skin (Hahn, 2005). Drug ineffectiveness has been argued to be associated with inappropriate translation of drug doses from one animal species to another (Reagan-Shaw *et al*., 2008).

3.3 SJL/J Mice

3.3.1 Origin of the SJL/J Model

The SJL mouse model was developed by James Lambert at The Jackson Laboratory in 1955 from three different sources of Swiss Webster mice. Dr ED Murphy of the Jackson Laboratory, Bar Harbor, Maine, reported in 1962 the development of spontaneous lymphomas resembling Hodgkin's disease in an inbred strain of SJL/J mice (Murphy, 1963). In 1967, Yumoto and Dmochowski described the strain as follows: "The SJL/J strain is a new inbred strain, derived from non-inbred Swiss Webster stock by brother-to-sister matings for over 46 generations. Spontaneous lymphomas occur in over 90% of the mice at an average age of 13 months". Murphy also observed lesions which he described as 'of striking histologic similarity' to human Hodgkin's disease in the SJL/J strain (Murphy, 1963).

The SJL mouse strain has been reported to be susceptible to many induced autoimmune diseases such as experimental autoimmune encephalitis (EAE) and inflammatory muscle disease (Bernard and Carnegie, 1975; Rosenberg *et al*., 1987). In addition, skeletal muscle of the SJL mice has been shown to have an increased regenerative capacity (Grounds and McGeachie, 1989; Mitchell *et al*., 1992), and to demonstrate a spontaneous occurrence of what has been designated an 'inflammatory

myopathy, accompanied by loss of strength' (Hohlfeld *et al*., 1988; Weller *et al*., 1997). Only about a decade later, Bittner and co-workers, 1999, identified a reduction in dysferlin protein in SJL/J mice, consistent with the dysferlin reduction in dysferlinopathy patients with a missense mutation. This novel spontaneous mutation in the dysferlin gene in the SJL/J mice strain, results in a reduction of dysferlin to approximately 15% of levels detected in dysferlin-competent animals and human samples (Bittner *et al*., 1999). Therefore this model is ideal for research in the field of dysferlinopathy.

3.3.2 General Information on the SJL/J Strain

The SJL model has been found to display a very high incidence of reticulum cell sarcomas resembling Hodgkin's disease by approximately one year of age (www.jax.org/jaxmice). Sarcomas first appear in the Peyer's patches and mesenteric lymph nodes and later in the spleen, liver, thymus and other lymph nodes. Most of the tumors are mixed-cell types classified as type B reticulum cell neoplasms, but a few are type A histiocytomas. This strain is also characterized by extreme aggression in males and its susceptibility to experimental autoimmune encephalomyelitis (EAE) for multiple sclerosis research. The SJL/J strain develops a spontaneous myopathy resulting from a splice-site mutation in the dysferlin gene (Bittner *et al*., 1999). This *Dysf*^{jm} allele has been shown to result in decreased levels of dysferlin protein in SJL/J mice and makes this mouse a good model for the dysferlinopathies (www.jax.org/jaxmice).

The spontaneous myopathy is characterized by a progressive loss of muscle mass and strength corresponding with an increase in muscle pathology. Pathological changes include muscle fibers with central nuclei, size variation, splitting, inflammatory infiltrate, necrosis and eventual replacement of muscle fiber with fat (Weller *et al*., 1997; Bittner *et al*., 1999; Suzuki *et al*., 2005). While muscle weakness can be detected as early as three weeks of age, the greatest pathology occurs after 6 months of age (Bittner *et al*., 1999; Fox, 2007; www.jax.org/jaxmice). A 171 bp in-frame deletion in the encoded mRNA of the SJL/J mouse is predicted to remove 57 amino acids from the corresponding protein (www.jax.org/jaxmice). The region corresponds to most of the fourth C2 domain of the protein, and the deletion has been reported likely to results in instability of the protein (www.jax.org/jaxmice). The molecular basis for the mutation is the result of a splicing mutation in the gene, resulting from a deletion of a small tandem repeat (www.jax.org/jaxmice). While western blotting was found to show a reduction of dysferlin protein in SJL mice, microarray data was found to show an upregulation of dysferlin mRNA (Suzuki *et al*., 2005).

3.3.3 Justification of the Model for Dysferlinopathy Studies

Histopathological examinations of muscles in SJL mice of different ages and different sources (SJL/J, SJL/Olac) by Bittner and co-workers, 1999, disclosed features compatible with a progressive muscular dystrophy. These features included degenerative and regenerative changes of muscle fibers and a progressive fibrosis. Proximal muscle groups were found to be primarily affected by these changes, whereas the distal muscles remained less affected (Bittner *et al*., 1999). The first histological changes could be observed as early as three weeks of age. By seven months, the dystrophic changes included inflammatory foci, as well as the appearance of fatty and fibrotic tissue (Bittner *et al*., 1999). The team of Bittner associated morphological alterations with signs of slowly progressive muscle weakness, which was detected as early as three weeks after birth, when mice were suspended by their tails. Subsequent breeding experiments revealed this muscular dystrophy phenotype to be inherited as an autosomal recessive trait (Bittner *et al*., 1999). Following genetic analysis, it came to light that the mutation mapped to the region syntenic with chromosome 2p13 in humans; the region where the gene mutated in human dysferlinopathy has been mapped (Bashir *et al*., 1998; Liu *et al*., 1998; Bittner *et al*., 1999). Immunoblot analysis with the NCL-hamlet antibody, displayed a reduction of dysferlin-specific bands of muscle samples from different sources of SJL mice. The reduction corresponds to that found in humans with a missense mutation in the dysferlin gene. The team of Bittner suggested that this mouse will become a useful animal model for studying the pathomechanisms of dysferlin deficiency and, moreover, for applying therapeutic rescue strategies.

Since SJL descends from a wild derived strain of Swiss mice which are genetically distinct from the common laboratory mouse, there is not an appropriate control strain available (Von der Haag *et al*., 2005), Therefore the SWR/J strain were found most suitable to use as a negative control in the present study. The SWR inbred strain was developed by Clara J Lynch at the Rockefeller institute who obtained Swiss mice from A de Coulon of Lausanne, Switzerland and began inbreeding around 1929. The strain was transferred to Raymond Parker at the University of Toronto who supplied them to The Jackson Laboratory in 1947 at F28+. The SWR/J mice are widely used in research as a general purpose strain (www.jax.org/jaxmice).

3.3.4 Histological Characteristics of the SJL/J Model

Muscle disease has been found to develop spontaneously in every member of the SJL/J mouse strain (Weller *et al*., 1997). Weller and co-workers, 1997, described that the disease has a very predictable

course and onset, and can be divided into three distinct phases (Weller *et al*., 1997). In young adult SJL/J mice (age 2 to 4 months), muscle functions normally and pathologic abnormalities are minimal although present. At age 6 to 8 months, the active phase begins, and all mice develop extensive muscle necrosis accompanied by the presence of immune cells and myophagocytosis. Strength is decreased, although muscular atrophy is still not present. In the final, degenerative phase of the disease, at age 10 to 16 months, muscle necrosis continues, strength loss accelerates, and muscle mass is progressively lost. By 16 months of age, SJL/J mice have lost half of their young adult muscle mass and strength (Weller *et al*., 1997). Weller and co-workers found that by 6 months of age, muscle from most SJL/J mice exhibited active myopathy, defined as 10 or more necrotic fibers per quadriceps, containing mononuclear cells.

Between different groups of 6 month old SJL/J mice, the percentage of individual animals with active disease varied from 60% to 100%, averaging 78% of mice overall. The typical pattern consisted of scattered single involved fibers, and as many as 100 such fibers per quadriceps cross section were counted. Involved fibers consisted of relatively intact muscle fibers with mononuclear cells beginning to enter the membrane, necrotic fibers completely filled with cells, and fiber remnants still containing a few cells (Weller *et al*., 1997). An increase in muscle fiber size variation was also found and areas of more widespread endomysial involvement typical of later disease stages were sometimes present and reported to appear especially near perimysial fascia (Weller *et al*., 1997). Mild perivascular inflammation was commonly observed, although not found to be predominant, while the histological pattern of fiber involvement at the age of 6 months appeared to be identical to autoimmune myositis (EAM) in this mouse strain, and like EAM the inflammatory infiltrate consisted primarily of macrophages (Weller *et al*., 1997). This evidence provided a platform for decision making of the age at which animals in the present study should be entered into the program. Considering evidence of full disease onset in the SJL/J model at six months of age, and the belief that 90 days of antioxidant supplementation will be best to determine the cellular effect brought about by the supplementation, it was decided to enter 14 week-old SJL/J mice into a 90 day program.

3.3.5 Muscle Regenerative Capacity

The muscle of young SJL/J mice has been found to be capable of vigorous regeneration (Grounds and McGeachie, 1989; Mitchell *et al*., 1992). Two-month-old SJL/J muscle regenerates almost completely *in vivo* after a crush injury, whereas injured Balb/c muscle forms scar tissue and few myotubes (Grounds and McGeachie, 1989). Weller and co-workers, 1997, found that young SJL/J mice are also

stronger than young Balb/c mice, suggesting a correlation with the higher muscle regenerative capacity. The inference was made that faster and better myotube formation compensates for early muscle damage in young mice, but eventually falls behind as more and more muscle fibers are destroyed (Weller *et al*., 1997). Mitchell and co-workers, 1992, found the formation of new myotubes in SJL/J muscle after crush injury to be associated with efficient clearance of muscle debris by macrophages. These cells were later reported to play the major clearance role in spontaneous myopathy in this strain (Weller *et al*., 1997). The superior regeneration of SJL/J muscles were found to be due to a faster and more extensive inflammatory response combined with a greater capacity for myotube formation (Mitchell *et al*., 1992). Intrinsic muscle factors were suggested to play a central role rather than the genotype of the bone marrow-derived macrophages (Mitchell *et al*., 1995).

3.4 The Animal Study

3.4.1 Design and Layout

This chapter provides a detailed outline of the animal study and study design that preceded the outcome of the research objectives concerned in the present study.

Sixty female animals with a mean weight of ≈20g were imported from the Jackson Laboratory, Bar Harbor, USA for the present study. An import permit was obtained from the Department of Veterinary Services, Ministry of Agriculture, and the conditions stipulated therein were strictly adhered to. Sample size was determined in collaboration with a biostatistician (Medical Research Council (MRC), South Africa), following convention in ANOVA studies that error degrees of freedom be at least 30. Ten of these animals were of the SWR/J strain and served as the negative control in the study, while the other 50 animals were SJL/J mice. On the date of shipment the SWR/J mice were nine weeks old and the SJL/J mice were eight weeks old. Forty days were allowed for acclimatization before commencement of the study.

Animals were housed at the laboratory animal facility of the University of Pretoria's Biomedical Research Centre (UPBRC) at Onderstepoort (Pretoria, South Africa). The barrier unit was free of all major pathogens as serology, bacteriology and parasitology tests, performed around the same time as the present study, all conformed to the facility's quality assurance policy. Animals were maintained in the facility's barrier unit in Tecniplast IVC cages (Figure 3.1), (Eurostandard type II L) with individual air supply, equipped with a pre-filter and HEPA filter system.

Sterile Agrebe basic 55cm^2, cotton sheet laboratory animal cloth bedding (for boxes Type II/III Ll) were used as bedding and changed once weekly. The main air supply system consisted of a 50% Fresh Air Primary filter and a Secondary Bag filter with 10 to 15 air changes per hour. A constant temperature of 23 to 24°C, a relative humidity of 40 to 60%, and a 12-hour dark-light cycle were maintained. The animals were allowed free access to JL Rat and Mouse 6F-IRRAD food (PMI Nutrition International, LLC) and water *ad libitum*.

Figure 3.1: Tecniplast IVC cage with individual air supply (top removed, lying on the left).

When a decrease and slowing in the mobility of the animals was observed, as a result of disease progression, it was decided to supplement the animals' diet by addition of Nestlé Cerelac sterile baby cereal on the floor of each cage, from day 50 until termination. The food supplementation did not contain any of the two products tested in the present study and were not expected to influence the results. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee.

On day 0 of the 90 day trial, the animals were marked by means of earclipping according to the German identification system for laboratory animals. Accurate identification is of utmost importance when a large number of animals are used and housed collectively in order to avoid replicate procedures in the same animal. Identification is also important for the documentation of clinical observations and condition assessment of the animals, as each animal was assessed individually, and observations documented for each individual. At the age of 14 weeks and 15 weeks for SJL/J and SWR/J mice, respectively, the animals entered a 90 day experimental study. On this day, six SJL/J animals were terminated (as discussed in termination procedures below), for comparison of age-related changes with muscle tissue of 27 week-old SJL/J mice. The six animals were all 14 weeks of age, and represented the age control group.

The 54 animals that entered the study on day one of the 90 day trial were divided into the following groups, and caged communally in group order (Table 3.1). Ten SWR/J mice received a placebo for 90 days in the same volume as the antioxidants tested in the study, and represented the negative

control group. Eight SJL/J mice received a placebo for 90 days in the same volume as the antioxidants tested in the study, and represented the positive control group. Nine SJL/J mice received 60mg/kg resveratrol per day, and represented the resveratrol group. Nine SJL/J mice received 40mg/kg CoQ10 per day, and represented the low CoQ10 group. Nine SJL/J mice received 120mg/kg CoQ10 per day, and represented the high CoQ10 group, and nine SJL/J mice received a combination of 40mg/kg CoQ10 plus 60mg/kg resveratrol per day, simultaneously administered by oral dosing, and represented the resveratrol/CoQ10 combination group. Oral dosing was performed for all substances in all groups, using a 1000µl syringe with a mouse oral gavage needle nr. 20*g*. All animals were weighed on day one, and thereafter twice weekly.

Table 3.1 Summary of subject classification in group order

Group	Strain	Treatment	Concentration (mg/kg/day)	Age at Termination (in weeks)
Negative control	SWR/J	None	-	**28**
Positive control	SJL/J	None	-	**27**
Resveratrol	SJL/J	**Resveratrol**	60	**27**
CoQ10 (low)	SJL/J	**Low CoQ10**	40	**27**
CoQ10 (high)	SJL/J	**High CoQ10**	120	**27**
Resveratrol/CoQ10 combination	SJL/J	**Resveratrol/CoQ10**	60/40	**27**
Age control	SJL/J	None	-	**14**

Doses were calculated according to the BSA normalization method (Formula 3.1) for more appropriate conversion of drug doses from animal studies to human studies (Reagan-Shaw *et al*., 2008). Body surface area correlates well with parameters of mammalian biology, which makes BSA normalization logical for allometric scaling of drug doses between species, given that the activity of most drugs corresponds to the relationship between the drug and some physiological processes or functions (Reagan-Shaw *et al*., 2008). It was therefore decided to use the formula for dose translation based on BSA (formula 3.1), suggested by Reagan-Shaw and co-workers, 2008, to calculate the appropriate doses of CoQ10 and resveratrol to be administered in mice.

Formula 3.1: Dose translation based on BSA

$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \times \frac{\text{Animal } K_m}{\text{Human } K_m}$$

Where,

HED	Human equivalent dose in mg per kg
K_m	The K_m factor, body weight (kg) divided by BSA (m^2), is used to convert the mg/kg dose used in a study to an mg/m^2 dose
Animal K_m	3 (mouse)
Human K_m	37 (human weighing 60kg)

3.4.2 Dose Calculation, Dose Preparation, Supplements, and Solvents

The motivation behind the use of the BSA normalization method was that it has been justified as a reliable method for accurate dose translation from animal to human studies (Reagan-Shaw *et al*., 2008). In order to obtain doses high enough to provide a reliable indication of the antioxidant effectiveness in mice, comparable to a predicted effect in humans, BSA-based dose calculation was found to be the most appropriate method and has been reported to be far superior to the simple conversion based on body weight only (Reagan-Shaw *et al*., 2008).

To convert the dose used in a mouse to a dose based on surface area for humans, formula 3.1 multiply the mouse dose in mg/kg by the K_m factor for a mouse (3), divided by the K_m factor for a human (37). This calculation results in a human equivalent dose for a 60 kg person (Reagan-Shaw *et al*., 2008). The body weight approach has historically been the most common general approach to scaling in toxicology, particularly in regulatory toxicology testing, and the 'ideal' weight used in the BSA-based calculations (60kg), was presumably adopted from this system. There are several ways to perform a scaling operation on a body weight basis; the most often employed being to simply calculate a conversion factor (K). A difficulty with this approach is that the body weights of any population of animals or people change throughout life, and even a common age will present considerable variation. Custom is therefore, to use an 'ideal person' (70kg for men and 50kg for women, now set as a 'standard' of 60kg) or 'ideal' human weight (for which there is considerable less consensus) (Gad, 2006).

For the present study, it was decided to test two different concentrations of CoQ10, one relatively low, and the other three times higher, and one concentration of resveratrol. Two hundred mg per

day, as human physiological equivalent level, was decided on as the lower dose for CoQ10, and therefore the higher dose of 600mg/day. Resveratrol was administered at 300mg per day, as the human physiological equivalent level. Therefore, in order to obtain the animal equivalent dose (AED), formula 3.2 was derived from formula 3.1:

Formula 3.2: Dose translation from human to mouse doses

$$\text{AED (mg/kg)} = \text{HED (mg/kg)} \times \frac{K_m \text{ (human)}}{K_m \text{ (mouse)}}$$

Where,

AED	Animal equivalent dose in mg per kg
HED	Human equivalent dose in mg per kg
K_m	The K_m factor, body weight (kg) divided by BSA (m^2), is used to convert the mg/kg dose used in a study to an mg/m^2 dose.
Mouse K_m	3
Human K_m	37 (human weighing 60kg)

The concentrations incorporated in the study resulted in the following derived AEDs:

i. resveratrol group - 6omg/kg of resveratrol per day

ii. low CoQ10 group - 40mg/kg of CoQ10 per day

iii. high CoQ10 group - 120mg/kg of CoQ10 per day,

iv. resveratrol/CoQ10 combination group - 60mg/kg resveratrol plus 40mg/kg CoQ10 per day.

All supplement preparations were made up fresh every week in milliQ water, homogenized by ultrasonic treatment (as was done by Kettawan and co-workers, 2007), and stored at room temperature.

Resveratrol was obtained in a commercially available gel capsule at 50mg units, 98% pure, prepared from *Polygonum cuspidatum* (manufactured for CHM/RD Pty Ltd, and distributed by Blueskygreenearth Herbs Pty Ltd, Australia). The capsules were emptied, and powder was dissolved in appropriate volumes of milliQ water to obtain the desired concentration. No heat was added, the solution was activated by mild shaking. CoQ10 was obtained in the commercially available Q-Gel® formulation at 100mg per unit, in a softgel capsule. Each capsule contained 150 international units (IU) natural vitamin E (as d-alpha tocopheryl acetateUSP/NF) and 100mg CoQ10 (ubidecarenone

USP) (manufactured for Nutraceutical Sciences Institute; distributed by vitacost.com, USA). After addition of the CoQ10 to milliQ water, the container was placed in a waterbath at 60°C for 10 to 20 seconds and then removed to be mildly shaken. The procedure was repeated two to three times, until the solution was homogenous. In order to allow adaption to the strength of the antioxidants, half of the dose of each separate preparation was administered, however, the volume was maintained. These doses were administered for the first 14 days of the 90 day study, and thereafter, full doses were administered until the end of the study.

3.4.3 Routine Procedures and Observations During the Course of the 90 day Study

3.4.3.1 Dosing

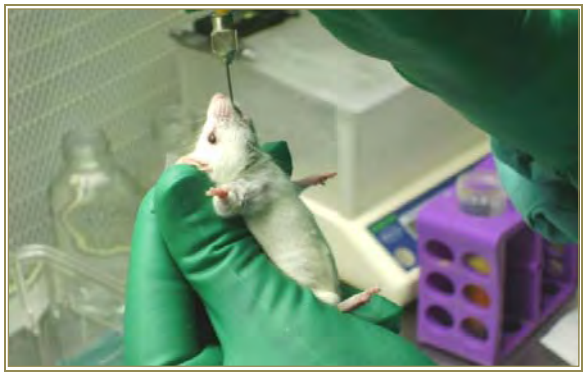

Figure 3.2: Oral dosing.

Administration (Figure 3.2) of specific concentrations was done at constant volume (200µl) (Figure 3.3) over all the groups, at different concentrations, once per day by oral dosing, for 90 days. The two control groups, (positive and negative), received 200µl of milliQ water as placebo.

Administration of supplements in all the groups was done in a single oral dose once daily. The same veterinary technologist performed the dosing procedure every day for the duration of the trial.

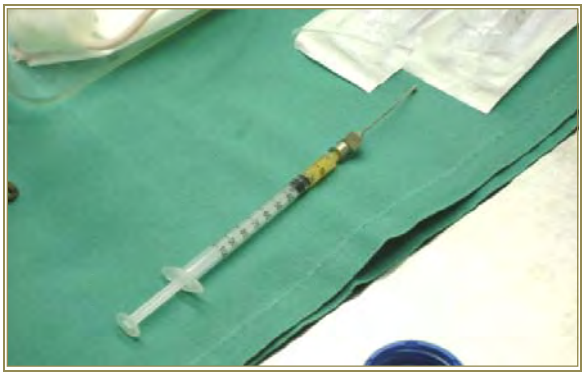

Figure 3.3: Syringe used for oral dosing at 200µl.

3.4.3.2 Weighing

Weights were measured twice weekly, mainly as indicative factor for overall well-being. Each animal was removed from the cage in a flow cabinet and placed on a balance. The animals were handled with care and allowed to adapt to the different environment (Figure 3.4). As soon as the animal sat still on the middle of the balance, the weight value in grams was recorded. Statistical analyses were done on weights recorded on the following days of the 90 day trial: day 1, as baseline weight; day 52, after additional supplementation with Nestlé Cerelac baby cereal was started; day 59, when definite weight gain in all groups was documented by the veterinary technologist; days 66, 73 and 80, as the days on which tensile strength was measured; day 90 as the last day of the 90 day trial. Statistic results are presented in section 4.3.1.

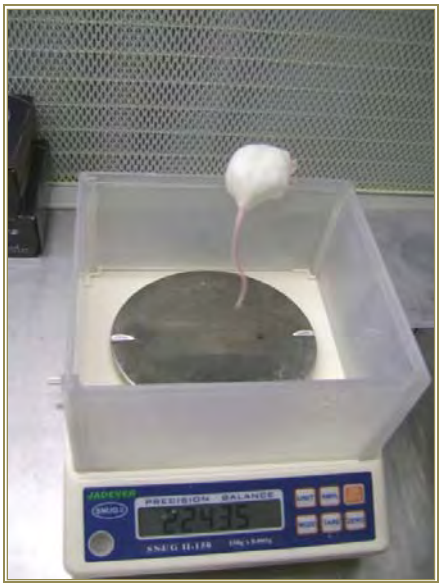

Figure 3.4: Weighing of an SJL/J mouse.

3.4.3.3 Tensile Strength Test

From week 7 to week 11, of the 90 day trail, the animals were assessed for total strength, by measuring the tensile strength on a custom built tensile strength meter (Figure 3.5). The tensile strength meter consisted of a load cell (from Emfuleni Scales, Vanderbijlpark, South Africa), allowing tensile force to be measured in grams. The system uses a smooth metal grid (custom made) (grid size 15 x 15 mm, wire diameter 2 mm) supported by a t-bar that is attached to the load cell at a 0° angle. The grid served as platform for the animal to grasp with the paws, while being pulled by the tail slowly backwards in the horizontal plane. The force applied to the bar the moment the grasp is released, represented the peak tension measured in grams. The load cell was connected to a

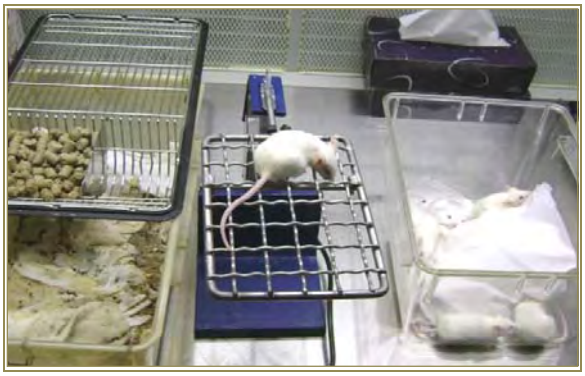

Figure 3.5: The tensile strength test meter.

computer with Digicator software which provided a peak hold function with an mv-RS 232 converter, that served as the sensor, recording the peak tensile strength afforded by each individual animal.

Maurissen and co-workers, 2003, recommended that grip performance data be collected using consistent techniques and with a thorough understanding of how the equipment of the test system functions. To ensure consistency in the present study, the procedure was done by the same veterinary technologist every time. The technologist was informed on the operational aspects and functions of system components upon installation of the tensile strength test meter. Calibration of the system was done upon installation, and every six months thereafter. For evaluation purposes every animal was taken from the cage, individually and put on the metal grid, then left to adapt (Figure 3.5).

As soon as the animal orientated itself in the correct position (tail orientated towards the direction in which the load cell affords a positive value), it was pulled slowly and steadily backwards by the tail, until the animal released the grasp (Figure 3.6).

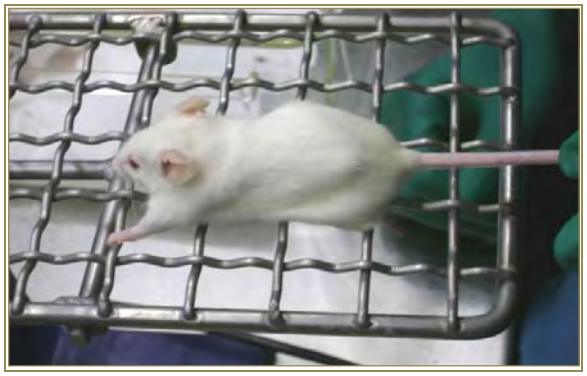

Figure 3.6: Tensile strength test.

The value of strength in grams was recorded at the exact time point where the grasp broke. Animals were never forced to grasp the bars of the metal grid, nor were they forced to align in the position for strength measurement. For each animal the test procedure was repeated thrice, and values were recorded manually as displayed by the Digicator. The highest value of the three repetitions was recorded as the animal's grip strength for that session. And the final value was calculated as the mean strongest grip strength over the 5 week period for every group. Mice were not trained prior to testing and each mouse was tested once (3 consecutive times). If an animal showed no cooperation, by premature releasing of its grip or by walking backwards on the metal grid, as soon as force was exerted to the animal by pulling the tail, the animal was put back in the cage. No further assessments were attempted for that specific animal again on that specific day, in order to avoid any unnecessary exhaustion and stressing. Animals were allowed to grasp with all four paws simultaneously. The tensile strength meter was cleaned every week before and after every tensile strength test procedure, using 70% ethanol and

sterile tissue paper. The sensor was zeroed before and after every individual measurement taken. After each group was tested, the animals were assessed and scored for behaviour and activity.

3.4.3.4 Food Supplementation

As the animals served the imperative part of this study, and also in other research of this nature, apart from standard ethical consideration, all necessary measures were taken to ensure the comfort and maintenance of condition of all animals at all times. Therefore, when the veterinary technologist observed a decrease and slowing in the mobility of the animals (recorded on day 48), as a result of disease progression, it was expected that the animals might find it difficult to reach for their food, that was provided in the cage top (Figures 3.1 and 3.5). Condition assessment in the preceding two weeks revealed nothing other than progressive weakness, in addition to lethargy in some animals. Fighting between cage mates were often observed in this time and it appeared as if the animals were stressed. It was decided to supplement the animals' diet from day 50 of the 90 day trial by addition of Nestlé Cerelac, sterile baby cereal on the floor of each cage.

3.4.3.5 Observations

Routine observations were made every day at the same time, before and after oral dosing. Since the study did not include any invasive procedures, no pain or suffering was expected in the animals. However, due to the nature of the animal model itself, it was possible that episodes of discomfort could present. A pain score sheet was set up to evaluate the pain of the animals in order to ensure humane treatment at all times. If the pain score appeared to be altered above acceptable levels, euthanasia by cervical dislocation would have been performed, followed by an autopsy to determine the exact origin and cause of the detected condition. Scoring for diarrhoea was done on the following scale: 0 = normal; 1 = loose faeces on floor; 2 = pools of faeces on floor; 3 = running out on handling; +m = mucus; b = blood (in stools). The overall condition of animals were examined by assessing each individual daily for activity, scratching, anxiety, pinched face, ruffled coat, nose bleed, bite marks and diarrhoea. Overall condition were scored with limits being 1 = emaciated, and 4 = normal. A summary of key events during the course of the 90 day trial are provided in Table 3.2.

3.4.4 Findings, Difficulties and Limitations

All essential obligations for ensuring the well-being (freedom from discomfort, distress, and pain) were met at all times. With the exception of a few difficulties and the regretful, but unavoidable loss of four animals during the course of the animal study, all went according to plan, resulting in the

successful collection of the tissue of interest following termination. At termination all animals were still in a good condition.

One event that needed attention, was the resveratrol preparation used at the beginning of the study. The supplement was obtained in a commercially available vegetable capsules at 200mg *Polygonum cuspidatum* per unit, providing 50% (100mg) resveratrol (manufactured for Solgar Vitamin and Herb, UK, Herts). The product was a plant extraction preparation. After dissolving the capsules in milliQ water, the solution had a dark brown colour and a strong herbal smell. The animals treated with this preparation, showed resistance as aggression was observed in the concerned groups. On the 27th day nose bleeds were observed in these groups, mainly after dosing. On day 29 nose bleeds started as the veterinary technologist opened the container with the resveratrol before dosing, indicating a possible fear for the taste of the antioxidant. A new 98% pure formulation of resveratrol (a chemical preparation) was obtained and prepared in milliQ water. The colour of the preparation was milky-white, and it was tasteless and odourless. The behaviour of the animals changed to normal after administration of the new preparation, and no nose bleeds related to dosing were reported in these groups again.

The most common side-effects for CoQ10 has been reported to only involve the gastrointestinal system and included nausea, diarrhoea, appetite suppression, heartburn and epigastric discomfort (Rozen *et al*., 2002). In large studies the incidence of gastrointestinal side-effects is less than 1% (Rozen *et al*., 2002). No known side effects have been reported for resveratrol. To provide the animals with comfort at all times, it was decided to start the study with half the original doses, to prevent any gastrointestinal instability. Even at this concentration, diarrhoea was noticed in the groups treated with CoQ10. Time was allowed for the animals to adapt, and following toleration of the half concentrations, full doses were administered. No diarrhoea was reported after this change, and all the animals seemed to have tolerated the higher doses well. After day 9, no incidence of diarrhoea was reported for the CoQ10 group again.

Another common incident was fighting between cage mates. The fighting occurred in all antioxidant treated groups, and it became apparent that it was more frequent after the tensile strength test was performed. The strain of the procedure resulted in severe irritation and tension of the animals a short while after the procedure was performed. This resulted in cage mates biting each other. The reason why female animals were used in the present study is because they can be caged communally, while males are too aggressive. From the observations made in the present study, it

seems that although female SJL/J animals are more mildly tempered than what has been reported for males, they fight as a result of tension caused during a test procedure.

All four animals that died during the study were sent to the Golden Vetpath Laboratory (Pretoria, South Africa) for autopsy. Morphological diagnosis in animal 8 was found to be 'aspiration pneumonia'. The pathologist found the mouse to be in good body condition. The abdomen appeared distended and opening of the abdominal cavity, revealed severe gas accumulation within the intestine leading to the presence of dilated gas-filled intestinal loop. The finding was likely to be due to anorexia before death, since the stomach also did not contain notable amounts of food. The lungs appeared mildly congested with multifocal dark red areas that had a congested appearance with increased consistency. The areas were present all along the edges of the lobes and represented areas of suspected pneumonia. Mild sinusoidal congestion and leukostasis were found in liver histology. The most notable lesion was the multifocal necrogranulomatous bronchopneumonia.

In animal 33, moderate pulmonary congestion, with protein-rich lung edema was detected. Moderate congestion and blood pooling in hepatic sinusoids, with no presence of inflammation were found. Moderate autolysis in trachea and oesophagus were detected with thymic lymphoid tissue found to be well-populated with lymphocytic cells.

In animal 34, moderate congestion with blood pooling in the larger blood vessels as well as the capillary walls of the lungs were found. Mild extramedullary haemopoiesis in the spleen, but normal lymphoid tissue in the splenic white pulp was reported. Moderate congestion and blood pooling was observed in the liver, although no inflammation was detected.

In animal 42, histological evaluation was indicative of acute aspiration pneumonia with a purulent infiltrate affecting both lung lobes. No other specific lesions were observed. The carcass was found to be in good condition, the intestinal tract was gas-dilated and the stomach contained food. Moderate lymphoid hyperplasia was detected in histological assessment of the spleen. No findings in any of the autopsies could be related to any of the procedures or the antioxidant treatments used in the study.

Table 3.2 Important events and observations during the 90 day trail

Event	Day	Remark
Mice arrived from Jackson Laboratory, Bar Harbor, USA. Period of acclimatization started.	40 days before day 1	
Mice - earclipped and weighed.	*0*	
Termination of the age control group (at 14 weeks of age) First day of dosing. All doses are administered at half the concentration.	*1*	Half dose concentrations to allow adaptation.
The high CoQ10 and resveratrol/CoQ10 combination groups had diarrhoea; mucus present in faeces, faeces has an orange tint.	*3*	Faeces stick to bedding and to the side of the cage. No blood could be detected in the faeces.
Animals in all antioxidant supplemented groups drink more water than animals in the positive and negative control groups.	*4*	More water consumption (30–50 ml more) observed in antioxidant supplemented groups.
In the high CoQ10 and resveratrol/CoQ10 combination groups very soft faeces with a yellow-orange tint was observed.	*5*	Assessment for hydration, body weight, and body condition found animals to be in good condition.
In the high CoQ10 and resveratrol/CoQ10 combination groups – faeces were still soft, with yellow–orange tint	*6*	Animals maintain body condition. A slight decrease in activity was found in the concerned groups.
Faeces in both the high CoQ10 and resveratrol/CoQ10 combination groups were more formed, with the presence of a yellow-orange tint	*7*	Animals' bodies start to get use to the concentration of the antioxidants.
Faeces were more formed in the high CoQ10 and resveratrol/CoQ10 combination groups.	*8*	Overall condition of the concerned groups appeared improved. Animals' activity increased.
All groups had normal faeces.	*9*	
Animal 50 was not well; had an injury around anus; and seemed unsteady; weighed 19.97g	*12*	No weight loss in animal 50. No sign of dehydration detected.
Animal 42 died. Weight = 19.82g.	*14*	A 0.77g weight loss recorded for animal 42. Carcass sent for autopsy.
Full doses were administered in the resveratrol and low CoQ10 groups.	*15*	All animals displayed overall good condition.
Animal 30 had a swollen right eye. Resveratrol group presented with diarrhoea.	*16*	No dehydration or weight loss could be detected in any of the animals.
Animal 34 presented with a bitemark on abdomen; ±2mm upper right from vagina.	*17*	
Animal 30 still had a sore right eye. The eye was flushed with luke-warm water.	*19*	
Full doses were administered in the high CoQ10 and resveratrol/CoQ10 combination groups.	*22*	
Nose bleeds were observed in the resveratrol and resveratrol/CoQ10 combination groups. Animals 26, 27, 28, 30 & 52 presented with nose bleeds directly after dosing. Fighting was observed in the low CoQ10 group.	*27*	
Animals 33 & 34 were not well. They presented with pilo-erection and a hunched posture.	*28*	
Animals 33 & 34 were found dead in the cage. Resveratrol group-nose bleeds started directly before dosing. Resveratrol/CoQ10 combination group – only one nose bleed.	*29*	It is clear that the animals were being stressed by the dosing with resveratrol. The preparation smells and tastes bad. Carcasses sent for autopsy.
Started dosing a new preparation of resveratrol, prepared from a 98% pure form that was tasteless and odourless. Half doses of resveratrol were administered.	*30*	
New formulation of resveratrol was found to be handled well. No nose bleeds reported.	*31*	It is clear that the taste and smell of the previous resveratrol preparation caused severe stress in these groups.

Event	Day	Remark
Fighting occurred in the resveratrol and high CoQ10 groups.	32	
New resveratrol formulation administered at FULL doses.	36	Half doses of the new formulation of resveratrol seemed to be well-tolerated by all animals. Progress to full doses.
Fighting occurred in the resveratrol and resveratrol/CoQ10 combination groups.	38	
Animal 8 was not well. Presented with pilo-erection and a hunched posture. Erratic breathing was present.	39	
Animal 8 has recovered. Mice in the low CoQ10 group seemed to be fighting.	40	
Fighting amongst individuals in the low CoQ10 group continued.	41	
Animal 8 died. Animal 32 were badly bitten on hind-quarters.	42	Carcass of animal 8 sent for autopsy.
Animal 32 had a nose bleed.	43	
Animal 1 was slightly unstable after oral dosing.	44	
Progressive weakening over the past two weeks was noted in all SJL/J mice groups. Almost as if the animals drag their hind quarters. Animals appeared much more lethargic.	48	SJL/J mice were 21 weeks of age. Lethargy serves as an important clinical indicator of illness. In this case onset of muscular dystrophy. Food supplementation is considered.
Supplementation of all groups with Cerelac baby food on cage floors.	50	
First tensile strength test were performed in all groups. In all antioxidant supplemented groups, animals were fighting after tensile strength test procedures.	52	Aggression was noted amongst individuals of treated groups after tensile strength test was performed.
2nd Tensile strength test were performed. Less fighting in all the groups that were previously fighting. All groups showed weight gain.	59	
3rd Tensile strength test were performed. Fighting occurred in all antioxidant supplemented groups after testing.	66	Aggression was noted again after tensile strength test amongst individuals of treated groups.
4th Tensile strength test were performed. Fighting occurred in all antioxidant supplemented groups after testing.	73	
Final Tensile strength test were performed.	80	Final tensile strength test, in order to avoid possible influence with CK and LDH levels, as well as possible muscle injury.
Animals 32 & 38 were fighting. Bitemarks were present on their backs.	81	
Resveratrol group – mice seemed to look 'scruffy' (ruffled coat).	84	No sign of dehydration or body weight loss. Animals were closely monitored.
Resveratrol group – mice still looked unwell (ruffled coat). Animals 22 & 30 had heavy nose bleeds.	85	
Animal 30 had a nose bleed. Mice in the high CoQ10 group seemed to be fighting.	88	
Mice 43, 45 and 48 were fighting.	89	
Mice in the high CoQ10 group still seemed to be fighting. SWR/J mice were 28 weeks old. SJL/J mice were 27 weeks old.	90	
Termination	91	

3.4.5 HEALTH GUIDELINES

Health guidelines as described by Foltz and Ullman-Cullere, 1999 were followed and animals presenting with any of the obvious health problems were also assessed for the other. Subtle health problems (Foltz and Ullman-Cullere, 1999) that animals were examined for on a daily basis include:

i) *Activity/behaviour*: When opening a cage the behaviour of the mice was examined. Were the animals curious (demonstrating normal open-field activity), did any seem thin, runted, lethargic, or in pain (especially in comparison to cage mates)? Were any of the mice hunched up or having difficulty breathing? Were they grooming, over-grooming, scratching, licking or mutilating themselves? All these criteria were used as indications for closer examination and evaluation.

ii) *Dehydration*: Dehydrated mice will have eyes that appear recessed in their heads. Facial fur will appear fuzzier due to pilo-erection. Once one lifts the mouse's skin over the shoulder blades, it will not return quickly to its original shape, and will remain bunched. Euthanasia was indicated if any of the animals were positive for dehydration.

iii) *Diarrhoea*: mice with diarrhoea often don't have fluid faeces, but faeces might be moist and sticky. Bedding and sides of the cages were investigated daily. Faeces and the area around the anus were examined for the presence of blood in case of the presence of moist and sticky faeces. Diligent assessment of hydration, body weight and body condition was performed on all animals of a cage where diarrhoea was diagnosed.

Additional health observations (Foltz and Ullman-Cullere, 1999) were made under the following guidelines:

i) *Lethargy*: a lethargic mouse may exhibit sluggish behaviour, stupor, coma, hypoactivity, prostration, or a hunched posture. These were considered important clinical indicators of a serious illness. Supportive care or euthanasia was indicated.

ii) *Ruffled fur*: an unkempt and ungroomed appearance is indicative of a mouse that is not feeling well. If noticed along with other signs, such as dehydration or loss of body condition, this was considered an indication for euthanasia.

These guidelines were strictly adhered to during the course of the study and found to be very helpful in decision making in order to ensure that the well-being of all animals were maintained at all times. Fortunately, no incidence called for early intervention (euthanasia) in the present study and animals could be maintained optimally until termination.

3.5 TERMINATION PROCEDURES

The termination procedures (Figure 3.7) took place on day 91, the day after the 90 day trial ended. At this point animals from the SJL/J strain were 27 weeks old and animals from the SWR/J strain were 28 weeks old.

The animals were anaesthetized by isofluorane inhalation, and terminated by cardiac puncture (Figure 3.7 A). Blood was obtained from the cardiac puncture and blood smears were made, three microscopic slides for each individual animal. Blood was collected in 1ml Microtainer serum tubes (Figure 3.7 B), and transported within 2 hours after termination to the laboratory (Department of Companion Animal Clinical Studies, Section of Clinical Pathology, Faculty of Veterinary Science), for analysis of serum CK and LDH levels.

Quadriceps and gastrocnemius muscles (from the right limbs) were isolated by the laboratory veterinarian and washed once in phosphate buffered saline (PBS), and thereafter immediately snap frozen in liquid nitrogen (Figure 3.7 C), before the samples were stored in 2 ml cryotubes, in liquid nitrogen, until analysis.

Quadriceps and gastrocnemius muscles from the left limbs were isolated and samples (Figure 3.7 D) appropriately sized for chemical fixation (Figure 3.7 E) were dissected from the mid-belly region of the muscle. Three different samples from each muscle, approximately 2 x 3 mm in size, were isolated. From each animal, one sample was fixed in a 2.5% formaldehyde, 0.075 M phosphate buffered solution for light microscopy work, the other two samples for electron microscopy work were fixed in a 2.5% glutaraldehyde/2.5% formaldehyde, 0.075 M phosphate buffered solution. Chemical fixation was done overnight.

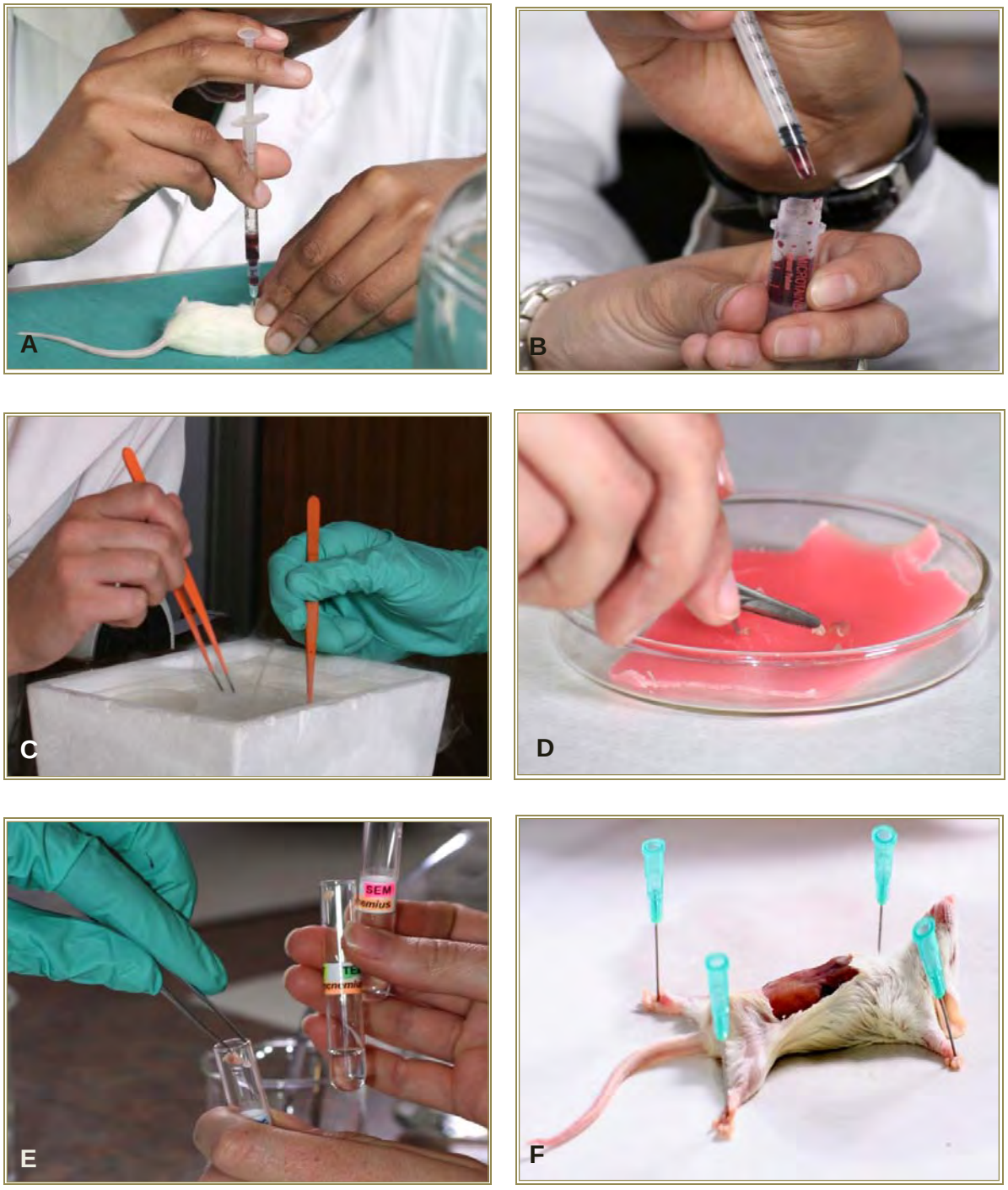

Figure 3.7: The termination procedure. A) Blood are collected from cardiac puncture; B) in a serum tube; C) Muscle tissue are snap-frozen in liquid nitrogen; D) Tissue sampling for microscopic analysis; E) Tissue collection in glass vials containing fixative; F) Mouse prior to dissection.

Chapter 4

Measuring non-specific parameters not directly related to muscle cell structure, and not directly affecting histopathology in dysferlin-deficient muscular dystrophy

4.1 INTRODUCTION

Weights and physical strength

The body weight of the mouse is a measure of muscle mass as well as of overall well-being (Li *et al*., 2005).It is usually monitored weekly or monthly throughout chronic experiments as an index of general health (Grounds *et al*., 2008). A weight loss of 10 to 15% within a few days or an overall weight loss of 20% is a decisive factor for euthanasia (Foltz and Ullman-Cullere, 1999).

Strength-assessing methods such as the length of time an animal can hang from a cage top or swim in a tank, are not very quantitative and can be very hard on the animal (Weller *et al*., 1997). Force transduction strength testing is a non-invasive procedure that can be used to test an animal frequently without great distress (Weller *et al*., 1997). It is a behavioural test that was eventually introduced into regulatory test batteries to screen for neurobehavioral toxicity (Maurissen *et al*., 2003). This extremely quantitative method allows for accurate assessment of treatment (Weller *et al*., 1997). Typically, a grip strength apparatus consists of a grasping device or platform that is connected to a strain gauge or load cell. In general, the test measurement is conducted by allowing the animal to grasp the device and then pulling it away until its grip is broken (Maurissen *et al*., 2003). Maurissen and coworkers, 2003, identified factors that can affect grip strength testing. These factors include parametric factors, peripheral sensory nervous system damage and diet restriction-induced changes in body weight and muscle mass. Changes in grip performance due to loss of body weight has been found to be reversible and positively correlate with changes in hind limb muscle mass (Maurissen *et al*., 2003).

Blood enzymes

The amount of ATP within the muscle fiber at any one time is sufficient for only about eight twitches (Silverthorn, 2004). As a backup energy source, muscles contain phosphocreatine. The high-energy phosphate bonds in this molecule are created from creatine and ATP when muscles are at rest (Silverthorn, 2004). When muscles become active, the high-energy phosphate group of

phosphocreatine is transferred to ADP, creating more ATP to power the muscles (Silverthorn, 2004). The enzyme responsible for transferring the phosphate group is creatine kinase (CK), also known as creatine phosphokinase (CPK) (Silverthorn, 2004). Muscle cells contain large amounts of this enzyme. Consequently, elevated blood levels of CK usually indicate damage to skeletal or cardiac muscle (Silverthorn, 2004).

Miyoshi, 1967, reported four patients from two consanguineous families that presented with recessively inherited late-onset distal myopathy associated with clear-cut muscular dystrophy and significantly elevated CK levels. Elevated CK levels are not specific in themselves (Urtizberea *et al*., 2008). In early stages of dysferlinopathy, they are usually markedly elevated, up to 50 to 100 times over normal values (Urtizberea *et al*., 2008). In the context of a patient presenting with distal motor symptoms, this massive increase is quite suggestive of Miyoshi myopathy (MM) and therefore of dysferlinopathy. Over time, CK levels tend to decline and lose their informative value (Urtizberea *et al*., 2008).

In a study by Nemoto and co-workers, 2007, serum CK levels of SJL/J mice were found to increase along with exacerbation of the inflammatory changes, when compared to B10 mice. In younger SJL/J mice (animals were approximately 14 weeks old) CK values were found to be already elevated (mean CK was 522.0 ± 219.1 IU), though there were a few inflammatory changes. As early as 1960, activities of the enzymes CK and lactic dehydrogenase (LDH) were found to be elevated in the blood of patients with Duchenne's muscular dystrophy (DMD) (Thomson *et al*., 1960; Cohen and Morgan, 1976). Reductions in increased levels of these enzymes had been used in earlier studies as an indicator of the efficacy of experimental drug treatments in DMD (Cohen *et al*., 1976; Chazot *et al*., 1972). Massively elevated serum levels of muscle enzymes, including CK, LDH, and aldolase, have also been reported in early stage dysferlinopathy (reviewed by Urtizberea *et al*., 2008).

Haematology

One of the most important functions of blood is transport of humoral agents and cells of the immune system that protect the body from pathogenic agents, foreign proteins, and transformed cells (Ross *et al*., 2003). Blood cells and their derivatives include: red blood cells (erythrocytes), white blood cells (leukocytes), and platelets (Ross *et al*., 2003). Leukocytes are subclassified into two general groups: granulocytes, including neutrophils, eosinophils, and basophils, and agranulocytes, including, lymphocytes and monocytes (Leeson *et al*., 1988; Ross *et al*., 2003). De Gruchy expressed a conviction of most haematologists when he stated that the examination of the blood smear is 'the most important single investigation in the anaemic patient' (De Gruchy, 1965). Hattersley and

Ragusa, 1965, added that the skilful examination of a well-prepared blood smear is one of the most important examinations in non-anaemic patients.

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*). Leukocytes are functional only to a small extent in the bloodstream, their greatest activity being exhibited in the tissues (Leeson *et al.,* 1988). These cells 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. Increased demand for particular leukocytes in various sites is reflected by increased numbers in the circulation. The absolute and differential white cell count is therefore a useful pointer to diagnosis (Young *et al*., 2006).

The antioxidant properties of CoQ10 and resveratrol are of interest to the present study as this chapter aim to determine whether or not its administration will have any effect on non-specific parameters in SJL/J mice. Non-specific parameters are regarded as factors not directly related to muscle cell structure, and not directly affecting histopathology in dysferlin-deficient muscular dystrophy. These parameters include body weight, physical strength, the levels of inflammatory leukocytes at the haematopoietic level, and CK and LDH levels in blood. A quantitative analysis was performed on white blood cell counts, blood CK and LDH levels, as well as the weight change and tensile strength of animals.

4.2 Materials and Methods

4.2.1 Weights and Tensile Strength

The weight of each individual animal was recorded twice weekly, as described in section 3.4.3.2 throughout the course of the study. The average weights of the assessed mice, for each experimental group, are presented in Figure 4.1. The repeated weight measurements of each SJL/J mouse were compared between the six experimental groups via repeated measures ANOVA. A level of significance ($P \leq 0.05$) was utilized during the statistical analysis conducted. For physical strength analysis, the tensile strength of each individual animal was measured, as described in section 3.4.3.3, once per week for weeks 7 to 11 of the 90 day trial. For each mouse, the five tensile strength measurements acquired were utilized to determine the change in physical strength over time relative to the animals' starting physical strength, measured on day 52. The relative physical strengths, for each of the four occasions, were then compared separately between the six groups, (positive control, negative control, resveratrol, low CoQ10, high CoQ10 and resveratrol/CoQ10

combination groups) with the utilization of one-way ANOVA. A level of significance ($P \leq 0.05$) was utilized during the statistical analysis conducted. The recorded weights and tensile strengths of each SJL/J mouse measured during the course of the trial were assessed together, to determine if a relationship existed between the two factors. The assessment was conducted, for each group of mice over a 5 week period, via the calculation of the coefficient of determination.

4.2.2 Haematological Analysis

Three blood smears were prepared 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 with a Nikon Optiphot transmitted light microscope equipped with a Nikon digital camera DXM 1200F, for each of five animals within all the groups under study (positive control, negative control, age control, resveratrol, low CoQ10, high CoQ10 and resveratrol/CoQ10 combination groups). The purpose of the evaluation was to manually quantify each cell of the leukocyte species (Figure 4.8); namely lymphocytes, monocytes, neutrophils, basophils and eosinophils; counting up to 100 leukocytes per slide in a total of three zones. Each slide was analyzed until 100 white blood cells were observed. Each white blood cell 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.

4.2.3 Laboratory Tests

Blood samples, drawn at termination from each mouse, were evaluated for their serum CK and LDH levels. Analysis of serum CK and LDH levels were performed by Department of Companion Animal Clinical Studies, Section of Clinical Pathology, Faculty of Veterinary Science, University of Pretoria. Blood was collected in 1ml Microtainer serum tubes at termination and transported within 2 hours after termination to the laboratory. For determination of CK levels, the laboratory used a modification of the optimised standard method according to the recommendations of the IFCC. The Alfa Wassermann Creatine Kinase Reagent is intended for the quantitative determination of the Creatine Kinase activity in serum and plasma using the Alfa Wassermann Clinical Chemistry systems (ACE® and the NExCT™).

Principle of the Procedure:

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{CK} \text{Creatine} + \text{ATP}$$

$$\text{Glucose} + \text{ATP} \xrightarrow{HK} \text{Glucose-6-P} + \text{ADP}$$

$$\text{Glucose-6-P} + \text{NADP}^{+} \xrightarrow{\text{G-6-PDH}} \text{Gluconate-6-P} + \text{NADPH} + \text{H}^{+}$$

The reaction rate is measured at 340 nm.

For determinations of LDH levels the laboratory used a modification of the optimised standard method according to the recommendations of the Deutsche Gesellschaft Fur Klinische Chemie. The Alfa Wassermann Lactate Dehydrogenase Reagent is intended for the quantitative determination of Lactate Dehydrogenase (LDH) activity in serum and plasma using the Alfa Wassermann Clinical Chemistry systems (ACE® & NExCT™).

Principle of the Procedure: Pyruvate + NADH + H^+ ^{LDH} L-Lactate + NAD^+

The rate of the reaction is measured at 340 nm.

The levels of CK and LDH were expressed as units per liter (U/l). The quantified serum CK and LDH levels were compared between the six experimental groups via a one-way ANOVA and a non-parametric Kruskal-Wallis one-way ANOVA, respectively. A level of significance ($P \leq 0.05$) was utilized during the statistical analysis. The statistical program, NCSS, was utilized in order to perform statistical analysis.

4.3 Results and Discussion

Information regarding animal age at termination, and dosages of antioxidants supplemented in the respective groups, were summarized in Table 3.1.

4.3.1 Body Weight

Table 4.1 expresses the days chosen for weight assessment as well as the reason for its utilization. Through the use of the Anderson-Darling test for normality the data within each group was found to assume a normal distribution, allowing for the utilization of the parametric repeated measures ANOVA test. The repeated measures ANOVA test revealed that there was no statically significant difference between weights of the assessed groups over the entire study ($P = 0.74$) (Figure 4.1), although, there was a significant difference found to exist between the recorded weights on each of the assessed days (Table 4.1) within all the groups ($P < 0.00001$) (Figure 4.2). The subsequent use of the Tukey-Kramer Multiple-Comparison Test revealed that general weight alteration patterns existed within each of the groups. Weights increased significantly from day 1 to day 90 (Figure 4.2), although the weights recorded on days 66, 73 and 80 were all smaller than that of day 90 and larger than that of day 1. They were not significantly different from each other.

Table 4.1 Days chosen for weight assessment and the reason for its utilization

Day	Reason
1	Baseline weight; beginning of 90 day trial.
52	Food supplementation with Cerelac baby cereal. 1st tensile strength test.
59	Definite weight gain recorded for all the groups. 2nd tensile strength test.
66	3rd tensile strength test.
73	4th tensile strength test.
80	5th tensile strength test.
90	End-point weight. Last day of the study.

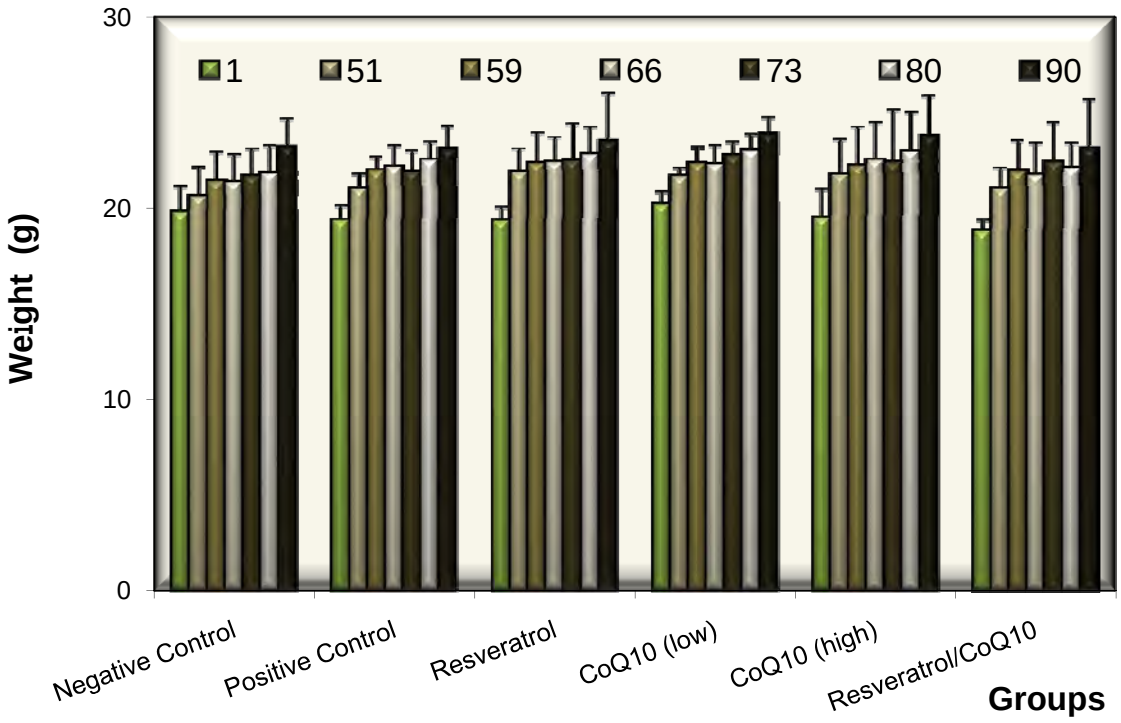

Figure 4.1: Average weights of experimental groups on specific dates as stipulated in Table 4.1 with error bars representing the standard deviation (SD).

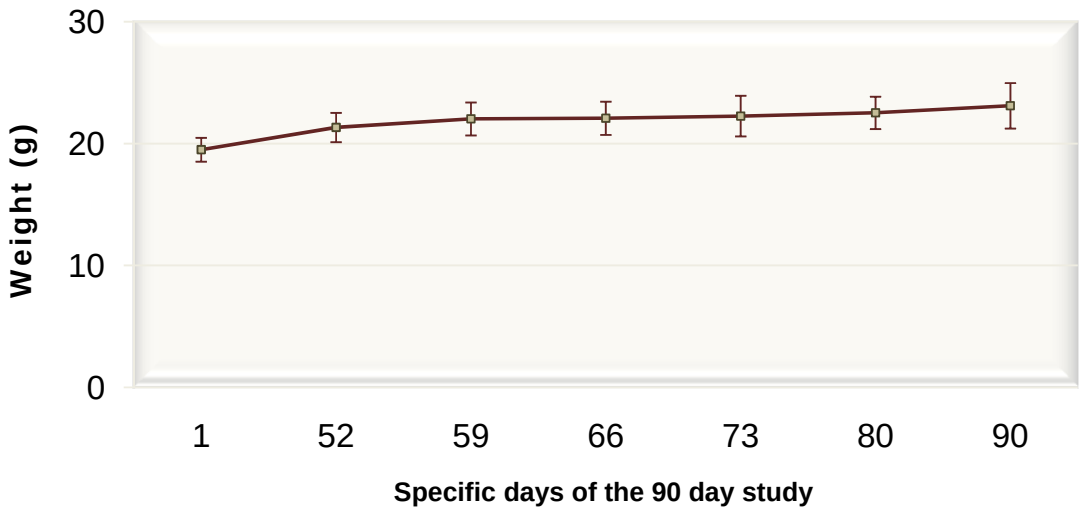

Figure 4.2: Animal weights increased significantly (P < 0.00001) in all groups from day 1 to day 90 with error bars representing the standard deviation (SD).

In establishing criteria for assessing health and endpoint determinations, a common descriptive ground for monitoring criteria were met in collaboration with the veterinary staff of the UPBRC. When an animal has lost 20% or more of its body weight, an experimental endpoint would be reached, allowing for early intervention, in this case euthanasia by cervical dislocation. The weight loss can be sudden or over a few days, while closely monitoring the condition of the concerned animal. If an animal presented with deteriorating condition in the form of ruffled coat, dehydration, excessive diarrhoea, decreased food or water consumption, and decreased activity, the weight of such an animal was closely monitored every day before dosing.

Weight change was used as the main indicative factor of the overall condition and well-being of the SJL/J and SWR/J mice in the present study. Food was initially provided in a container on the cage top (Figure 4.5, B) where mice had to reach on their hind limbs to feed. As weakness set in, nutritional health of the animals became a matter for concern. Supplementary food was provided from day 50 of the 90 day trial, until termination, on cage floors to ensure that the animals were well-fed. It was therefore expected that the added nutrition would result in a weight gain, as was recorded in all groups (Figure 4.2), 9 days after supplementation started.

Weights differ significantly between day 1 and day 90 in all assessed groups (P < 0.00001) (Figure 4.1), but no significant differences were found between groups (P = 0.74) (Figure 4.2). The significant increase in weights between day 1 and day 90 could be attributed to the supplementary food provided.

4.3.2 Physical Strength

The muscle tensile strength of each of the groups was measured on 5 separate occasions during week 7 to 11 of the 90-day trial. Up to three measurements were performed, if possible, on each occasion, with the largest of these utilized in subsequent statistical analysis as it was considered to be the maximum muscle tensile strength for that individual mouse at that particular point in time. Through the use of Shapiro-Wilk W test for normality the data within each group was found to assume a normal distribution, thus allowing for the utilization of the parametric repeated measures ANOVA test. The repeated measures ANOVA test revealed that there was no statically significant difference between the assessed days for the various groups (P = 0.89) (Figure 4.3) although over the entire 5 week period there was a statically significant difference between certain groups (Figure 4.4) in terms of their tensile strengths (P = 0.0036).

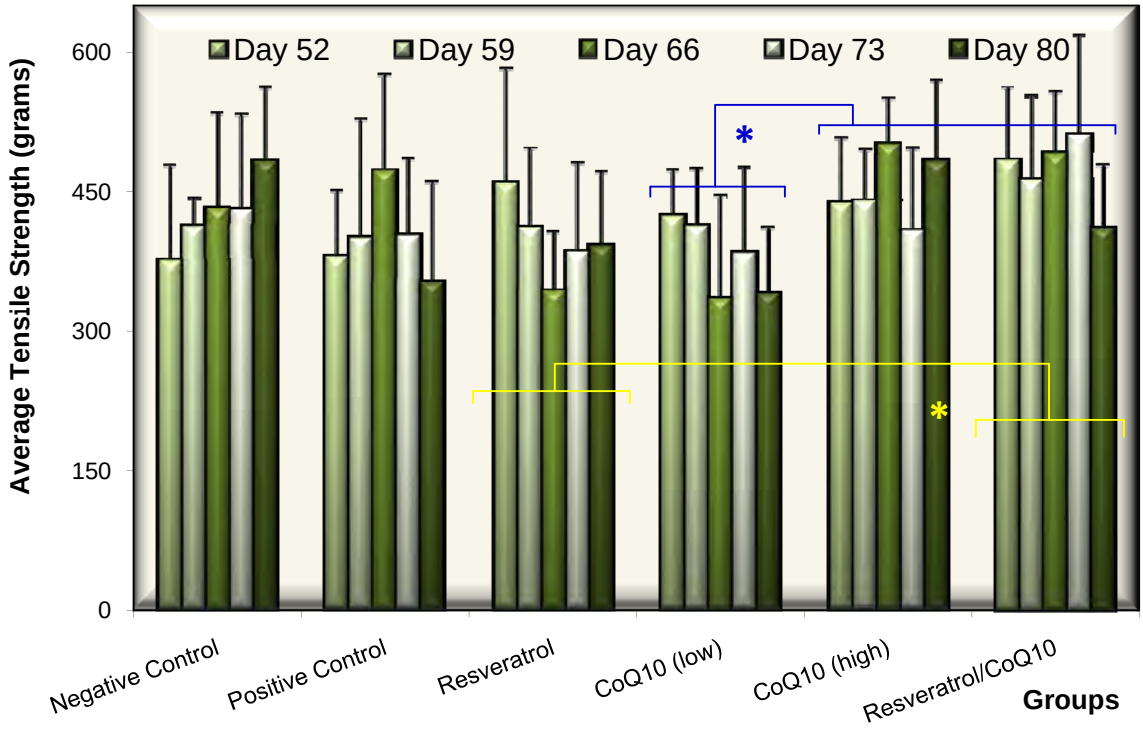

Figure 4.3: Average tensile strength of mice for five weeks in the 90-day trial. There was no statically significant difference between the assessed days for the various groups (P = 0.89). Standard deviation (SD) is represented by the error bars.

The use of the Tukey-Kramer Multiple-Comparison Test revealed that the low CoQ10 group (379.3 ± 40.6g) was physically significantly weaker than both the high CoQ10 (454.1 ± 37.1g) and resveratrol/CoQ10 combination (472.1 ± 38.3g) groups. The resveratrol group (398.8 ± 42g) was

significantly weaker than the resveratrol/CoQ10 combination group. The positive control group (402.13 ± 44.4g) displayed slightly higher values than both the low CoQ10 and the resveratrol groups, although not significant. The negative control group (427.47 ± 38.8g) displayed greater physical strength than what was observed in the positive control, low CoQ10 and resveratrol groups, although lower than that of the high CoQ10 and resveratrol/CoQ10 combination groups. Weller and co-workers, 1997, found that young SJL/J mice are also stronger than young Balb/c mice.

Within each experimental group no relationship could be determined between the assessed factors of weight and tensile strength when assessment was conducted for each group via the calculation of the coefficient of determination (r^2). It can therefore be concluded that these two factors appear to have no association, at least statistically, with each other. (Negative control r^2 = 0.0403; Positive control r^2 = 0.0304; Reveratrol r^2 = 0.0710; Low CoQ10 r^2 = 0.0356; High CoQ10 r^2 = $2x10^{-7}$; CoQ10 + Resveratrol r^2 = 0.0705).

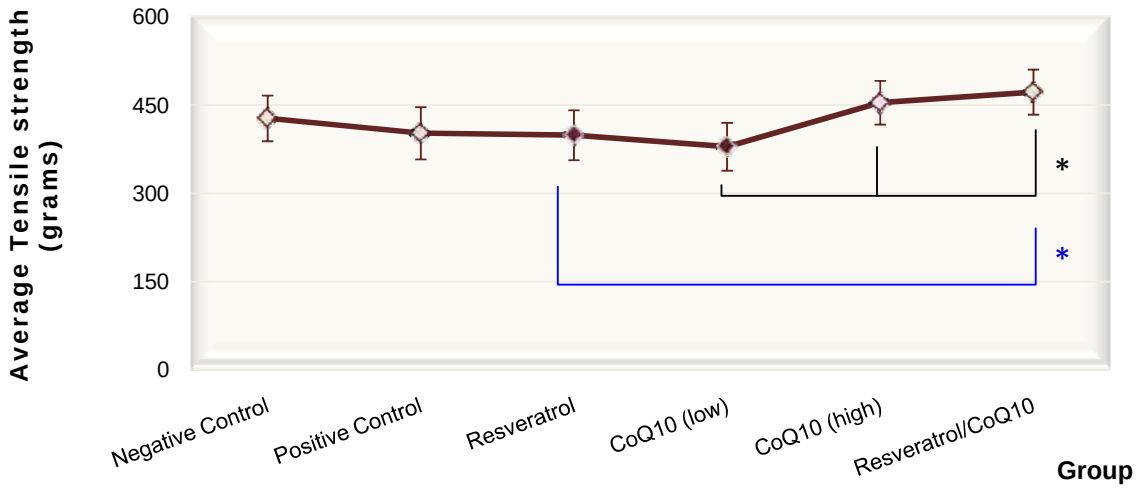

Figure 4.4: The trend in average tensile strength of experimental groups over the 5 week period, following supplementation with antioxidants. Statically significant differences (*) between certain groups in terms of their tensile strengths (P = 0.0036) occurred. The average strength of the low CoQ10 group was found to be significantly smaller than the high CoQ10 and resveratrol/CoQ10 combination groups. The resveratrol group was only found to be significantly smaller than the resveratrol/CoQ10 combination group. Error bars represent standard deviation (SD).

Strength loss can be due to multiple factors. Weller and co-workers, 1997 reported that the loss of strength can be attributed to the normal aging process. Weller's team found that this loss of strength appeared to be accounted for by loss of muscle mass relative to body weight, indicating a correlation between body weight and strength. Interpretation of grip performance data must critically consider

the fact that factors such as loss of body weight, changes in sensory function, and other non-motor (operational, systemic and behavioural) changes contribute to the overall grip performance measurement (Maurissen *et al*., 2003). It was expected that the supplementation of the animals' diet in the present study with Nestlé Cerelac sterile baby cereal, and the weight gain recorded as a result thereof, could have an effect on the strength measured. Calculation of the coefficient of determination showed that no association between these two parameters could be detected in the present study.

In SJL/J mice, Weller and co-workers found that strength in SJL/J mice does not decline steadily but in distinct drops which are correlated in time to specific pathological events. An early strength drop (at 6 and 8 months) was suggested to be attributed to the active process of early muscle disease, and is the only strength loss that could not be accounted for by loss of muscle tissue (Weller *et al*., 1997). One factor that could have contributed to the results obtained was the fact that animals from all groups in the present study showed adaptive behaviour (Figure 4.5), a non-motor change that may influence the grip performance test (Maurissen *et al*., 2003). Instead of pulling the grid bars with their paws, when force was exerted at the tail end, in the horizontal plane away from the grid, these animals started walking backwards. Sometimes they even turned around, to avoid the demanding task. Although this behaviour was observed in all groups in the present study, it is likely that some animals who showed adaptive behaviour found the task to hold on to the bar, until their grip was broken, too strenuous. Therefore an animal could release the grip prematurely and turned around. There was no way an animal would be forced to grip the grid bars, and usually if this behaviour occurred with the first pull, it continued, and for that specific day, that animal did not provide any data.

After the tensile strength test was performed, aggression and fighting occurred amongst individuals in most of the groups (chapter 3, table 3.1). Therefore, it was of the essence to cause the least amount of strain and distress on the animals during the procedure. Animals were not subjected to gripping more than three times on a given day, as this presumably might have been harmful to their stress levels. No strength testing was done in the last week before termination in order to rule out any possible muscle injury or trauma.

The results from the tensile strength test suggest that high dose antioxidant supplementation offered preservation of physical strength in 6 month-old SJL/J mice, to levels slightly higher than that measured in healthy SWR/J mice of the same age. These strengths were not dependent on the weight gain recorded in these groups.

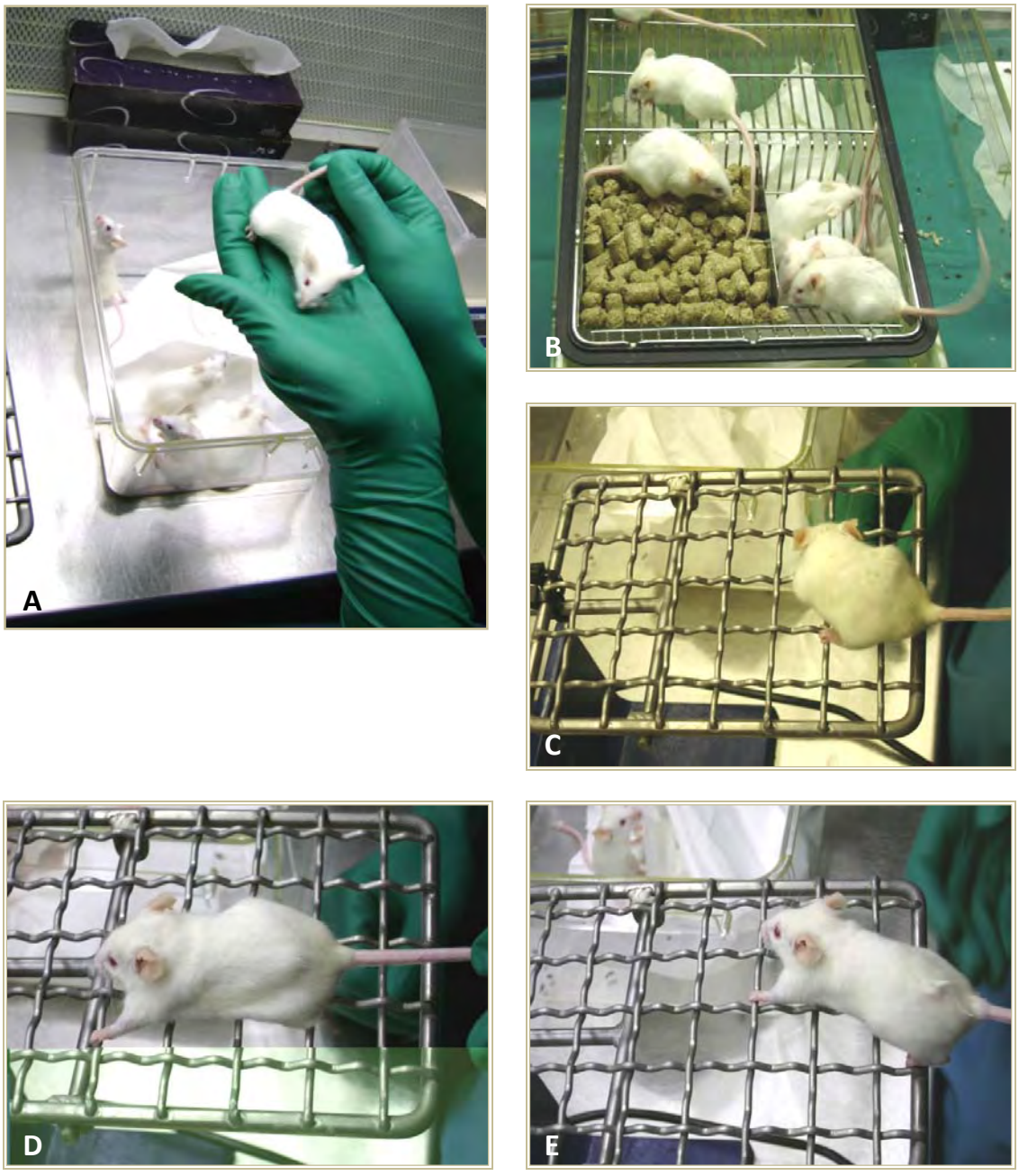

Figure 4.5: The condition of every animal was monitored before (A) and after (B) every tensile strength test. C) An animal whose grip broke, just after performing the tensile strength test. D) An animal with a firm grip during tensile strength testing. E) An animal showing adaptive behaviour. The animal prematurely released its grip every time force was exerted to the tail, and orientates its body in a side-ways direction on the grid.

4.3.3 Blood Enzyme Levels (CK & LDH)

As equal sample size existed in each of the experimental groups considered, as seen from the variance of the groups, it was considered to be equal for both enzymes. For the analysis of derived CK values a parametric one-way ANOVA could be utilized, as the random and independent data possessed a normal distribution. The results of this parametric test are presented in Table 4.2. The parametric one-way ANOVA test could only be utilized for the CK results, as the random and independent LDH observations did not meet a normal distribution pattern. In the case of the LDH analysis a non-parametric equivalent, the Kruskal-Wallis one-way ANOVA was run instead and the result is presented in Table 4.2. Both the CK and LDH levels were not significantly different between the six different groups, where significance was set at $P \leq 0.05$. The CK and LDH values of SJL/J mice at the age of 27 weeks (≈190 days) in the present study are given in table 4.3, as represented in Figure 4.6.

Table 4.2 Statistical comparison serum CK and LDH levels of the six experimental groups

Statistical Test	Enzyme	P value
One-Way ANOVA	**CK**	0.41
Kruskal-Wallis One-Way ANOVA	**LDH**	0.27

P-value = level of significance

Table 4.3 Mean CK and LDH levels ± standard deviation (SD), and standard error (SE) for CK and LDH data

Group	CK (U/l 37°C) ± SD	SE (CK)	LDH (U/l 37°C) ± SD	SE (LDH)
Negative control (n = 6)	5228.83 ± 4434.88	***2134.66***	2425.00 ± 1017.16	***990.00***
Positive control (n = 6)	1700.67 ± 1382.78	***694.30***	2000.83 ± 533.82	***816.84***
Resveratrol (n = 6)	2346.00 ± 2053.55	***957.75***	2220.17 ± 787.86	***906.38***
Low CoQ10 (n = 6)	2171.50 ± 2433.60	***886.51***	1972.00 ± 661.51	***805.07***
High CoQ10 (n = 6)	2308.67 ± 2467.74	***942.51***	2343.33 ± 682.76	***956.66***
Resveratrol/CoQ10 combination (n = 6)	3208.83 ± 2421.47	***1310.00***	3957.17 ± 2430.73	***1615.51***

SE = SD/√*n*, and *n* represent the sample size within the group

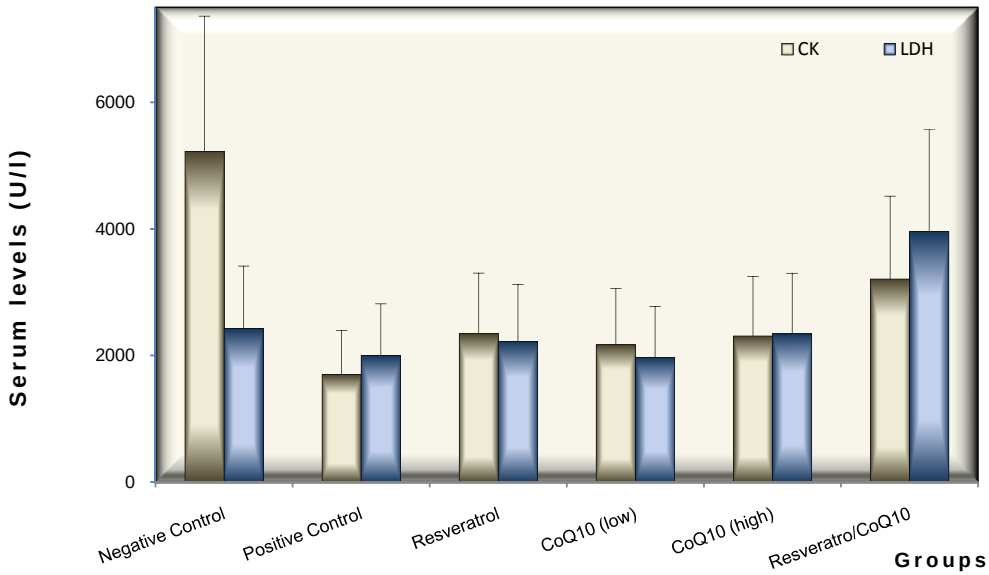

Figure 4.6: Mean serum CK and LDH levels (U/l 37°C) with error bars representing standard error (SE).

Nemoto and coworkers found an age related increase in CK levels of SJL/J mice. The current study revealed an increase in CK levels in all the groups treated with antioxidant therapy, when compared to the untreated positive control group. Also, all the CK values of the present study in SJL/J mice, treated and untreated, were found to be lower than that of the negative control SWR/J mice, in which values were not expected to be elevated. None of the differences between the groups, revealed any statistical significance for either CK or LDH level analysis. The LDH assay has previously been reported to be less satisfactory as a measure of blood muscle enzyme levels than CK. The reason being, any haemolysis that occurred during the preparation of the samples can cause elevated LDH (Morgan *et al*., 1981).

The results of laboratory CK and LDH levels from the present study displayed abnormally high values obtained in the negative control groups for both CK and LDH assays. The very large difference between the data points and their means are emphasized by the abnormally high SD's. To formulate an inference from the CK and LDH data, SE bars were utilized, to get an indication of the region where the mean of the whole possible set of results or the whole population can be expected to lie (Figure 4.6; Table 4.2). Standard error provides a measure of how variable the mean will be, if the study is repeated many times (Cumming *et al*., 2007). Even subsequent to calculation of the SE, the data points were found to be not constant enough to provide a reliable representation of the region where the mean could be expected for the whole possible set of results. Because of the very high serum CK value displayed by the negative control, no pattern of the elevation trend could be related

to the disease condition, or the antioxidant supplementation. Similar to the CK values, the serum LDH data was considered rather contentious and not reliably indicative of a significant tendency. The sample size and single assay procedures in the present study can be reasoned as cause for the inconclusive results. With a small sample size, any outliers in such a population will influence a mean to an extent where the results can be found inconclusive.

4.3.4 Differential White Blood Cell Count

Between each of the seven groups the comparison of monocyte-, lymphocyte- and neutrophil-counts were facilitated via one-way ANOVA, as all the necessary assumptions for these parametric tests were met. The comparisons of eosinophil and basophil counts from the seven groups 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 4.4 expresses the statistical tests run for each white blood cell species and the outcomes thereof where significance was set at $p \leq 0.05$.

Table 4.4 Statistical comparison performed upon the various leukocyte cell species derived from the blood of the assessed groups

Leukocyte species	Test utilized	P value	Difference between compared groups determined, if applicable, with the aid of Tukey-Kramer Multiple-Comparison Test's
Monocytes	One-way ANOVA	0.961	No significant difference existed between any group's monocyte counts.
Lymphocyte	One-way ANOVA	0.857	No significant difference existed between any group's lymphocyte counts.
Eosinophil	Kruskal-Wallis one-way ANOVA	0.0104	The eosinophil count for the age control group was significantly higher than that of all the other groups assessed except for the negative control group.
Neutrophil	One-way ANOVA	0.0454	The neutrophil count for the age control group was significantly higher than that of the resveratrol/CoQ10 combination group.
Basophil	Kruskal-Wallis one-way ANOVA	0.0654	No significant difference existed between any group's basophil counts.

Table 4.5 Percentage leukocyte species per group

Group	*Lymphocytes*	*Monocytes*	*Neutrophils*	*Basophils*	*Eosinophils*
Negative control	47.8	44.6	2.6	0.8	4.2
Positive control	52	45.2	1.6	0	1.2
Resveratrol	57.4	40.6	0.8	0.4	0.8
CoQ10 (low)	54.6	43	1.2	0.4	0.8
CoQ10 (high)	49.4	46	2.8	0	1.8
Resveratrol/CoQ10 combination	63.6	34.8	0.6	0.2	0.8
Age control (14 weeks)	48.4	38.8	5.2	1	6.6

Table 4.5 gives the percentage of leukocyte species counted for each group. Eosinophil counts were significantly higher in the age control group than all other groups assessed except for the negative control group (P = 0.0104). Neutrophil counts of the age control group were significantly higher only when compared to the resveratrol/CoQ10 combination group (P = 0.0454). Both monocytes and macrophages circulating in the bloodstream were counted as monocytes.

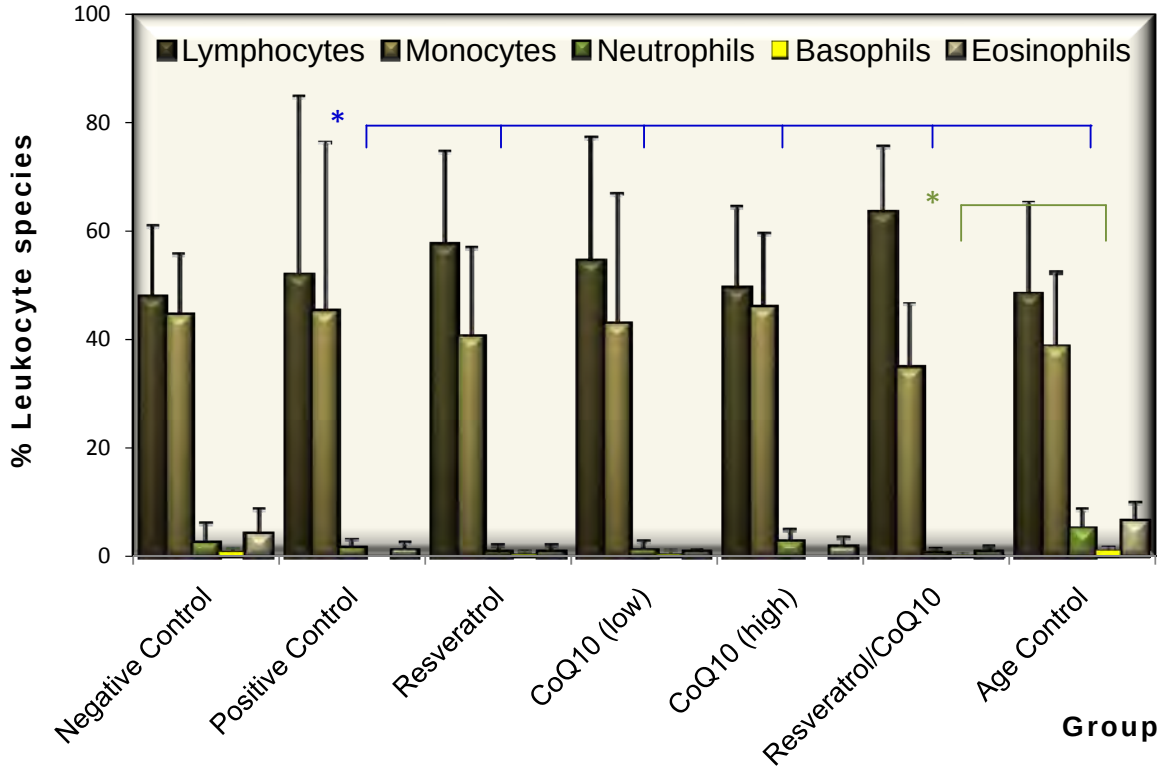

Figure 4.7: Average percentage of leukocyte species assessed per group. Statically significant differences (*) between certain groups in terms of their eosinophil counts (blue line) (P = 0.0104) and neutrophil counts (yellow line) (P = 0.0454) occurred. Standard deviation (SD) is given by the error bars.

From Figure 4.7, it can be seen that the resveratrol/CoQ10 combination group displayed the highest lymphocyte count. The lowest monocyte, neutrophil and eosinophil count, as well as the lowest non-zero basophil count were also detected in this group. In addition, this group displayed the highest lymphocyte count. It is therefore predicted that this group will display the least inflammation at the tissue level. The age control group displayed the highest neutrophil, eosinophil and basophil count. The high CoQ10 group displayed the lowest lymphocyte count, as well as the lowest (zero) basophil count, in conjunction with the positive control group. It is worth mentioning that the high CoQ10 and the positive control groups, with a basophil count of zero displayed the highest monocyte count

overall. Further, the high CoQ10 group had the smallest lymphocyte to monocyte ratio of the experimental groups, in conjunction with the negative control group.

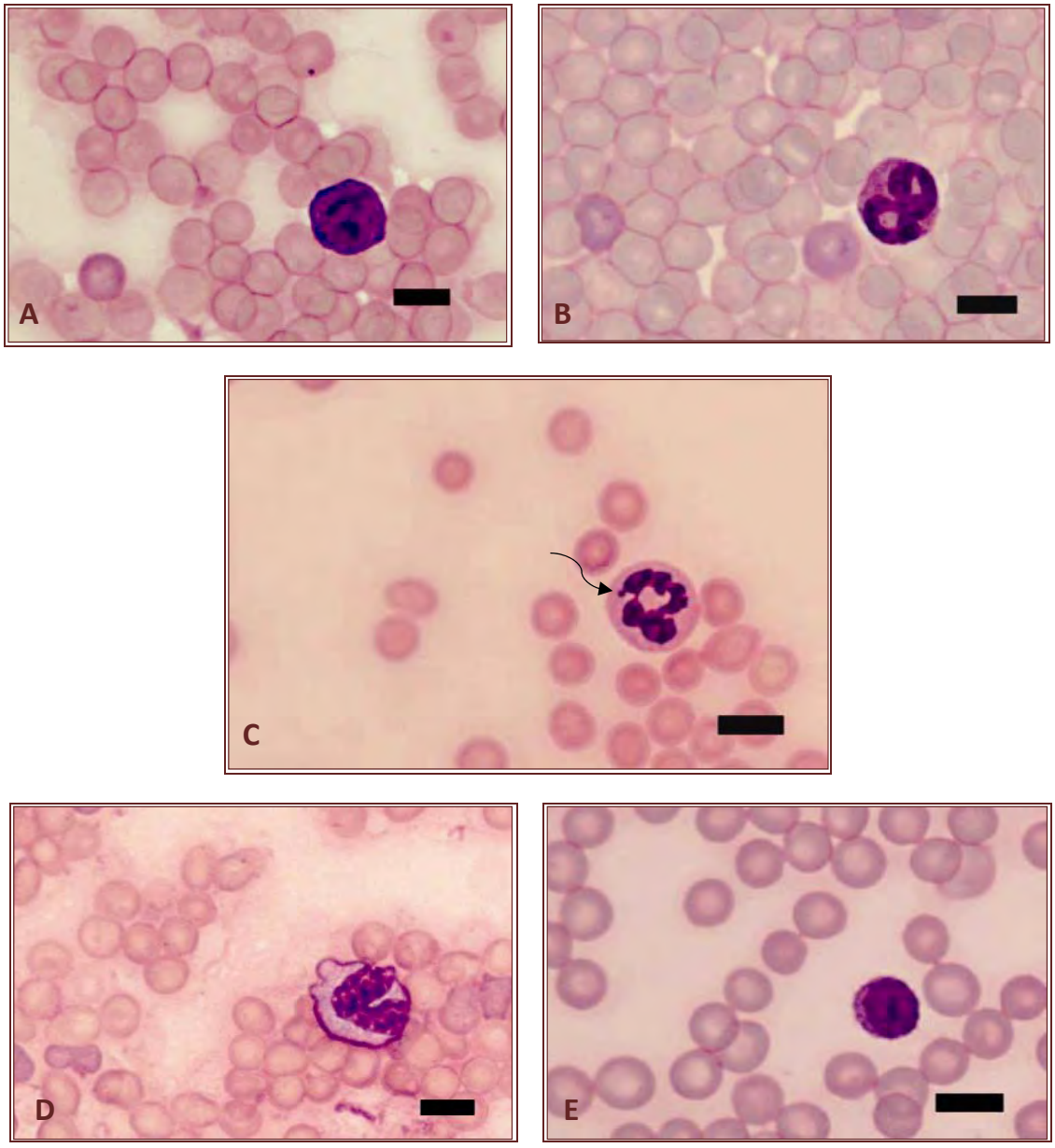

Figure 4.8: Blood smears stained with Wright's stain. Leukocytes from SJL/J mouse blood, **a**) Basophil; **b**) Eosinophil; **c**) Neutrophil; **d**) Monocyte; **e**) Lymphocyte. Scale bar = 10 µm

Neutrophils are attracted by chemotactic factors (chemotaxins) released from damaged tissue or generated by the interaction of antibodies with antigens on the surface of the microorganisms

(Young *et al*., 2006). They are amoeboid cells that squeeze through the capillary walls and enter the tissue fluid where they phagocytise foreign material (Ross *et al*., 2003).

Eosinophils are able to modulate inflammatory responses at several levels and have a central role in the induction and maintenance of inflammatory responses due to allergy (Young *et al*., 2006). Eosinophils can act as pro-inflammatory leukocytes and are able to modulate local immune responses. Mast cells and basophils act as effector cells in allergic disorders mediated by IgE and T helper lymphocytes as well as in the immune response to parasites (Young *et al*., 2006). Monocytes appear to have little function in circulating blood. These cells are motile phagocytic cells and are the precursors of macrophages found in peripheral tissues and organs (Young *et al*., 2006). They respond by chemotaxis to the presence of factors from damaged tissue, micro-organisms and inflammation by migration into the tissues and differentiation into macrophages. With their capacity for phagocytosis and content of hydrolytic enzymes, they engulf and destroy tissue debris and foreign material as part of the process of healing (Young *et al*., 2006).

Text box 4.1

The 'drumstick appearance'

The 'drumstick appearance' of the sex chromatin was found in a neutrophil from the present study (Figure 4.8 c, arrow). This appearance is observed in 3% of human female neutrophils, and represents the heterochromatin of one of the two X chromosomes of the female. Although presumably present in all neutrophils, it is often closely packed with one of the lobes of the nucleus in most cells and is therefore obscured (Kelly *et al*., 1984).

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. Lymphocytes play the central role in all immunological defence mechanisms where they circulate between various lymphoid tissues and all other tissues of the body via the blood and lymphatic vessels (Young *et al*., 2006). There is a constant recirculation of lymphoid cells through tissues and back to the circulation as part of immune surveillance. Large lymphocytes represent either B and T lymphocytes (activated lymphocytes which possess surface receptors that interact with a specific antigen) or natural killer (NK) lymphocytes (programmed to kill certain types of transformed cells). They are *en route* to the tissues where they will become antibody-secreting plasma cells (Young *et al*., 2006; Ross *et al*., 2003).

Raised neutrophil counts are indicative of an acute inflammatory response and are especially seen in association with bacterial infections. Raised eosinophil counts are seen in response to allergy and in infections with certain parasites, as eosinophils are able to modulate inflammatory responses at several levels. The highest lymphocyte counts were observed in the resveratrol and resveratrol/CoQ10 combination groups, suggesting a possible mechanism of resveratrol in

modulating the immunological response. The lowest monocyte, neutrophil and eosinophil counts, as well as the lowest non-zero basophil count were observed in the resveratrol/CoQ10 group. This observation leads to the hypothesis that this group will display the least inflammation at the tissue level. On the contrary, the highest neutrophil, eosinophil and basophil counts observed in the age control group indicate an aggressive inflammatory response at the tissue level. Histopathological assessment in chapter 5 will shed more light on this observation. The high CoQ10 group displayed the lowest lymphocyte counts, with the smallest lymphocyte to monocyte ratio in conjunction with the negative control group. This group further displayed lymphocyte, monocyte and neutrophil counts, with the highest similarity to that observed in the negative control group. In addition, the highest eosinophil count, compared to other antioxidant supplemented groups and the positive control group were detected in the high CoQ10 group. This observation suggests a decreased inflammatory reaction in the high CoQ10 group.

4.4 Concluding Remarks

The good condition maintained in animals in the present study, suggest that supplementary food provided at a more convenient location, may be a good practice to employ as standard, when maintaining muscular dystrophy murine models beyond the point of full disease onset.

Significant stronger tensile strength tests were performed by mice from the high CoQ10 and resveratrol/CoQ10 groups, compared to low CoQ10 and resveratrol groups. Although stronger than the control groups, differences were not significant. The results suggest that the antioxidant supplementation in low CoQ10 and resveratrol groups were not sufficient in protecting muscle against pathological changes that lead to a strength loss. The results further suggest that a higher concentration antioxidant supplementation, as administered in the high CoQ10 and resveratrol/CoQ10 combination groups, increase the limb strength in SJL/J mice.

From the CK and LDH data, the degree of difference from the average CK value for the specific populations, represented by each group in the study, was very large. It is therefore concluded that the CK and LDH values in the present study might not be indicative or representative enough of the disease or the effect of antioxidant supplementation in the SJL/J mouse. It can be concluded that in order to obtain more informative values, the assays should be repeated more times, and preferably include a larger sample size/population.

At the earlier stages of dysferlinopathy at the age of 14 weeks, as represented by the age control group, the increase in eosinophil counts could very likely be a compensatory mechanism to decrease

initial inflammation in the muscle tissues of the dysferlinopathic mice. Considering no significant difference in number between the negative control group and the 14 week-old age control group, it is possible that as disease progressed, the production of eosinophils was down regulated in SJL/J mice in an age-dependent manner. In addition, as the inflammatory process increased with age, the initial possible compensatory mechanism offered by eosinophils is likely not to be able to keep up with the ever increasing inflammatory progression. Neutrophils are linked to early inflammatory responses and often sensitised in self-antigen recognition characteristic to autoimmune disease, a known complication in the SJL/J strain (www.jax.org/jaxmice). Thus the higher neutrophil count in the age control group is probably related to inflammation at disease onset, but may also be indicative of autoimmunity; whereas the eosinophil count may possibly play a more definite role in the pathogenesis of dysferlinopathy.

The hypothesis that the resveratrol/CoQ10 combination group will display the least inflammation at the tissue level, due to the lowest monocyte, neutrophil, basophil and eosinophil numbers measured in this group are further supported by the observation that this group also displayed the greatest physical strength. In addition to this observation, a similar decrease in tissue inflammation is expected in the high CoQ10 group, due to similar white blood cell counts to the negative control group. Histopathological studies in chapter 5 will provide more insight to these observations.