

Differential Leukocyte Counts of SJL/J Mice with Dysferlinopathy Treated with Resveratrol and Coenzyme Q₁₀

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Summary

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

Introduction

The dysferlinopathies are a set of myopathies in which a mutation in the dysferlin gene is inherited in an autosomal recessive pattern. They include Limb-Girdle Muscular Dystrophy Type 2B (LGMD2B) and Miyoshi Myopathy (MM). The dysferlinopathies mostly affect muscles of the shoulder

and pelvic girdles (*Angelini, 2002*); LGMD2B first exhibits weakness in the proximal muscles and MM exhibits weakness in the distal muscles. These myopathies display a prominent inflammatory response (*Angelini, 2002; Diers et al., 2007*).

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

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ogy between the human and mouse dysferlin genes (Vafiadaki *et al.*, 2000). As a negative control, the SWR/J mouse with normal muscle is recommended as a general purpose strain.

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

The antioxidant and sequentially anti-inflammatory properties of these two products are of interest to the present study as it is our aim to determine whether or not their administration will affect the levels of inflammatory leukocytes at the haematopoietic level. Leukocytes are involved in the cellular and humoral defences of an animal against foreign materials. Leukocytes are functional only to a small extent in the bloodstream, their greatest activity being exhibited in the tissues (Leeson *et al.*, 1988). They are capable of amoeboid movement, enabling them to travel out of the circulatory system in order to elicit an immune response in the tissues where such response is needed.

Leukocytes of two main types exist, namely granulocytes and agranulocytes. The granulocytes have a cytoplasm containing numerous characteristic granular bodies and possess nuclei which exhibit considerable variation in shape. Neutrophils, baso-

phils and eosinophils are classified as granulocytes. The agranulocytes are characterised by a clear, homogenous, slightly basophilic cytoplasm. The nuclei of these leukocytes are spherical to reniform in shape. Lymphocytes and monocytes are classified as agranulocytes (Leeson *et al.*, 1988).

Materials and Methods

Animals

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

Animals were housed at the laboratory animal facility of the UPBRC at Onderstepoort (University of Pretoria, Pretoria, South Africa). The animals were maintained in the facility's barrier unit in Tecniplast IVC cages, Eurostandard type II L with individual air supply, equipped with a pre-filter and HEPA filter system. The barrier unit was free of all major pathogens as serology, bacteriology and parasitology tests performed were all negative. Agrebe basic, 55cm², cotton sheet laboratory animal cloth bedding for boxes Type II/III L1 were used as bedding and changed once weekly. The main air supply consisted of a 50% Fresh Air Primary filter and a Secondary Bag filter with 10-15 air changes per hour. A constant temperature of 23-24°C, a relative humidity of 40-60%, and a 12-hour dark-light cycle were maintained. The animals were provided JL Rat and Mouse 6F-IRRAD food (PMI Nutrition International, LLC) and water *ad libitum*. As a decrease and slowing in the mobility of the animals was observed with disease progression, it was decided to supplement the animals' diet by addition of Cerelac, sterile baby cereal on the floor of each cage, from day 50 of the 90-day trial.

At study commencement, the animals were 14- and

15-weeks old respectively for SJL/J and SWR/J mice. Six SJL/J animals were terminated for baseline results at this time. The remainder of the animals were entered into a 90-day experimental period, after which the study was concluded once the animals had reached approximately 27- and 28-weeks of age respectively for SJL/J and SWR/J mice. All experimental procedures were carried out in strict accordance with the requirements of the University of Pretoria's Animal Use and Care Committee. Ethical clearance was obtained from the University of Pretoria's Biomedical Research Center (UPBRC).

Administration of Test Agents

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

Blood Smears

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

Statistical Analysis

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

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

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

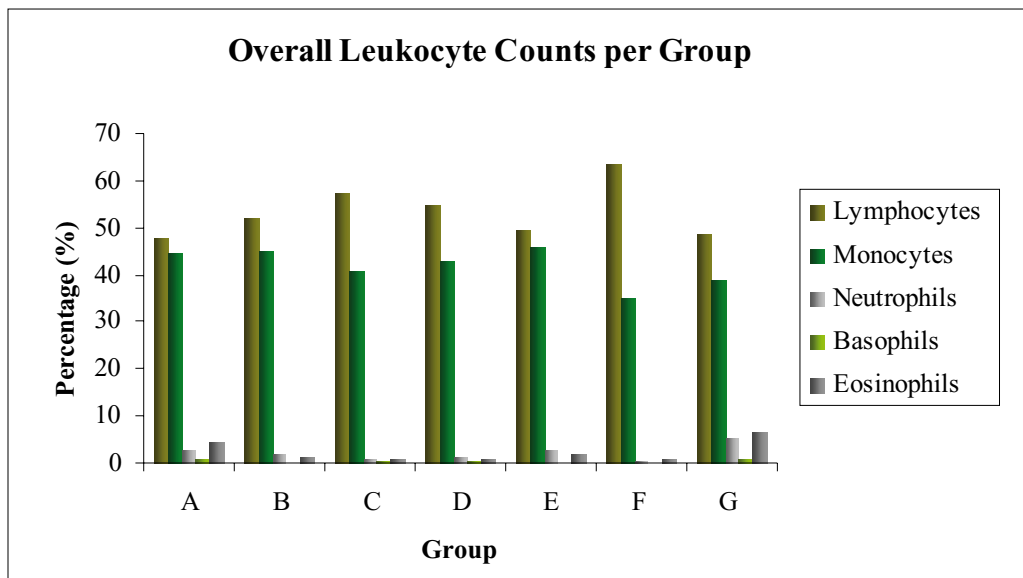


Figure 1. Mean leukocyte counts of five animals assessed per group A-G.

Results

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

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

est (zero) basophil count, in conjunction with the positive control group B. It is noteworthy that the high CoQ₁₀ group E and the positive control group B, with basophil counts of zero, have the highest monocyte counts overall. Further, the high CoQ₁₀ group E has the smallest lymphocyte to monocyte ratio of the experimental groups, in conjunction with the negative controls group A.

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

Monocyte, lymphocyte and basophil counts displayed no significant differences ($P > 0.05$) between any of the groups assessed. Eosinophil counts were significantly higher ($P < 0.05$) in the baseline group

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

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

* Significance was set at a level of 0.05

G than all other experimental groups assessed except for the negative control SWR/J mice of group A. Neutrophil counts of the baseline termination group were significantly higher ($P < 0.05$) only when compared to the combination group F.

Discussion

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

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

Monocytes mature continuously into macrophages upon migration into the tissues and are most abundant in the connective tissues, but continuously recirculate using the bloodstream as transport medium. Neutrophils are short-lived (only a few days) and

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

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

Eosinophils are thought to be important primarily

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

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

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

Basophils, like eosinophils, are present at the sites of inflammatory reactions. They are normally present in very low numbers in circulation and seem to have a similar role to that of eosinophils in the de-

fence against pathogens, as they are also recruited to sites of allergic reactions.

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

Conclusion

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

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

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

References

- Angelini C: Advances and Perspectives in Muscular Dystrophies. *Basic Appl Myol* 2002, 12 (1), 17-25.
- Bittner RE, LVB Anderson, E Burkhardt, R Bashir, E

- Vafiadaki, S Ivanova, T Raffelsberger, I Maerk, H Höger, M Jung, M Karabasiyan, M Storch, H Lassmann JA Moss, K Davison, R Harrison, KMD Bushby, A Reis*: Dysferlin deletion in SJL mice (SJL-Dysf) defines a natural model for limb girdle muscular dystrophy 2B. *Nat Genet* 1999, 23, 141-142.
- Diers A, M Carl, G Stoltenburg-Didinger, M Vorg-erd, S Spuler*: Painful enlargement of the calf muscles in Limb-Girdle Muscular Dystrophy Type 2B (LGMD2B) with a novel compound heterozygous mutation in DYSF. *Neuromuscul Disord* 2007, 17, 157-162.
- Eniva Corporation*: Co-Q10. 2006. Available online at: <http://www.enivamembers.com> [Accessed: 02/03/2008]
- Folkers K & R Simonsen*: Two successful double-blind trials with coenzyme Q10 (vitamin Q10) on muscular dystrophies and Neurogenic atrophies. *Biochim Biophys Acta* 1995, 1217, 281-286.
- Jain Foundation Inc*: Research into Miyoshi/LGMD2B. 2008. Available online at: <http://www.jain-foundation.org/projects.php> [Accessed: 16/04/2008]
- Janeway CA Jr, P Travers, M Walport, MJ Shlom-chik*: Immunobiology, the immune system in health and disease. 6th edition. Churchill Livingstone, London 2005.
- Leeson TS, CR Leeson, AA Paparo*: Text/Atlas of Histology. W.B. Saunders Company, Canada 1988.
- McElderry MQB*: Grape Expectations: The Resveratrol Story. 1999. Available online at: <http://www.quackwatch.org> [Accessed: 16/10/2008]
- Mitchell T*: Resveratrol: cutting-edge technology available today. Life Extension Foundation. 2008. Available online at: <http://www.lef.org> [Accessed: 16/10/2008]
- Roy H & S Lundy*: Resveratrol. Pennington Nutrition Series 2005, Number 7
- Suzuki N, MAoki, Y Hinuma, T Takahashi, Y Onodera, A Ishigaki, M Kato, H Warita, M Tateyama, Y Itoyama*: Expression profiling with progression of dystrophic change in dysferlin-deficient mice (SJL). *Neurosci Res* 2005, 52, 47-60.
- Thomas MS, AL Miller, NW Lukacs*: Chemokines and chemokine receptors in pulmonary disease. *Current Topics in Membranes* 2005, 55, 189-222.
- Vafiadaki E, A Reis, S Keers, R Harrison, LVB Anderson, T Raffelsberger, S Ivanova, H Hoger, RE Bittner, K Bushby, R Bashir*: Cloning of the mouse dysferlin gene and genomic characterization of the SJL-Dysf mutation. *Neuroreport* 2001, 12 (3), 625-629.