Chapter 7

Oxidative stress in the SJL/J mouse and the effect of Coenzyme Q10 and Resveratrol supplementation

7.1 INTRODUCTION

The body's antioxidant system is made up of a variety of components of both endogenous and exogenous origins (Jacob, 1995). These include small molecule antioxidants which neutralize free radicals, like enzymes which catalyze radical and peroxide quenching reactions, and metal binding proteins which sequester iron and copper ions (Jacob, 1995). The diversity of physiologic antioxidant protection apparently extends beyond endogenous-, dietary-, and metal binding protein antioxidants (Jacob, 1995). The endogenous antioxidant compounds and enzymes insure that antioxidant defence is not lacking when dietary intake of antioxidant enzymes are not sufficient (Jacob, 1995). There is compelling evidence that components of the antioxidant system interact to provide diversity and depth to the body's antioxidant protection system (Jacob, 1995).

Tidball and Wehling-Henricks, 2005, reported that although defects in the membrane repair may be a primary pathogenic mechanism in dysferlinopathy, the prominent inflammatory infiltrate in dysferlin-deficient muscle suggests that inflammation may play a significant but unexplored role in promoting muscle damage. Fanin and Angelini, 2002 reported an increased myosin heavy chain class I (MHC-1) positive reaction in association with the presence of macrophages localized to the cytoplasm of regenerating fibers in dysferlinopathy patients. This was also occasionally observed on the surface of non-necrotic and non-regenerating fibers, suggesting that the inflammatory response might precede necrosis in dysferlin-deficient fibers (Fanin and Angelini, 2002). Linnane and co-workers, 2002, predicted that small metabolic imbalances, which may not have short-term untoward effects, may lead to systemic disease when maintained over long periods of time.

The decline of mitochondrial energy production resulting in increased oxidative stress and apoptosis does play a significant role in degenerative diseases and ageing (Cenacchi *et al*., 2007). Freeman and Crapo, 1982, documented that oxidative stress can alter virtually any aspect of cellular physiology. Among the earliest evidence in support of the hypothesis that the muscular dystrophies are diseases of oxidative injury, were the findings that dietary deficiency of vitamin E, leads to many symptoms of the inherited muscular dystrophies (Murphy and Kehrer, 1989). This is indicative of oxidative

damage to the components of muscle membranes, rather than the cytoplasm, as an underlying basis of tissue damage (Murphy and Kehrer, 1989).

Oxidative stress develops when an imbalance between pro-oxidants and antioxidants exist, where an excess of pro-oxidants consumes antioxidants (Lindschinger *et al*., 2004). This imbalance is associated with a ROS attack on polyunsaturated fatty acids (PUFAs) and other biomolecules (Lindschinger *et al*., 2004). The radical induced chain reaction is called lipid peroxidation (Lindschinger *et al*., 2004). Lipid peroxidation is amongst other things, responsible for the breakdown of PUFAs, resulting in the formation of a great variety of aldehydes (Esterbauer and Cheesman, 1990). The formation of these breakdown products may react with proteins and DNA and as a result are toxic and mutagenic (Marnett, 1999).

Reactive oxygen species (ROS) can attack vital cell components, damage cell membranes, inactivate enzymes, and damage the genetic material in the cell nucleus (Jacob, 1995). Oxidative damage to body cells and molecules has been implicated in a wide variety of diseases, including degenerative diseases such as heart disease, cancer, multiple sclerosis and others (Jacob, 1995). Oxyradicals can attack proteins, thereby changing their structure and ability to function (Jacob, 1995). Increasing evidence suggests that the degenerative processes in dystrophic muscle may be due to oxidative stress (Ragusa *et al*., 1997). Ragusa and co-workers, 1997 provided indirect evidence for oxidative stress in dystrophin-deficient muscle and suggested that oxidative stress may be constitutively present in mdx mouse muscle, but may not be the principle pathogenic mechanism. Disatnik and co-workers, 1998, found evidence of increasing oxidative stress preceding the onset of muscle cell death in dystrophin-deficient mice. These results support the hypothesis of Murphy and Kehrer, 1989 that free radical-mediated injury may contribute to the pathogenesis of muscle necrosis in the muscular dystrophies (Disatnik *et al*., 1998).

Rando, 2002, reported that with any muscle sample analysed, there would be many secondary processes occurring that accompany any muscle degeneration, most notably the infiltration of inflammatory cells reflecting the immune response to tissue damage. Therefore, any, most or all the biochemical changes indicative of oxidative damage can reflect this secondary process (Rando, 2002). Rando, further stated that oxidative damage due to inflammatory cell infiltration is not insignificant given the evidence of secondary damage caused by the immune cells themselves. This can contribute to oxidative stress or to cellular damage by the release of specific cytokines (Rando, 2002). Therefore, the secondary effects should necessarily be distinguished from the primary pathogenic mechanisms that lead to cell death (Rando, 2002).

Disatnik and co-workers, 1998, reported an age-dependent increase in lipid oxidation products and compensatory induction of antioxidant enzymes, supporting oxidative stress as a primary pathogenic mechanism in the mdx mouse. Because secondary processes may confound biochemical analysis of actively degenerating tissue, studies of muscle before the onset of degeneration provide a more definitive assessment of primary pathophysiological mechanisms (Rando, 2002). The present chapter therefore paid specific attention to how the parameters assessed here differ between SJL/J mice at 14 weeks of age and 27 weeks of age. In both these age groups, the pathology has already set in, therefore the analysis of the present chapter will focus on how the parameters assessed here, were influenced as age progressed.

Although the significance and precise extent of the oxidative stress contribution in the degenerative process of dystrophic muscle is poorly understood, there is increasing evidence that the degenerative process may indeed be due to oxidative stress (Niebrój-Dobosz and Hausmanowa-Petrusewicz, 2005). The ultrastructural findings in chapter 6 of the present study provided evidence consistent with ultrastructural changes to muscle mitochondria of human skeletal muscle, experimentally subjected to oxidative damage by ROS. The findings reported in chapter 6 suggested that mitochondria in SJL/J mouse muscle are probably subjected to oxidative stress damage. Although others (Disatnik *et al.*, 1998) have confirmed oxidative stress injury as the primary pathogenic mechanism of muscle degeneration in dystrophin-deficient muscle in the mdx model, no evidence could be found from literature that dysferlin-deficient muscle is subjected to the same insult. To address the notion that muscle pathology in dysferlin-deficient muscle may be free radical-mediated, the lipid peroxidation content of quadriceps muscle in SJL/J mice, was evaluated in the present chapter. In addition, the antioxidant activity in quadriceps muscle was evaluated as the total antioxidant status (TAS), in all SJL/J mice groups. These two parameters were compared and expressed as an oxidative stress index.

7.2 Materials and Methods

After the 90 day animal study, SWR/J mice were terminated at the age of 28 weeks, SJL/J mice were 27 weeks of age. The age control group that was terminated at the beginning of the 90 day trial was 14 weeks of age. Quadriceps muscles from all groups were assessed for levels of lipid peroxidation and all SJL/J mouse groups were assessed for total antioxidant status. One quadriceps muscle from each animal was isolated and frozen in liquid nitrogen. Tissue was stored in liquid nitrogen until the assay procedures were performed. Prior to the assays, muscles were cut in half and weighed. One half was processed for TAS analysis while the other was processed for lipid peroxidation

determination. Before assay procedures were conducted, tissue was stored at -70°C, until analysis. Animals, groups, sample size, treatments and results from the assay procedures are outlined in Table 7.1.

7.2.1 Total Antioxidant Status

Total antioxidant status activity was measured by means of the Randox Kit (Antrim, United Kingdom). The kit is in the form of a colorimetric assay of which the principle is based on the inhibition of the absorbance of the radical cation of 2,2'-azinobis(3-ethylbenzothiazoline sulphonate) ($ABTS^+$) by antioxidants present in the tissue. The $ABTS^+$ radical cation forms when ABTS interacts with the ferrylmyoglobin radical species, generated by the activation of metmyoglobin with H_2O_2. The $ABTS^+$ radical cation is colorimetrically detected by its blue-green colour, and was measured on a spectrophotometer set at 595nm at 37°C. The antioxidants present in the sample suppress the formation of the radical cation to an extent and on a time scale that is proportional to the antioxidant concentration (Rice-Evans and Miller, 1994). Quadriceps muscle from each animal was homogenized individually using a Janke & Kunkel homogenizer, (IKA Werk; Ultra-Turrax) in 1 ml ice cold phosphate buffered saline (PBS). The homogenates were centrifuged at 800g for 10 min and the resultant supernatant used for TAS analysis, as per manufacturer's instruction. The protein concentration for every sample was determined with the aid of the Pierce® BCA Protein Assay Kit (Thermo Scientific) and calculated from a standard curve (Figure 7.1) using a polynomial regression analysis. The TAS of muscle samples was expressed as millimoles per gram (mM/g) of proteins for each individual. Results are given as mean TAS per group ± standard deviation (SD).

7.2.2 Lipid Peroxidation

Lipid peroxidation was measured by the thiobarbituric acid (TBA) assay, according to the method by Draper and Hadley, 1990, and Kaczor and co-workers, 2007. The assay was used to measure the level of cellular malondialdehyde (MDA) in quadriceps muscle tissue, which is proportional to the degree of lipid peroxidation (Disatnik *et al.*, 1998). The quadriceps muscles were weighed, and homogenized individually using a Janke & Kunkel homogenizer, (IKA Werk; Ultra-Turrax) in 1ml buffer containing 50mM K_2PO_4, 1mM EDTA, 1mM DTT, 1.15% KCl, and 1mM butylated hydroxytoluene (BHT) (Supelco Analytical, Bellefonte, PA, USA). The addition of BHT to the reaction mixture before processing has the ability to extensively reduce or even eliminate the oxidation of PUFAs, a process that might artefactitiously occur during the MDA determination procedures (Draper and Hadley, 1990). The homogenates were centrifuged at 8000 rpm for 10 min at 4°C. The resultant supernatant was retained and 100µl of the sample was mixed with 2 volumes (200µl) of ice cold 10% (w/v)

trichloroacetic acid (TCA) (Merck). The samples were incubated for 5 min to allow proteins to precipitate The samples were then reacted with an equal volume (300µl) of a 0.067% (w/v) solution of TBA (Sigma-Aldrich) and incubated in a boiling water bath for 10 min. Samples were cooled on ice for 5 min, and centrifuged at 7000 rpm for 1 min to remove dense particles from the supernatant. The resultant clear supernatant (200µl) was transferred to a micro plate and absorbance was measured at 550nm. The concentration of MDA was calculated using the MDA standard curve with 1,1,3,3-tetramethoxypropane (Aldrich) as a standard (Figure 7.1). A 10mM stock solution was prepared, and from the stock solution dilutions were made to range from 0 to 20µM. The standards were treated the same as the samples and the absorbance measured at 550nm. A linear regression analysis was performed and MDA tissue levels were expressed as nanomoles per gram wet weight muscle. Results are given as mean MDA concentration per group ± SD.

7.2.3 Oxidative Stress Index

Oxidative stress index is given by the ratio percentage of total lipid peroxidation to total antioxidant status. The result is an indicator of the degree of oxidative stress (Harma *et al*., 2003; Horoz *et al*., 2005) and is given in arbitrary units (AU) ± SD.

7.2.4 Statistical Analysis

TAS Assay

The TAS assay, in duplicate and total protein concentration from the quadriceps muscle of the SJL/J mice assays was preformed and utilized for the calculation of the total antioxidant capacity per gram of protein. The average total antioxidant capacity per gram of protein for each experimental group is presented in Table 7.1. The calculated total millimolar antioxidant capacity per gram of protein values was statistically compared between the various experimental groups, via a one-way ANOVA in NCSS. A significance level of 0.05 was used.

TBA Assay

The TBA assay, in triplicate, was performed upon a weighed mass of wet muscle derived from quadriceps muscle of experimental mice. The data was utilized in order to calculate the quantity of MDA present per gram of muscle tissue. The average MDA content per gram of wet muscle mass, for each experimental group, is presented in Table 7.1. The quantified MDA levels were statistically compared between the various experimental groups, via a Kruskal-Wallis one-way ANOVA in NCSS due to the lack of normal data distribution. A significance level of 0.05 was used.

OSI

Unequal sample size could be utilized during this statistical assessment as a Modified-Levene Equal-Variance Test proved all the OSI data possessed equal variance. Due to the data failing to meet a normal distribution pattern, the non-parametric Kruskal-Wallis One-way ANOVA was utilized. A significant difference (P = 0.004082) was found to exist between two or more of these groups. The Tukey-Kramer Multiple-Comparison *pos hoc* test was employed to determine where this difference lay. A significance level of 0.05 was used.

7.3 Results and Discussion

The mean values for antioxidant activity, levels of lipid peroxidation detected, and oxidative stress index in each group are presented in Table 7.1.

Table 7.1 A summary of groups assessed for antioxidant status, lipid peroxidation, and oxidative stress index. Specification of animal age, treatment doses, and results from the TAS and TBA assays, as well as the OSI calculations are presented

	Group	Age (weeks)	Treatment	TAS ± SD (mmol/g)	MDA ± SD (nmol/g)	OSI (AU)
Control groups	**Negative control** (SWR/J mice) (n = 6)	*28*	Placebo	-	248.49 ± 78.91	
	Positive control (n = 6)	*27*	Placebo	0.24 ± 0.04	480.02 ± 199.89	0.22 ± 0.13
	Age control (n = 3)	*14*	No	0.11 ± 0.02	941.81 ± 93.14	0.86 ± 0.15
Treatment groups	**Resveratrol** (n = 6)	*27*	Resveratrol 60mg/kg/day	0.28 ± 0.08	292.04 ± 73.14	0.11 ± 0.05
	CoQ10 (low) (n = 5)	*27*	CoQ10 40mg/kg/day	0.32 ± 0.05	303.60 ± 83.97	0.10 ± 0.02
	CoQ10 (high) (n = 6)	*27*	CoQ10 120mg/kg/day	0.28 ± 0.08	328.47 ± 148.01	0.12 ± 0.08
	Resveratrol/CoQ10 combination (n = 6)	*27*	Resveratrol (60mg/kg/day) + CoQ10 (40mg/kg/day)	0.21 ± 0.03	302.74 ± 65.00	0.15 ± 0.03

OSI = Oxidative stress index; AU = arbitrary unit; MDA = Malondialdehyde; TAS = total antioxidant status; TBA = thiobarbituric acid

Figure 7.1: Standard curves for protein and malondialdehyde (MDA) standards. The formulas on the graphs were used for polynomial and linear regression analysis, respectively.

7.3.1 Total Antioxidant Status

To determine whether the supplementation of SJL/J mice with various concentrations of antioxidants will result in elevated levels of antioxidant activity in the muscle tissue, the total antioxidant status was determined in quadriceps muscle from SJL/J mice (Figure 7.2). An increase in TAS was observed in the resveratrol (0.28 ± 0.08mmol/g), low CoQ10 (0.32 ± 0.05mmol/g) and high CoQ10 (0.28 ± 0.08mmol/g) groups when compared to the positive control (0.24 ± 0.04mmol/g) group. The age control (0.11 ± 0.02mmol/g) group showed the lowest level of TAS compared to all the other groups. The resveratrol/CoQ10 combination (0.21 ± 0.03mmol/g) group displayed the lowest TAS compared to the other antioxidant supplemented groups, as well as the positive control group.

A significant difference (P = 0.000477) was found in terms of the total millimolar antioxidant activity per gram of protein between the six experimental groups when assessed by means of a one-way ANOVA. The data was normally distributed and although of unequal sample size was proved to be of equal variance with the use of the Modified-Levene Equal-Variance Test. The use of Tukey-Kramer Multiple-Comparison *pos hoc* test revealed that the age control group showed a significantly smaller total antioxidant activity per gram of protein than all the other experimental groups except the resveratrol/CoQ10 combination group. The resveratrol/CoQ10 combination group was significantly smaller, in terms of its total antioxidant activity, than the low CoQ10 group. All the other experimental groups were statistically indifferent to each other.

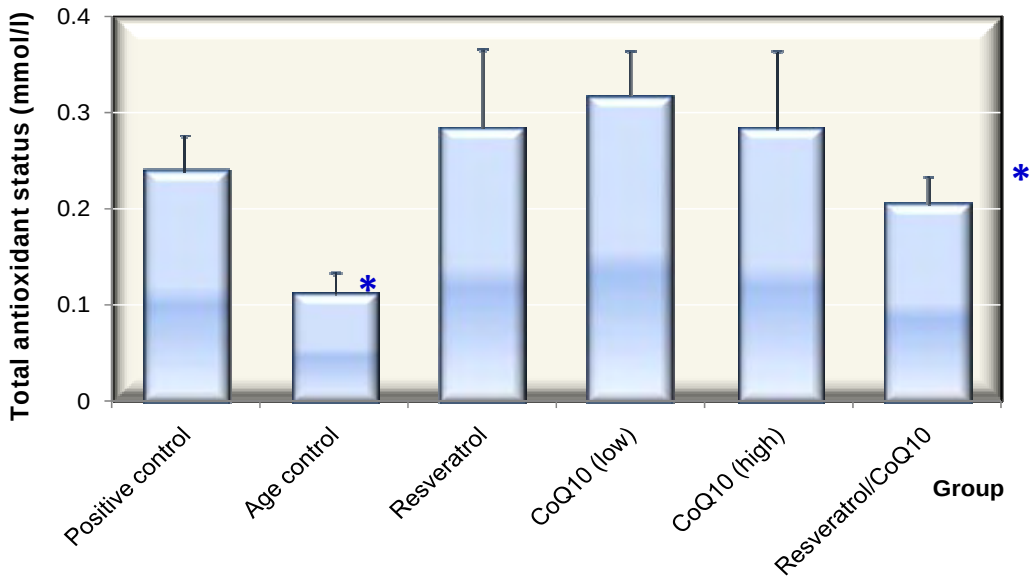

Figure 7.2: Levels of total antioxidant status in quadriceps muscles of SJL/J mice. The lowest antioxidant activity is observed in muscle samples from the age control group. The resveratrol/CoQ10 combination group shows a significantly lower antioxidant activity than the low CoQ10 group. The resveratrol and both CoQ10 groups all display higher antioxidant activity than what is observed in the untreated positive control group, although this difference is not significant. Significance at a level of 0.05 is indicated by *

7.3.2 Lipid Peroxidation

To directly test for the presence of oxidative stress in the SJL/J mice after the 90 days of supplementation with various concentrations of antioxidants, the level of lipid peroxidation was evaluated for each group. The TBA assay, to assess oxidative injury, was performed on quadriceps muscle from all groups and measured for each individual animal (Figure 7.3). The positive control (480.02 ± 199.89nmol/g) and age control (941.81 ± 93.14nmol/g) groups displayed markedly elevated levels of tissue MDA, compared to the antioxidant treated groups, as well as the negative control group.

All the antioxidant supplemented groups displayed levels of tissue MDA, comparable to that found in the negative control group (Figure 7.3). The resveratrol group (292.04 ± 73.14nmol/g), the low CoQ10 group (303.60 ± 83.97nmol/g), and resveratrol/CoQ10 combination group (302.74 ± 65.00nmol/g) showed the lowest levels of MDA compared to the positive control group. In the high CoQ10 group (328.47 ± 148.01nmol/g), MDA levels is only slightly higher than that detected in the

negative control group and other treatment groups, but still much lower than that detected in both the positive and age control groups.

Unequal sample size could be utilized during this statistical assessment as a Modified-Levene Equal-Variance Test which proved that all the data possessed equal variance. Due to the data failing to meet a normal distribution pattern, the non-parametric Kruskal-Wallis One-way ANOVA was utilized. A significant difference (P = 0.0190911) was found in terms of the total nanomolar MDA content per gram of wet muscle mass between the experiential groups. The use of Tukey-Kramer Multiple-Comparison *pos hoc* tests revealed that the age control group showed a significantly higher MDA content within their quadriceps muscles in comparison to all the other groups assessed. The negative control group showed significantly lower MDA values than the positive control. All the other experimental groups were statistically indifferent to one another.

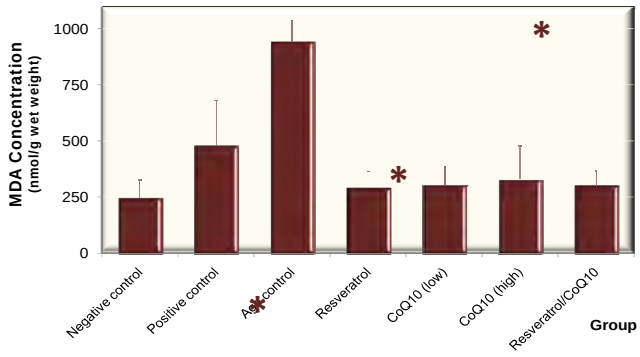

Figure 7.3: Levels of lipid peroxidation in quadriceps muscles of all groups in the present study. An increase in MDA levels was detected in the positive and age control groups, when compared to the negative control and the treatment groups. All groups supplemented with antioxidants showed decreased levels of MDA when compared to the positive and age control groups and similar to MDA levels in the negative control group. Significance at a level of 0.05 is indicated by *

Figure 7.3 shows the level of MDA to be greater in the untreated positive and age control groups, than in the negative control and antioxidant treated groups. The level of MDA was markedly elevated in the younger SJL/J mice (14 weeks of age). These results indicate that oxidative stress

levels in 14 week-old SJL/J quadriceps muscles are higher than that detected in older animals. The data suggest that these levels can be detected from 14 weeks of age, and perhaps earlier. This is in contrast with the findings of Disatnik and co-workers, 1998, who found an age dependent increase in lipid oxidation products in mdx mice.

7.3.3 Degree of Oxidative Stress

An enormous increase in the degree of oxidative stress in the age control group (0.86 ± 0.15), compared to all other groups was shown by calculation of the OSI. The Tukey-Kramer Multiple-Comparison *pos hoc* test revealed that the age control group showed a significantly higher OSI in comparison to all the other groups assessed. All groups treated with antioxidants; the resveratrol (0.11 ± 0.05), the low CoQ10 (0.1 ± 0.02), the high CoQ10 (0.12 ± 0.08), and the resvertrol/CoQ10 combination (0.15 ± 0.03) groups, all showed reduced levels of the degree of oxidative stress when compared to the positive control (0.22 ± 0.13) group, although not statistically significant.

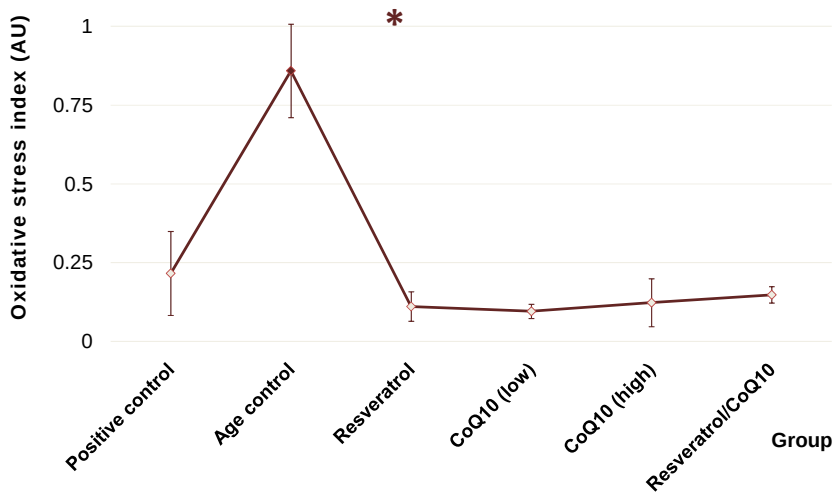

Figure 7.4: The tendency in the degree of oxidative stress in SJL/J mice untreated and supplemented with antioxidants. The age control group showed a significantly higher OSI in comparison to all the other groups assessed. OSI is expressed in arbitrary units (AU). Significance at a level of 0.05 is indicated by *

7.3. 4 JUSTIFICATION OF THE APPROACH FOLLOWED

A complex variety of products, many of which are reactive electrophiles are generated by lipid peroxidation (Marnett, 1999). The basic reactions of lipid peroxidation result in the formation of isoprostanes and MDA (Marnett, 1999). MDA occurs in biological samples in the free state and in various covalently bound forms (Draper and Hadley, 1990). Its levels can be estimated by the TBA reaction which yields a group of chromophores known as TBA reactive substances (TBARS) (Murphy and Kehrer, 1989).

Murphy and Kehrer, 1989, reviewed the role of oxidative stress in dystrophic muscle and reported that many studies have found dystrophic human-, mouse-, and chicken muscle to contain higher levels of TBARS than normal subjects. The study further revealed that the presence of TBARS and other indexes of oxidative stress in inherited muscular dystrophies is probably not due to the secondary pathological effects of inherited muscular dystrophy. It more likely indicates oxidative stress ongoing in the muscle tissue (Murphy and Kehrer, 1989).

The validity of MDA as an index of lipid peroxidation has been clouded by controversy regarding its formation as an artefact of analysis, and as a product of enzyme reactions, its occurrence in various bound forms, and the specificity of methods used for its measurement (Draper and Hadley, 1990). Nevertheless, widespread interest has been attracted to the determination of MDA in biological samples as it appears to offer a facile means of assessing lipid peroxidation in biological materials (Draper and Hadley, 1990). It is furthermore one of the most frequently used indicators of lipid peroxidation (Nielsen *et al*., 1997). Nielsen and co-workers suggested that MDA be used only as a biomarker for the degree of lipid peroxidation on a group basis.

Methods for measurement of the antioxidant activity of fluids are all essentially inhibition methods; a free radical species is generated, there is an endpoint by which the presence of the radical is detected, and the antioxidant activity of the added sample inhibits the end point by scavenging the free radical (Rice-Evans and Miller, 1994). These methods have been reported to vary greatly as to the radical that is generated, the reproducibility of the generation process, and the endpoint that is used (Rice-Evans and Miller, 1994). Since there is not yet an accepted 'gold standard' reference method for measuring TAS (Hubel, 1999; Horoz *et al*., 2005), it was decided to determine the antioxidant activity of quadriceps muscle with aid of the Randox TAS kit. The kit is one of the widely used methods for antioxidant activity/response measurement (Horoz *et al*., 2005). Lantos and co-workers, 1997, suggested TAS to be a valuable and reproducible method for measurement of the actual antioxidant status in different diseases and as a tool to monitor treatment.

The findings of mitochondrial alterations in chapter 6 indicated that the muscle tissue of SJL/J mice is subjected to oxidative damage. Therefore, analysis of lipid peroxidation levels in quadriceps muscles was performed, as a parameter for the presence of oxidative stress, in all groups in the present study. Lindschinger and co-workers, 2004, suggested that a statement about oxidative stress should be based on an antioxidant status, in the context of multiple oxidative stress parameters. In conjunction with determination of MDA levels, the TAS in muscle tissue was determined. These two parameters were used to calculate the OSI, which represents the degree of oxidative stress in the muscle tissue.

7.3.5 Denotation of the Results

The highest levels of MDA were detected in the age control group, while the lowest antioxidant activity was present in this group. The positive control group displayed markedly lower levels of MDA than that detected in the 14 week-old mice. The TAS levels in the positive control group were not significantly different from that detected in the antioxidant treatment groups. On the contrary the levels of MDA in the positive control group were markedly elevated compared to the antioxidant treatment groups, and the negative control group.

The data showed a relation between the antioxidant status and the amount of lipid peroxidation in the SJL/J mouse consistent with the findings of Lindschinger and co-workers, 2004, who reported that a decrease in TAS indicates an excess of ROS and therefore a consumption of antioxidants. At age 14 weeks, the very low levels of antioxidants are associated with the very high levels of lipid peroxidation in the age control group. These findings suggest that at the age of 14 weeks, the pathological changes has already set in and resulted in production of oxidative stress in the quadriceps muscles of SJL/J mice. Even though the dystrophic changes were only minimally present in the age control group, as observed by morphological and ultrastructural analysis in chapters 5 and 6. It can be further expected that these very high levels of oxidative stress will initiate severe tissue disruption that will manifest at the cellular level as age progress, consistent with the histopathology described for the positive control group in chapter 5.

Seeing that age control group received no antioxidant treatment, a decreased antioxidant status was expected. The antioxidant status in the age control group was nevertheless, significantly lower than the TAS measured in all the other SJL/J mice. This might indicate that at the age of 14 weeks, the endogenous antioxidant system has not yet been activated to address the high levels of oxidative stress in the tissue, or it is not yet responding. While in the positive control group, the TAS values

were similar to that of the treatment groups. This observation indicates that since this group did not receive any antioxidant supplementation, the endogenous antioxidant system must have been responding to high levels of oxidative stress. Induction of antioxidant enzymes is a common cellular response to increased levels of oxidative stress (Harris, 1992).Therefore the endogenous antioxidant response should be responsible for the increased antioxidant activity observed in 27 week-old untreated SJL/J mice. These results might further suggest that there is a delayed activation or response of the endogenous antioxidant system in the SJL/J mouse. As an effect of this late response, increasing oxidative stress levels resulted in the tissue deterioration, and in fact the ongoing dystrophic processes seen in the untreated 27 week-old SJL/J mice.

A similarity in MDA levels was observed in the negative control and antioxidant treatment groups. These results are indicative of the ability of antioxidant supplementation to address the degree of lipid peroxidation, and thereby, decrease the levels of MDA. It appears from the results that the decrease in MDA levels were independent of the dose of antioxidants administered. Structural analysis of quadriceps showed major differences between groups (chapter 5). It is therefore possible that the antioxidant supplementation at lower doses (resveratrol and low CoQ10 groups), lowered the levels of oxidative stress present in the muscle tissue. However, these concentrations were unable to delay the progression of tissue damage that was initiated by the presence of high oxidative stress levels, before the supplementation started.

It can therefore be stated that the onset of disease on a molecular level, occurred before 14 weeks of age in the SJL/J mouse, as the highest oxidative stress levels were measured in this group. The results are further suggesting that when oxidative stress in muscle tissue reaches a specific level, the endogenous antioxidant systems are activated to address this imbalance. This suggestion explains the reduced levels of MDA and increased levels of antioxidant activity measured in the untreated positive control group, when compared to the age control group. These results also suggest that if supplementation with antioxidants begins after the onset of this molecular cascade of events, responsible for oxidative stress increase in muscle, the degree of tissue damage will not be decreased by lower doses of antioxidant supplementation.

On the contrary, a lower oxidative stress profile was observed in groups treated with high doses of antioxidants (high CoQ10 and resvertarol/CoQ10 combination groups). This observation supports the findings of a lesser degree of tissue damage observed at microscopic level (chapter 5). The levels of MDA in antioxidant treatment groups suggest that the reduction of lipid peroxidation in the muscle is independent on the concentration of antioxidants administered. But histopathological

investigations (chapter 5) revealed that only very high levels of antioxidant supplementation were able to reduce the cellular disruption. This observation revisits the suggestion that a delayed activation or response of the endogenous antioxidant system might be present in the SJL/J mouse. It is therefore suggested that the higher doses of antioxidant supplementation might compensate for this delayed response, resulting in sparing of muscle tissue.

To evaluate the relationship between the levels of oxidative stress, and antioxidant response in the SJL/J mice in the present study, the two parameters assessed were compared to determine the OSI for each group. As an indicator of the degree of oxidative stress, calculation of the OSI revealed that the oxidative/antioxidative balance shifted towards antioxidative balance in 27 week-old untreated SJL/J mice compared to 14 week-old counterparts. This is probably the result of an activated endogenous antioxidant system. Furthermore, another shift was observed towards antioxidative balance in all 27 week-old SJL/J mice supplemented with antioxidants compared to the untreated 27 week-old SJL/J mice. These results showed that supplementation of SJL/J mice with antioxidants from an age of 14 weeks for 90 days result in a shift towards antioxidant balance.

7.4 Concluding Remarks

The current chapter investigated the presence of oxidative stress in the quadriceps muscle tissue as an additional parameter of the cellular effects afforded by CoQ10 and resveratrol supplementation. The benefit for dystrophic muscle from any individual antioxidant treatment would depend on the actual nature of the oxidative stress occurring in the muscle tissue (Rando, 2002). Rando, 2002, stated that different susceptibilities to oxidative stress are not identical. Even if oxidative stress is indeed the primary pathophysiological process leading to muscle cell death in the dystrophies, effective treatment with antioxidants will need to be targeted to the specific deficit in antioxidant defence (Rando, 2002). From the results obtained here, it is evident that there is a relationship between the very high levels of oxidative stress in 14 week-old SJL/J mice and the degree of pathology in 27 week-old counterparts. In addition, the results suggested that the possibility for a delayed endogenous antioxidant response exist, and that this delay is in part responsible for the severity of dystrophic progression. It is therefore possible that the specific deficit in antioxidant defence in SJL/J mice are this delayed antioxidant response. Therefore, application of low dose antioxidant supplementation might permeate a protective effect onto the cellular level, if supplementation is started before onset of the disease.

The results obtained in chapter 6 proposed the mitochondria as an organelle particularly susceptible to oxidative stress. It might not be the only organelle, but the ultrastructural alterations to the mitochondria were more pronounced. CoQ10 is present in the innermembrane of mitochondria, where the major part of ATP production occurs (Crane, 2001). The unique function of CoQ10 as an essential part of the cellular machinery is given by its ability to transfer electrons from the primary substrates to the oxidase system. At the same time CoQ10 transfers protons to the outside of the mitochondrial membrane (Crane, 2001). This results in a proton gradient across the membrane. As the protons return to the interior through the enzymatic machinery for making ATP, they drive the formation of ATP (Crane, 2001). CoQ10 is therefore the antioxidant of choice to target the specific organelle proposed to be deficient in antioxidant defence, in SJL/J quadriceps muscle.

Since the ultrastructural analysis (chapter 6) only provided qualitative information, it was decided to analyse SJL/J quadriceps muscle for the presence of oxidative stress, represented by the level of MDA present per gram of muscle, and quantify the findings. These results suggest that the endogenous antioxidant defence system in tissue subjected to oxidative stress insult is activated in response to the tissue damage in subjects not supplemented with antioxidants. The large amount of MDA detected in 14 week-old SJL/J mice showed that quadriceps muscle in these animals is subjected to oxidative stress injury before this age. These results further suggest that once oxidative stress is present in the muscle, it will result in dystrophic changes in the tissue that cannot effectively be reduced by lower antioxidant doses.

It was established that the supplementation with CoQ10 and resveratrol resulted in an increase in antioxidant activity and a decrease in lipid peroxidation levels in the quadriceps muscle of SJL/J mice. This gave rise to a shift in the oxidative/antioxidative balance towards an antioxidative balance in 27 week-old SJL/J mice.

It is evident from the present findings that the ultrastructural mitochondrial alterations in the SJL/J mouse are largely due to oxidative stress insult. The OSI suggests that antioxidant supplementation at all doses has the ability to shift oxidative/antioxidative balance towards an antioxidative balance. However, only higher doses of antioxidants (CoQ10 or a combination of resveratrol/CoQ10) are able to permeate this effect onto the cellular level.

The OSI might become a useful tool for oxidative stress assessment in the dysferlinopathies, the MDs, and other conditions, especially the lifestyle diseases.

From the results of this chapter, evidence is provided supporting the notion that metabolic disturbances are brought about by the increased levels of oxidative stress. In addition, very high levels of oxidative stress are present even before inflammation could be detected at the cellular level. This is indicative that the generation of oxidative stress in quadriceps muscle is preceding the inflammatory events in SJL/J mice. The ultrastructural changes to mitochondria, suggestive of oxidative stress injury (chapter 6) are most likely the result of oxidative stress present in the muscle. The level of oxidative stress was successfully reduced in SJL/J mice by all doses of antioxidants used in the present study, but transformation at the cellular level were afforded only by the higher antioxidant doses. This observation supports the suggestion that endogenous antioxidant response might be delayed in the SJL/J mouse. Therefore only very high levels of antioxidants will afford protection against tissue deterioration after onset of disease.

Future research might be conducted to establish whether antioxidant supplementation from a younger age will provide a more pronounced protective effect in dysferlin-deficient muscle tissue. Also, if antioxidant supplementation is started at a pre-pathological stage in the SJL/J mouse model, whether lower levels of antioxidants will then be able to maintain muscle integrity at cellular level.

Chapter 8

Concluding Discussion

The dysferlinopathies encompass a group of disorders that are not responsible for early mortality, but for a devastating deterioration of mobility in the affected. During the course of this disease this loss of ambulation results in an escalating decreased quality of life. To date, there is no curative treatment for this group of inherited disorders.

With the identification of the novel skeletal muscle gene dysferlin that is mutated in the dysferlinopathies (Liu *et al*., 1998; Bashir *et al*., 1998), the need for a therapy to restore the expression of the protein dysferlin has become indispensable. Rando, 2002, reported that although the genetic basis of many of the muscular dystrophies has been known for over a decade, the pathogenic mechanisms leading to muscle cell death in the dystrophies remain a mystery. The gene mutation responsible for the clinical pathology in the dysferlinopathies has been identified a decade ago (Bashir *et al*., 1999; Liu *et al*., 1999). However, the exact pathogenic mechanism remains to be elucidated and therefore the effective application of therapeutic strategies, lingers.

The decision to employ CoQ10 as an antioxidant was based on its known potent antioxidant potential, bearing in mind that oxidative stress may contribute to the pathological events in this disease. The decision to employ resveratrol, a rather weak antioxidant (Hu *et al*., 2007), in the same study for the same application, was based on its known ability to mediate inhibition of the detrimental effects caused by amongst others, oxidative stress ((Fauconneau *et al*., 1997; Kirimlioglu *et al*., 2008; Robb *et al*., 2008).

It has previously been shown that presymptomatic supplementation with CoQ10 in primary CoQ10 deficiency has revealed an unparalleled outcome (Montini *et al*., 2008). No clear benefit has been found in clinical trials of antioxidant therapy in human DMD that included treatments with ascorbate, penicillamine, tocopherols and SOD (Murphy and Kehrer, 1989; Walton and Nattrass, 1954; Stern *et al*., 1982; Roelofs *et al*., 1979; Fenichel *et al*., 1988). However, these trials have been limited in size and duration and almost always included subjects with advanced disease progression. On the contrary, Folkers and Simonsen, 1995, reported definite increase in physical performance of patients with various muscular dystrophies following treatment with CoQ10.

Murphy and Kehrer, 1989, clearly stated that if lipid- and water-soluble antioxidants are applied as treatment strategy in the muscular dystrophies, such treatments would only be symptomatic. They added that the underlying biochemical defect of muscular dystrophy, as with nearly all inherited diseases, can only be cured if techniques are developed to repair or replace the defective genetic material.

As early as 1989, mounting evidence revealed that oxidative processes play a fundamental role in the inherited dystrophies, either directly or indirectly (Murphy and Kehrer, 1989). Disatnik and co-workers, 1998, stated that the beneficial effect of antioxidant therapy would be compelling support of the hypothesis that oxidative stress contributes to the initiation of muscle cell necrosis in the muscular dystrophies. The present study, by the use of various parameters, confirmed that not only is oxidative stress present in skeletal muscle of dysferlinopathic mice, but it is very likely responsible for the initiation of the dystrophic process.

Assessment of non-specific parameters in chapter 4 provided valuable additional knowledge that strengthens the observations made on a cellular level in this study. Evaluation of animal weight in the present study provided an effective means of condition evaluation, to ensure animal well-being was maintained at all times. The animal weights of all experimental groups increased over the 90 day period due to supplementary food provided for their convenience. Weight increase was independent of the antioxidant supplementation.

No significant conclusion on the effect of antioxidant supplementation in SJL/J mouse model could be derived from the CK and LDH blood levels in the present study. The reason being, abnormally high values in the negative control groups could not be explained. This was in part due to the sample size utilized for these laboratory tests.

Significantly higher eosinophil counts were measured in the age control group compared to all other groups assessed. In addition, this group displayed significantly higher neutrophil counts compared to the resveratrol group, and the highest basophil count was also detected in this group. This finding correlates with the highest levels of MDA measured in this group, as well as the lowest antioxidant status. These results indicate that the highest inflammatory response was present at the age of 14 weeks and can be related to the highest degree of oxidative stress, measured for this group. On the contrary, the histopathological analysis of 14 week-old animals displayed only minimal to mild dystrophic changes. This leads to the suggestion that the deployment of inflammatory cells at the

age of 14 weeks might be in response to the high levels of oxidative stress. This inflammatory reaction, not yet detectable at cellular level at the age of 14 weeks, suggests that this cascade of events is activated around the age of 14 weeks, and give rise to the histopathology observed in untreated 27 week-old SJL/J mice.

The lowest monocyte, neutrophil, eosinophil and non-zero basophil counts, were detected in the resveratrol/CoQ10 group. This observation gave rise to the hypothesis that the lowest inflammatory activities would be observed on tissue level in this group. Consistent with the histopathological observations from this group, that displayed, from a qualitative perspective, the least inflammatory changes, the hypothesis could not be rejected. The observation of the highest physical strength displayed by this group supported this statement. Although not statistically significant, this group showed the highest tensile strength, in addition to the high CoQ10 group, compared to all other groups. A decrease in the degree of oxidative stress compared to the positive control and age control groups further strengthens the suggestion that supplementation with resveratrol and CoQ10 in combination, was able to afford physical and cellular improvement of the dystrophic alteration pattern in the SJL/J mouse.

Haematological analysis of the high CoQ10 group showed the highest similarity to that of the negative control group. At microscopic level, tissue analysis revealed a striking similarity to what was observed in the resveratrol/CoQ10 combination group, with reduced levels of inflammatory changes compared to other groups. The physical strength measured for animals on high dose CoQ10 supplementation was also stronger compared to all other groups, in addition to the resveratrol/CoQ10 combination group. The OSI measured about half of that in the positive control group. These results support the suggestion that supplementation with high doses of CoQ10 was also able to afford physical and cellular improvement of the dystrophic alteration pattern in the SJL/J mouse. This improvement was afforded almost to the same extent as that seen with resveratrol/CoQ10 combination treatment.

Morphometric assessment suggested that active regeneration occurred in the high CoQ10 and resveratrol/COQ10 combination groups, followed by maturation of fibers towards larger fiber diameters. Other groups did not display this observation. These results indicate that while quadriceps muscle fibers had a tendency towards smaller diameters in untreated and low dose antioxidant supplemented groups, CoQ10 at high dose and resveratrol/CoQ10 in combination allow for fiber maturation, indicated by the tendency of fibers towards larger diameters.

The differences in white blood cell counts detected between the high CoQ10 and resveratrol/CoQ10 combination groups, probably suggest that the mechanism whereby enhancement in condition of SJL/J mice was brought about, differs between these two supplementation strategies. The presence of minimal to mild inflammatory changes at cellular level of these two groups suggests that the dystrophic progression will probably be delayed and not reversed, following antioxidant treatment.

Resveratrol alone and low dose CoQ10 supplementation resulted in similar reduction of oxidative stress levels than that afforded by high CoQ10 doses and resveratrol/CoQ10 in combination. These two antioxidants at low doses were also able to increase antioxidant status in the muscle tissue to the same extent as high doses of antioxidants. Nevertheless, the supplementation of SJL/J mice with resveratrol alone or low dose CoQ10 was not able to decrease dystrophic alterations at cellular level to the same extent than what was afforded by high dose CoQ10 and resvertarol/CoQ10 in combination. Consistent with the histopathology data obtained from these two groups, was the reduction in physical strength that was lower than all other experimental groups. Collectively, the results from the resveratrol and low CoQ10 groups suggest that antioxidant supplementation at these doses will not result in enhanced physical condition in the SJL/J mouse. Neither will it afford protection at the cellular level. It is therefore possible that supplementation from the age of 14 weeks was too late to result in beneficial effects from these antioxidant doses. This notion further implicates that only high doses of CoQ10 or low doses of resveratrol and CoQ10 in combination will be effective in slowing disease progression in the SJL/J mouse, if supplementation is started after onset of disease.

Ultrastructural analysis of SJL/J quadriceps muscles confirmed the hallmarks of the disease at this level, previously described by others (Selcen *et al.*, 2001; Cennachi *et al*., 2005). Focussing on non-specific ultrastructural alterations in the present study resulted in the valuable observation that non-specific ultrastructural changes in muscle mitochondria of SJL/J mice are indicative of oxidative stress injury. This observation provided useful evidence that oxidative stress might also be, in addition to the deficit in membrane repair, and chemotactic response, a causative mechanism for the ongoing muscle cell necrosis in the SJL/J mouse. It can also not be excluded that oxidative stress, might even be the primary pathogenic mechanism in the SJL/J mouse. This finding directed the assessment of oxidative stress levels in quadriceps muscles of SJL/J mice, and how these levels were affected by the administration of different concentrations of antioxidants.

The results from oxidative stress assessment revealed significantly higher levels of oxidative stress in 14 week-old animals, while in the 27 week-old counter parts only about half this level was measured. Calculation of the oxidative stress index revealed that the degree of oxidative stress in 27 week-old SJL/J mice is about a quarter of that detected in 14 week-old animals. These results provide strong evidence that the endogenous antioxidant system respond to the initial elevated oxidative stress levels. When comparing the high OSI in the age control group to the histopathological data of the positive control group, it was revealed that this initial elevated OSI can be correlated to the severe disruption of tissue integrity at cellular level. This finding indicates that oxidative stress is accumulating in muscle tissue of the SJL/J mouse before the age of 14 weeks. Thereafter it reaches peak levels at around this age, triggering the endogenous antioxidant system to respond. A matter of concern with this observation is that although the OSI in 27 week-old animals is much lower, the tissue disruption at cellular level is most severe. This provides evidence that the endogenous antioxidant system might respond too late to prevent the onset of tissue damage. From these results it can be suggested that very high levels of oxidative stress at the age of 14 weeks leads to tissue deterioratiom, as displayed by histopathology data of untreated 27 week-old animals. Therefore, it is probable that the initiation of the necrotic process in the SJL/J mouse is due to very high levels of oxidative stress, in conjunction with a delayed endogenous antioxidant response.

The results from this research provide evidence that high levels of CoQ10 and a combination of resveratrol/CoQ10 are able to manipulate this defect, and as a result delay the dystrophic progression. Antioxidant supplementations at these two concentrations lead to:

- White blood cell counts, reflecting i) similarity to that observed in the negative control group, and ii) levels suggestive of very low inflammatory response at the tissue level.
- Stronger physical strength, where tensile strength exceeded that measured in the negative control group.
- A reduced occurrence of inflammatory incidence and dystrophic changes at cellular level, resulting in enhanced tissue quality.
- A reduced oxidative stress index, where levels of lipid peroxidation were lowered to a profile similar to that measured for the negative control group. In addition, TAS was elevated, compared to the positive control group.

It can be concluded that increased oxidative stress levels are present in the SJL/J mouse. All antioxidant supplementation doses were able to decrease the levels of oxidative stress following 90 days of supplementation. Only a high dose of CoQ10 and/or resveratrol/CoQ10 in combination were able to permeate this effect onto a cellular level. This statement is supported by the observation of i) decreased leukocytes in the circulation of the high dose combination group; ii) higher physical strength observations with both the single antioxidant at high dose as well as the combination of antioxidants at high dose; iii) decreased levels of dystrophic markers observed at the light microscopic level, and iv) a reduced oxidative stress index.

The hypothesis of this study stating that, the supplementation of SJL/J mice with the antioxidants CoQ10 and resveratrol would be beneficial to the animals, and that the beneficial effect would be evident at cellular level, was not rejected.

Oxidative stress may also be viewed as a condition in which the production of oxidative products exceeds their removal by cellular repair mechanisms, which may lead to acute cellular metabolic disturbances and even cell death, if such changes accumulate (Rando, 2002). From the results obtained it can be suggested that the current therapeutic strategy might hold significant value in combination with cell-based therapies in the dysferlinopathies. It is suggested that the antioxidant protection offered by high dose CoQ10 and resveratrol/CoQ10 in combination, will afford modulation of the internal cellular environment in dysferlin-deficient tissue. This adjustment at cellular level creates a host environment where there is a reduced level of oxidative stress, and therefore a more favourable environment to accommodate transplanted cells.

This thesis provides evidence to create a new platform for combination therapeutic strategies. The supplementation of subjects with antioxidants is unlikely to reverse the pathological process. However, high doses afford the manipulation of dystrophic progression. In combination with cell transplantation therapeutic strategies, high dose antioxidant supplementation might provide an optimal environment for donor cell differentiation and maturation in dysferlin-deficient muscle tissue.

This thesis therefore concludes with the proposition that the unfavourable state in muscle tissue, brought about by inevitable, and to date, irreversible dystrophic processes that sets in after disease onset in the dysferlinopathies and progress gradually over the lifespan, might be manipulated by supplementation with high dose antioxidants. Presumably, the effect will be superior if

supplementation starts before the pathology sets in. In addition, this supplementation might offer a favourable environment for donor cell maturation and differentiation in cell transplantation therapies.

This thesis harmonizes the statement of Bermúdez-Crespo and López, 2007, that it takes a broad, comprehensive and systematic approach to understand biology that is generally unbiased and not dependent on existing knowledge. This study succeeded in manipulating at least three alterations in the dysferlinopathies. By application of high dose antioxidant supplementation, higher physical strength, reduced dystrophic changes at cellular level, and a reduced oxidative stress index were attained in the SJL/J mouse model. The supplementation of CoQ10 at 120mg/kg/day and resveratrol/CoQ10 in combination at 40 and 60mg/kg/day, respectively, directed dystrophic changes at cellular and physical levels towards a normal phenotype. The present study succeeds in providing an alternative treatment strategy to address metabolic derangements at cellular level, and provides a new platform for advances in cell-based therapies.