**Grape-seed extract (oligomeric proanthocyanidin) or N-acetylcysteine antioxidant supplementation several days before and after an acute bout of plyometric exercise**

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***Master of Science (Exercise Science)***

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# DECLARATION

*I, the undersigned, hereby declare that the work contained in this thesis is my own work and that I have not previously in its entirety or in part submitted it at any university for a degree.*

Signature: .....................................

Date: .....................................

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# DEDICATION

This thesis is presented in dedication to my supervisor: Professor Kathryn H. Myburgh and co-supervisor: Professor Carine Smith, for allowing me the opportunity, sharing their knowledge and imparting a love for science.

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# ABSTRACT

This thesis aims to determine whether supplementation with a grape-seed derived antioxidant, oligomeric proanthocyanidin (PCO) or the glutathione precursor, N-acetylcysteine (NAC) may prove beneficial as treatment for exercise induced muscle damage (EIMD) in athletes. In this double-blind cohort study, 21 healthy, uninjured male rugby-players in mid-season training phase, aged between 18 and 25 years were randomly divided into three treatment groups. Participants received 210 mg PCO, NAC or placebo treatment for 9 consecutive days. The study comprised a 6-day wash-out period (protocol days: -12 to -7), followed by a 6-day supplement loading period (protocol days: -6 to -1) a plyometric exercise intervention (protocol day 0) and continued supplementation for 2 days (protocol days: 1 to 2). The exercise intervention comprised 15 sets of 10 near maximal, vertical plyometric squat jumps. Blood samples and delayed onset of muscle soreness (DOMS) scores were collected on protocol days: -6, 0, 1 and 2. Assessments included serum creatine kinase (CK) activity, oxygen radical absorbance capacity (ORAC), malondialdehyde (MDA) and soluble vascular cell adhesion molecule-1 (sVCAM-1) concentrations over time as well as a differential circulating leukocyte count (neutrophils, lymphocytes, monocytes, eosinophils and basophils). Data analysis of CK activity revealed no significant differences between groups. However, PCO treatment prevented a significant peak in the CK response at 24 h (as seen in the placebo and NAC groups) when compared to baseline, pre and post readings ($p<0.05$). NAC supplementation significantly improved serum ORAC after the exercise intervention. By 48 h, serum ORAC had improved significantly from readings taken immediately post exercise ($p<0.05$) only in the NAC group. For all groups, absolute neutrophil counts peaked at 6 h post exercise from baseline or pre readings ($p<0.05$). In both NAC and placebo treated groups, neutrophil counts had decreased significantly in circulation by 24 h post exercise from the 6 h time-point ($p<0.05$). However, neutrophil counts only reached significantly lower levels by 48 h post exercise ($p<0.05$) in the group supplemented with PCO. The monocyte count also peaked significantly at 6 h post exercise when compared with other time-points before and after the exercise intervention ($p<0.05$) in all treatment groups. Neither antioxidant treatment significantly altered the responses of other leukocyte sub-populations, MDA or sVCAM-1 concentrations where main effects of plyometric exercise was evident. Although not statistically significant, a trend toward diminished sVCAM-1 expression with either antioxidant supplementation was apparent. These findings suggest that PCO supplementation (210mg/d) which includes a 7 day loading period may diminish plyometric EIMD by limiting (but not completely inhibiting) the neutrophil response. Secondary muscle

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damage may be prevented by partially blunting neutrophil infiltration, rather than only quenching free radicals released during the neutrophil oxidative burst. Furthermore, the finding that NAC supplementation improves serum ORAC only after exercise may provide added benefit when administered in combination with PCO.

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# OPSOMMING

Hierde tesis is daarop gerig om vas te stel of aanvulling met ‘n druifsaadekstrak (DSE) gederiveerde antioksidant: pro-antosianiedoliese oligomeer (PSO), of die glutathione voorloopermolekule, N-asetielsistien (NAS) voordelig beskou kan word as behandeling vir atlete onderhewig aan spierskade veroorsaak deur oefening. Gedurende hierdie dubbelblinde kohort studie is 21 gesonde, manlike rugbyspelers sonder beserings tussen die ouderdom van 18 en 25 jaar in middel-seison fase ewekansig in drie behandelingsgroepe verdeel. Deelneemers het elk 210 mg PSO, NAS of placebo-aanvulling geneem vir nege agtereenvolgende dae. Die studie het bestaan uit ‘n 6-dag uitwasperiode (protokoldae: -12 tot -7), as ook ‘n 6-dag aanvullings periode (protokoldae: -6 tot -1), gevolg deur ‘n pliometriese oefeningsintervensie (protokol dag 0) en verdere aanvulling tot en met 2 dae na die oefening (protokol dae: 1 tot 2). Die oefeningsintervensie het 15 stelle van 10 naastenby maksimale, vertikale pliometriese hurksponge behels. Bloedmonsters en vertraagde aanvang spierseerheid (VAS) tellings is op protokoldae: -6, 0, 1 en 2 geneem. Analiese het serum kreatien kinase (KK) aktiwiteit, suurstof radikaal absorpsie kapasiteit (SRAK), Malondialdahied (MDA) en oplospare vaskulêresel adhesie molekule-1 (oVAM-1) konsentrasie bepalings asook ‘n differentiële sirkulerende leukosiet seltelling ingesluit. KK aktiewiteit het geen merkwaardige verskil tussen groepe getoon nie. PSO aanvulling het wel gelei tot die voorkoming van ‘n merkwaardige piek in die KK response soos in die placebo en NAC behandelde groepe bevind is by die 24 h tydspunt in vergelyking met basislyn-, voor- en na-oefeningslesings ($p<0.05$). NAS het ‘n merkwaardige verbetering in serum SRAK getoon, maar eers teen 48 h na oefening. Slegs die NAS behandelde groep het op hierdie tydspunt ‘n betekenisvolle verbetering in SRAK getoon in vergelyking met lesings direk na oefening ($p<0.05$). Vir alle groepe is ‘n betekenisvolle toename in absolute neutrophiltellings waargeneem 6 h na oefening in vergelyking met basislyn- en vooroefeningslesings ($p<0.05$). Beide NAS en placebo-behandelde groepe het ‘n betekenisvolle afname in neutrophiltellings teen 24 h na oefening getoon in vergelyking met die 6 h tydspunt ($p<0.05$) maar met die PSO-behandelde groep word hierde afname eers teen 48 h waargeneem ($p<0.05$). Monosiettellings het in alle groepe 6 h na oefening ‘n betekinsvolle piek getoon ($p<0.05$). Waar slegs die hoofeffek van die pliometriese oefening betekenisvol was, het nie een van die twee antioksidant aanvullings ‘n merkwaardige verandering aan die respons van ander leukosiet sub-populasies, MDA of oVAM-1 konsentrasies getoon nie. Al kon statistiese beduidenheid nie bewys word nie, wil dit blyk dat ‘n verminderde oVAM-1 uitdrukking onstaan het in die geval van beide antioksidant-behandelde groepe. Tesame stel hierdie bevindinge voor dat PSO toediening (210mg/d)

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insluitende ‘n 7-dag aanvullingsperiode die vermoë verleen om die neutrophielrespons gedeeltelik te onderdruk (sonder om dit heeltemal te inhibeer) en sodoende spierskade verminder. Dus word verdere spierskade moontlik verlaag deur die voorkoming van neutrophil weefsel infiltrasie eerder as verwydering van reaktiewe spesies wat vrygestel word tydens oefening. Die bevinding dat NAS aanvulling serum SRAK eers na oefening merkwaardig verbeter, kan as voordelig beskou word, veral wanneer toegedien in samewerking met PSO om verdere spierskade te voorkom en herstelling vinniger te bewerkstellig.

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# TABLE OF CONTENTS

DECLARATION ..... i

DEDICATION ..... ii

ABSTRACT ..... iii

OPSOMMING ..... v

TABLE OF CONTENTS ..... vii

LIST OF FIGURES ..... xii

LIST OF TABLES ..... xiv

GLOSSARY ..... xv

CHAPTER 1

Background

1.1 Introduction to Rugby Union ..... 1

1.2 Plyometric Exercise ..... 3

1.2.1 Skeletal Muscle Physiology ..... 4

1.2.2 Skeletal Muscle Damage ..... 5

1.2.3 Exercise-Induced Muscle Damage ..... 5

1.2.4 Recovery ..... 8

1.3 Exercise Immunology ..... 9

1.3.1 The Inflammatory Response ..... 9

1.3.2 Exercise-Induced Inflammation ..... 9

1.3.3 Systems of Cell Recruitment ..... 12

1.3.4 Neutrophil and Macrophage Recruitment ..... 13

1.3.5 Satellite Cell Recruitment ..... 14

1.4 Neutrophils ..... 15

1.4.1 Hematopoiesis ..... 15

1.4.2 Extravasation ........................................................................................ 16

1.4.3 Functions.................................................................................................. 19

1.5 Oxidative Stress......................................................................................... 20

1.5.1 Secondary Muscle Damage........................................................................ 20

CHAPTER 2

Literature Review

2.1 Treatment Modalities ................................................................................ 22

2.2 Traditional treatments ............................................................................... 23

2.2.1 Anti-inflammatory treatments.................................................................... 23

2.2.2 Alternative Therapies................................................................................. 24

2.3 Antioxidants .............................................................................................. 25

2.4 Glutathione and N-Acetylcysteine ............................................................ 27

2.5 Grape-Seed Extract ................................................................................... 31

2.5.1 Introduction to Polyphenols ...................................................................... 31

2.5.2 Flavanoids.................................................................................................. 32

2.5.3 Proanthocyanidins...................................................................................... 34

2.5.4 Resveratrol.................................................................................................. 36

2.5.5 *Oxiprovin*™ ............................................................................................. 37

2.6 Summary of Literature Review.................................................................. 43

2.7 Hypotheses and Aims of the Current Study............................................... 43

2.7.1 Summary of Research Problem.................................................................. 43

2.7.2 Principal Hypothesis.................................................................................. 44

2.7.3 Additional Hypotheses............................................................................... 45

2.7.4 Aims of This Study .................................................................................... 45

2.7.5 Implications and Potential Clinical Application of Outcomes .................. 46

Stellenbosch University http://scholar.sun.ac.za

CHAPTER 3

Materials and Methods

3.1 Study Design ..... 47
3.2 Participants ..... 47
3.2.1 Inclusion Criteria ..... 48
3.2.2 Exclusion Criteria ..... 48
3.2.3 Ethical Considerations ..... 49
3.2.4 Instructions to Participants: ..... 49
3.3 Study Procedure ..... 50
3.4 Experimental Protocol (Time-Course) ..... 51
3.4.1 Protocol Day -12 to -6 (Wash-Out Period) ..... 52
3.4.2 Protocol Day -6 (Visit 1: Baseline Measurements) ..... 52
3.4.3 Protocol days -5 to -1 (No Visit: Treatment Loading Phase) ..... 52
3.4.4 Protocol days 0 (Visit 2: Plyometric Exercise Intervention) ..... 52
3.4.5 Protocol days 0 (Visit 3: 6 Hours Post Exercise) ..... 54
3.4.6 Protocol Days 1 and 2 (Visits 4 and 5: 24 and 48 Hours Post Exercise) ..... 54
3.5 Intervention protocols ..... 54
3.5.1 Induction of Muscle Damage ..... 54
3.5.2 Supplementation ..... 58
3.6 Data Collection ..... 60
3.6.1 Information Collection ..... 60
3.6.2 Whole-Blood Sample Collection ..... 61
3.7 Sample Analysis ..... 63
3.8 Statistical Analysis ..... 63

Stellenbosch University http://scholar.sun.ac.za

CHAPTER 4

Results

4.1 Sample Population Profile and Baseline Measurements ........ 64
4.1.1 Training Habits ........ 65
4.1.2 Health Status ........ 66
4.2 Non-Specific Indicators of Muscle Damage ........ 67
4.3 Serum ORAC and MDA Concentrations ........ 69
4.4 Serum Soluble VCAM-1 Concentrations ........ 69
4.5 Leukocyte Count ........ 72

CHAPTER 5

Discussion

5.1 Extent of Muscle Damage ........ 75
5.2 Antioxidant Status ........ 77
5.3 Oxidative Stress ........ 78
5.4 Soluble Adhesion Molecules ........ 79
5.5 Time-Course and Magnitude of Neutrophil Infiltration ........ 80
5.6 Limitations ........ 81

CHAPTER 6

Synthesis

6.1 Evaluation of Aims and Hypotheses ........ 84
6.2 Speculation ........ 85
6.3 Conclusion ........ 86
6.4 Implications and Clinical Applications of the Current Findings ........ 86
6.5 Directions for Future Research ........ 87

References ........ 88

Appendices ........ 110

8.1 Appendix A: *Oxiprovin*™ Package Insert ........ 110
8.2 Appendix B: *Warren Chem Specialites (Pty) Ltd* ISO 9001 Registration Form .... 111
8.3 Appendix C: HREC Response to Ethics Application ........ 112
8.4 Appendix D: Letter Indicating Amendments to Application for Ethical Approval... 113
8.5 Appendix E: Approval of Amendment to Application for Ethical Approval ........ 115
8.6 Appendix F: Participant Recruitment Letter ........ 116
8.7 Appendix G: Participant Information Leaflet and Consent Form ........ 118
8.8 Appendix H: Participant Personal Information Forms ........ 128
8.9 Appendix I: Participant Diet and Exercise Log ........ 132
8.10 Appendix J: Retrospective Supplement Log ........ 133
8.11 Appendix K: Visual Analog Scale (VAS) for Pain Assessment ........ 135
8.12 Appendix L: Protocol Used for the ORAC Assay ........ 136
8.13 Appendix M: Serum TBARS Method Protocol ........ 139

Notes ........ 140

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# LIST OF FIGURES

## Chapter 1

**Figure 1.1:** Designated positions and player numbers of a rugby union team.................... 2

**Figure 1.2:** The Crusaders performing plyometric jumping drills using XLR8 PlyoHurdles (50 cm) as part of their training regime........................................................ 4

**Figure 1.3:** A minor and moderate partial muscle tear in relation to the anatomical structures: the primary and secondary fascicles respectively .......................................... 7

**Figure 1.4:** Driving regeneration: the interaction between muscle and immune cells. ......12

**Figure 1.5:** Time-course of inflammatory immune cells after injury .................................14

**Figure 1.6:** Peripheral blood smear including a large neutrophil granulocyte...................15

**Figure 1.7:** The Multistep model of leukocyte extravasation ..........................................19

## Chapter 2

**Figure 2.1:** Cellular Antioxidant Protective Mechanisms................................................27

**Figure 2.2:** Schematic representation of potential biochemical processes involved in the fatigue sparing effects of compounds that increase the muscle glutathione pool ....29

**Figure 2.3:** Over-the-counter products that contain NAC as active agent ........................30

**Figure 2.4:** The commercially available grape-seed derived antioxidant supplement; Oxiprovin™ ..........................................................................................37

**Figure 2.5:** A qualitative comparison of muscle recovery after chronic vs. acute PCO supplementation ............................................................................42

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## Chapter 3

**Figure 3.1:** Visual representation of the 15-day experimental protocol ............................51

**Figure 3.2:** Participants performing the plyometric jump exercise protocol while being monitored (protocol day 0)..........................................................................53

**Figure 3.3:** Correct squat technique ......................................................................56

**Figure 3.4:** The contractile phases of a plyometric squat jump .......................................57

**Figure 3.5:** Identical OxiprovinTM (left), NAC (middle) and microcrystalline placebo powder (right) capsules. Neither of the treatment groups could be characterised by a scent, flavour or appearance. ..........................................................59

## Chapter 4

**Figure 4.1:** The effect of plyometric exercise on non-specific markers of muscle damage for all treatment groups over time ..........................................................68

**Figure 4.2:** The effect of plyometric exercise on the serum ORAC of all treatment groups over time ..........................................................................................70

**Figure 4.3:** The effect of plyometric exercise on numbers of circulating neutrophils and monocytes in all treatment groups over time ..........................................73

Stellenbosch University http://scholar.sun.ac.za

# LIST OF TABLES

## Chapter 1

**Table 1.1:** Involvement of various cell types in inflammation and muscle damage..........11

## Chapter 4

**Table 4.1:** Sample population profile (denoted as mean ± SD) .......................................65

**Table 4.2:** Distribution of grouped player positions within treatment groups.....................65

**Table 4.3:** Data obtained for sVCAM-1, ORAC and MDA analyses ................................71

**Table 4.4:** Data obtained after performing a full leukocyte count of samples from all treatment groups at all time-points.....................................................................74

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# GLOSSARY

| | |
|---|---|
| **AA** | arachidonic acid |
| **ATA** | atmospheres absolute |
| **ATP** | adenosine triphosphate |
| **BM** | basement membrane |
| **C5** | complement components |
| **CAM** | cellular adhesion molecule |
| **CD** | cluster of differentiation |
| **CHD** | coronary heart disease |
| **CINC-1** | cytokine-induced neutrophil chemoattractant |
| **CK** | creatine kinase |
| **COX** | cyclooxygenase |
| **CRP** | C-reactive protein |
| **DMSO** | dimethylsulfoxide |
| **DNA** | deoxyribonucleic acid |
| **DOMS** | delayed onset of muscle soreness |
| **EIMD** | exercise-induced muscle damage |
| **ELISA** | enzyme-linked immunosorbent assay |
| **E-selectin** | endothelial-specific selectin |
| **FABP** | fatty acid binding protein |
| **G-CSF** | granulocyte colony-stimulating |
| **GF** | growth factor |
| **GH** | growth hormone |

| | |
|---|---|
| **GM-CSF** | granulocyte-macrophage colony-stimulating factor |
| **GPCR** | G-protein coupled receptor |
| **GPx** | glutathione peroxidase |
| **GSE** | grape-seed extract |
| **GST-α-1** | glutathione-S-transferase-alpha-one |
| **GTH** | glutathione |
| **h** | hour/s |
| **•HO** | hydroxyl radicals |
| **$H_2O_2$** | hydrogen peroxide |
| **HBOT** | hyperbaric oxygen therapy |
| **$HO^-$** | hydroxide ion (in neutral form) |
| **HREC** | Human Research Ethics Committee |
| **HSVEC** | human saphenous vein endothelial cell/s |
| **HUVEC** | human umbilical vein endothelial cell/s |
| **ICAM-1** | intercellular adhesion molecule one |
| **ICAM-2** | intercellular adhesion molecule two |
| **IFN-y** | interferon gamma |
| **IL-12** | interleukin twelve |
| **IL-1β** | interleukin one beta |
| **IL-6** | interleukin six |
| **IL-8** | interleukin eight |
| **IRB** | International Rugby Board |
| **LFA-1** | leukocyte function antigen one |
| **L-selectin** | leukocyte-specific selectin |

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| | |
|---|---|
| **LTB4** | leukotrienes |
| **M1** | pro-inflammatory mactrophage subtype one |
| **M2** | anti-inflammatory macrophage subtype two |
| **MAdCAM-1** | mucosal addressin cellular adhesion molecule |
| **MAPK** | mitogen activating protein kinase |
| **Mb** | myoglobin |
| **MCC** | Medicines Control Council |
| **MCP-1** | monocyte chemotactic protein one |
| **MHCf** | foetal myosin heavy chain |
| **MIP-1α** | macrophage inflammatory protein one alpha |
| **MPC** | myogenic precursor cell |
| **MPO** | myeloperoxidase |
| **MRC** | Medical Research Council |
| **MRF** | myogenic regulatory factor |
| **mRNA** | messenger ribonucleic acid |
| **n** | number of subjects |
| **NAC** | N-acetylcysteine |
| **NADPH** | nicotinamideadenine dinucleotide phosphate |
| **NFκB** | nuclear factor-kappa B |
| **NO** | nitric oxide |
| **NSAIDs** | non-steroidal anti-inflammatory drugs |
| **$O_2^-$** | superoxide anion |
| **$ONOO^-$** | peroxynitrite |
| **ORAC** | oxygen radical absorbance capacity |

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| | |
|---|---|
| **PCO** | proanthocyanidin oligomer |
| **PDTC** | pyrrolidine dithiocarbamate |
| **PECAM-1** | platelet endothelial cellular adhesion molecule one |
| **PG** | prostaglandins |
| **PGE** | pro-inflammatory prostaglandins |
| **PLA** | placebo |
| **P-selectin** | platelet-specific selectin |
| **RICE** | rest, ice, compression and elevation |
| **RNS** | reactive nitrogen species |
| **ROS** | reactive oxygen species |
| **SC** | satellite cell |
| **SD** | standard deviation |
| **SOD** | superoxide dismutase |
| **SSC** | stretch-shortening cycle |
| **SSc** | systemic sclerosis |
| **sVCAM-1** | soluble vascular cellular adhesion molecule one |
| **TBARS** | thiobarbituric acid reactive substances |
| **TGF-β** | transforming growth factor beta |
| **TNF-α** | tumour necrosis factor alpha |
| **TxA2** | thromboxane A2 |
| **VAS** | visual analog scale |
| **VCAM-1** | vascular cellular adhesion molecule one |
| **VLA-4** | very-late antigen four |
| **WADA** | World Anti-Doping Association |

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# CHAPTER 1:

# BACKGROUND

## 1.1 INTRODUCTION TO RUGBY UNION

Participation in physical activity or exercise, particularly in the form of sport, has been prominent through the ages in many cultural groups (31). A noteworthy example is the Olympic Games, which began in Greece and continues to take place today, with the next event taking place in Rio de Janeiro in 2016 (224). Exercise-conferred health benefits and socio-cultural implications have prompted the development of sport to previously unforeseen levels in recent years and professional sport has advanced into a lucrative industry (31).

Since union rugby turned professional in 1995, the sport has grown momentously and the 2007 World Cup attracted a total audience of over four-billion viewers (87, 169, 174). As the industry surrounding the sport continues to grow, so do the financial incentives and other pressures on club and national-level teams. This places vast stress on the physical development of players and their swift recovery after games and return to activity after injury (87). Rugby union is a contact sport that involves kicking, ball-passing and vigorous sprinting while embracing impact with other players. During the 80 minutes of game-play, each of the 15 players (per side) has a designated position and number as outlined by the *International Rugby Board* (IRB) (105). These positions are grouped (as forwards or backs) according to the physical demands placed on the players (see Figure 1.1) (203). Gameplay requires athletes to regularly perform manoeuvres that result in the swift adjustment of direction, in order to evade opposition players. The change of direction (also known as a cutting task) necessitates rapid player deceleration using one leg followed by acceleration using the other (87). The leg used to decelerate the athlete needs to generate significant stability while the other needs to rapidly decelerate the load received and quickly accelerate this weight into a new direction. This is a form of eccentric contraction and the tactic propels the player along a different course (87).

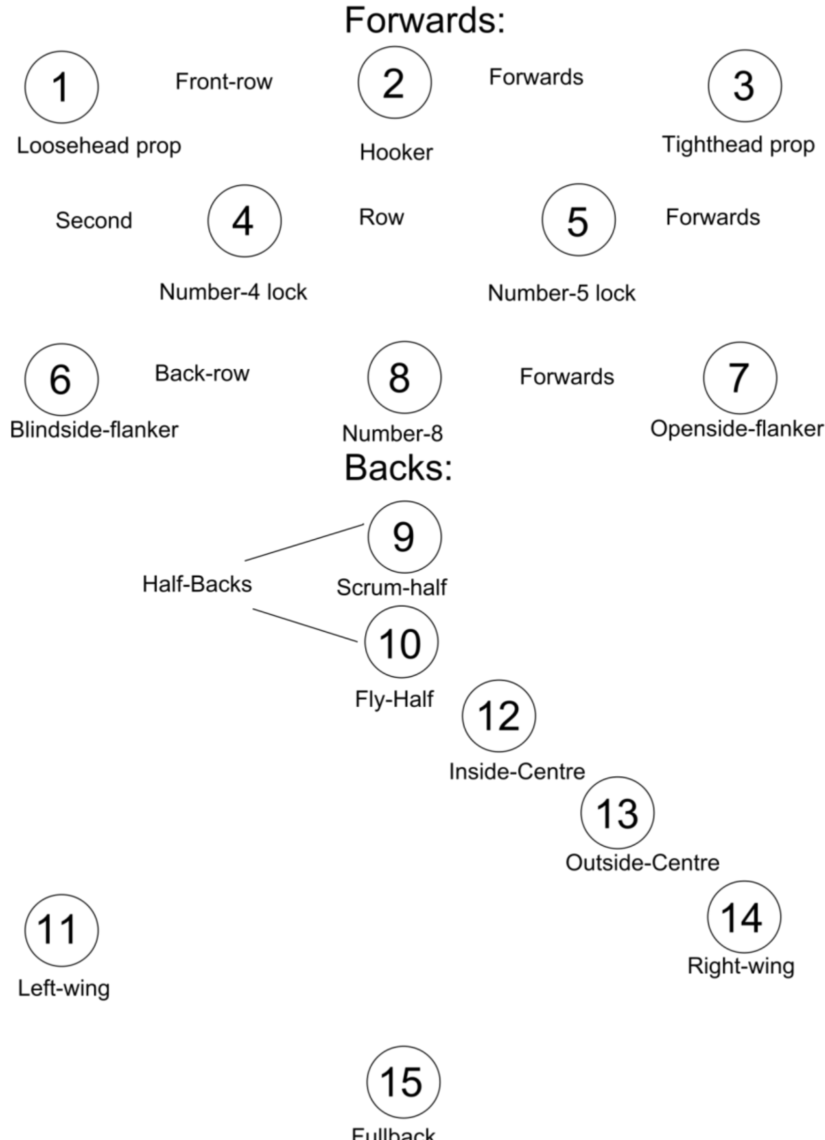


***Figure 1.1: Designated positions and player numbers of a rugby union team.*** *Adapted from Outsports Online (203).*

For developing and elite players, physical preparation is considered the most important contributor to success (68). The physical requirements include muscle strength (maximal force generating capacity at a given speed) and power (the product of the velocity at which the force is generated) (67, 124). In particular these have been used as selection criteria for the forwards taking part in scrums, rucks and mauls (67) . Given that muscle strength is crucial during these contact situations, traditional belief holds that forwards should possess greater strength than backs (67, 135, 217). However, a recent evaluation comprising several strength tests indicated that both collegiate forwards and backs performed similarly on all tests (266). Strength and power training are thus essential to players of all positions and plyometric training has received special consideration in rugby training and developmental models.

## 1.2 Plyometric Exercise

Studies have confirmed that training regimes incorporating plyometric exercises (such as skipping, stiff ankle hops, sprint-specific bounding, drop and counter-movement jumps as well as vertical jumps) may improve muscle power and strength, sprinting speed and agility (87, 223, 260). The fact that plyometric drills activate similar stretch-shortening processes in the M. quadriceps to those that occur during cutting tasks implies that this training develops sport-specific strength and power (106, 265). However, the intense impact and loading experienced during the ground contact phase of plyometric jumping may induce skeletal muscle micro-damage (68).

Plyometric exercise encompasses a maximal stretch followed by the generation of a forceful muscle contraction, repeatedly. A distinctive characteristic is the dynamic conversion between eccentric and concentric action which inadvertently causes the exercising muscle to experience an extreme shearing force. Thus the repeated fast switch from an eccentric to a concentric contraction may accentuate (micro-) damage caused to the active muscle when compared with that caused by other modes of training. Plyometric exercise is said to comprise three phases, collectively termed the stretch-shortening cycle (SSC) (126, 127). From rest, or during landing, the eccentric phase entails the motion during which the muscle stretches (or lengthens) in preparation for the next jump while synchronously producing force to control the movement. Upon completion of the eccentric phase, a transitory period of quick change from an eccentric to a concentric contraction occurs and is known as the amortization phase. This transition period is thought to be the principal phase for plyometric exercise-induced muscle damage (EIMD) because of the extreme shearing forces that are generated. The final phase, the concentric phase, entails forceful concentric muscular contraction which finally brings about the desired movement (propulsion/jump) (265)

In light of the distinctiveness of this workout, plyometric exercise is typically employed with the end-goal of improving maximal power out-put. Also, adaptation to this type of training may promote quicker rehabilitation and prevent muscle damage (68). Plyometric jumping protocols have been incorporated into the training regimes of several other elite sports teams including soccer, volleyball and basketball, and have been employed by runners, and cyclists including Lance Armstrong, and *The Crusaders* (49, 106, 255). *The Crusaders* are a professional rugby union team based in Christchurch, New Zealand. They are the most successful team in the history of the *Super Rugby* competition having earned seven titles during the decade preceding 2008 (Figure 1.2) (49).

However, because most plyometric jump exercise protocols requires athletes to perform several sets of repeated muscle contractions, delayed onset of muscle soreness (DOMS) often results (282). This is accompanied by escalations in serum indices of collagen breakdown (265), which occur in conjunction with the morphological changes (258). This phenomenon is particularly prominent in individuals who are untrained, or have not previously adapted by performing similar exercises.

***Figure 1.2: The Crusaders performing plyometric jumping drills using XLR8 Plyo Hurdles (50 cm) as part of their training regime.*** *Photograph obtained from R80Rugby Online (212).*

### 1.2.1 Skeletal Muscle Physiology

Skeletal muscles cells (muscle fibres) consist of myofibrils. Myofibrils are comprised of several proteins including contractile proteins (actin and myosin), accessory proteins (titin and nebulin) and the regulatory proteins (troponin and tropomyosin). These are organised into sarcomeres, the smallest functional (contractile) units of muscle (246). During concentric contractions the sarcomeres shorten. During isometric contractions they remain of static length, while during eccentric contractions sarcomeres lengthen and are exposed to considerably more risk of muscle damage than during the other phases, despite a lower metabolic demand (45).

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### 1.2.2 Skeletal Muscle Damage

Skeletal muscle damage (comprising myofibrillar damage) may occur in several ways. Contusion injuries are frequently reported during accidents or sport-play and result from external causes. Contusion injuries occur when the impact (opposite force) of a moving, but non-penetrating object (e.g. cricket ball, hockey stick, fist or other bony body-part of a team-mate or opponent), causes the rupture of muscle fibres at or adjacent to the area of impact (109). Internal causes, such as repeated bouts of contraction (or a sudden forceful contraction) may also induce muscle damage tears, especially during fatigue (151).

### 1.2.3 Exercise-Induced Muscle Damage

EIMD has become a prominent theme in exercise physiology (206). Numerous studies and publications have presented and reviewed evidence that exercise may result in muscle damage and inflammation in otherwise healthy skeletal muscle [see review by Paulsen *et al.* (206)]. EIMD is associated with transient impairment of muscle function (force- and power generating capacity) post exercise (206). However, severe muscle damage, inflammation and a prolonged period of recovery (>1 week) may be inflicted by extreme exercise protocols such as those including maximal eccentric exercise across a large range of motion (such as plyometric jumping), in an unaccustomed individual (206). The exercise type, intensity, angular velocity, speed, duration, as well as the trained-state and individual athletes' genetic traits (such as fibre length) will primarily determine the extent of muscle damage (198). Therefore, substantial inter-individual variation may be expected in response to a specific exercise and during subsequent processes (206).

The initial priorities after EIMD include the removal of debris and the restoration or replacement of damaged tissue. Therefore, EIMD is associated with phagocytic leucocyte accumulation in the affected muscle and surrounding tissue and satellite cell (SC) activation (206). The extent to which this occurs, as well as the magnitude of the cytokine response, depends on the degree of muscle damage incurred. However, the systemic cytokine response may be linked more closely to the metabolic demands of exercise rather than muscle damage (206). Cytokines are small cellular signalling proteins that are produced by several cell types; although they are primarily known to originate from immune cells and muscle cells. They recruit immune cells to the site of injury, contribute to the regulation of SCs and are crucial to the restoration of muscle structure. Specifically interleukin six (IL-6), interleukin one beta (IL-1β) and tumour necrosis factor alpha (TNF-α) have been shown to be involved in regeneration (248). A comprehensive review by Smith *et al.* discusses

cytokines with pro- and anti-inflammatory properties, their cellular sources and major activities (248). For the purpose of this thesis, we will focus on the muscle damage caused by the eccentric component of exercise.

#### 1.2.3.1 Eccentric Exercise-Induced Muscle Damage

From as early as the 19th century, the physiological responses to eccentric exercise have been investigated in human studies (8, 206). Since then several researchers, most notably Gordon, Huxley, Julian (85, 86) and Morgan (111, 112), further elucidated the muscle, sarcomere and myofibrillar changes induced by contraction accompanied by stretch (206). The ‘popping sarcomere hypothesis’, initially described by Morgan and Proske (183) aimed to explain the disruption of myofibrillar structures after eccentric contractions (206). It is now well established that eccentric exercise may result in the acute breakdown of skeletal muscle. The severity of the damage is proportional to the size of the tear (see Figure 1.3). Thus, tearing of a single fibre (average diameter of 60 μm) remains without clinical relevance. However, the involvement of adjacent connective tissues (including the endomysium, perimysium, epimysium and the fascia) may also contribute to the severity, and differentiate minor from moderate partial muscle tears. The magnitude of the disruption is said to be dependent upon several factors including exercise intensity or duration and adaptation (44, 82, 126, 127).

The mechanical basis for muscle damage caused by eccentric exercise is explained in terms of stretch-induced strain imposed on the fibre while contracting against gravitational force (79). In addition, it has been shown that the eccentric component of muscle contractions employ fewer motor units (less muscle fibres) which predictably subjects the dedicated fibres to greater stress during this phase. The muscle damage caused by the eccentric component of exercise may be characterised by pervasive micro-damage of the fibres that were specifically engaged during the action. This holds true for all fibre types, although currently it is believed that injury is more likely to affect fast-twitch fibres (79). Thus the eccentric exercise-induced partial tears (both moderate and minor), compromise the structural and so also the functional integrity of the muscle (185). Thus, the resultant decrease in functional capability (maximal power out-put and force generating capacity) is said to be in direct proportion to myofibrillar disruption (44, 127). The morphological degeneration of myofibres is associated with fatigue, DOMS, inflammation and the release of a plethora of chemical mediators and both contractile and cytoplasmic proteins into the circulation. (44, 82, 103, 204, 282).

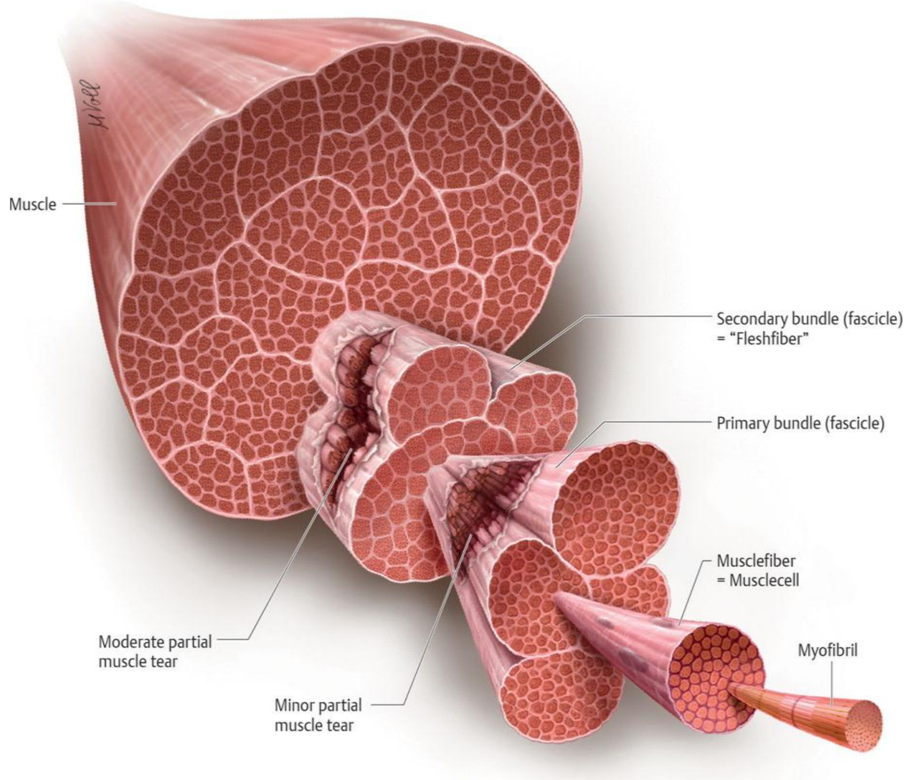


***Figure 1.3: A minor and moderate partial muscle tear in relation to the anatomical structures: the primary and secondary fascicles respectively.*** *Adapted from: Mueller-Wohlfahrt, et al.* (185).

#### 1.2.3.2 Markers of Muscle Damage

Myofibrillar disruption is accompanied by the escape of several proteins and enzymes, which consequently results in their elevation in circulation. These may serve as (non-specific) serum/plasma markers of muscle damage. The circulatory increase of such analytes varies according to the severity of the damage and time elapsed since onset. Also, depending on the known functions of these molecules, their peak concentrations are known to occur at different times and their use as markers of muscle damage are therefore selected accordingly. Although considered non-specific, commonly used markers include creatine kinase (CK), C-reactive protein (CRP), Myoglobin (Mb), and fatty acid binding protein (FABP). Despite recent controversy regarding its use, the measure of CK activity continues to serve as the standard circulatory marker of muscle damage (192). However, the measurement of muscle function as reflected by force-generating capacity (e.g. maximal concentric or isometric strength) is generally considered to be more reliable and valid for assessing the degree of muscle damage (206). Recent evaluation of the validity of power out-put as a more accurate functional marker of muscle damage presented no substantial evidence in favour of this postulate (214).

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#### 1.2.3.3 Extent of Damage

Muscle damage may be categorized as severe, moderate or mild according to loss of function and clinical impairment (109). Severe strains/contusion injuries result in nearly complete impediment of function, and are characterised by tears which extend across a significant area of the muscle. This type of injury may have various clinical implications and often requires invasive procedures for successful recovery. However, injuries that result in a partial tear across the muscle (secondary fascicles) is classified as moderate. A mild strain (or contusion) is associated with minimal or no loss of function accompanied by slight swelling and discomfort. Only a few muscle fibres (primary fascicles) are torn (185). The latter are more frequently reported in sport and exercise related injuries.

### 1.2.4 Recovery

Following skeletal muscle damage, several mechanisms encompassing interactions between cells, extra-cellularly secreted factors and the cellular matrix are involved in the regulation of the regeneration process. These processes include the degeneration of damaged myofibres, the removal of debris and myogenesis (38), while the regulatory factors involved primarily include cytokines, growth factors (GFs), and myogenic regulatory factors (MRFs). Growth factors also contribute to SC activation, proliferation and/or differentiation, by influencing the balance between proliferation and differentiation. Although cytokines and GFs are crucial to SC activation, migration and the proliferation of the SC-derived myogenic precursor cells (MPCs), the entire process of muscle regeneration is essentially regulated by the MRFs (78, 89). These proteins (MyoD, Myf5, myogenin and MRF4) contribute to both the positive and negative regulation of SC activation, proliferation and differentiation (279). SCs are located beneath the myofibrillar basement membrane (BM) (22, 89) and under normal circumstances remain quiescent within mature myofibres. However, in response to stimuli (such as injury), they may be activated, re-enter the cell cycle and proliferate to form new myoblasts. The newly formed myoblast may differentiate and fuse with damaged myofibres or each other, forming multi-nucleated myofibres (93). Thus SCs function to repair muscle fibre ultrastructure either by replacing or repairing damaged muscle fibres (48, 235). Although SC activation is usually limited to the affected area, they may be recruited from adjacent fibres when the connective tissue between the affected fibres is also compromised by the injury (234). However, SC activation also contributes to the formation of potentially harmful reactive species (235). Factors contributing to the production of reactive species, and in particular reactive oxygen species (ROS) will be highlighted through-out this thesis.

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The natural progression of events subsequent to muscle damage (including tissue repair and remodelling processes) are carefully modulated by an intricate scheme of interactions between the immune and musculoskeletal systems (207), as discussed below.

## 1.3 Exercise Immunology

The multifaceted healing process succeeding skeletal muscle damage includes a central role for the immune system in exacerbating the existing tissue damage, augmenting regeneration and influencing fibrosis (248).

### 1.3.1 The Inflammatory Response

The immune system encompasses several structural components (spleen, bone-marrow and the lymphatic system), immune cells (leukocytes), and a vast number of cytokines, all of which are involved with the onset or resolution of inflammation. These reactions are clinically characterised by several symptoms most notably including increased sensitivity, pain, redness and swelling of the affected area. Feverishness has also been reported, although less frequently. These symptoms are mainly caused by vasodilation, an increase in vascular permeability, oedema, the appearance of acute phase proteins and further downstream effects of histamine (52). Finally, subduing these processes following a rapid and effective inflammatory stimulus is essential for tissue regeneration to proceed successfully (248).

### 1.3.2 Exercise-Induced Inflammation

Even an rest, both myocytes and immune cells maintain low levels of cytokine production (serum TNF-α: ±3.5 pg/ml and IL-6: ±2 pg/ml and) so that cellular functions may be regulated and homeostasis maintained (148). However, during the immediate events following injury and during the regeneration process, the relative levels of muscular and systemic cytokines fluctuate as a dynamic scheme of communication occurs between muscle and inflammatory cells. The proportion to which each of these cell types are responsible for the injury-related cytokine release is not yet known (71). For example, increases to circulatory IL-6 were believed to result from the infiltrated immune cells at the affected area following a contusion injury or eccentric EIMD (94, 207). However, it has since been shown that large amounts of IL-6 may be produced and secreted merely by the contraction of a muscle (253). Regardless of the source of cytokine production and release,

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there is consensus that there is interaction between cytokines, growth factors, muscle tissue and various immune cells and that this will influence the following:

- the time-to-onset of the inflammatory response,
- the duration of the immune response (short- vs. longer-term adaptation) and
- whether a change in favour of a particular response (pro- vs. anti-inflammatory) will compromise the other.

EIMD is associated with a discernible upsurge in the release of pro-inflammatory cytokines, including IL-6, IL-1β and TNF-α (154). The levels of pro-inflammatory cytokines correlate with the activation of the oxidative-stress sensitive nuclear factor-kappa B (NFκB) (23). Resident macrophages also release pro-inflammatory cytokines (IL-1β and TNF-α), thereby recruiting monocytes and promoting SC activation, ultimately promoting muscle regeneration (32, 261).

The early phase of recovery is characterised by the release of pro-inflammatory cytokines (and subsequently additional tissue damage), while the concentrations of the anti-inflammatory cytokines typically increase later. The anti-inflammatory cytokines, also released by both muscle and immune cells are generally associated with the stimulation of tissue repair (158). For details see Table 1.1.

***Table 1.1: Involvement of various cell types in inflammation and muscle damage.***

| Cell type | Cytokines/ Growth factors | Injury-related activity | Reference |
|---|---|---|---|
| **Neutrophils** | IL-1<br>IL-6<br>IL-8<br>TGF-β<br>TNF-α | Source of pro-inflammatory cytokines<br>First leukocyte to infiltrate injury site<br>Phagocytosis of necrotic myofibres and cellular debris | (32)<br>(32)<br>(261) |
| **Monocytes/ Macrophages** | FGF-2<br>IGF-1<br>IL-1<br>IL-6<br>TGF-β<br>TNF-α<br>LIF | Source of growth factors, cytokines and reactive species<br>Sending survival factors to regenerative cells<br>Promote muscle injury or proliferation *in vitro* and *in vivo* | (261)<br>(32) |
| **Fibroblasts** | IL-1<br>IL-6<br>IL-8 | Produce chemotactic signals for circulating inflammatory cells<br>Help formation of connective tissue scar | (110) |
| **T lymphocytes** | IL-1<br>IL-2<br>IL-6<br>TNF-α<br>TGF-β<br>MIF<br>IFN-γ<br>TNF-β | Involved in immediate hypersensitivity via IL-1 and 6<br>Involved in delayed sensitivity reactions via IFN-γ, TNF-β and IL-2 | (242) |
| **B lymphocytes** | IL-1<br>IL-2<br>IL-6<br>TNF-β | Involved in antibody formation | (196) |
| **NK cells** | IL-1<br>TNF-β<br>IFN-γ | Causes an increase in lymphocyte concentration | (29) |
| **Eosinophils** | IL-6 | Capable of generating reactive oxygen species | (189) |
| **Platelets** | TGF-β | Secretion of adherence factors (P-selectin) to help neutrophils gain access to site of injury | (157)<br>(242) |
| **Injured skeletal muscle cells** | IL-1<br>IL-6<br>TGF-β<br>FGF<br>IGF<br>HGF<br>LIF | Release growth factors and cytokines which helps to activate regeneration | (132)<br>(110) |

**Table adapted with permission from Kruger MSc Thesis* (130)

### 1.3.3 Systems of Cell Recruitment

Capillaries located at or around the affected muscle fibres may rupture upon injury, resulting in exposed collagen. Platelets (present in circulation) may become activated as result of binding to the exposed collagen and subsequently release pro-inflammatory mediators including 5-hydroxy tryptamine (serotonin), thromboxane A2 (TxA2), and histamine. The latter is also secreted by surrounding mast cells and increases blood flow to the affected region (109, 207). Thereby, immune cells including neutrophils and primary macrophages (M1) may gain direct access to the injury zone (262) (Figure 1.4). The permeability of the surrounding (healthy) capillaries is improved, thereby allowing plasma proteins and immune cells access to the surrounding tissue seconds after injury (52, 243). Additionally, chemotactic factors (released as part of the initial inflammatory response) contribute to the attraction of these immune cells although the dynamics of the process of attraction changes with time elapsed since injury (262).

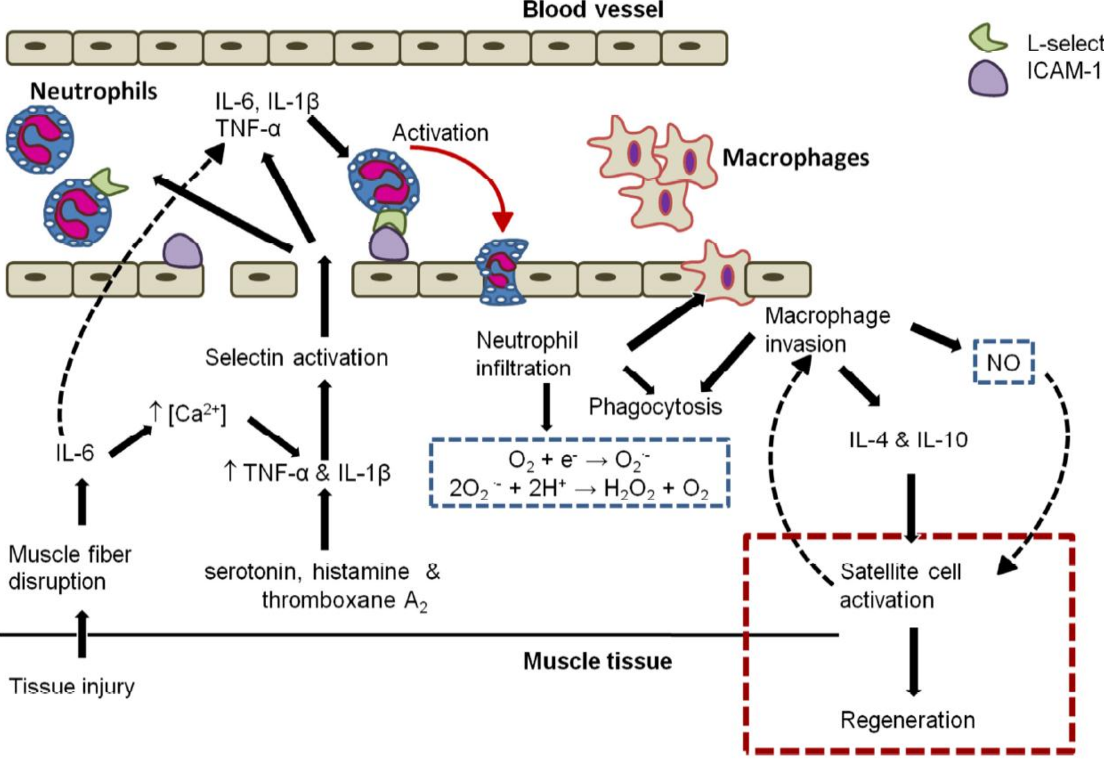


***Figure 1.4: Driving regeneration: the interaction between muscle and immune cells.*** *Adapted from Kruger Ph.D. thesis* (129).

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### 1.3.4 Neutrophil and Macrophage Recruitment

Neutrophils are the most abundant immune cells at the damaged site during the first couple of hours and days after injury. Pro-inflammatory mediators (released by the affected tissue) elicit an escalation in the release of neutrophils from storage sites; principally the spleen and bone-marrow. Their mobilization dramatically increases the circulating neutrophil count, thus allowing a high rate of neutrophil infiltration during the first few hours after injury (243, 261). The specific time-point at which an increase in circulating neutrophils becomes evident differs according to the type and severity of the damage incurred (152, 153, 211). However, their removal from circulation (either due to infiltration into damaged tissue, or phagocytic action of other immune cells) has been shown to be similar in various exercise trials (154, 252). The neutrophil response (represented by number of circulating neutrophils) peaks at 24 hours (h) post injury, after which the count decreases and returns to normal at approximately 72 h as inflammation is gradually resolved (248). The decreasing neutrophil response coincides with the infiltration of mononuclear cells and appearance in tissue of primary (M1) and secondary (M2) macrophages (144). See Figure 1.5 for the chronological series of events.

Within one hour post injury, neutrophils begin to phagocytose debris (263). However, they also play other roles such as communicating with the myofibres progenitor cells. IL-6, TNF-α, IFN-γ, IL-1β, interleukin 12 (IL-12) and transforming growth factor beta (TGF-β) (35), all influence myoblast differentiation and/or proliferation *in vitro* (93, 137). Also, because the neutrophil α4-integrin receptor binds to vascular adhesion molecule-1 (VCAM-1), which is expressed by both myoblast and myotubes *in vivo*, neutrophils may be involved directly with the activation of SCs (236). Neutrophils play another proactive role in the facilitation of muscle repair (3, 9, 35, 180) by promoting the accumulation of macrophages (178). Macrophages start to infiltrate the affected tissue roughly 24 - 48 h post injury (63).

The early responding macrophages (M1) exacerbate the inflammatory process, while those recruited during the later response (M2) aid in muscle regeneration mainly by the distinctly different factors released by each sub-type. Additionally, M1 macrophages can convert to the M2 phenotype although the mechanism that regulates this change remains to be elucidated (220, 262).

Although neutrophils and macrophages are both phagocytic, their roles in inflammation differ substantially. During phagocytosis, both produce reactive species to aid with the removal of debris and dead or damaged tissue from the necrotic area. However, each is recruited to the affected area at the appropriate time-point for that cell type.

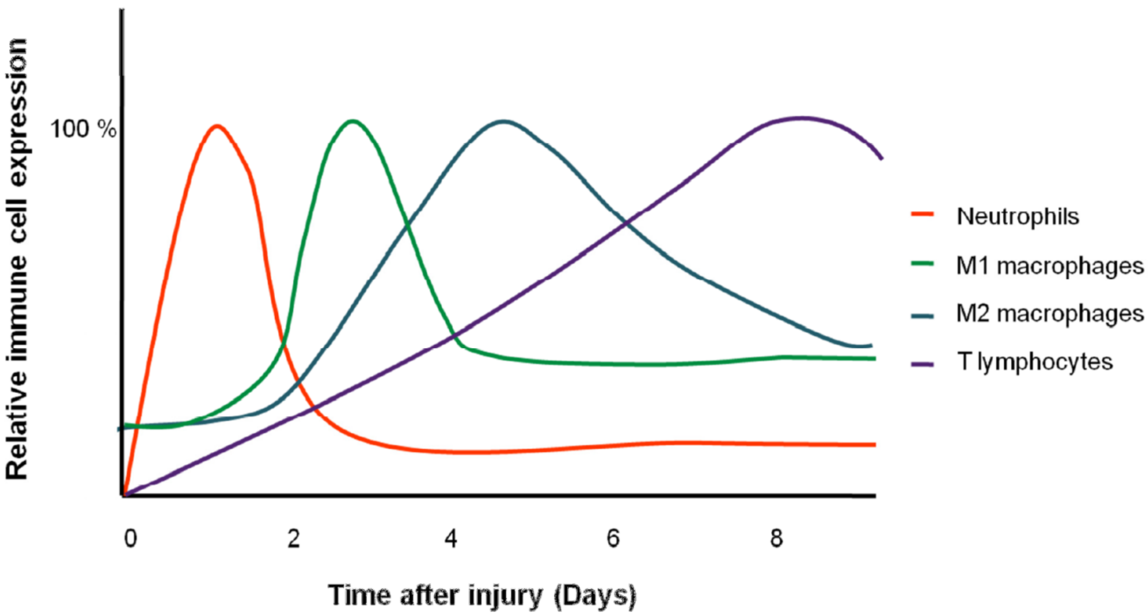


***Figure 1.5: Time-course of inflammatory immune cells after injury.*** *100% indicates peak response to injury. Adapted from Smith et al.* (248)

### 1.3.5 Satellite Cell Recruitment

Myogenesis is impaired in the absence of macrophage infiltration and it is accepted that macrophages contribute distinctively to muscle regeneration (141). Macrophages stimulate SC proliferation and maturation, and recruit additional macrophages by producing large amounts of nitric oxide (NO). The local macrophage count is proportionate to the recruitment and activation of more SCs (5, 40).

Immune stimulation of SCs further reinforces the presence of macrophages. Chazaud *et al.* (40) demonstrated *in vitro* that SCs may attract monocytes (specifically and selectively) through a layer of endothelial cells and that this ability is dependent on the stage of SC proliferation and differentiation (40). Soon after their release from quiescence, individual SC chemotactic ability is most potent, but declines progressively through to terminal SC differentiation. The final chemotactic power is similar to that of other mature cell types (40). Because of the added chemotactic capacity of SCs, the recruitment of immune and other cells is further reinforced.

In summary, interactions between the affected muscle, vascular tissue, immune cells and local and systemic factors ultimately facilitate repair. It is important to highlight that recruitment of phagocytic immune cells (and other cells vital to the recovery process) takes place by means of chemoattraction, activation of a pro-inflammatory response and the

subsequent formation of a positive feedback loop. Neutrophils and macrophages contribute to both additional damage and recovery. For the purpose of this thesis, the following section will pay specific attention to the activation (including infiltration) and functional role that neutrophils play during EIMD and inflammation.

## 1.4 NEUTROPHILS

### 1.4.1 HEMATOPOIESIS

Neutrophils are large multi-lobulated blood-borne leukocytes (see Figure 1.6) that originate in the bone marrow from multipotent hematopoetic stem cells (haematoblasts). These granulocytes form part of the innate immune system. The mean lifespan of a neutrophil is 180 hours (4.5 days) from their release into circulation. Since excessive ROS-production by neutrophils exacerbates the initial muscle damage, it is important to have a clear understanding of neutrophil functioning and the processes regulating neutrophil recruitment to the affected area before treatments can be considered. The following section will explain the steps of neutrophil extravasation from peripheral circulation (including those recruited from bone-marrow and the spleen) to the site of damage.

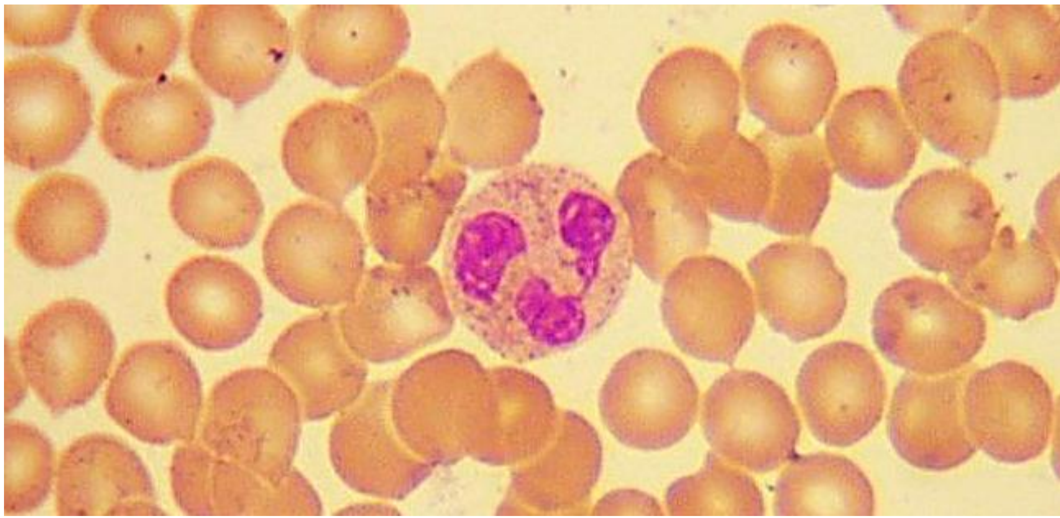

***Figure 1.6: Peripheral blood smear including a large neutrophil granulocyte.*** *Stained using MayGrunwald Giemsa (100x oil immersion). Photograph by: Tommaso Leonardi (30/10/2005). Obtained from: Histology-World Online* (98).

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### 1.4.2 EXTRAVASATION

As soon as one hour after injury, neutrophils accumulate at the site of acute inflammation (caused by injury or infection) as result of recruitment by chemoattractants, activation, and binding with cellular adhesion molecules (CAMs) expressed on the surfaces of the endothelial cells near the damaged muscle tissue (145). Neutrophils, while still in circulation, are activated and roll along the surface of the endothelial wall (by weak association with endothelial selectins) before adhering to areas of intact capillary endothelium via high-affinity binding with integrins. This is followed by their transmigration (diapedisis) across the BM and sarcolemma; allowing neutrophils to form the predominant immune cell infiltrate (Figure 1.7). The following section will describe the process comprising over-lapping stages of chemotaxis, activation, rolling-adhesion, tight-adhesion, and diapedisis.

#### 1.4.2.1 CHEMOTAXIS

In order for leukocytes to enter the affected tissue from circulation, communication must originate from the injured myofibres (75). A variety of chemoattractants including cytokines and CC and CXC chemokines are released by myofibres in order to promote neutrophil chemotaxis (4, 51, 188, 202, 219). EIMD stimulates the production and systemic release of pro-inflammatory cytokines that mediate the chemoattraction of leukocytes. Monocytes and neutrophils in turn also produce chemoattractant cytokines (273). Chemoattractants allow neutrophils to accumulate near the site of inflammation depending on the change in chemotactic gradient (17, 18, 140, 149).

Studies have demonstrated that after the mechanical loading of skeletal muscle, neutrophil migration may be influenced by IL-1, IL-6, IL-8, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF), either directly or indirectly (35, 278). For example, IL-8 binding to the CXCR1 and CXCR2 G-protein coupled receptors (GPCR) induces neutrophil mobilisation, increasing the number of circulating neutrophils (17, 18, 131, 140, 149). Granulocyte colony-stimulating factor (G-CSF) enhances neutrophil motility, thereby increasing the ability of neutrophils to migrate across the vascular endothelium (191). However, TNF-α acts indirectly by upregulating production of the neutrophil-specific chemotactic factor, cytokine-induced neutrophil chemoattractant (CINC-1) which promotes neutrophil migration (46). The expression of some leukocyte adhesion molecules is also dependent on CINC-1 (92).

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#### 1.4.2.2 Selectin-Dependant Capture (Rolling Adhesion, Tethering and Activation)

Upon inflammation, endothelial cells express all three members of the selectin family, viz. platelet-specific selectin (P-selectin), endothelial-specific selectin (E-selectin) and leukocyte-specific selectin (L-selectin) (20, 37). The ligand of the later is expressed by nearly all leukocytes (barring a subpopulation of memory lymphocytes) (251). The selectins function as leukocyte-endothelial adhesion molecules that allow for low affinity binding between circulating leukocytes and the inner wall of the vessel. Thus, the constant formation and breakage of relatively weak associations between selectin ligands (expressed by circulating leukocytes) and the selectins (expressed by the endothelial cells) cause the flow of these cells to slow down. The constant formation and breakage of these low affinity bonds result in the leukocytes rolling along the inner wall of the capillary, with the flow of the blood (37, 139).

#### 1.4.2.3 Activation

The now rolling and selectin-tethered leukocytes are activated by the binding of their G-protein coupled receptors (GPCRs) to chemokines presented by the endothelial surface. Once activated, integrins (expressed by the leukocytes themselves) undergo change from the inactive to active form, while the leukocytes shed their selectin ligands (113, 143). The now active leukocyte-expressed integrins bind with high affinity to compatible ligands expressed by the endothelial cells. Thus, there is a change from weak (leukocyte expressed) selectin-dependant adhesion, to robust (endothelial expressed) integrin-dependant adhesion.

#### 1.4.2.4 integrin-Dependant Capture (Tight Adhesion)

The firm capture or tight adhesion of leukocytes is necessary for the immobilization of leukocytes, their subsequent transmigration through the endothelial barrier and BM, and finally gaining access to the inflamed area (37). The pro-migratory/chemotactic response is furthered by the expression of several such ligands (or CAMs) and the rearrangement of the actin cytoskeleton (34).

The immunoglobulin superfamily of endothelial-expressed CAMs include the platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule 1 and 2 (or ICAM-1 and ICAM-2 respectively), vascular cellular adhesion molecule 1 (VCAM-1) and the mucosal addressin (MAdCAM-1) (34). Among these, VCAM-1, ICAM-1 and ICAM-2 are the principal endothelial receptors for β2 integrins (expressed by neutrophils) (251). ICAM-1 is specifically involved in neutrophil migration to an inflammatory lesion, through interaction

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with β2 integrins (see next paragraph) (77). Several studies have shown that pro-inflammatory cytokines (IL-1β, TNF-α, and IFN-γ), promote the expression of ICAM-1 (66, 208). The relative adhesion molecule expression may thus be dependent on the cause of inflammation as well as the type and extent of tissue damage in the case of injury.

Neutrophils express both β1- and β2-integrins (209). Neutrophils have been characterised specifically by their expression of β2-integrins including Mac-1, leukocyte function antigen-1 (LFA-1) and very-late antigen 4 (VLA-4) (80, 250). Studies have shown that Mac-1 expression may be upregulated in response to TNF-α (275, 281).

#### 1.4.2.5 Diapedisis (Protrusion and Transmigration)

While integrin-bound, neutrophils remain influenced by the stimulation of their GPCRs by cytokines including CC and CXC chemokines released from the site of damage and by other immune cells in the vicinity (4, 51, 219, 243, 261). Upon firm integrin-mediated binding, other chemical mediators are released at the affected area, promoting neutrophil diapedisis (243, 261). These mediators include complement components (C5), pro-inflammatory prostaglandins ($PGE_2$ and $PGF_{2\alpha}$) and leukotrienes (LTB4) (241). Furthermore, the activated platelets (previously discussed) release TxA2, serotonin and histamine which promote neutrophil locomotion (159). In consequence, neutrophils extend pseudopodia and pass through gaps between endothelial cells. Neutrophils migrate across both the BM (which is partly degraded by proteases) and the sarcolemma, and enter the injured tissue according the increasing chemical gradient (175).

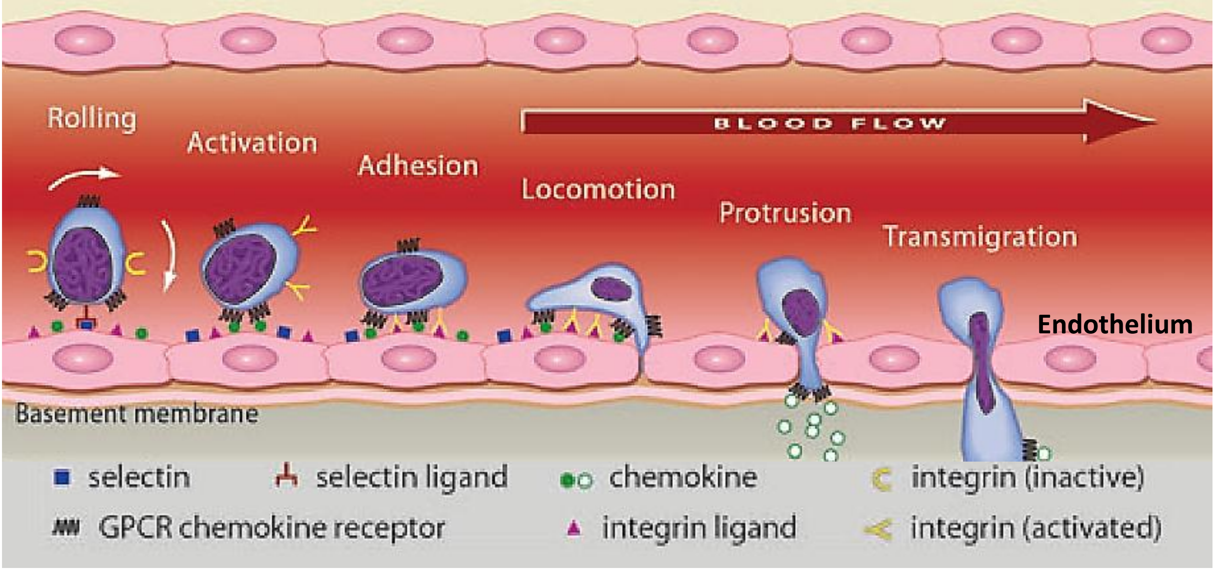


***Figure 1.7: The Multistep model of leukocyte extravasation.*** *See text for detailed explanation. Adapted from: Man et al.* (155).

### 1.4.3 Functions

Once activated and present in the area of damage, neutrophils may survive for up to 48 hours before their removal by macrophages. While present in the affected tissue, neutrophils fulfil two functions: phagocytosis of dead tissue and promotion of the inflammatory process (263).

Firstly, neutrophils clear the wound of necrotic material and blood-derived fibrin by phagocytosis (109, 263). Each phagocytic event results in formation of a phagosome into which ROS and hydrolytic enzymes [such as myeloperoxidase (MPO), a highly reactive oxidising agent] are secreted from their granule stores. The activity of these enzymes leads to the production of several oxidants such as superoxide anion, hydrogen peroxide and hypochlorous acid (267, 268) all of which contribute to lysis of phagocytic targets through superoxide-dependent mechanisms (160). The superoxide-dependent mechanism of phagocytosis has been confirmed both *in vitro* (160) and *in vivo* (193) .

Secondly, neutrophils amplify the inflammatory response by releasing pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α (32, 261) (as previously discussed). This further strengthens inflammatory reactions of other cell types, causing positive reinforcement of inflammation, which effectively leads to the recruitment and activation of additional neutrophils and ultimately increased activation of the nicotinamideadenine dinucleotide phosphate (NADPH) oxidase enzyme. This enzyme is expressed on the surface of activated neutrophils and is responsible for the release of reactive oxygen species by allowing the

neutrophil to rupture. Thus, both functions of neutrophils contribute to the production and release of reactive oxygen species, which has undesired effects by contributing to oxidative stress as will be discussed in the next section.

## 1.5 Oxidative Stress

The cellular production of ROS occurs even in the absence of skeletal muscle damage or pathology. Basal ROS production by myofibrillar mitochondria stimulates local adaptive responses (186). Significantly more ROS is generated by several activated biochemical pathways in response to exercise or other primary insults like a contusion injury (14). During exercise, aerobic energy production results in the excessive generation and escape of ROS from mitochondria (36, 213). Thus, during exercise cellular ROS may increase up to twenty-fold (247). Such conditions may result in a state where oxidant generation subjugates the cell's endogenous antioxidant defence mechanisms. This phenomenon is termed 'oxidative stress', and is now considered a major contributor to inflammation (167). Oxidative stress occurs when reactive nitrogen species (RNS) or ROS overwhelm either the cellular enzymatic or substrate antioxidants (167). The role of antioxidants in this regard will be discussed later.

ROS generation within the myofibre and skeletal muscle tissue has been studied both *in vitro* (215) and *in vivo* (201). Cellular damage caused by exercise-induced oxidative stress includes one or all of the following: lipid peroxidation (162), protein oxidation (232), and DNA damage (163). An increased lipid and protein oxidation rate results in impaired cell viability and an acute phase inflammatory response due to the compromised structural integrity of the sarcolemma (14). The generation of free ROS is important for the early phase inflammatory response to muscle damage, but ROS may worsen necrosis by extending the injury zone to include neighbouring myofibres and tissue (147, 162, 210).

### 1.5.1 Secondary Muscle Damage

Neutrophil recruitment during the early acute immune response after injury is required for removal of debris and critical to the success of the subsequent repair process (70, 261). Cytokines contribute to neutrophil activation by activating the potent enzyme NADPH oxidase (26). This enzyme causes neutrophils to erupt and splurge ROS to the surrounding tissue; a phenomenon universally known to as the 'neutrophil respiratory burst'. The ROS released include hydrogen peroxide ($H_20_2$), hydroxyl radicals (•HO), the neutral form of the hydroxide ion ($HO^-$), and superoxide anions ($0_2^-$) (2). The later reacts rapidly with NO to form

peroxynitrite ($ONOO^-$). Finkel and Holbrook (2000) postulated that oxidative stress originates mainly from the copious production of peroxynitrite ($ONOO^-$), which exacerbates tissue damage in the already adversely affected area (76). This successive stint of muscle damage is termed “secondary muscle damage” and characteristically occurs within 48 hours of the primary insult (70, 261). Thus, while the inflammatory and phagocytic responses are critical to recovery from injury, ROS released by ruptured neutrophils contribute to a second bout of muscle damage by worsening the already manifest state of oxidative stress (248). Macrophages may also generate ROS following muscle damage, however macrophages are perceived as distinctly pro-survival, especially once the shift from a pro-inflammatory (M1) to an anti-inflammatory macrophage population has occurred (M2) (248).

Although skeletal muscle tissue has a pro-survival aptitude, cellular defences could be overwhelmed under the noxious conditions following the respiratory burst of neutrophils, allowing necrosis to include the healthy surrounding tissue (210, 261). It has been shown that skeletal muscle damage may be reduced by up to 40% under conditions where neutrophils have been depleted prior to ischemia-reperfusion injury (133). This finding is in agreement with a study which demonstrated that the neutrophil respiratory burst and discharged ROS are vital in mediating the removal of debris (damaged muscle tissue) after injury, but that the unnecessarily plentiful release of ROS results in the propagation of tissue damage (263). Therefore, it is evident that if one could limit oxidative stress, one could also limit the magnitude of inflammation and associated damage in the injured area, and potentially improve muscle recovery, such that normal muscle structure and function are restored sooner.

Taken together, it would seem that there are two requirements for preventing the above described secondary muscle damage:

- increased ability to scavenge free radicals, and
- a decreased production of free radicals

This premise has lead to many investigations of possible means of enhancing antioxidant status during or after exercise, including this thesis.

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# CHAPTER 2:

# LITERATURE REVIEW

This section will review the literature covering the potential of various treatment modalities (primarily antioxidants) for preventing oxidative stress post injury. More specifically attention will be paid to previous studies that have intended to restrain leukocyte access to the injured area and the exacerbation of tissue damage. Inhibition of molecules liable for promoting neutrophil migration into skeletal muscle (post exercise) may lead to treatment options for alleviating neutrophil-mediated muscle damage. However, it is also possible that the complete inhibition of neutrophil infiltration and oxidative stress will result in insufficient phagocytosis and too little activation of repair, fibrosis and tissue scarring, thereby amounting to longer-term declines in functionality (81). Thus the review will cover the potential of several treatments for both their free radical scavenging capacity, and ability to limit ROS production by impeding neutrophil infiltration as both of these conditions may contribute toward earlier, more efficient recovery. Antioxidant supplementation conveys protection against ROS and may thus limit secondary damage while promoting sooner muscle recovery. However, some antioxidants, such as those derived from grape-seed extract (GSE), may also present with other beneficial properties which may be associated with a tapered leukocyte response. As a result, supplementation using anti-oxidants such as those derived from GSE may provide a suitable treatment modality.

## 2.1 TREATMENT MODALITIES

Because skeletal muscle injuries are so frequently reported in association with sport and exercise, several preventative measures and post-injury treatment therapies have been researched. The experience of discomfort, pain, loss of flexibility, strength, the impairment of muscle function and the accompanying period of disability is widely associated with excessive training. Furthermore, incomplete (or severely delayed) recovery may result when a muscle injury is inappropriately treated, or if the athlete returns to action and reinjures the affected area. Thus, it would be helpful to devise therapeutic strategies that optimise the regeneration process and accelerate the restoration of function, thereby abridging time absent from training and match-play.

Most therapeutic interventions are intended for use post injury, rather than as a means of prevention. Currently, these treatment modalities are primarily focussed on the earlier

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restoration of skeletal muscle function and restraining the inflammatory response following EIMD (239). When administered post injury, agents that promote growth in humans may restore muscle function by accelerating muscle regeneration and increasing both the number and (cross-sectional) size of existing and newly generated fibres (116). Of these growth promoting agents, anabolic steroids, β2-adrenoceptor agonists, GFs [such as insulin-like growth factor-I (IGF-1)] and growth hormone (GH) are the most noteworthy. However, there are two major difficulties. Firstly, the use of several effective treatments is prohibited by the *World Anti-Doping Association* (WADA), because of their ability to provide an unfair competitive advantage or to deleteriously affect health. Secondly, the usefulness of several treatment modalities is questionable as their effectiveness is often measured by their capacity to relieve or mask the symptoms of EIMD, without sufficient convincing evidence of curbing further tissue damage or inflammation. Thus, the ideal treatment for skeletal muscle injuries is still lacking. Several therapies reviewed in this section present with both positive and negative aspects for consideration. Simultaneous occurrence of several underlying processes involved in recovery, varying severity of damage induced, and the non-specificity of symptoms together contribute to the intricacies of the research (103, 109).

## 2.2 TRADITIONAL TREATMENTS

These modalities may be classified as anti-inflammatory treatments (steroids and anti-inflammatory drugs) and alternative treatments (including the use of therapeutic ultrasound, hyperbaric oxygen, exercise, growth promoting agents and cryotherapy.

### 2.2.1 ANTI-INFLAMMATORY TREATMENTS

Pharmaceutical anti-inflammatory medications are not the focus of this thesis, and will only be discussed briefly. Short-term corticosteroid treatment is known to benefit muscle regeneration and alleviate pain (16). However, longer-term use may intensify the inflammatory reaction and inhibit the healing process, resulting in exacerbation of the damage and may place athletes at risk of tendon rupture due to an escalation in muscle mass (240).

The notion that non-steroidal anti-inflammatory drugs (NSAIDs) are able to blunt the inflammatory response post injury while facilitating sooner recovery is controversial (27). When administered at low doses, NSAIDs have shown some potential in limiting inflammation. The beneficial properties of NSAIDs in this context are principally based on the inhibition of prostaglandin synthesis [by cyclooxygenase (COX)] which has direct influences

of muscle recovery (27). High doses of NSAIDs have presented with deleterious effects. Regardless of these negative effects and the limited evidence in support of enhanced healing, NSAIDs are still recommended and prescribed by practitioners for their analgesic purposes.

### 2.2.2 Alternative Therapies

Other well-established treatment modalities include the use of cryotherapy, therapeutic ultrasound, hyperbaric oxygen, exercise and some growth promoting agents.

#### 2.2.2.1 RICE (Rest, Ice, Compression, Elevation)

Strategies that are non-invasive, or do not require supplementation are often used in combination. It follows that rest, ice, compression and elevation (RICE) treatments have become a default treatment of skeletal muscle and soft tissue injury. Concise understanding of the exact contribution and mechanism of each component is obscured by their use in combination. However, it is known that when applied immediately post injury, ice reduces metabolism, circulation and thus neutrophil infiltration, thereby minimizing hypoxic injury (52, 123). On the other hand, lowering muscle temperature to below 25°C, may cause vasculature to dilate, which may exacerbate the inflammatory response by causing haemorrhage (119). Nevertheless, cryotherapy in combination with compression restricts circulation and reduces pain while compression alone does not present with any benefit (142). Resting (with the injured limb elevated) may reducing swelling and pain by decreasing the accumulation of blood in the affected area (283).

#### 2.2.2.2 Therapeutic Ultrasound

Therapeutic ultrasound employs high-frequency sound waves (227, 276) that generate friction and heat (3-4°C higher than tissue temperature) (60) on the surface of tissues, leading to a “micro-massage” effect (110). Silveira *et al.* (245) showed in a rat gastrocnemius contusion injury model that therapeutic pulsed ultrasound (6 min duration, frequency of 1.0 MHz, intensity of 0.8 W/cm2) at several time-points after trauma holds no beneficial effect of its own. However, when used in combination with dimethylsulfoxide (DMSO, 15 mg/kg) antioxidant supplementation, therapeutic ultrasound treatment did reduce markers of muscle damage and oxidative stress after injury (245).

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#### 2.2.2.3 Hyperbaric oxygen Therapy (HBOT)

Hyperbaric oxygen therapy (HBOT) is known to reduce oedema, improve oxygen delivery and inhibit leukocyte adhesion [26, 27]. In a study by Nylander *et al.* (200) rat hindlimb ischemia was induced by limiting circulation for three hours, while the contralateral uninjured leg was used as control. A significant (p less than 0.001) postischemic edema was evident in the ischemic leg four up to 48 hours after restoration of circulation. However, a group of rats received hyperbaric oxygen treatment at 2.5 atmospheres absolute (ATA) for 45 minutes after restoration of circulation. In this group, postischemic edema was significantly reduced (p less than 0.001) and the reduction persisted for 40 hours [26]. However, because of disrupted circulation and the limited oxygen supply to the affected areas after severe injuries, treatment using HBOT is often deemed inappropriate [28]. Furthermore, treatment may be expensive and may be relatively unavailable for use as a routine intervention.

#### 2.2.2.4 Exercise

Light exercise (such as walking) or early mobilization of the injured limb after a short period of immobilization is commonly prescribed by clinicians as treatment for musculoskeletal injuries (88). This form of treatment has been proven beneficial for the regeneration of injured muscles as indicated by faster disappearance of haematoma and inflammatory cells and a reduction in DOMS, followed by a more rapid and organized myofibrillar regeneration. However, as reviewed by Järvinen, premature mobilisation of the affected limb may cause fibrosis and re-injury of the muscle. Thus, exercise as treatment modality should be implemented with caution.

The remainder of this literature review will direct attention to several antioxidants that have potential for the treatment or prevention of EIMD.

## 2.3 Antioxidants

As reviewed in the previous chapter, an imbalance between ROS and cellular antioxidant defences results in oxidative stress (272). Regardless of the model used for the induction of EIMD, the resultant oxidative stress may cause secondary muscle damage. Endogenous antioxidant defence mechanisms, viz. enzymatic or non-enzymatic systems, complement each other in diminishing the quantity of harmful oxidants in the cell (Figure 2.1). The former class comprises the enzymes: catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) (24). The later system includes copious amounts of non-enzymatic antioxidants, including: ubiquinone, uibiquinol-10, albumin, ceruloplasmin (copper

component), ferritin, vitamin A (retinol or its precursor, β-carotene), vitamin E (tocopherols, tocotrienols), vitamin C (ascorbic acid), glutathione (GTH), and the flavanoid family (15, 24, 72, 221). After injury (or any other situations where ROS production is increased such as an intense inflammatory response), these natural antioxidant reserves may be depleted and unable to scavenge the excessively generated free radicals (24). Therefore, dietary anti-oxidants may support endogenous enzymatic and non-enzymatic systems, by further scavenging the superfluous amounts of ROS generated.

Dietary antioxidants including β-carotene, ascorbic acid and alpha-tocopherol are effective in scavenging free radicals and thereby partially preventing oxidative stress (195). Although not in an exercise model, Brown *et al.* (28) demonstrated a link between acute dietary antioxidant supplementation and a decrease in oxidative stress as indicated by blunted lipid peroxidation in smokers (28). Furthermore, findings by Basu (15) could link oxidative stress related conditions with deficiencies of certain antioxidants (15). Alongside an increase in the clinical application of antioxidants, some studies have concentrated on antioxidant supplementation in the context of sport and muscle damage in otherwise healthy athletes. Because exercise-induced oxidative stress is associated with the increased permeability of the sarcolemma, allowing the leakage of CK, changes to the circulating concentration of CK is commonly used as an indirect marker of muscle damage. Therefore, a trend towards a blunted CK response after exercise in antioxidant-supplemented athlete groups indirectly suggests a protective function associated with the antioxidants (42, 204). The most widely researched antioxidants in this context are vitamins C and E. However, since high doses of these vitamins (and other antioxidants) may present toxic effects, athletes may be at risk of toxicity if acute intake is increased as treatment for muscle damage when already supplementing chronically (as an “immune booster”) (226). Therefore, in the context of muscle injury treatment, an ideal antioxidant supplement would merely require acute post-injury administration as opposed to pre-exercise (acute or chronic preventative) supplementation.

Previous literature by Niki *et al.* (195) wholly summarised the results of studies that have employed anti-oxidants used commonly by athletes (including Vitamins A, C and E) and will therefore not be repeated in this thesis. Rather, the context and premise of the current study requires an understanding of the role of the non-enzymatic, naturally occurring cellular antioxidant; GTH and its precursor amino-acids.

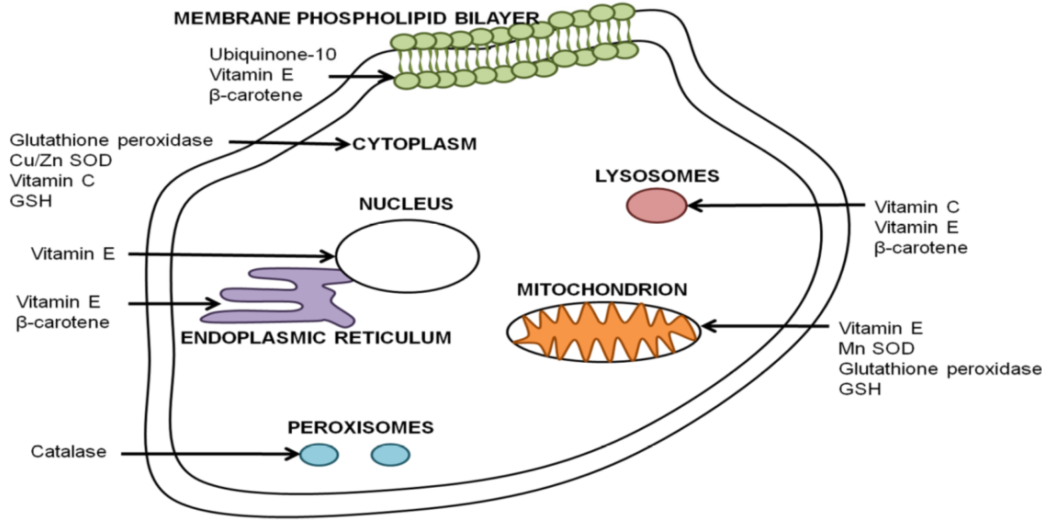


***Figure 2.1: Cellular Antioxidant Protective Mechanisms***

Abbreviations: Cu, copper; GSH, glutathione peroxidase (abbreviated in other literature as GPx); Mn, manganese; SOD, superoxide dismutase; Zn, zinc. Adapted from Machlin *et al.* (150).

## 2.4 GLUTATHIONE AND N-ACETYLCYSTEINE

As previously discussed, low concentrations of free radicals (oxidants) are detectable in resting muscle tissue while higher concentrations are evident during exercise. These muscle-derived free radicals include both ROS and RNS. The former has been identified as a contributing factor toward fatigue by impairing muscle function, while the latter is associated with a weakened force production without contributing to fatigue in healthy muscle, as reviewed by Ferreira and Reid (72). This literature review will focus on the pathways related to muscle-derived ROS production.

Antioxidants that oppose thiol oxidation may improve functional skeletal muscle performance. GTH is a thiol-donor and the most abundant and ubiquitous cellular antioxidant. It is responsible for the maintenance of oxidative equilibrium in the cell by reducing cellular ROS and RNS levels (53, 72). The quenching of free radicals may occur directly by thiol transfer, and the formation and breakdown of adducts or indirectly by means of a NADPH–dependent reaction which is catalyzed by GPx (53, 72). During this reaction GTH serves as substrate to GPx in the enzymatic breakdown of peroxides, including $H_2O_2$ and lipid hydro-peroxides. Furthermore, exercise-induced fatigue results in the oxidation of GTH. Therefore, by regulating the thiol redox status of myofibres, GTH (as thiol donor) is capable of inhibiting muscle fatigue (72).

Pharmacological inhibitors of GTH metabolism have been shown to accelerate muscle fatigue in an animal study (182). The phenomenon of GTH attenuated fatigue was adequately illustrated by Novelli *et al.* (199) when mice that had received direct administration of GTH exhibited improved endurance during a swimming exercise (199). Thus, the outcomes of the aforementioned experiments are consistent with the postulate that the sarcomere GTH redox state influences fatigue. Lands *et al.* (136) conducted a study in which healthy volunteers were supplemented with a whey-based thiol-donor. After 90 days subjects presented with a 13% increase in peak muscle power and endurance (as measured by isokinetic testing) (136).

Although beneficial to some extent, oral administration of GTH may not suffice in providing muscle tissue with quantities necessary to elicit improved performance (173, 237). This comes as result of rapid GTH hydrolysis by enzymatic action in the liver and small intestine (277). As direct methods of glutathione administration have therefore proved inadequate in restoring GTH levels, a plausible alternative may be the stimulation of GTH synthesis by the administration of its substrates. GTH is synthesized from its three constituent amino acids in two sequential adenosine triphosphate (ATP)-dependent reactions that are catalyzed by the rate-determining enzymes GTH synthetase and γ-glutamylcysteine synthetase (122, 172). As mentioned, GTH comprises three amino acid components, namely glutamate, glycine, and cysteine (72). Of these, the availability of cysteine is the rate-limiting factor in the naturally occurring process of cellular GTH synthesis. The two commonly used compounds for the improvement of cysteine availability (and subsequently enhanced GTH synthesis) are N-acetylcysteine (NAC) and α-lipoic acid (47, 72, 91, 120, 237). However, α-lipoic acid does not affect fatigue (47). NAC is the N-isomer of acetylated derivative of cysteine. It is a pharmaceutically produced amino-acid that supports GTH biosynthesis by functioning as a donor of the cysteine component (a reduced thiol). In addition, because of this amino-acid's sulfhydryl residue, NAC itself confers non-specific antioxidant properties. Therefore, NAC has the capacity to quench ROS and RNS along with a variety of other biological oxidants, directly (72, 237). Thus supplementation with NAC has become the standard method of supporting the GTH cycle in exercised muscle. Oral or intravenous NAC supplementation is not subject to enzymatic degradation, resulting in increased circulating cysteine and ultimately GTH concentrations (53, 100, 138).

An *in situ* study by Shindoh *et al.* (244) in 1997 was the first to show with compelling evidence that NAC inhibits muscular fatigue, as illustrated using rabbit diaphragms. Because this was the first finding of its kind, NAC was the first amino-acid reported to have this ability

(244). The study by Shindoh *et al.* (244) further revealed that oxidative stress plays a causal role in the onset of fatigue, rather than being a secondary outcome of muscular activity (as was previously believed) (72, 244). At a similar time, an *in situ* finding by Supinski (257) reinforced Shindoh *et al.*'s notion by showing that NAC is capable of inhibiting muscular fatigue by up to 40%, also in an animal model (257). Several *in vivo* studies, using isolated muscle preparations validated this finding (56, 72, 121, 181). See Figure 2.2.

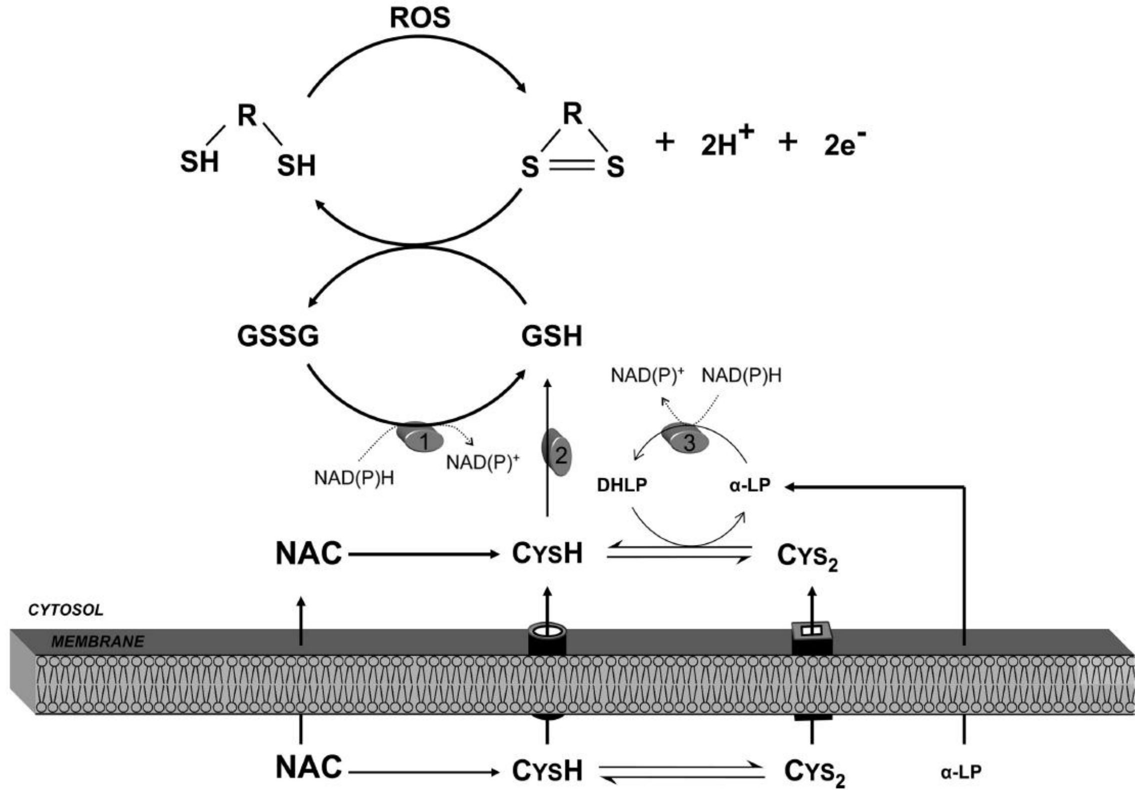


***Figure 2.2: Schematic representation of potential biochemical processes involved in the fatigue sparing effects of compounds that increase the muscle glutathione pool.*** *Image adapted from: Ferreira et al.* (72)

Although approved for clinical use, NAC is available without prescription, most commonly in form of effervescent tablets, powder-filled sachets or capsules. Brands include *ACC200*™ [*Hexal Pharma* (SA) (Pty) Ltd.], *Solmucol*™, [*Lagamed* (Pty) Ltd] and *AMUCO200*™ [*Camox Pharmaceuticals* (Pty) (Ltd)] as seen in Figure 2.3. NAC is a mucolytic agent used for conditions associated with excessive viscous mucous production, but is also employed in the management of paracetamol poisoning or over-dose (53, 233). Furthermore, clinical trials involving treatment with NAC have found some efficacy in patients with Alzheimer's disease

(53). Other beneficial effects of NAC supplementation have been reported in studies focused on nephropathy and chronic obstructive pulmonary disease (53, 57). NAC's capacity as an antioxidant and glutathione precursor allows it to play a role in the anti-inflammatory response, by interfering with signalling pathways activated by free radicals (6, 233). NAC also has the capacity to reduce ROS directly, by the reduction of the hydroxyl radical (•HO) and hypochlorous acid (6).

***Figure 2.3: Over-the-counter products that contain NAC as active agent***
*Solmucol*™, and *AMUCO200*™.

The wide-spread over-the-counter availability of NAC has allowed for innovative exercise-related experiments in humans. Several human trials have shown that NAC supplementation (which included a loading phase), prolonged time-to-task-failure during volitional workout protocols and improved the performance of limb and respiratory muscles during exhaustion protocols (72, 125, 165, 216, 269). Reid *et al.* (216) provided the evidence to confirm that these effects were peripheral and not via changes to the central nervous system (CNS) by using electrical-stimulation studies in humans (216). In 2006, a study by McKenna *et al.* (168) showed that NAC supplementation could effectively delay the onset of fatigue during whole body exercise such as swimming, cycling or running in endurance-trained individuals (168). With NAC supplementation, time-to-task-failure improved nearly 25% during a near-maximal cycling protocol (170, 171). However, recent controversy has been elicited after a report by Childs *et al.* (43) who suggested that NAC and Vitamin C may act as pro-oxidants under inflammatory conditions in humans (43).

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## 2.5 Grape-Seed Extract

### 2.5.1 Introduction to Polyphenols

Many studies have shown beneficial effects of supplementing with plant extracts. Relevant to this study are those potentially cable of promoting wound healing such as arnica, astragalus, calendula, hyssop, myrrh, paud'arco, angelica sinensis and the leaf extract; eucommia ulmoides (146, 280). However, our specific interest lies not only in improved recovery, but rather the ability of a naturally synthesised plant extract to reduce the amount of reactive species generated following muscle damage, and thereby curb subsequent muscle damage. Such a compound potentiates a natural means of reducing the severity of sport and exercise related muscle injuries, shortening recovery time, and allowing sooner return to practice or match-play.

Previous studies that employed polyphenol supplementation mostly made use of the form extracted from grape-seeds, namely proanthocyanidin (discussed later), or the blueberry derivative; anthocyanidin. As most polyphenol studies conducted within the past two decades were aimed at the treatment of pathologies, few studies presented with substantial evidence in favour of an improved recovery process after an acute muscle damaging insult. Nevertheless, their findings were significant (30, 59, 99, 117, 190). After an *in vivo* rat study, Kato *et al.* (117) reported that after being subject to a treadmill-induced exercise activity, polyphenol treated rats exhibited significantly lower force deficit and earlier recovery (in comparison with a control group) due to the suppression of oxidative stress within the skeletal muscle (117). Similarly, both Buetler *et al.* (30) and Dorchies *et al.* (59) reported an elevation in twitch tension with a reduction in muscle necrosis using a similar model using mdx mice (30, 59). Hofmann *et al.* (99) clearly illustrated an improved free radical scavenging capacity with a subsequent reduction of oxidative stress in polyphenol treated rats vs. controls (99). This finding was later confirmed in a mouse study by Nakazato *et al.* (190) whom showed that after lengthening contraction exercise, significantly lower levels of oxidative damage [as indicated by serum thiobarbituric acid reactive substances (TBARS)] were evident in polyphenol treated mice when compared with the control group (190). An mRNA analyses using samples obtained from the same study, revealed a significant elevation in glutathione-S-transferase-α-1 (GST-α-1) in the polyphenol treatment group, again confirming an enhanced oxidative capacity in the polyphenol treated group (190).

Shifting our focus to a human model Morillas-Ruiz *et al.* (184) reported that after a strenuous sub-maximal aerobic exercise trial, trained cyclists presented with lower lipid oxidation (as an indicator of oxidative stress) and a low CK response (a non-specific marker of muscle damage), when supplementing with a polyphenols during exercise (184). Thus it is suggested that polyphenol supplementation may have a favourable effect in curbing secondary muscle damage by protecting against exercise-induced oxidative stress. In 2010, an *in vitro* study by Hurst *et al.* (104) involving the exposure of an undifferentiated skeletal muscle cell line to agents that simulate exercise-induced oxidative stress pathways, polyphenols (obtained from blueberries) were able to suppress the activation of such pathways (104). Whilst being co-incubated with the blueberry polyphenol extract and subject to oxidative stress inducing agents, the undifferentiated skeletal muscle myotubes released less enzymes than those subject to identical conditions without polyphenols (104). This is indicative of a polyphenol-associated cellular protection effect.

### 2.5.2 Flavanoids

Flavanoids are biologically active plant-phenolics of which plenteous quantities are found in most red (and some white) wines, tea (camellia sinensis), most fruit types, green vegetables, onions and berries (156). During the 1980s, the *French Paradox* was formulated by French epidemiologists after observing low mortality rates associated with coronary heart disease (CHD), despite reported diets containing high saturated fat and cholesterol contents. The paradox was attributed in part to high levels of red wine consumption (25g/daily) (74, 218) although this argument is currently surrounded by much debate (62, 83, 177). Regardless, since the discovery, considerable research focus has been placed on the polyphenols abounding in most red wines (depending on the cultivar and production process). These polyphenols include, for the most part, the flavanoids: quercetin and proanthocyanidin, but also resveratrol (another classification of polyphenols). For this reason grape-seeds conventionally disregarded by the viniculture industry are now considered an abundant source for the isolation of profitable dietary supplements.

As with other plant phenolics, flavanoids are potent scavengers of free radicals. They also present with numerous other biological properties with potential beneficial effects (11, 12, 41, 231), including the following:

- cardioprotection (231),
- anti-allergic (179),
- vasodilatory (61, 231),
- anticarcinogenic (21, 95, 96) and
- anti-inflammatory effects (11, 21, 33, 128, 179, 187, 222, 229)

As previously discussed, it is vital that free radical scavenging capacity is increased, if secondary muscle damage is to be reduced. Thus, flavanoids are qualified by their ability to be oxidised by radicals, resulting in a less reactive, more stable radical (90). Furthermore, some flavanoids may quench superoxides, while other flavanoids become oxidised by the highly reactive oxygen-derived radical, peroxynitrite, which is rapidly produced after the neutrophil (oxidative) burst (90).

Injury and the resultant oxidative stress also cause activation of several arachidonic acid (AA) pathway enzymes, such as phospholipase A2, lipoxygenase and cyclooxygenase, glutathione reductase and the xanthine oxidase pathway (11, 21, 33, 179, 222, 229). Because these may hinder adequate muscle healing, their inhibition by flavanoids make this compound undeniably appealing in the treatment of skeletal muscle damage. Although the aforementioned benefits of flavanoid treatment have been studied in the context of cardiovascular diseases, many of these conclusions may be applicable in a skeletal muscle injury model. From this perspective, the most noteworthy effects include the anti-inflammatory, free radical scavenging, and vasodilatory properties. Thus, flavanoids may potentially limit the time required for muscle regeneration by both scavenging free radicals and suppressing enzymes which may otherwise hinder adequate muscle healing (97).

The flavanoid family can be further classified into several subclasses according to their chemical structure. These include flavanone, flavonol, flavone, anthocyanidins, and the flavanols [including monomeric flavanol, catechin, epicatechin, and the oligomeric proanthocyanidins (PCO)]. For interest of the current study, we will review the relevant literature for PCO.

### 2.5.3 PROANTHOCYANIDINS

When compared with extracts of other plants (mostly fruit and vegetables), grape-seeds contain an extraordinarily high amount of naturally synthesised, biologically active proanthocyanidin monomers, dimers and oligomers. These proanthocyanidins belong to the family of polyphenolic bioflavanoids which have previously shown to present with a potent antioxidant capacity (231). Furthermore (with the exception of quercitin) no adverse properties of proanthocyanidin supplementation have been reported to date. In 1998, an *in vivo* mouse study, Bagchi *et al.* (11) showed that oral gavage with 100 mg/kg of PCO derived from GSE demonstrated more potent antioxidant properties than vitamin C (100mg/kg), vitamin E succinate (100 mg/kg) or β-carotene (50 mg/kg) when comparing the abilities of each to constrain tissue damage caused by oxidative stress (11). Masuda *et al.* (164) showed that PCO's potent antioxidant capacity is present at both circulatory and tissue levels. This was done by determining the concentration of superoxide anions present in both the plasma and muscle homogenate of PCO vs. control rats by means of electron spin resonance (ESR) (164). Other than for its free radical scavenging capacity, PCO containing supplements have been beneficially employed in the treatment of cardiovascular ailments, inflammation and cancer (or their accompanying conditions), as reported by at least two *in vivo* studies (65, 284).

Much of the research conducted using PCO supplementation has been focussed on manipulating several anti-inflammatory pathways relevant to treatment and prevention of cardiovascular disease or atherosclerosis. Sen *et al.* (238) evaluated the effects of GSE on TNFα-induced ICAM-1 and VCAM-1 expression in primary human umbilical vein endothelial cells (HUVEC). It was found that dosages between one and five µg/ml down-regulated TNFα-induced VCAM-1 expression but not ICAM-1 expression. This was also observed at the mRNA expression level. A cell-cell co-culture assay was done to validate whether the inhibitory effect of GSE on VCAM-1 expression was also effective in down-regulating the interaction between leukocytes and endothelial cells. GSE treatment significantly decreased TNFα-induced adherence of T-cells to HUVEC (238). Therefore it would seem that PCO may have the potential to mediate the inflammatory process by exerting a negative effect on the migration of immune cells from circulation to the area of injury by modulating adhesion molecule expression on the lumen of blood vessels. Similarly, in a publication by Badia *et al.* (10) it was reported that a 28-day period of moderate red wine consumption affected plasma CRP and IL-1β levels while diminishing both VCAM-1 and ICAM-1 expression. This randomised cross-over trial analyzed the effects of red wine (high polyphenolic content) and

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gin (low polyphenolic content) on human monocyte adhesion to an endothelial cell line, in eight healthy men. Measurement of human monocytes adhesion to endothelial cells was performed under TNF-α stimulated and basal conditions. The study showed that TNF-α-induced adhesion was only partially reduced after gin consumption, but that after red wine consumption, adhesion was almost entirely abolished [101].

Estruch *et al.* (69) performed a randomized, crossover, single-blinded trial in which 40 healthy men consumed wine or gin for 28 days to evaluate the effects of these beverages on inflammatory biomarkers of atherosclerosis. Both interventions showed anti-inflammatory effects by reducing plasma fibrinogen and IL-1α levels. However, only wine decreased plasma CRP, ICAM-1 and VCAM-1 [102]. In both of these studies, monocytic cells that had been isolated from healthy males were incapable of endothelial adhesion *in vitro* [101] *and in vivo* [102]. Furthermore, proanthocyanidins have presented with favourable effects on these vascular endothelial functions including the synthesis and release of nitric oxide (NO) (55, 64). NO is necessary for vasodilation, thereby increasing platelet aggregation, and the influx of acute phase proteins into the injured area (205). Thus, patients with coronary artery disease (with impaired endothelial function) presented with improved endothelium-dependent vasodilation after receiving grape juice (purple) (254). In addition, proanthocyanidins are specifically recognized for their ability to support and protect metabolically active tissue by inhibiting the proteolytic activity of collagenase and elastase. Both of these enzymes may contribute to fibrosis (13, 264).

Most of the studies discussed above investigated the positive effects of proanthocyanidins for cardiovascular diseases. In the research on cardiovascular disease, resveratrol features most frequently.

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### 2.5.4 RESVERATROL

Resveratrol is also classified as a polyphenol. It is a phytoalexin and one of the most researched grape-seed derived antioxidants commonly found in red wine (73). A review by de la Lastra and Villegas (50), summarised the mechanisms (and dosage) by which resveratrol may exert a therapeutic effect (50). Two particularly noteworthy mechanisms are the:

- inhibitory effect on activation of primarily neutrophils and macrophages and an
- inhibitory effect on the production of the pro-inflammatory cytokines, IL-8 and IL-6 (58)

Most relevant to this thesis, are the studies where supplementation with resveratrol directly inhibited the expression of both ICAM-1 and VCAM-1, both of which are adhesion molecules involved with immune cell adhesion and migration from circulation across the endothelial cell layer. Several studies were conducted using stimulated HUVEC and the results documented by both Ferrero *et al.* (73) and Bertelli *et al*. (19). In addition, Rotondo *et al.* (228) showed that resveratrol decreased the expression of Mac-1, suggesting that resveratrol curbs the migration of neutrophils by bring about a reduction in β2 integrin expression on neutrophils themselves, rather than, or in addition to modulating the expression of endothelial adhesion molecules (228). In their review of resveratrol's anti-inflammatory effect, Lastra and Villegas (50) also mentioned an alteration to the synthesis of eicosanoids. A previously published rodent study reported that prostaglandin (PG) synthesis is inhibited following the direct inhibition of the cyclooxygenase (COX) activity, specifically COX-1, but not COX-2, in a carrageenan-induced paw oedema (108). Conversely, a study on human mammary epithelial cells by Subbaramaiah *et al.* (256) revealed that the expression of prostaglandin was unambiguously affected via the COX-2 pathway (256). Nevertheless, common to all studies are reports that resveratrol presents consistently with anti-inflammatory properties.

### 2.5.5 OXIPROVIN™

*Oxiprovin*™ is a registered *Proudly South African* and commercially available antioxidant supplement prepared by *Brenn-O-Kem* (Pty) Ltd, Wolseley, South Africa. *Oxiprovin*™ is a 99% pure GSE, packaged in capsule form (70 mg PCO per capsule) with micro-crystalline powder (Figure 2.4). The separation procedure involves an ethanol and water extraction process, by which the active ingredient (PCO) is isolated from grape-seeds of local origin (25). The process does not involve the use of organic solvents, further meriting the extract as safe for oral consumption as a dietary supplement. *Oxiprovin*™ contains 45% PCO and less than 5% oligomeric monomers. The remainder of the extract comprises long chain sugars and oligomer-bound glycosides. *Oxiprovin*™ contains no colourants, preservatives or flavourants and to date, no side-effects have been reported. As per the package insert, the manufacturer recommends a dosage of one capsule (70 mg PCO), taken twice daily with water (equating to 140 mg GSE per day) for adults, and 1 capsule daily with (or dissolved in) water for children. Pharmacologically, *Oxiprovin*™ has been classified as a western herbal dietary supplement with antioxidant properties. However, it holds no scheduling status. Nevertheless, its use as a supplement is acclaimed as beneficial for the treatment of arthritis, enhancing the immune-system, circulation and health of blood vessels, countering allergies, retarding ageing (39), and reducing free radicals present in both circulation and at tissue level. Refer to the package insert for more details (Appendix A).

***Figure 2.4: The commercially available grape-seed derived antioxidant supplement; Oxiprovin™.***

*Oxiprovin*™'s ability to prevent muscle damage, and improve recovery from muscle injury has not yet been studied to the extent that it may be recommended as such. However, according to Kruger *et al.* (129) the following are prerequisite properties of a natural (plant-derived) product for consideration as an effective therapy in the treatment or prevention of skeletal muscle damage. The product should:

- promote strengthening of plasma membranes, such that less damage may occur, and healing would occur more promptly.
- promote earlier satellite cell activation such that recovery may commence readily.
- promote an enhanced anti-inflammatory state (increase of anti-inflammatory cytokines), while the release of pro-inflammatory cytokines are attenuated.
- limit neutrophil infiltration to the affected area, while allowing the recruitment of macrophages to remain unaffected or improved.
- diminish ROS at both tissue and circulatory level by improving oxygen radical absorbance capacity (ORAC), thereby averting damage resulting from oxidative stress.

*Oxiprovin*™ is one of several GSE products available commercially in South Africa. Others include *Procydin*™ [*Value Added Life Health Products* (Pty) Ltd] and *Salute Santé* grape-seed oil (*Food & Vin*e, Inc.). Proanthocyanidin oligomers (PCO) are potent naturally occurring antioxidants, protecting against reactive species and free radical damage. They are known to strengthen and protect living tissue (25). However, they are not regarded by all researchers as the flavonoid primarily responsible for the beneficial effects of GSE supplementation as they are not as readily absorbed as monomers (225). Proanthocyanidin monomers (present in small quantities in *Oxiprovin*™) are rapidly absorbed and bind with heavy metal ions present in circulation (84, 230).

In 2004, Mastaloudis *et al.* (163) showed that grape-seed extract (PCO) supplementation inhibited lipid peroxidation and fatigue after extreme exercise, suggesting a proanthocyanidinic ability to enhance recovery, in a human model (163). Lafay *et al.* (134) demonstrated that supplementation with grape-seed extract improved antioxidant status and physical performance while diminishing oxidative stress in a group of 20 elite sportsmen (134). During this specific trial several parameters were assayed in urine and plasma samples taken throughout one month of GSE or placebo supplementation (400mg daily). Explosive muscle power and levels of fatigue were also measured throughout. Although, the subjects in this study presented with a decrease in CK activity across all treatment groups, a significant improvement in performance was evident in certain players (134).

To investigate the short-term effects of oral PCO (*Oxiprovin*™) supplementation on recovery from skeletal muscle micro-damage following an acute bout of plyometric exercise in trained rugby players, a study by Viljoen *et al.* (274) supplemented a group of 20 trained male rugby-players with either *Oxiprovin*™ (140 mg/d) or a placebo treatment. Supplementation commenced 30 minutes prior to the performance of a strenuous bout of plyometric exercise (10 sets of 10 maximal squat jumps). Supplementation continued for four days during recovery. In this thesis (unpublished) it was shown that *Oxiprovin*™ completely conserved muscle power measured during vertical jumps comparing pre- and post-intervention time-points (post time-points: significantly conserved at 0 and 36 h). Although this supports the prospect of short-term *Oxiprovin*™ use as a viable strategy for athletes during periods of heavy training and/or competition, its effectiveness in limiting neutrophil mediated muscle damage, was not studied. Indeed, no significant differences were reported for the circulating neutrophil count between the placebo and *Oxiprovin*™ supplemented groups (140 mg/d, as recommended by the package insert). Furthermore, no significant differences were evident between groups with regards to CK activity (274).

Using an *in vivo*, rat model of non-invasive contusion injury to assess the effect of pre-injury chronic *Oxiprovin*™ administered by oral gavage, Myburgh *et al.* (187) presented various changes induced by PCO which indicated that recovery was accelerated. It was histologically shown that GSE-supplementation resulted in the earlier activation of satellite cells and formation of newly generated muscle fibres (as seen through H&E staining, and $MHC_f$ labelling). Also, CD56 staining showed accelerated activation of SC in the *Oxiprovin*™ supplemented group. ORAC results showed that the *Oxiprovin*™ supplemented group presented with a greater free radical scavenging effect. This, in conjunction with lower plasma CK activity than seen in the placebo treated group, allowed for the conclusion that *Oxiprovin*™ supplementation (20 mg/kg/day) protected skeletal muscle against secondary damage after a contusion injury (130).

Since the occurrence of an injury cannot be predicted, the issue of chronic as opposed to acute supplementation warranted investigation. For this purpose, Kruger *et al.* (129) used a rat hind-limb contusion injury model. Both chronic and acute PCO supplementation resulted in an earlier onset of muscle recovery, verified by an earlier satellite cell response and foetal myosin heavy chain ($MHC_f$) synthesis compared to a placebo control group. It was also found that chronic *Oxiprovin*™ supplementation presents with an anti-inflammatory cytokine reaction (as seen by a significant increase in IL-10, 3 days post-injury), whilst blunting the circulatory pro-inflammatory cytokine response (TNF-α and IL-6). Since it is known that both of these are important activators of the NFκB, AA or mitogen activating protein kinase (MAPK) inflammatory pathways, it has been suggested that PCO functions by diminishing or

completely inhibiting one or more of these signalling pathways. After H&E staining of sectioned muscles samples taken at the 14 day time-point (post injury), a qualitative comparison could be conducted between the treatment groups. This revealed that the group chronically supplemented with *Oxiprovin*™ had undergone earlier recovery with regards to muscle ultrastructure, than the group acutely supplemented post-injury as seen in Figure 2.5. Although both the chronic and acutely supplemented groups presented with superior ultra-structural muscle recovery when compared to placebo groups, samples from the chronically supplemented group had already undergone complete regeneration, while that taken from the acutely supplemented treatment group still presented a number of disorganised fibres. It is postulated that this may be as result of a residual oedema or an altered state of adhesion molecule expression, but also in part due to the time required for the absorption of PCO into circulation and the uptake into tissue (187).

A novel finding of the chronic supplementation study was that PCO diminished the neutrophil response whilst expediting earlier macrophage infiltration into the affected area. Furthermore, a change in macrophage phenotype had been illustrated between the chronically and placebo supplemented groups. On day five post injury, the former presented with a macrophage change from the M1 to M2, while the placebo group presented with high numbers of M1 (129). Although circulatory neutrophil numbers were elevated at both 12 and 24 h post injury in the *Oxiprovin*™ group, they were decreased by day 3 post injury. Surprisingly, there was no significant elevation in the number of neutrophils in the muscle on day 3 or any other time-point. This suggests that since neutrophil numbers decrease in circulation (but had not entered the injured or border areas) their mobilisation had been reduced as well. In contrast, in the placebo treated group, the lower circulatory neutrophil response (seen 24 h post injury) seemed to have occurred as a result of neutrophil infiltration into the injured and bordering muscle areas. The specific mechanism by which PCO supplementation limits the neutrophil response in this model is as yet unknown. Kalfin *et al.* (114) showed that proanthocyanidin supplementation reduced circulating concentrations of sICAM-1, sVCAM-1 and E-selectin in plasma samples taken from patients with systemic sclerosis (SSc) (114). Similarly, *in vitro* studies have shown that GTH, its precursor; NAC and the antioxidant pyrrolidine dithocarbamate all inhibited the expression of both ICAM-1 and VCAM-1 in various cell types that had been stimulated by cytokines or oxidants (118, 161). Ferrero *et al.* (73) showed by means of an *in vitro* study, that by adding resveratrol to two activated cell lines [(viz. TNF-a–stimulated HUVEC and lipopolysaccharide-stimulated human saphenous vein endothelial cells (HSVEC)], both the expression of ICAM-1 and VCAM-1 were significantly inhibited, respectively (73). They hypothesised that this is because resveratrol is a tyrosine kinase inhibitor, and that it would

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therefore be able to modify both ICAM-1 and VCAM-1 expression and consequently prevent both monocytes and granulocytes from adhering to the endothelial cells. These results suggest that resveratrol is an antioxidant and affects at least some cell signalling pathway. This warrants further investigation, particularly in the context of sport, exercise, and recovery from EIMD.

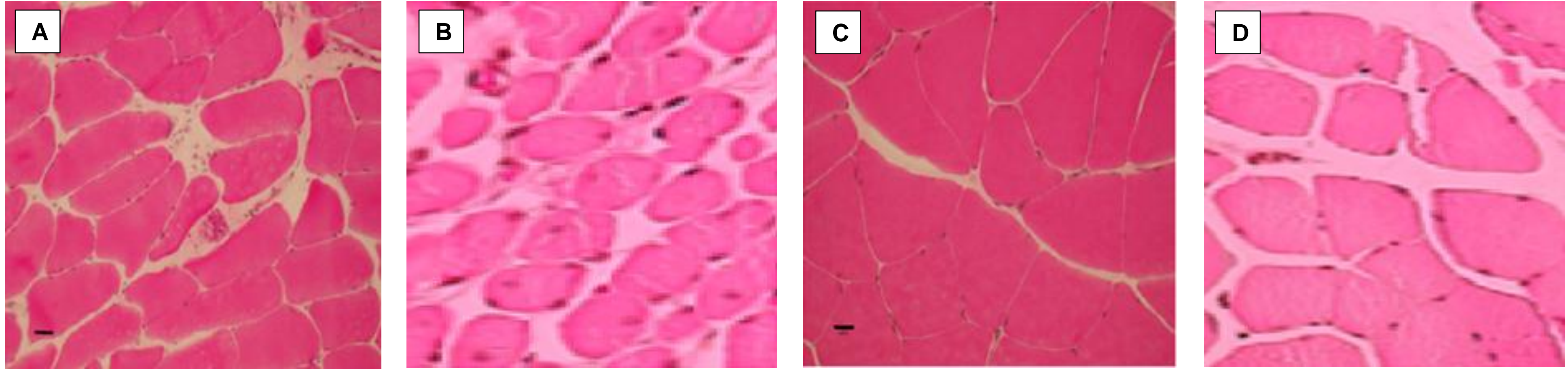


***Figure 2.5: A qualitative comparison of muscle recovery after chronic vs. acute PCO supplementation.*** *Earlier ultra-structural recovery after acute injury in a rat model. H&E stained muscle samples.* [A] *chronic placebo supplementation*. [B] *acute placebo supplementation.* [C] *chronic Oxiprovin™ supplementation.* [D] *acute Oxiprovin™ supplementation.* Adapted from Myburgh *et al.* (187).

## 2.6 Summary of Literature Review

Various antioxidants including the vitamins A, C, and E are effective in scavenging free radicals and thereby partially preventing oxidative stress. Other cellular antioxidants including GTH and its precursor NAC may both play a role in curbing the inflammatory response by interfering with signalling pathways activated by free radicals. Despite the described benefits of antioxidant supplementation, they are not able to entirely eliminate secondary muscle damage following exercise. Furthermore, the optimal timing and dosage of supplement administration still needs to be investigated. Thus, it is imperative that research continues in search of an optimal treatment which is easily administrable and can minimise fibrosis while modulating inflammation, leukocyte infiltration, ROS production, oxidative stress and a second stint of muscle damage. A viable option may be supplementation with PCO, such as GSE. In animal models, it has been shown that *Oxiprovin*™ supplementation may blunt the passage of neutrophils from circulation into the affected muscle area after exercise, a mechanism that could reduce neutrophil-mediated secondary muscle damage resulting from the oxidative burst. Yet, the mechanism by which *Oxiprovin*™ supplementation specifically limits the neutrophil response has not been directly identified. It has been suggested that *Oxiprovin*™ supplementation may modulate endothelial activation and neutrophil adhesion rather than neutrophil activation. This warrants further investigation, particularly in the context of sport (specifically union rugby), plyometric exercise and recovery from EIMD.

## 2.7 Hypotheses and Aims of the Current Study

### 2.7.1 Summary of Research Problem

Contemporary models for the treatment of EIMD range from the use of drugs to physiotherapy. However, the usefulness of several of these treatment modalities is questionable as their effectiveness is mostly determined by their ability to alleviate the symptoms of EIMD, rather than curbing further intramuscular damage or inflammation. To date, there is no treatment modality that prevents secondary muscle damage optimally. Thus, athletes and other relevant parties including coaches, team-members and financial stake-holders need to endure the wait for adequate recovery before sport-participation may resume. Depending on the type and severity of the injury, insufficient recovery and scar formation may occur, particularly if existing treatment modalities are employed inappropriately.

In animal models, GSE supplementation has successfully decreased healing time required after muscle injury. This phenomenon was even more pronounced when including a loading period (simulating chronic supplementation). Furthermore, because it is known that neutrophils may harm skeletal muscle by the excessive release of reactive oxygen species, PCO's mechanism of action may provide an unsurpassed strategy for preventing neutrophil-mediated secondary muscle damage. Thus, antioxidants such as those found in grape-seeds may prevent secondary muscle damage by scavenging reactive species, in addition to preventing their release by limiting neutrophil infiltration into tissue. However, the specific mechanisms responsible are unknown, and therefore GSE cannot (yet) be recommended for use in the treatment or prevention of EIMD.

Since plyometric exercise forms an integral part of the training regime of many elite athletes (and in particular union rugby players), it has recently been demonstrated that an acute bout of plyometric exercise results in non-severe transient muscle damage (without loss of ambulation), with or without acute GSE supplementation (at low dosage). However, it is not known whether more optimal results may have been obtained had participants been supplemented chronically at a higher dose. Thus, this thesis aims to elucidate the possible mechanism by which GSE supplementation may prove beneficial as treatment for EIMD, such that sportspersons may recover within the minimum time.

### 2.7.2 Principal Hypothesis

Chronic supplementation with a high dose of *Oxiprovin*™ will prevent secondary muscle damage succeeding plyometric exercise-induced muscle damage in non-professional, healthy, male rugby-players aged between 18 and 25 years.

Indirect evidence of this effect will include an accelerated conversion from a pro- to an anti-inflammatory state and fewer neutrophils undergoing an oxidative burst. A further principal hypothesis is that the free radical quenching effects will be of a similar extent to that of chronic supplementation using the GTH precursor, NAC. Due to additional polyphenol-related advantages of *Oxiprovin*™, chronic supplementation using NAC will present with a lesser effect on all other measures.

### 2.7.3 Additional Hypotheses

*Oxiprovin*™ will:

- limit the circulating neutrophil count,
- reduce the activated neutrophil count,
- interfere with mechanisms for neutrophil migration into the injured tissue through inhibition of chemotaxis by:
  - decreasing pro-inflammatory cytokine expression (such as IL-6, and TNF-α) and
  - decreasing other chemotactic factors (CXC and CC chemokines) and finally
- reduce evidence of adhesion molecule expression (such as ICAM-1 and VCAM-1)

### 2.7.4 Aims of This Study

- To determine the free radical scavenging ability of the polyphenol-containing supplement. *Oxiprovin*™
- To determine if *Oxiprovin*™ prevents secondary muscle damage following an acute bout of plyometric exercise.
- To compare these results with those obtained from an antioxidant without polyphenolic properties, (in this case, NAC).

The study design allows for a comparison of chronic oral *Oxiprovin*™ supplementation or chronic NAC vs. placebo supplementation after an acute bout of plyometric exercise, to determine if either or both of the supplements will:

- change circulating CK activity (as non-specific marker of muscle damage) and pain scores as indicator of extent of muscle damage and resolution during recovery,
- prevent oxidative stress and accumulation of malondialdehyde (MDA), a by-product of lipid peroxidation,
- change the systemic ORAC,
- change the numbers and ratios of circulating erythrocytes, leukocytes and leukocyte sub-populations (neutrophils, basophils, eosinophils, monocytes and lymphocytes),
- change the magnitude and time-course of neutrophil disappearance from circulation,
- change circulating concentrations of pro- and anti-inflammatory cytokines and
- change the concentration of circulating adhesion molecules

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The above should aid the understanding of the effects of chronic oral *Oxiprovin*™ supplementation in comparison with those of chronic NAC supplementation after an acute bout of plyometric exercise and could describe PCO effects of *Oxiprovin*™ supplementation, over and above its free-radical scavenging capacity.

### 2.7.5 Implications and Potential Clinical Application of Outcomes

The results of this study may allow for greater insights into *Oxiprovin*™ or other antioxidants' use for the prevention or earlier recovery from EIMD. Further studies and registration for a clinical trial with the Medicines Control Council (MCC) may lead to the possible registration of *Oxiprovin*™ for additional indication, in prevention or treatment of muscle damage.

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# CHAPTER 3:

# MATERIALS AND METHODS

## 3.1 STUDY DESIGN

In order to elucidate the mechanism(s) by which *Oxiprovin*™ limits secondary muscle damage and promotes recovery after EIMD in a human model, a carefully designed and very well-controlled study had to be conducted. Also, because the current investigation includes sport-specific application, strict inclusion and exclusion criteria had to be followed. This investigation complied with the requirements of the Human Research Ethics Committee (HREC), Stellenbosch University, and was conducted in accordance with the Declaration of Helsinki, with good clinical and laboratory practice according to the guidelines of the Medical Research Council (MRC). In this cohort study a randomised control trial was performed. The study was both observational and longitudinal (in order to avert recall bias), and made use of three parallel treatment groups all of which were subject to two interventions. The study was also performed as double-blind to control for observational or investigator bias. Only after the protocol and all analyses had been completed was it made known which treatment group participants had been assigned to.

## 3.2 PARTICIPANTS

24 healthy, uninjured male rugby-players in mid-season training phase, aged between 18 and 25 years were recruited for this research study [such that the subject number (n) = 8 for each treatment group]. Recruitment took place by advertising the study within various teams participating in a local university league (see Appendix F). Candidates were granted admittance to the study based on compliance with the all inclusion criteria or were declined admittance if they complied with at least one of the exclusion criteria. The conditions were as follows:

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### 3.2.1 Inclusion Criteria

The candidate must:

(a) have read, understood and signed the informed consent form as provided by the HREC (see Appendix G),
(b) be listed as a local or recreational rugby-club player,
(c) be in mid-season training phase,
(d) have been injury free for the previous 6 months,
(e) be aged between 18 and 25 years

### 3.2.2 Exclusion Criteria

The candidate:

(a) has experienced a sport or muscle injury within the last six months,
(b) was currently receiving treatment or had been using anti-inflammatory drugs, NSAIDS or corticosteroid-containing medication (including inhaled forms) within the previous two months,
(c) was currently supplementing with dietary antioxidants in any form,
(d) expressed a history of gastrointestinal disorders or disturbances such as peptic ulcerations,
(e) was an elite athlete,
(f) had match-play scheduled within seven days after the date of the plyometric exercise intervention,
(g) has a history of anaphylaxis,
(h) suffers from hay-fever,
(i) is a smoker,
(j) presents with asthma,
(k) was feeling ill, or had presented with symptoms of a common cold or fever within the previous two months

Although 24 participants had agreed to partake, only 22 completed the study, while the data gather from another was removed after non-compliance was reported (final n = 21). Previous studies by our group using similar models for EIMD (viz. downhill running) in sample populations with an even smaller subject number have yielded significant statistical power. This was attributed to consistently (very) low baseline values. All participants received compensation on a pro-rata basis for transport and meal costs as well as their time investment in the study.

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### 3.2.3 Ethical Considerations

The study design and protocol was approved by the HREC (sub-committee II), Stellenbosch University (study reference no: N10/02/060, amendment no. 3). See Appendix C, D and E. All information has been kept strictly confidential. Only the principal and selected other investigators had access to personal information and to the experimental data. All research was conducted in accordance with the Declaration of Helsinki, using Good Clinical and Laboratory Practise, and according to the guidelines of the MRC.

All potential participants were informed of the details of the research both verbally and in writing using an adapted information sheet (version 2.1) as provided by the HREC (Appendix G). Experimentation did not commence without the consent of the participant. Participation was entirely voluntary and participants were free to withdraw from the study at any time. There was no indication that participants would be negatively affected by their participation in the study and the level of risk involved was evidently low. The possible contraindications of NAC supplementation were limited by enforcing stricter exclusion criteria, and eliminating candidates most likely at risk. The orthopedic risk was reduced by only recruiting healthy trained male rugby-players. In the unlikely event that a participant would suffer a negative event as a result of participation, the researchers and study doctor were on standby to provide the necessary information or remedial therapy. Participants gave written permission for footage (including photographs and videos) collected during the study to be used for both academic and publication purposes.

### 3.2.4 Instructions to Participants

Various restrictions were applied for the full 15-day duration of the study (protocol days: -12 – 2). This was done in order to prevent additional muscle damage, earlier recovery, enhanced performance or irregular readings in free radical absorbance capacity or markers of oxidative stress. Since the local university league had reached the mid-season break two weeks prior to the commencement of the current study, all routine rugby practice and rugby games had ceased. Regardless, participants were required to elude any and all forms of exercise and unusual physical activities besides those required for daily activity, such as walking. Furthermore, restrictions included medication indicated for pain-relief or inflammation, and all other treatment modalities such as physiotherapy and ice-treatments. All forms of supplementation with dietary antioxidants, immune-boosters, multivitamins, performance enhancers or recovery agents had to be ceased. Participants were required to abstain from smoking as well as alcohol and caffeine consumption. All the above mentioned

restrictions were mandatory for the duration of the study (a total of 15 days) and until the last blood sample was taken. Participants were instructed to log their daily diets and activities according to the template provided (Appendix I) and were supplied with instructions for the oral administration of the various treatments. A detailed schedule of the study program including arrangements for the fixed laboratory visits was provided. All participants were instructed to report to the testing site (*Exercise Laboratory, Department of Physiological Sciences, Stellenbosch University*) at the determined time of day for a total of 5 times on specified days, viz. protocol day -7, 0, 1 and 2 (twice on protocol day 0).

## 3.3 Study Procedure

Participants were informed of all details of the research and signed informed consent forms. Baseline measurements were performed and participants were randomly divided into three treatment groups by an academic not involved in the study. Group 1 received the GSE (*Oxiprovin*™) as treatment. Group 2 received the pharmaceutically produced GTH precursor; NAC as treatment for use as an anti-oxidant control group. Group 3 received placebo (microcrystalline-filled) capsules with no nutritional value. All procedures performed were identical for each of the treatment groups. The study comprised a six-day wash-out period (protocol days: -12 to -7), followed by the collection of baseline measurements, a six-day supplement loading period (protocol days: -6 to -1) before a muscle-damaging plyometric exercise intervention was performed by all participants (protocol day 0). Supplementation continued for an additional 2 days (protocol days: 1 to 2). Blood samples were acquired and various other parameters were measured throughout the study period. Vertical jump performance and all other measures were identical for all treatment groups. No muscle biopsies were taken during this study. The need for isokinetic testing had been eliminated as findings from previous studies employing these methods were suffice in presenting a loss in muscle power and function in a more adapted population after a less strenuous plyometric exercise protocol (274). The concise research protocol is explained below followed by a detailed account of the specific procedures. See Figure 3.1 for an overview of the experimental protocol).

## 3.4 EXPERIMENTAL PROTOCOL (TIME-COURSE)

The time-course of the study is depicted in Figure 3.1 and will be described in the following section.

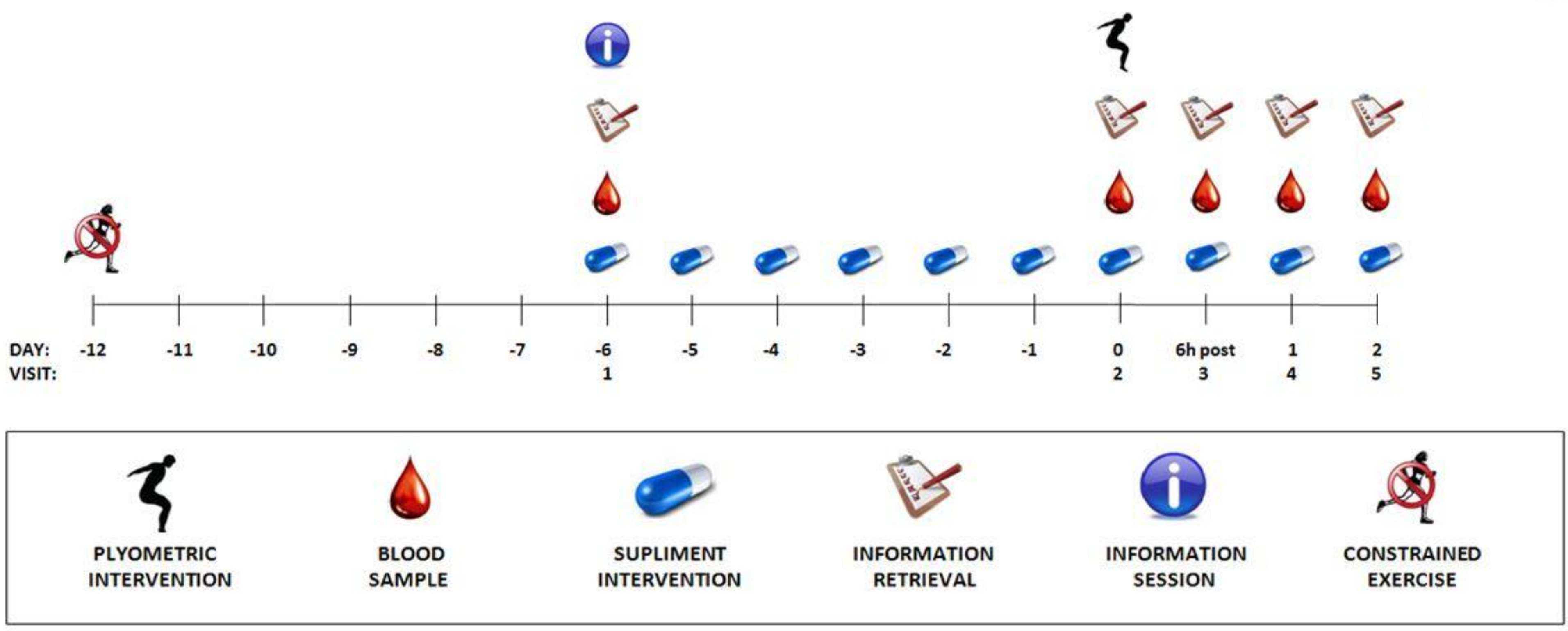


***Figure 3.1: Visual representation of the 15-day experimental protocol.*** *Participants were required to visit the laboratory on five occasions during this period. Blood draws were taken at six time-points, viz. -7 days (baseline), pre- and post-exercise, as well as at 6 h, 24 h and 48 h after the plyometric exercise intervention.*

### 3.4.1 Protocol Day -12 to -6 (Wash-Out Period)

On protocol day -12 (i.e. one week prior to the exercise intervention) all participants were telephonically reminded of their restrictions and the investigator's expectations. These restrictions were required for the duration of the study, i.e. until the last blood sample is taken in order to control for additional muscle damage, earlier recovery, enhance performance or deviant readings in free radical absorbance capacity. Where applicable, deviations from restrictions were logged accordingly by the participant and were considered when interpreting the results. No visit to the testing site was required until protocol day -6, i.e. after a supplement loading phase period of one week.

### 3.4.2 Protocol Day -6 (Visit 1: Baseline Measurements)

Upon the first laboratory visit, participants were again informed of the nature of the experimental procedure, and offered the opportunity to discuss and ask questions. Thereafter, participants completed questionnaires concerning personal particulars, medical history, sporting history and exercise habits (Appendix H). Participants completed a pain assessment questionnaire according to a visual analog scale (VAS) in response to a squat test (as later described). Baseline measurements were taken including height, weight, resting blood pressure and heart rate, followed by the first blood sampling. Participants were randomly assigned to treatment groups as previously explained and received their supplement regimes as well as instructions concerning administration, viz. 3 capsules orally with a full glass of water (approximately 330 mL) after breakfast, prior to each subsequent visit.

### 3.4.3 Protocol days -5 to -1 (No Visit: Treatment Loading Phase)

This period constituted the loading phase. Participants continued supplementation (as explained) while at home. There was no need for laboratory visits during this time.

### 3.4.4 Protocol days 0 (Visit 2: Plyometric Exercise Intervention)

A blood sample was collected after which the participants completed the VAS for pain assessment. Participants performed a 10-minute jogging warm-up session and an assessment of maximal vertical jump performance was conducted. Each participant was assigned to a wall section on which the height representing 90% of that particular participant's maximal vertical jump height had clearly been marked using red tape. This may

be visualised in Figure 3.2. During the plyometric exercise protocol, participants stood against the wall while facing an investigator. Investigators stood on elevated surfaces such that the red tape representing the participant's target jump height would be at the investigator's eye level. This was done to prevent possible error of parallax during the investigator's evaluation of each jump performed during the plyometric protocol.

***Figure 3.2: Participants performing the plyometric jump exercise protocol while being monitored (protocol day 0).***

Participants were asked to perform 15 sets of 10 near maximal vertical plyometric squat jumps with 60 second rest intervals between sets. Investigators would keep record of all failed attempts, and the count combined into a 16th set. However, in the case of this study, all participants successfully completed all of their jumps on first attempt and it was not necessary for a 16th set. A blood sample was taken immediately post exercise and participants were again asked to rate their perceived level of pain using the VAS.

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#### 3.4.5 Protocol days 0 (Visit 3: 6 hours post exercise)

At 6 h post exercise, a third blood sample was collected and a perceived muscle soreness assessment questionnaire was completed.

#### 3.4.6 Protocol Days 1 and 2 (Visits 4 and 5: 24 and 48 hours post exercise)

A single blood sample was collected from each participant on the morning after the exercise intervention (24 h post exercise intervention) and participants were asked to complete the perceived muscle soreness assessment questionnaire. Similarly, this protocol was repeated at 48 h post-exercise (protocol day 2). On the last day of the study, participants were asked to complete the retrospective medicine, supplement and exercise questionnaire (Appendix J) and return their diet logs (Appendix I) that had been recorded daily by the participants from protocol day -7.

### 3.5 INTERVENTION PROTOCOLS

This study included two intervention protocols, namely the supplementation (with either *Oxiprovin*™, NAC or a placebo) and a non-severe plyometric EIMD model.

#### 3.5.1 INDUCTION OF MUSCLE DAMAGE

A non-invasive model of exercise-induced injury was identified as a most suitable model for use in the current study. Due to the elimination of surgical procedures, the model excluded the possibility of infection, and additional immune system activation which may have resulted following tissue damage such as incised skin and muscle biopsies.

#### 3.5.1.1 Warm up protocol:

To improve circulation and minimise risk of injury or strain, a pre-exercise warm-up protocol had been included in the study design. The warm up will consisted of a five-minute jogging activity, five minutes of stretching and three sets of five knee bends. This took place prior to any other form of physical exertion, including determination of each participant's maximum jump height.

#### 3.5.1.2 Maximum Jump Height Assessment:

Valuation of each participant's maximal vertical jump height was determined after completion of the warm-up protocol, in preparation for the plyometric exercise protocol. All participants were asked to perform 3 consecutive maximal vertical squat jumps while facing a wall. During each jump, participants made a chalk mark on the wall at the apex of the jump as attained by the fingertips. The highest of the three marks were chosen as the maximum vertical jump height. For each participant, the distance between the ground and their highest mark was used to calculate the target height required for the plyometric exercise intervention protocol.

#### 3.5.1.3 Plyometric Exercise Protocol

To induce non-severe, transient EIMD to the M. quadriceps (rectus femoris, vastus lateralis, vastus medialis and vastus intermedius), participants performed fifteen sets of 10 vertical squat jumps while maintaining at least 90% of maximal vertical jump height on all attempts. The sets were separated by a 60-second recovery interval during which participants were allowed to move around freely, sit down do light stretching and rehydrate.

The mechanism by which a plyometric model for EIMD is uniquely characterised may be summarised as the fast change between maximal eccentric and concentric actions (also known as the amortization phase). During the landing phase, the eccentric muscle contraction involves a lengthening motion while a 90° knee angle is adopted (against gravity) (Figure 3.3). This is followed immediately by a very fast transition (15-120 ms) to a maximal concentric contraction which will propel the participant into a jump while maintaining an upright trunk. As this motion differs from routine exercises, unaccustomed non-severe, transient muscle damage is incurred in the M. quadriceps. See Figure 3.4 for a visual representation of the various contractile phases of a plyometric jump.

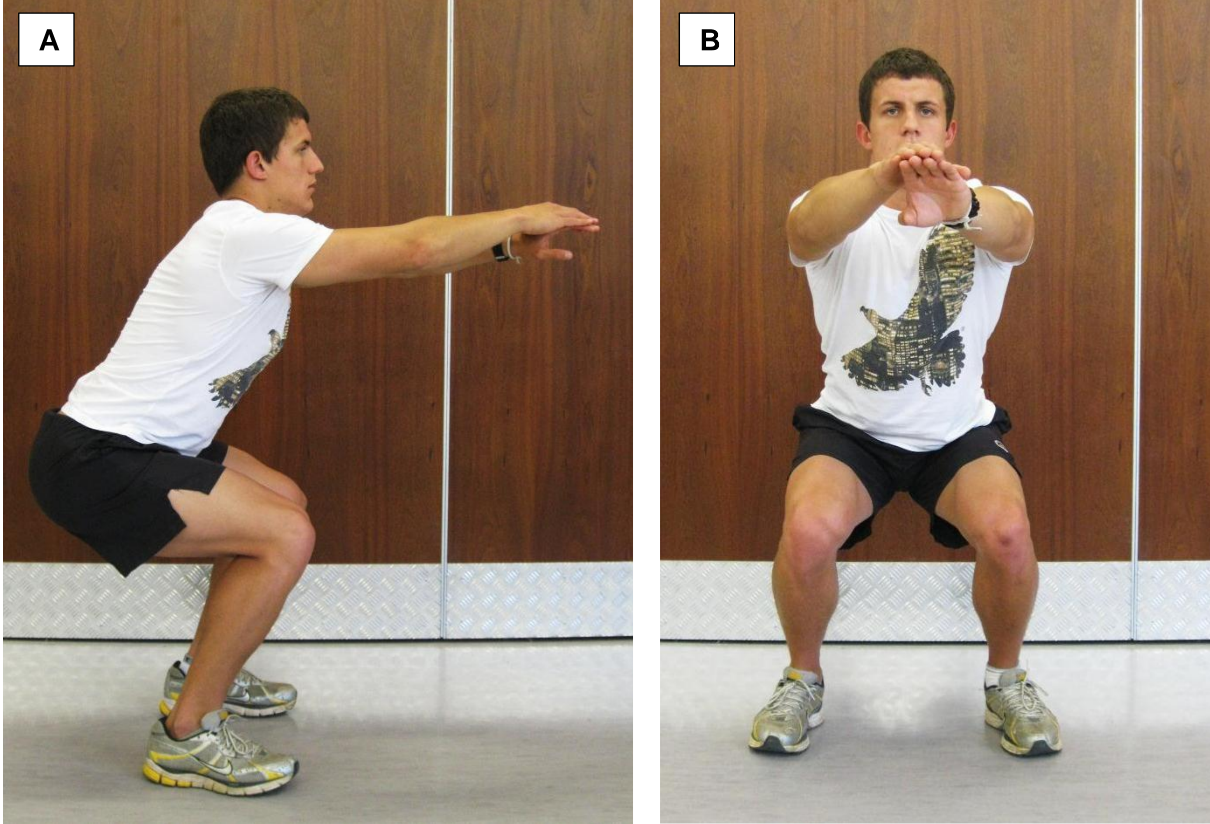


***Figure 3.3: Correct squat technique.*** *[A] lateral view. [B] anterior view.*

#### 3.5.1.4 Injury prevention:

The exercise regime occurred in a controlled environment with emphasis on appropriate technique and with continuous monitoring by capable investigators. Participants whom had previously sustained moderate to severe injuries to their lower extremities (ankle, feet, and knee, upper and or lower leg) were screened before participation in the study. If evidence of insufficient rehabilitation post-injury had been presented, or if the injury had occurred within the previous 6 months, the potential participant would have been excluded.

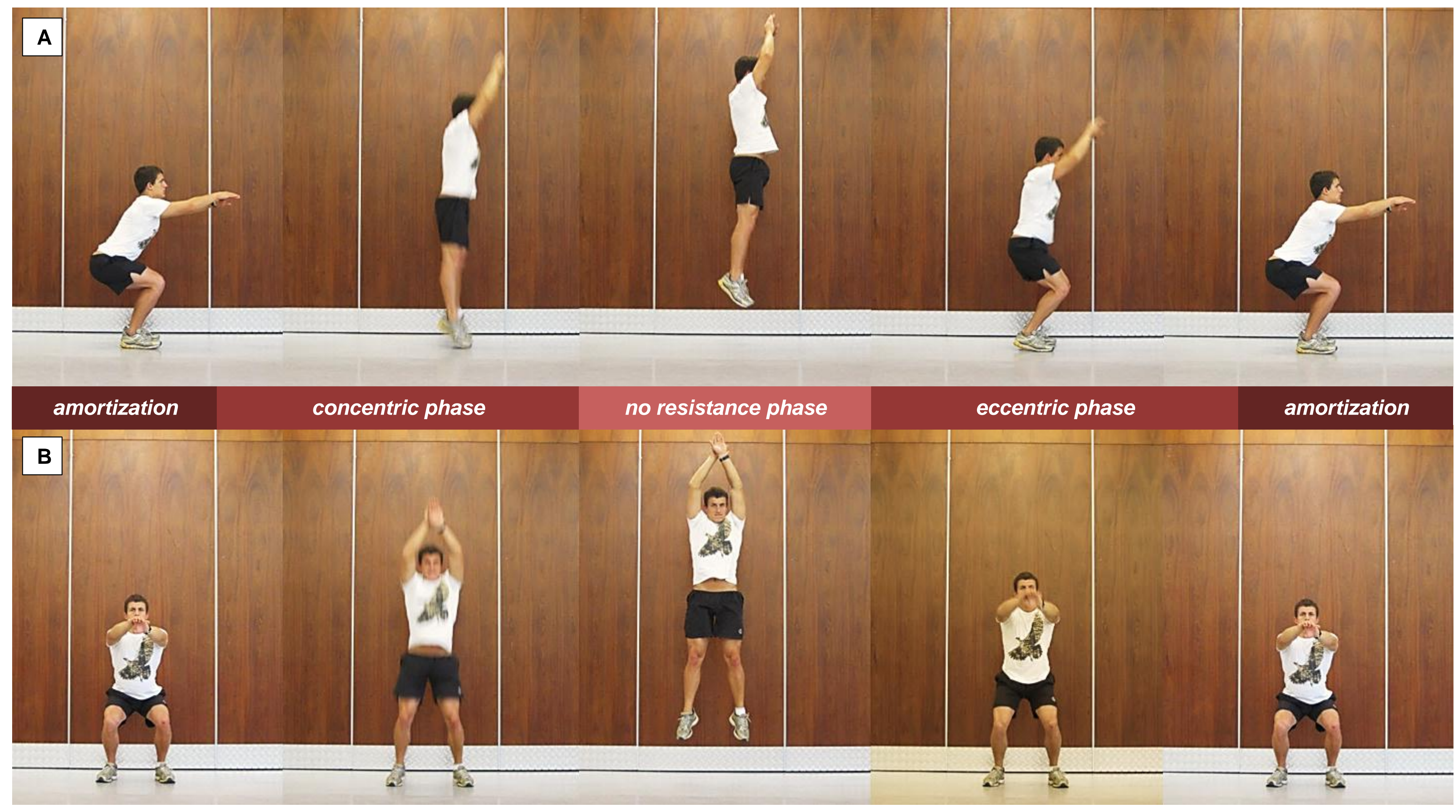


***Figure 3.4: The contractile phases of a plyometric squat jump.*** *[A] lateral view. [B] anterior view.*

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### 3.5.2 Supplementation

All participants were randomly assigned to one of the three equally sized treatment groups. These were *Oxiprovin*™ (GSE), NAC (control anti-oxidant) or placebo (microcrystalline powder) treatment groups. Because the study was performed as double-blind, neither investigator nor participant was aware which of the three treatment groups a participant belonged to. Treatments were assigned to participants by an academic not involved in the study using coded identifiers which only the academic had access to. Treatments seemed identical in terms of both internal and external appearance, taste and smell. Consequently, the specific treatment group were not recognisable. The dosage of the active agent for all treatment groups was equivalent. For all groups, each capsule contained 70 mg of the active agent (*Oxiprovin*™, NAC or microcrystalline powder). To match the *Oxiprovin*™ treatment regime (the consumption of three capsules daily for nine consecutive days), all treatment groups were instructed to do likewise. Therefore, all participants received 27 capsules of their respective treatment. Participants were instructed to swallow three capsules daily with at least 330mL (approximately one glass) of water, after breakfast. Supplementation commenced seven days prior to the exercise protocol (as part of the previously described loading phase) and continued until the day of the last blood draw (two days after the exercise intervention). On the day of the exercise intervention (or days that blood samples were taken) supplementation was to occur at least 30 minutes prior to the laboratory visit. In the event that any negative effect should have occurred as result of use of either supplement, treatment would have been terminated immediately. The study doctor would have been informed and the participant would exit the study at no detriment to themselves.

#### 3.5.2.1 *Oxiprovin*™ Supplementation

It is recommended that one capsule be taken twice daily (with or dissolved in water) as per the package insert (see Appendix A). A previous study has shown that chronic supplementation (or protocols including a loading phase) produce more significant results than acute supplementation (129). In some cases trends could be detected, but were of poor significance, possibly due to insufficient amounts of supplementation. Thus a higher dose administration may be more feasible in determining a significant proanthycyanidic effect with particular regards to limiting the neutrophil response after EIMD. Therefore, participants supplemented with three 70 mg capsules (thus a total of 210mg *Oxiprovin*™) daily. This dosage is still considered safe for ingestion according to an assessment of the risks and safety of polyphenol consumption (176).

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#### 3.5.2.2 N-acetylcysteine Supplementation

100% pure NAC powder was specially produced by *Warren Chem Specialities Pty Ltd* (11 Mansell Road, Killarney Gardens, Cape Town, *ISO 9001: 2008*) for use in this study (see Appendix B). The NAC powder was capsulated by qualified pharmacists at *Coetzenburg Pharmacy* (*Langehoven Student Centre*, Stellenbosch) using empty capsules as provided by *Brenn-O-Kem*, (Wolseley, South Africa), the manufacturers of *Oxiprovin*™. The end-product obtained was capsules identical to *Oxiprovin*™ and the placebo capsules, but with each capsule containing 70mg of 100% pure NAC power. The amount of NAC per capsule was determined such that there would be an equal antioxidant capacity between the *Oxiprovin*™ and NAC supplemented groups. Thus supplementation comprised a total of 210mg NAC (3 capsules) per day, which is substantially lower than the dosage approved by the MCC. Nevertheless, notification had been submitted to the MCC that NAC is being used outside of its licenced indication, and it was not necessary to register the study as a clinical trial.

#### 3.5.2.3 Placebo (Microcrystalline Powder) Supplementation

The third group received a placebo treatment. The placebo capsules were specially prepared by the manufacturers of *Oxiprovin*™ (*Brenn-O-Kem*, Wolseley, South Africa) using microcrystalline cellulose (which presents no strong scent or flavour). The capsules used were identical to those used for the *Oxiprovin*™ and NAC treatment groups and was therefore identical in appearance both internally and externally with even the contents resembling that of *Oxiprovin*™ and NAC powder.

***Figure 3.5:*** *Identical OxiprovinTM (left), NAC (middle) and microcrystalline placebo powder (right) capsules. Neither of the treatment groups could be characterised by a scent, flavour or appearance.*

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## 3.6 Data Collection

Data was collected in the form of information retrieved from participants by the completion of forms and questionnaires. All other data was obtained from whole blood samples taken at relevant time-points throughout the study. Both methods are detailed below.

### 3.6.1 Information Collection

#### 3.6.1.1 Personal Particulars

Prior to participating in the study, participants completed the prerequisite documentation necessitating them to detail their personal particulars (such as age and nationality), medical history (including current medical conditions or medications). Participants also had to complete an injury history questionnaire in which they could indicate the anatomical area(s) where they are currently injured or had experienced injury within the previous 12 months. Participants also completed a section describing their rugby history as well as their participation in other sports, training habits and other forms of exercise. If it was found that participants could not satisfy both the inclusion and exclusion criteria, they were excluded from the study. Please see Appendix H for the relevant documentation used.

#### 3.6.1.2 Baseline Measurements

Baseline measurements for the assessment of the general health status of participants included measurement of body mass, height, blood pressure and heart rate. These measurements were all performed by skilled investigators and using good clinical practice. All measurements were taken at the same time of day while participants were in a relaxed state.

#### 3.6.1.3 VAS Muscle Soreness and Fatigue Assessment (DOMS testing)

Perceived muscle soreness scores were obtained in order to subjectively determine pain, soreness and discomfort of the M. quadriceps muscle group throughout the study and at time-points corresponding with all other measured parameters. This was done using a VAS for pain assessment. The questionnaire (Appendix K) required participants to rate their perceived level of soreness, pain and discomfort in response to a squat-test. The squat test comprised three slow (two second) squats, while focusing on the possible sensation (pain, soreness and discomfort) experienced in the M. quadriceps. Ratings were indicated by means of a numeric scale similar to that of the Borg's rating scale of perceived exertion. The

scale was numbered from 0 to 10, with 0 being indicative of no pain, soreness and discomfort and 10 representing unbearable pain. These perceived muscle pain, soreness and discomfort scores were taken at all time-points immediately prior to blood sample collections.

#### 3.6.1.4 Exercise and Diet Log

Because nutrition and activity affect physiological profiles and so also the possible outcomes of this study, participants agreed to abstain from exercise, supplements and various medications. Ideally an investigator may wish to control the diet and activity level of participants to a greater extent, possibly by standardising meals; however, this would not reflect real-time scenarios where a healthy population with an uncontrolled diet is considered. For this reason, participants were required merely to log their daily dietary intake and activities using the exercise and diet log provided (Appendix I). All participants logged their dietary intake and activities daily for the duration of the study. The completed logs could then serve to provide a level of context from which to understand the results.

#### 3.6.1.5 Retrospective Medication and Supplement Log

Although participants were required to (and agreed to) abstain from the use of medication, health supplements, smoking and alcohol for the duration of the study, deviations may have occurred. Therefore participants were necessitated to complete a retrospective medication and supplement questionnaire (included as Appendix J) on the last day of the study. Participants were asked to describe the type of medications; vitamins, minerals, stimulant, performance or recovery supplements used and give the product name where applicable. They were also required to indicate the frequency and total amount of times that each was used during the study.

### 3.6.2 Whole-Blood Sample Collection

For each of the participants, a total of six whole blood samples were collected on four different days [protocol days: -6, 0 (three times), 1 and 2]. The first blood sample was collected on protocol day -6 (baseline). On the day of the exercise intervention (protocol day 0), three blood draws were taken (before, immediately after and again 6 h after the plyometric exercise protocol was performed). With the exception of the pre- and six h post-exercise blood draws on protocol day 0, all blood samples were taken consistently at the same time of day (08h00). This was intended as a control for diurnal changes such as

hormonal fluctuations. The plyometric exercise protocol was scheduled such that the sample taken immediately post exercise could occur at 08h00 in consistency with the baseline and following day's blood draws. Therefore, the pre-exercise blood samples were taken at 07h30 (before the warm-up exercise, maximal vertical jump performance and the plyometric exercise intervention). The 6 hours post exercise samples were taken at 14h00. All following blood samples (protocol days: 1 and 2) were taken at 08h00.

During draws, participants were required to rest in the supine position for the full duration of the procedure. Blood samples were primarily drawn from the brachial vein on the anterior surface of the elbow by qualified phlebotomists. A total sample volume of 14mL whole blood was collected per occasion. This was done using two 5mL *SST™ II Advance* blood collection tubes, and a single 4mL *EDTA™ (K2E 7.2mg)* blood collection tube. The former would be used for serum separation and the latter for analyses that require whole blood samples. Both used tube types are manufactured by *BD Vacutainer®, Becton Dickinson and Company*.

The procedures followed for each of the filled tubes are detailed below.

#### 3.6.2.1 *SST™* (2 x 5mL) tubes for Serum Separation

10mL whole blood was collected from each participant in two (5mL) serum separating *SST™ II Advance* blood collection tubes to ensure sufficient serum availability for the various analyses. The fresh samples were allowed to clot at room temp for 10 minutes before being centrifuged using an *Eppendorf Centrifuge 5804 R* (*Merck Chemicals [Pty] Ltd*). Centrifugation was performed at 1500g (or 3000 rpm) for 10 min at 4°C as per the protocol for good sample preparation provided by *BD Vacutainer®, Becton Dickinson and Company*. The serum samples were then aliquated into 5 ml eppendorf tubes and immediately frozen at -80°C for later analysis. The serum separating *SST™ II Advance* blood collection tubes containing the supernatant were discarded appropriately and marked for incineration.

#### 3.6.2.2 EDTA (1 x 4mL) Tubes for Whole Blood Sample Analysis

4mL whole blood was collected from each participant in one (4mL) *EDTA™ (K2E 7.2mg)* blood collection tube. This amount would be sufficient for a full blood cell count as well as a differential white blood cell count.

## 3.7 Sample Analysis

The analytical profile included the following procedures:

- Total serum CK activity was determined by the *Medi-Clinic PathCare Pathology Laboratory* (4 Saffraan Avenue, Stellenbosch, Western Cape, South Africa) using an enzymatic method by means of a *Beckman Coulter DXC/LX* system.
- The oxygen radical absorbance capacity (ORAC) assay was performed on all serum samples using a preheated reader (*$FL_X$800 Microplate Fluorescence Reader, Bio-Tek Instruments Inc.*) in accordance with the protocol (Appendix L) published by Huang *et al.* (101).
- The serum MDA analysis was performed at the *Antioxidant Research Unit* of the *Cape Peninsula University of Technology (CPUT)* Bellville Campus (Symphony Way, Bellville, Western Cape, South Africa) by spectrophotometry using the TBARS method (see Appendix M). Samples were run in triplicate, and the results expressed as the mean score of three readings (µM per litre of serum).
- An enzyme-linked immunosorbent assay (ELISA) was performed for the quantitative detection of human soluble vascular cellular adhesion molecule-1 (sVCAM-1) in serum samples. The analysis was performed using *Human sVCAM-1 Platinum ELISA* kits manufactured by *eBioscience* ® (product code: BMS232) in accordance with the protocol provided.
- Both a full blood cell count as well as a differential white blood cell count was performed by the *PathCare Haematology Unit* (PathCare Reference Laboratory and Business Centre, PathCare Park, Neels Bothma Street, N1 City, Goodwood, Cape Town, Western Cape) using a *Beckman Coulter LH 750* system.

## 3.8 Statistical Analysis

All statistical analyses were performed using *Statistica* version 10.0 (*StatSoft Software*). All data are presented as means ± standard deviation (SD) or mean (SD), unless otherwise stated. A p-value of less than 0.05 was regarded as statistically significant ($p < 0.05$). Data was assessed for normal distribution. Differences between time-points and treatment groups were analyzed using factorial analysis of variance (ANOVA). When significant differences were detected, these were evaluated by Bonferroni post hoc analysis. For parameters with low sample number (e.g. n=5 for the *Oxiprovin*™ treatment group), statistical power of the analysis was assessed, and the results interpreted and discussed in context.

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# CHAPTER 4:

# Results

After a characterisation of the sample population including their baseline measurements is presented, the following results will be presented:

- serum CK concentrations (for use as a non-specific serum markers of skeletal muscle damage and myofibrillar disruption),
- serum ORAC (for evaluation of antioxidant capacity),
- serum MDA concentrations (for use as an indicator of oxidative stress),
- serum sVCAM-1 concentrations (identified as most relevant adhesion molecule within the context of the current study),
- differential white blood cell count (comprising: neutrophils, lymphocytes, monocytes, eosinophils and basophils) expressed as cells/L and as percentages of the total white blood cell (leukocyte) count and
- perceived muscle soreness scores in response to a squat test

## 4.1 Sample Population Profile and Baseline Measurements

For both NAC and placebo treatment groups n = 8 participants per time-point. However, for the *Oxiprovin*™ Treatment group, n = 5, as two of the participants from this group had withdrawn after baseline measurements were taken, while the data gather from another was removed after non-compliance was reported. The baseline measurements for all participants (n = 21) that satisfactorily completed the study are presented in Table 4.1.

The 21 participants included 10 specifically backline players while 9 were forwards. The remaining two participants were unassigned. Furthermore, of the 15 players positions which typically comprise a rugby union team, the following player positions were covered, viz. (i) backline: scrum-half (one), fly-half (three), winger (two), centre (two), fullback (one); (ii) loose forwards: number 8 (two), flank (four); and (iii) tight forwards: lock (three), hooker (two), unassigned (two). This data is summarised in table 4.2.

***Table 4.1: Sample population profile (denoted as mean ± SD)***

| | |
|---|---|
| Age (years) | 21.05 ± 1.88 |
| Height (m) | 1.83 ± 0.05 |
| Mass (kg) | 86.12 ± 12.82 |
| BMI (kg/m²) | 25.70 ± 2.99 |
| Blood Pressure (Systolic) | 135.05 ± 12.57 |
| Blood Pressure (Diastolic) | 78.95 ± 6.73 |
| Heart Rate (B/M) | 62.54 ± 6.68 |
| Years of Competitive Rugby Experience | 8.53 ± 3.61 |

Furthermore, a comprehensive assessment of player position, training habits and injury status, revealed the following:

***Table 4.2: Distribution of grouped player positions within treatment groups***

| Treatment Group | Forward-Line Players | Back-Line Players | Unassigned Position |
|---|---|---|---|
| ***Oxiprovin™*** | 1 | 4 | 0 |
| **NAC** | 4 | 3 | 1 |
| **Placebo** | 4 | 3 | 1 |

### 4.1.1 Training Habits

Findings from the questionnaires disclosed that most participants engaged in regular cross-training at least two to three times per week in addition to their usual rugby practice and match play schedule. These additional forms of training mostly included running, swimming and weight-training. Although exercise habits varied between individuals, the level of activity was similar for all three treatment groups. During the study period, none of the participants partook in any form of exercise which could have negatively affected the outcome of this experiment.

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### 4.1.2 Health Status

None of the players had any form of injury which had not completely been resolved prior to their participation in the study. Of the 21 participants, 11 had sustained some form of injury in the previous 12 months (viz. two ankle sprains, two wrist/hand sprains, two shoulder injuries, a hamstring strain, a groin/hip strain, and three knee injuries), all of which had since resolved.

Of the 21 participants, two suffered from on-going medical conditions. One of the participants chronically received clomipramine hydrochloride, in its commercial form; *Anafranil®* (*Mallinckrodt Pharmaceuticals*) and fluoxetine hydrochloride in the commercially available form of *Lilly-Fluoxetine®* (*Eli Lilly and Company*). Both of these were for the treatment of Obsessive-compulsive disorder (OCD). The dosage taken of each varied as prescribed by the participant's health-care practitioner. This participant had randomly been assigned to the placebo group. Lastly, a participant presenting with ance vulgaris was receiving zinc picolinate and selenium together in the form *Zinplex®* capsules (manufactured by a highly reputable third party manufacturing company for and on behalf of *Zinplex®*). The participant had randomly been assigned to the treatment group that had received NAC supplementation. None of the participants that had randomly been assigned to the *Oxiprovin*™ treatment group were receiving any form of chronic medication.

The retrospective medication and supplement questionnaire revealed that several participants occasionally made use of additional supplementation during their participation in the study. For the most part, these were isolated incidents with the intention of improving performance on days of university examinations, or for the treatment of headaches. The timing of each could not be determined.

Supplementation and medication reported for the placebo-supplemented group:

- *ViralGuard*™ tablets (*Nutrilida*)
- *Vita-Thion®* Tonic (*Adcock Ingram Ltd*)
- *Panado-Co®* Tablets (*Adcock Ingram Ltd*)
- *Disprin®* effervescent tablets *(Reckitt Benekiser Healthcare [UK] Ltd*)

Medications and supplements reported by the NAC-supplemented group:

- *Pharmaton®* capsules (*Boehringer Ingelheim*)
- *PowerPlay™* energy drink (*The Coca-Cola Company*)
- *Disprin®* effervescent tablets *(Reckitt Benekiser Healthcare [UK] Ltd*)

The *Oxiprovin*™ supplemented group reported no medication use or additional supplementation. Although the use of medications and other supplements had clearly been restricted for the duration of the study, compliance could not be controlled. The above mentioned data needs to be considered when interpreting the following results.

## 4.2 Non-Specific Indicators of Muscle Damage

Data analysis of CK activity revealed no significant differences between groups. However, an ANOVA main effect of time was observed for the CK response ($p<0.05$). At the 24 h time-point, CK activity was significantly higher than at baseline, pre- and post-exercise, in both the Placebo and NAC treatment groups ($p<0.05$). However, the *Oxiprovin*™ treatment group presented with no significant change in CK activity before or after exercise. This data is presented in Figure 4.1 (Panel A). CK activity is expressed as IU/L (international units per litre). The normal range for human serum CK activity at 37°C is 15 – 195 IU/L.

Although no significant differences were found between groups, all groups displayed main effect of time ($p<0.05$) on DOMS experienced while squat test was performed (Figure 4.1 Panel B). For all groups, DOMS scores were significantly higher at 6 h, 24 h and 48 h after exercise when compared with baseline and pre-exercise scores. Furthermore, by 48 h after exercise, DOMS scores had increased significantly ($p<0.05$) from the scores taken immediately post and 6 h after the plyometric exercise intervention.

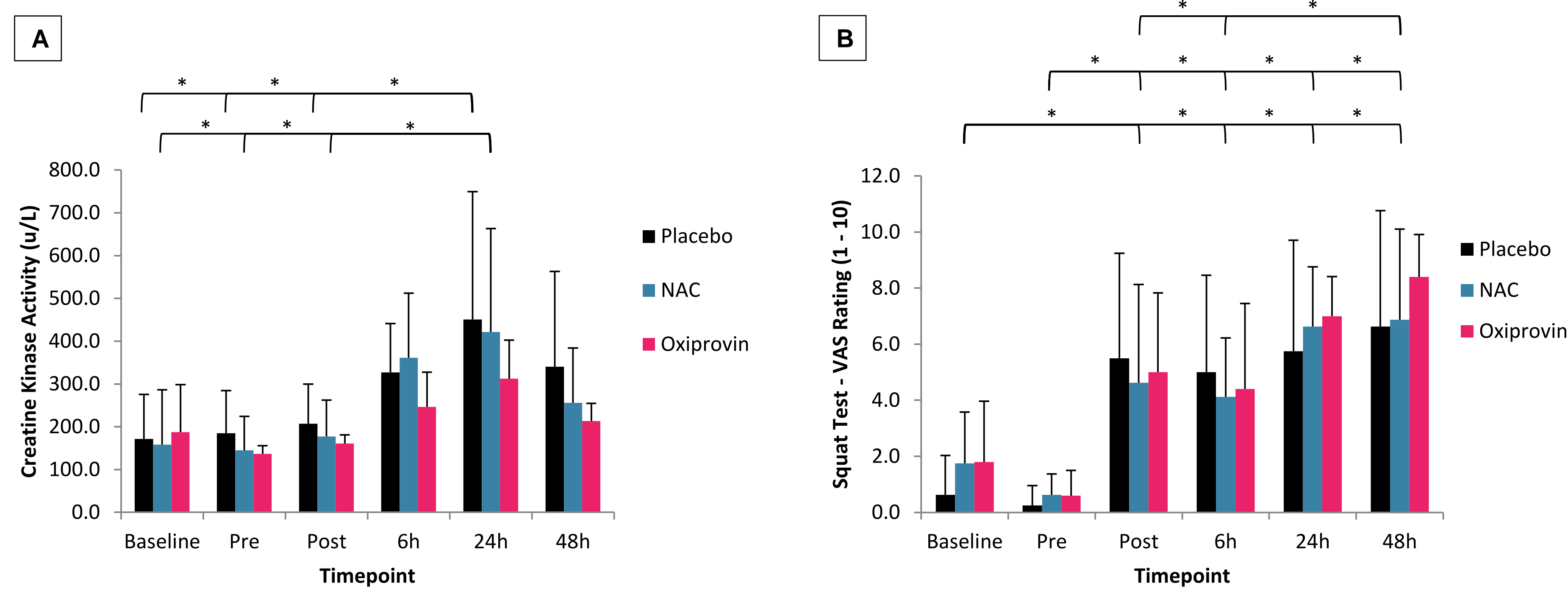


***Figure 4.1: The effect of plyometric exercise on non-specific markers of muscle damage for all treatment groups over time***

*Data analysis: Factorial analysis of variance (ANOVA) with Bonferonii post hoc test. Where main effect of time is evident, Bonferonii significance indicated in figures: *p<0.05. [A] CK Activity. Main effects of time was indicated by ANOVA (p<0.05). [B] Pain assessment. ANOVA main effect of time was significant and confirmed by post hoc analysis (*p<0.05).*

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## 4.3 SERUM ORAC AND MDA CONCENTRATIONS

Values are expressed as ORAC units (1 micro-M trolox equivalent). High inter-individual variability was evident for ORAC (mmol trolox equivalent/L) and large SDs were evident for all groups [Figure 4.2 (A) and Table 4.3]. Therefore ORAC values were recalculated as percentages of their respective baseline readings to allow for a more representative identification of treatment or time effect, or other interactions [Figure 4.2 (B)]. No significant differences were found between treatment groups across all time-points. However, in the *Oxiprovin*™ treatment group, the ORAC had decreased significantly by 6 h post exercise when compared with those readings taken immediately before exercise (ANOVA main effect of time, $p<0.05$) as seen in Figure 4.2 (A). Although main effect ANOVA did indicate interaction between treatment and time ($p<0.05$), this could not be confirmed using post-hoc tests. When readings are expressed as percentages of baseline readings, the NAC treatment group presented with a significantly increased ORAC at 48 h post exercise when compared with data obtained immediately post exercise [ANOVA main effect of time, $p<0.05$; Figure 4.2 (B)]. No other significant effects were found for treatment over time. Data analysis of serum MDA concentrations revealed no significant differences between treatment groups or within groups over time (Table 4.3).

## 4.4 SERUM SOLUBLE VCAM-1 CONCENTRATIONS

There were no significant differences in serum sVCAM-1 concentrations for groups over time or between treatment groups (Table 4.3). Although not significant, a trend towards a diminished sVCAM-1 response is evident in both NAC and *Oxiprovin*™ groups at all time-points after baseline. For both groups, mean values tended to be lower than those of the placebo treated group at all time-points after supplementation had commenced (viz. the pre, post, 6 h, 24 h and 48 h time-points). However, because of large variability between participants, this trend could not be established. Subsequently, a power analysis was performed which revealed a statistical power of 56.3% with the sample size available. This is also due to the high level of variability between participants (largely the placebo treated group). Thus in order for the differences observed between treatment groups to be a deemed significant, an subject number of at least 11 (per group) would be required in this population.

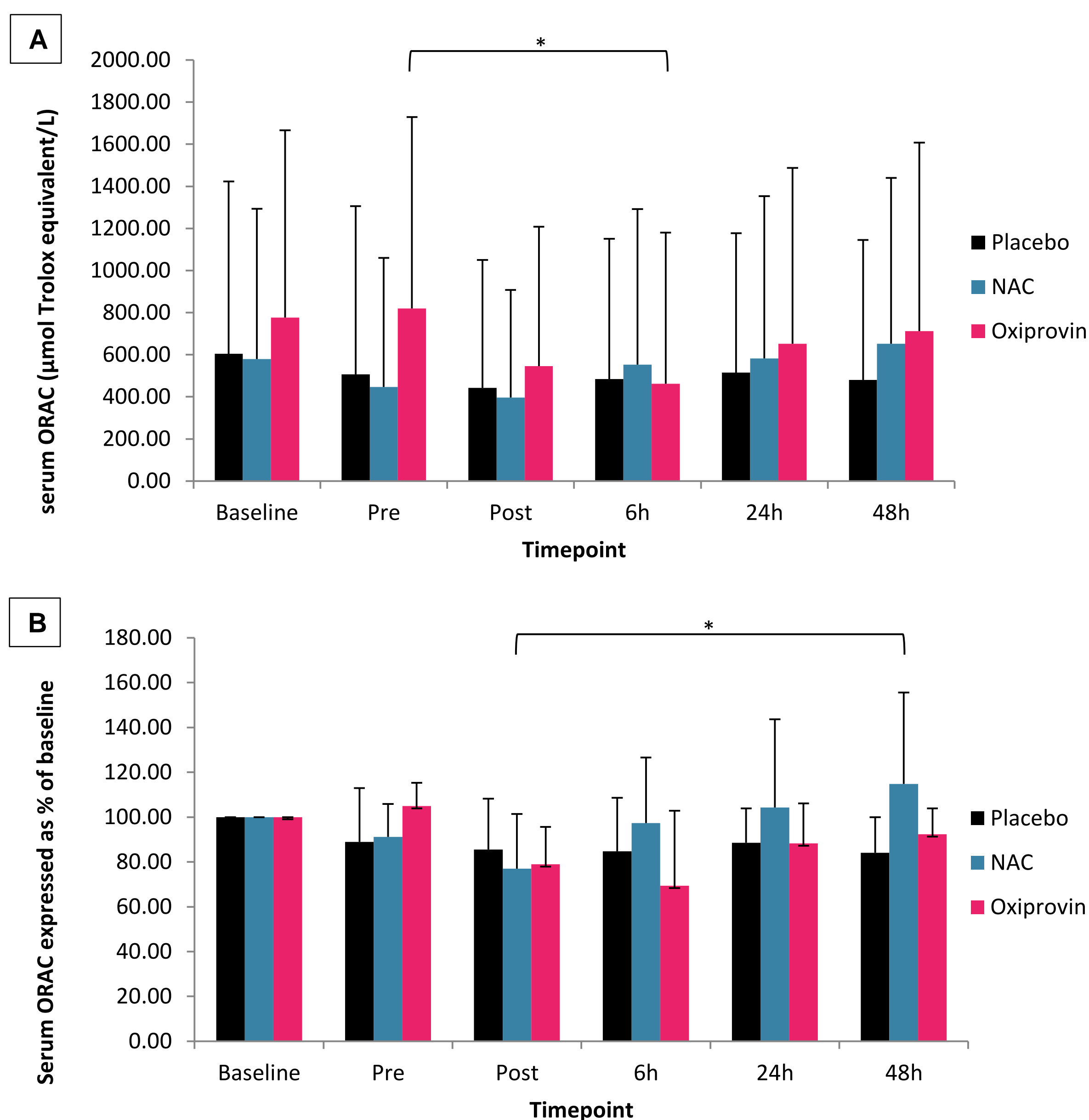


***Figure 4.2: The effect of plyometric exercise on the serum ORAC of all treatment groups over time***

**(A)** Comparison of serum ORAC over time (expressed as µmol Trolox equivalents per litre of serum). Factorial analysis of variance (ANOVA) with Bonferonii post hoc test (*p<0.05) indicates a significant time effect in the *Oxiprovin*™ group, but not in the placebo and NAC treatment groups.

**(B)** Change in serum ORAC (expressed as % of respective baseline values). ANOVA main effect of time was significant in the NAC group after post hoc analysis (*p<0.05).

***Table 4.3: Data obtained for sVCAM-1, ORAC and MDA analyses***

| | Treatment | Baseline | Pre | Post | 6 hours | 24 hours | 48 hours |
|---|---|---|---|---|---|---|---|
| **sVCAM-1** (pmol/L serum) | **Placebo** | 946 (135) | 938 (390) | 789 (156) | 1115 (632) | 884 (567) | 1048 (778) |
| | **NAC** | 881 (202) | 596 (165) | 855 (254) | 548 (189) | 558 (114) | 641 (212) |
| | ***Oxiprovin*™** | 832 (202) | 499 (165) | 819 (254) | 670 (189) | 653 (114) | 622 (212) |
| **ORAC** (µmol Trolox equivalents/L serum) | **Placebo** | 605 (818) | 507 (799) | 443 (607) | 484 (667) | 515 (662) | 480 (665) |
| | **NAC** | 580 (714) | 447 (613) | 396 (512) | 552 (740) | 583 (770) | 652 (788) |
| | ***Oxiprovin*™** | 777 (889) | 820 (910) | 546 (664) | 462 (717) | 653 (835) | 713 (895) |
| **MDA** (µM/L serum) | **Placebo** | 2.9 (0.6) | 3.1 (0.5) | 3.1 (0.4) | 2.8 (0.8) | 3.4 (0.6) | 3.0 (0.7) |
| | **NAC** | 3.1 (0.6) | 3.3 (0.9) | 3.1 (1.3) | 2.9 (0.6) | 3.3 (0.4) | 3.5 (0.8) |
| | ***Oxiprovin*™** | 3.9 (0.5) | 3.7 (0.7) | 3.5 (0.6) | 3.0 (1.2) | 3.6 (0.7) | 3.2 (0.8) |

*All data expressed as mean (SD).*

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## 4.5 Leukocyte Count

A significant increase in neutrophil and monocyte count was found over time and is shown in Figure 4.3. However, no treatment effect was reported between groups. The neutrophil count of all three treatment groups peaked at 6 h post exercise and returned to values similar to those at baseline by 48 h post plyometric intervention ($p<0.05$), as shown in Figure 4.3 (A). Similar findings were obtained even when the neutrophil count was expressed as a percentage of the total leukocyte count [Figure 4.3 (B)]. The monocyte count also peaked significantly at 6 h post exercise when compared with other time-points before and after the exercise intervention ($p<0.05$) in all treatment groups, as shown in Figure 4.3 (C). However, when expressed as a percentage of the total leukocyte count, the monocyte count does not peak significantly at the 6 h time-point in the *Oxiprovin*™ treatment group [Figure 4.3 (D)].

Lymphocyte counts increased significantly between baseline and pre-exercise measurements in both the NAC and *Oxiprovin*™ supplemented groups ($p<0.05$), but not in the placebo group. Basophils and eosinophils presented no relevant differences. The data sets for the total leukocyte count and remaining leukocyte sub-populations are summarised in Table 4.4 as mean (SD). All cell counts are expressed as cells/L of fresh human whole blood samples at 37°C. In the case of white blood cells, the sub-population counts were also expressed as a percentage of the total white blood cell count.

The normal expected range for the total white cell count is 4.0 – 11.0 x $10^9$ cells/L.

The normal range for each of the particular white blood cell sub-populations are listed below:

Neutrophils: 2.00 – 7.50 x $10^9$ cells/L

Lymphocytes: 1.00 – 4.00 x $10^9$ cells/L

Monocytes: 0.00 – 0.80 x $10^9$ cells/L

Eosinophils: 0.00 – 0.40 x $10^9$ cells/L

Basophils: 0.00 – 0.10 x $10^9$ cells/L

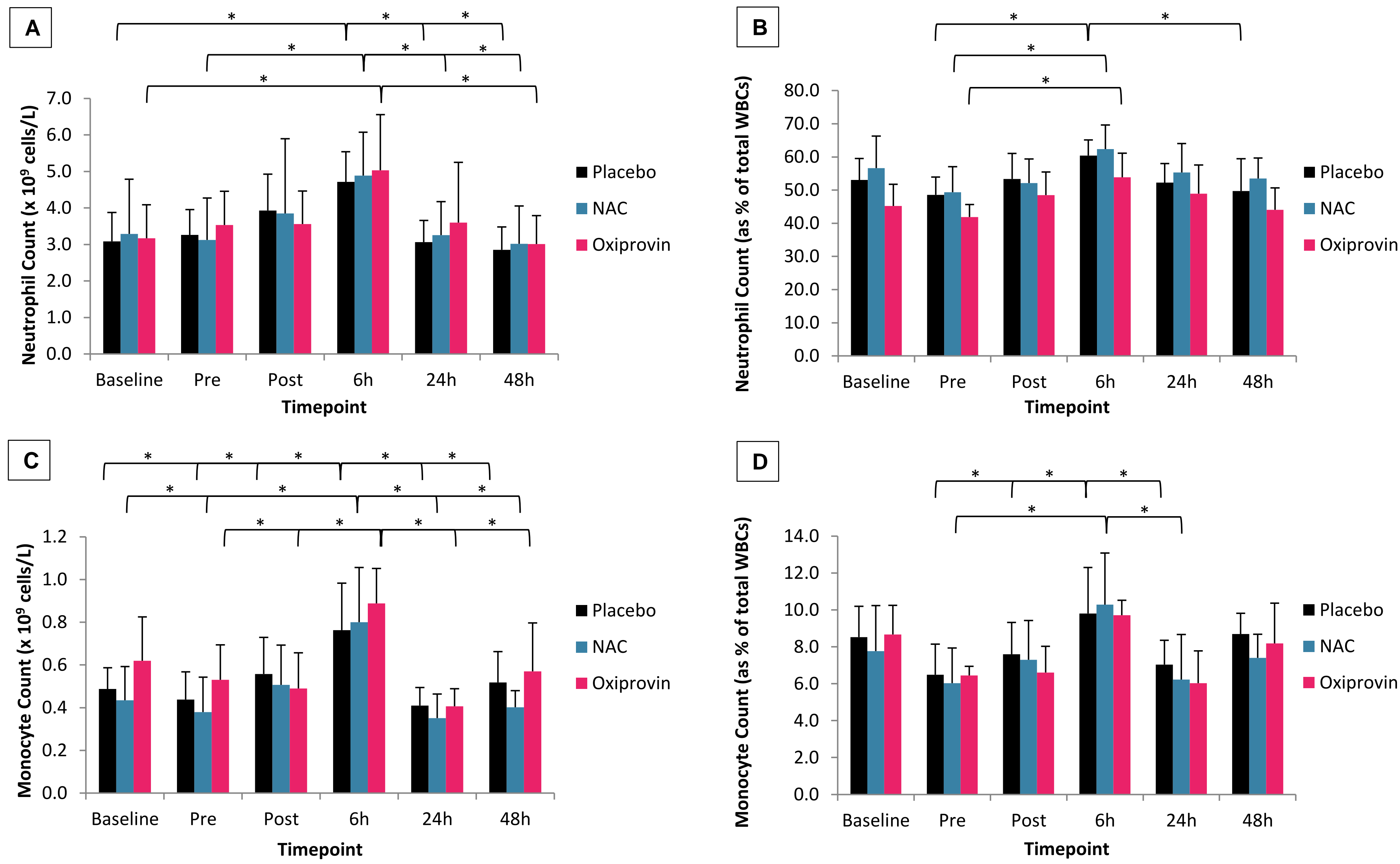


***Figure 4.3: The effect of plyometric exercise on numbers of circulating neutrophils and monocytes in all treatment groups over time.*** *[A] Neutrophils. [B] Neutrophils (as percentages of total leukocytes) [C] Monocytes [D] Monocytes (as percentages of total leukocytes). Factorial analysis of variance (ANOVA) with Bonferonii post hoc test (*p<0.05) indicates a significant time effect in A, B, C and D.*

***Table 4.4: Data obtained after performing a full leukocyte count of samples from all treatment groups at all time-points***

| | Treatment | Baseline | Pre | Post | 6 hours | 24 hours | 48 hours |
|---|---|---|---|---|---|---|---|
| Total WBC | Placebo | 5.8 (0.9) | 6.7 (1.1) | 7.4 (1.8) | 7.8 (0.8) | 5.9 (0.8) | 5.9 (1.4) |
| | NAC | 5.6 (1.6) | 6.2 (1.6) | 7.2 (3.1) | 7.8 (1.5) | 5.8 (0.9) | 5.6 (1.5) |
| | *Oxiprovin*™ | 7.0 (1.6) | 8.4 (1.9) | 7.3 (1.5) | 9.2 (1.8) | 7.1 (2.0) | 6.9 (1.7) |
| Lymphocytes | Placebo | 2.0 (0.3) | 2.7 (0.6) | 2.7 (1.1) | 2.0 (0.3) | 2.1 (0.4) | 2.2 (0.8) |
| | NAC | 1.8 (0.3) | 2.5 (0.6) | 2.7 (1.0) | 2.0 (0.5) | 2.0 (0.3) | 2.0 (0.5) |
| | *Oxiprovin*™ | 2.9 (0.6) | 4.0 (0.8) | 3.0 (0.5) | 3.0 (0.4) | 2.9 (0.4) | 3.0 (0.9) |
| Eosinophils | Placebo | 0.2 (0.2) | 0.3 (0.2) | 0.3 (0.3) | 0.2 (0.2) | 0.3 (0.3) | 0.3 (0.3) |
| | NAC | 0.2 (0.1) | 0.2 (0.1) | 0.2 (0.1) | 0.1 (0.1) | 0.2 (0.1) | 0.2 (0.1) |
| | *Oxiprovin*™ | 0.3 (0.2) | 0.3 (0.2) | 0.2 (0.2) | 0.2 (0.2) | 0.2 (0.1) | 0.2 (0.2) |
| Basophils | Placebo | 0.02 (0.03) | 0.01 (0.00) | 0.01 (0.00) | 0.01 (0.00) | 0.01 (0.01) | 0.03 (0.03) |
| | NAC | 0.01 (0.01) | 0.02 (0.01) | 0.02 (0.01) | 0.02 (0.03) | 0.02 (0.01) | 0.02 (0.01) |
| | *Oxiprovin*™ | 0.03 (0.04) | 0.03 (0.04) | 0.03 (0.04) | 0.01 (0.01) | 0.05 (0.04) | 0.03 (0.04) |

*Actual cell count = value x $10^9$ cells/L whole blood*

*All data expressed as mean (SD).*

# CHAPTER 5:

## DISCUSSION

The three principal findings in this study were:

- Firstly, chronic, high dose *Oxiprovin*™ treatment prevented a significant peak in the CK response that was seen in the placebo and NAC groups following a strenuous plyometric exercise intervention. This indicates that treatment using *Oxiprovin*™ impedes exercise-induced muscle damage. This has not previously been reported for *Oxiprovin*™ treatment.

- Secondly, NAC supplementation significantly improved serum ORAC although only after the exercise intervention and not in the loading phase. This is indicative of the cellular activation of GTH synthesis (from its constituent amino acids), but only in the presence of cellular stressors. This finding has also not previously been reported.

- Thirdly, chronic, high dose *Oxiprovin*™ prevented the relative neutrophil count from declining significantly at the 24 h time-point after peaking at 6 h post injury. This suggests an inability of neutrophils to enter the damaged tissue area. In both NAC and placebo treated groups, absolute neutrophil counts had decreased significantly in circulation by 24 h post exercise – a time-point which corresponds with a peak in neutrophil infiltration of the affected area in other studies. Furthermore, *Oxiprovin*™ supplementation did not decrease the systemic release of neutrophils. This was revealed by a significant elevation to similar levels across treatment groups at 6 h post exercise, from similar baseline values in the circulating neutrophil counts.

The discussion of general findings (and baseline measures) has been incorporated into the following sections.

### 5.1 EXTENT OF MUSCLE DAMAGE

Since a similar CK response was evident in all three treatment groups (peaking at 24 h post exercise), it clear that the exercise intervention employed induced a standard injury across treatment groups. This holds the implication that the observed differences between groups may be attributed to treatment only. The current study showed that CK activity was

significantly higher at the 24 h time-point, than at baseline, pre- and post-exercise, in both the placebo and NAC groups, but not the *Oxiprovin*™ supplemented group. Since CK activity serves as the standard indirect parameter for measuring muscle damage [46], the reflected ability of *Oxiprovin*™ supplementation to prevent substantial skeletal muscle damage after a strenuous plyometric exercise protocol may be beneficial to an athletic population. Furthermore, this finding suggests that *Oxiprovin*™ supplementation may be associated with a protective effect against EIMD, possibly by maintaining skeletal muscle membrane integrity, and preventing a leakage of enzymes (such as CK) into circulation. However, this difference between groups was not reflected in the assessment of DOMS scores. Regardless of the treatment group that participants had been assigned to, DOMS scores were significantly higher at all time-points after exercise when compared with baseline and pre-exercise scores. Furthermore, by 48 h after exercise, DOMS scores had increased significantly from the scores recorded immediately post and 6 h after the plyometric exercise intervention, even in the *Oxiprovin*™ supplemented group.

In an exercise study by Trombold *et al.* (270) recreationally active males were randomized into a crossover trial with either pomegranate extract which contained a polyphenol (ellagitannin) or placebo for nine days. To produce delayed-onset muscle soreness, subjects performed two sets of 20 single-arm maximal eccentric elbow flexion exercises. Muscle soreness and serum CK analysis at baseline and 2, 24, 48, 72, and 96 h post exercise did not differ significantly between groups despite improved recovery of isometric strength 24 and 48 h after exercise (270). In a follow-up trial, trained males supplemented with pomegranate juice performed six sets of 10 unilateral eccentric knee extension exercises. Again, muscle soreness scores were not significantly different between supplement and placebo groups (271).

DOMS may be affected by exacerbated tissue damage and the oxidative stress (7). This follows from the fact that neutrophil infiltration peaks at approximately 24 h post exercise and that they may survive within the tissue for up to 48 h before bursting (and causing secondary damage), or being removed by macrophages or other immune cells (248). However, this explanation for the prolonged sensation of pain is not in accordance with the postulate that the reduced CK response described may be attributed to diminished neutrophil infiltration. The range of factors that contribute to the sensation of DOMS remains to be elucidated (249). If it is not an accurate indirect measure of the extent of muscle damage, it may be related to reductions in strength and power or inflammation (197, 249).

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## 5.2 Antioxidant Status

It was hypothesised that antioxidant supplemented groups would present with greater ORAC in comparison to the control group, as supported by previous literature (101, 102). Thus, a decline in ORAC indicates a diminished ability of the antioxidants present in the serum to scavenge free radicals or a diminished presence of antioxidants. After strenuous exercise or in other situations of free radical overload, the ORAC also reflects the remaining ability to quench circulating free radicals. In the current study, the large SDs apparent in all three treatment groups are attributed to high variably between ORAC plate readings. The vast differences are due to the fact that samples were split amongst plates as a single plate does not have the enough wells to hold all samples. Differences between ORAC plate readings may be attributed to manufacturing differences, variability between ingredient batches, sample handling and timing. Consequently, ORAC values were recalculated as percentages of their respective baseline readings. When considering these values there seemed to be a trend towards a decreased ORAC across all three treatment groups at the time-points soonest after exercise (viz. post, and 6 h post). This suggests that serum quenching capacity had been utilised in extinguishing free radicals immediately after exercise. Also, it becomes clear that the quenching capacity of the NAC treatment group gradually increased from directly after exercise to significantly elevated levels by 48 h post exercise. Thus, NAC supplementation significantly improved serum ORAC although only after the exercise intervention. This may be explained by an up-regulation of GTH synthesis after the plyometric exercise intervention.

The locational specificity of the enzymes responsible for GSH synthesis provide the framework for the maintenance of intracellular tissue GSH concentrations and transport (259). Tissue GSH concentrations are influenced by diet and nutritional status and are subject to hormonal regulation and hormonal balance (259). The current study suggests that a strenuous bout of exercise or EIMD may contribute greatly toward the activation and continual stimulation of cellular GTH synthesis, a process which under physiological conditions is limited by the bioavailability of NAC, one of its amino-acid constituents (6, 173). Thus in the abundant presence of NAC, such as when chronically supplemented orally, GTH synthesis (as previously discussed) may continue and thereby improve the serum ORAC significantly by 48 h post exercise. In a study by Childs *et al.* (43), 14 young, healthy, untrained males received either 12.5 mg vitamin C and 10 mg NAC per kg body mass or placebo treatment for seven days following an eccentric arm exercise. The study did not include a supplement loading period. Subjects performed three sets of 10 repetitions using 80% of their maximum weight using only the non-dominant arm. Total antioxidant status of

the serum was measured prior to the injury and for seven days following exercise using methods developed by *Randox Laboratories* (Crumlin, UK). Supplemented subjects showed a significant increase in their total antioxidant status on days 2, 3, 4 and 7 while no significant change was reported for the placebo group (43). Because supplementation commenced after exercise, it is not clear whether the improved antioxidant capacity is as result of combined supplementation, the exercise intervention or both. Furthermore, the study concluded that combined supplementation (Vitamin C and NAC) exacerbated oxidative stress induced by exercise as indicated by elevated markers of oxidative stress and CK activities post exercise (43). The present data suggest that NAC supplementation on its own significantly improves total antioxidant status although only after the exercise intervention.

## 5.3 Oxidative Stress

As a by-product of lipid peroxidation, MDA serves as marker of oxidative stress in response to exercise most commonly by means of a TBARS assay (272). This method is non-specific and criticised for poor reproducibility and specificity that may lead to overestimations (54, 107). Thus there are no clinical standards for elevated MDA values (107, 115). In a study by Kanter *et al.* (115), pre and post exercise serum MDA levels were analyzed in endurance athletes (aged between 35 and 60 years) following an 80 km race (115). Post exercise MDA values were significantly elevated from resting values for all participants. The study reports 2.26 ± 0.39 µmol/L resting serum concentrations, and 4.0 ± 1.1 µmol/L MDA immediately after the race (mean ± SD). Maughan *et al.* (166) reported a peaked increase in MDA concentration (TBARS) 6 h after a 45 minute downhill-running exercise, with levels returning to baseline by 72 h post exercise (166). It has been hypothesised that increased levels of lipid peroxidation are due to macrophage reactions in tissue following eccentric exercise (274). In light of these findings serum MDA concentrations may be expected to peak immediately after exercise and again after immune cell infiltration and the subsequent oxidative burst (viz. 24 h or 48 h post exercise). The current study revealed no significant differences in serum MDA concentrations between treatment groups or within groups over time. It is possible that the degree of systemic free radical release was not sufficient to overwhelm serum quenching capacities and consequently induce oxidative stress even in the placebo group. In light of this, the contribution of neutrophils to oxidative stress induced secondary muscle damage may be less than previously suggested and *Oxiprovin*™ may diminish the extent of muscle damage by other means. Alternatively, the potentially beneficial effect of chronic, high dose NAC or *Oxiprovin*™ supplementation in preventing oxidative stress may also have been eclipsed by the extent of exercise-induced muscle damage, had the protocol been too strenuous. Thus, the plyometric model for inducing

muscle damage may have induced oxidative stress to the extent that the influence of either treatment in averting this response may have been thwarted. However, MDA (TBARS) analyses are known to produce conflicting results (272). In a study by Niess *et al.* (194), six trained and five untrained men completed an incremental treadmill test until exhaustion. Plasma MDA were not significantly affected in either group after exercise (194). Similarly, Alessio *et al.* (1) found no change in plasma MDA after repeated isometric contractions in 12 healthy male participants (also using the TBARS method) (1). These studies are of those in agreement with the current findings.

## 5.4 Soluble Adhesion Molecules

Serum sVCAM-1 concentrations revealed no significant differences between treatment groups or within groups over time. Despite not being significant, a trend towards a diminished sVCAM-1 response was evident in both the NAC and *Oxiprovin*™ treatment groups at all time-points after baseline. This trend suggests that either form of chronic antioxidant supplementation diminishes expression of sVCAM-1 within 1 week of supplementation. Secondly, this trend suggests that serum concentrations of sVCAM-1 remain consistently low, even after a plyometric exercise intervention. This may be indicative of an avenue that should be explored in future research as the alteration of adhesion molecule expression may be a likely means by which *Oxiprovin*™ or NAC exerts their beneficial effects in modulating muscle damage and inflammation. This explanation would also be consistent with other antioxidant studies mostly focused on diseased states [278-280]. One of these [Kalfin *et al.* (114)] evaluated whether *Activin*™ (also a GSE product), could reduce adhesion molecule expression in plasma during the inflammatory response of SSc patients. Patients received 100 mg daily (orally) for 30 days after which blood samples were taken. Blood was also taken from a SSc patient placebo control group and normal human volunteers. The study revealed up-regulation of ICAM-1, VCAM-1, E-selectin but not P-selectin in both SSc patient groups when compared with healthy volunteers. However, in comparison with the placebo group *Activin*™ treatment significantly attenuated the increased expression of these adhesion molecules and plasma MDA (114). The study did not evaluate *Activin*™'s effect on the healthy volunteers, did not include an exercise intervention and ORAC analysis. Marui *et al.* (161) suggest a molecular link between an antioxidant sensitive transcriptional regulatory mechanism and VCAM-1 gene expression. In their *in vitro* study, the antioxidants pyrrolidine dithiocarbamate (PDTC) and NAC repressed IL-1β activated VCAM-1 gene expression in cultured HUVEC (161). As mentioned in Chapter 4, power analysis revealed that with a statistical power of 56.3% the current study would need

to be repeated with a total n of at least 33 in order to establish statistically significant differences (or not). Furthermore, other adhesion molecules (such as ICAM-1 PECAM-1, E-selectin, P-selectin and the Mac-1 integrin) may also play a role and are thus equally worth investigation.

## 5.5 Time-Course and Magnitude of Neutrophil Infiltration

The relative proportions and absolute circulating leukocyte cell count provides vital insights to distributions within the body and the activation state of the immune system. In the current study, neither of the treatments lessened the release of neutrophils from systemic pools to circulation before or after exercise. However, the plyometric exercise intervention caused a significant increase in the circulating neutrophil count which peaked 6 h post exercise and returned to values similar to those at baseline by 48 h post plyometric intervention. This is consistent with a previous exercise study which reported inflammatory cell infiltration of the affected tissue, as indicated by muscle MPO activity [152]. MPO, NADPH oxidase and xanthine oxidase are present in activated neutrophils, and thus their presence is circulation is indicative of the respiratory burst.

Studies have associated peaked neutrophil infiltration evident at 24 h post injury with oxidative stress and myofibrilar tearing [135, 136]. This was consistent with the current study when considering the placebo and NAC treatment groups. However, the reduction of circulating neutrophils was more gradual in the *Oxiprovin*™ supplemented group. Therefoer, it may be said that the participants chronically supplemented with a high dose of *Oxiprovin*™ experienced a more gradual removal of neutrophils from circulation following a plyometric exercise intervention. In both the NAC and placebo treated groups, circulating neutrophils had already decreased significantly by 24 h post-exercise thus coinciding with the peak in neutrophil tissue infiltration previously reported. It is possible that in the *Oxiprovin*™ supplemented group neutrophils were unable to enter the damaged tissue area due to modulated activation or adhesion. This argument may also supported by an *in vivo* study by Ferrero *et al.* (73) in which resveratrol (1 μmol/L and 100 nmol/L) significantly inhibited ICAM-1 and VCAM-1 expression by TNF-α stimulated HUVECs and HSVECs, respectively (73). The fact that the number of circulating neutrophils remains relatively elevated suggests that binding does not take place, rather than the possibility that binding occurs at too great an affinity. Furthermore, all three treatment groups presented with similar peaks at the 6 h time-point, and neither treatment affected neutrophil recruitment from dormancy.

It is probable that the proposed inability for neutrophils to enter the damaged tissue area is in fact associated with a diminished expression of adhesion molecules on the endothelial surface or other interferences to neutrophil extravasation (selectin- or integrin-mediated binding), rather than thwarting diapedesis or transmigration. If this holds true, the exact mechanism may be elucidated by means of cell culture models which include the incubation of control and *Oxiprovin*™ treated neutrophils, and quantifying and comparing the receptors or adhesion molecules expressed. This may also be performed while including an endothelial cell layer in order to concomitantly evaluate endothelial receptors or adhesion molecules expression as suggested by Kruger et al. [29].

The primary mechanism by which *Oxiprovin*™ allows for a lower CK response associated with EIMD may be attributed to additional beneficial properties of this polyphenol. The current study was not able to elucidate these mechanisms.

## 5.6 LIMITATIONS

It is likely that this study may have reported many more significant findings, had the SDs of the groups been lower. Increasing the subject number, enforcing more strict inclusion and exclusion criteria as well as more controlled activity levels and diets, may have been helpful in this regard. Such measures would be recommended for performing clinical trials. However, enforcing the above mentioned controls would complicate participant recruitment, and would increase the financial burden of the study.

A recent review reinforces pronounced differences in the anthropometric and other physical characteristics of the forward- and back-line rugby players. The review describes forward-line players as typically taller and heavier than back-line players, with a greater proportion of body fat [6]. Furthermore, the direct exposure of front row players to high impact forces in the scrum necessitates superior strength than other forwards within the pack [6]. In light of this, it is important also to consider that although participants had been assigned to treatment groups at random, the *Oxiprovin*™ treatment group comprised mostly back-line players, while in the remaining groups, the distribution of player positions was approximately equal. Thus, the current study may have generated more specific results, with lower variability between groups, had only forward or back-lines players been recruited. Furthermore, variability in training (which may have provided protection against muscle damage) may have influenced results, despite thorough exclusion criteria. It would not be

appropriate to exclude candidates based on too many training criteria, since the objective of the study was to determine effect of the supplements in a group of rugby players.

With regards to treatment and the administration thereof, the following suggestions may be made in retrospect. Firstly, treatment dosages may rather have been determined for each individual based on their body mass. Thus a specific amount administered per kg body weight, for example: 4 mg/kg body mass. This is important when considering that the population group of interest presents with higher muscularity and thus body mass, than the data used for calculating the manufacturer's recommended dosages. This may also have provided better insights with regards to the appropriate dosage for use by athletes. However, calculating individual dosages may have further complicated (or even compromised) the double-blind nature of the study.

Although no deviation was reported, constant serum concentrations of the active agent may have been better controlled had participants been required to return unused capsules or log days that supplementation was missed.

The bioavailability of each of the respective treatments may have been improved had administration been spread across the day, rather than instructing administration at a single point in time, daily. In this regard, it may have been beneficial to advise participants to take one capsule with a full glass of water after breakfast, lunch and dinner, rather than taking all three capsules concomitantly after breakfast. Furthermore, no record was kept of the participants' water-consumption. Thus, it would have been valuable to assess the hydration state of each sample.

The inclusion of additional groups, not subject to the exercise protocol, may have enhanced interpretation. This is particularly true when considering ORAC results for the NAC treatment group, where ORAC improved significantly only after the plyometric exercise protocol.

Performing the test protocol and blood draws during early winter mornings complicated the study practically. Under cold conditions (and with restricted activity) vasoconstriction, lower heart-rates and other physiological factors complicated several blood draws. However, due to the nature of the study, no other time of day was suitable.

Regarding sample analyses, having included more markers of muscle damage such as Mb may have strengthened the current findings. Most notably, the performance of functional

testing, such as those described by Reeves *et al*. (214) (unpublished thesis), may have offered valuable insights with regards to force deficit, or preservation of muscle power and force-generating capacity within treatment groups over time [47]. This may be of particular importance when considering the possibly beneficial effects of *Oxiprovin*™ and NAC supplementation in the context of sport and exercise where maintained or improved performance even after strenuous exercise is greatly desirable. Furthermore, a recent review of commonly used markers of muscle damage reports that currently, only the measurement of force-generating capacity should be considered a reliable measure of muscle damage [30].

Since no immune cells were isolated, expression of adhesion molecules, cytokines and other factors could not be measured. Neither the macrophage nor circulating cytokine response were quantified as these would have expounded the scope of the already complex study. However, their roles are undeniably important and warrant further investigation.

Analysis of MPO concentrations may have offered valuable insights into neutrophils that may have undergone oxidative burst within circulation. However, as mentioned previously, MPO may also have been released by other immune cells, and thus compromised results.

Although deemed unnecessary for the aims of this study, muscle biopsies may have provided histological insights including fibre-typing, and allowed for the assessment of intramuscular ORAC and immune cell content, as well as the measurement of cytokine and chemokine production in cell culture. However, previous studies by have included analyses of the tissue compartment. These analyses returned with sufficient statistical power to confirm a blunted neutrophil infiltration and improved ORAC after injury in an animal model which employed both acute and chronic *Oxiprovin*™ supplementation (187).

Lastly, after injury the exact timing of the peak presence of the parameters of interest and secondary muscle damage varies according to the type and extent of the damage. Thus, it is possible that certain peak responses may have been missed. Of interest, the 4 h and 12 h time-points post exercise may have presented significant findings for several parameters. However, it was decided to restrict the number of time-points to avoid placing excessive strain on the participants, and because of the financial implications of including single time-point across all treatment groups for all parameters of interest.

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# CHAPTER 6:

## SYNTHESIS

Plyometric jumping drills are incorporated into the training programs of many sports teams, and have been shown to be particularly valuable in the context of union rugby. It is also known that plyometric exercise rigorously affects both skeletal muscle and immune cells. Although research has demonstrated several beneficial effects of *Oxiprovin*™ supplementation in both animal and human models, the underlying mechanisms have not been comprehensively investigated. Furthermore, it has been difficult to distinguish between effects conferred by PCO, rather than by antioxidant properties in general. Therefore, it was the aim of this thesis to determine the mechanism(s) of action by which chronic *Oxiprovin*™ supplementation may influence secondary muscle damage after an acute bout of strenuous plyometric jumping in a population of healthy male rugby players. This study is distinguished from other GSE studies as it included a control antioxidant group in addition to the placebo group, such that any additional effect (over-and-above the free radical scavenging ability) could be clearly demonstrated. Non-elite rugby players were recruited, so that participants were less adapted to plyometric exercise, when compared with the participants of previous studies. The current study was one of few that has assessed changes in soluble adhesion molecule concentration with and without GSE supplementation. Finally the present study used a higher dose of *Oxiprovin*™ supplementation including a one-week loading phase, with continued supplementation post exercise.

### 6.1 EVALUATION OF AIMS AND HYPOTHESES

Principally, we hypothesised that chronic supplementation with a high dose of *Oxiprovin*™ would prevent secondary muscle damage by means of blunting the disappearance of neutrophils from the circulation, following exercise-induced muscle damage. Outcome measures included:

(a) the extent of damage,
(b) lipid peroxidation,
(c) antioxidant status,
(d) circulating leukocyte counts,
(e) the time-course of neutrophil disappearance and
(f) soluble adhesion molecules in circulation

With regards to (a), it was found that the exercise intervention employed indeed induced a standard injury across treatment groups, with the implication that the observed differences between groups may be attributed to treatment only. There was some evidence, although not strong, that *Oxiprovin*™ treatment reduced skeletal muscle micro-damage after a strenuous plyometric exercise protocol, as reflected by a dulled CK response, but not DOMS.

Within the context of the current protocol, neither of the treatment groups presented with supressed oxidative stress. With regards to (c), the *Oxiprovin*™ treatment group displayed a somewhat surprisingly decreased ORAC by 6 h post exercise when compared with readings taken immediately before exercise, and when compared with the NAC and placebo groups. Furthermore (and potentially more noteworthy) was that only NAC supplementation significantly improved serum ORAC after the exercise intervention.

With regards to (d) and (e), the plyometric exercise intervention caused a significant increase in the circulating neutrophil count which peaked at 6 h post exercise and returned to values similar to those at baseline by 48 h post plyometric intervention. However participants chronically supplemented with a high dose of *Oxiprovin*™ presented with a prolonged elevation of circulating neutrophils after exercise, although not to a highly significant extent. This suggests the reduction of neutrophil infiltration into the damaged muscle tissue area.

With regards to (f), although not statistically significant, a trend towards a diminished sVCAM-1 response was evident in both the NAC and *Oxiprovin*™ treatment groups at all time-points after supplementation had commenced. This suggests a potential mechanism by which either form of antioxidant supplementation may moderate neutrophil infiltration and thwart subsequent oxidative stress and tissue damage.

## 6.2 SPECULATION

Thus, having accomplished the aims successfully, speculation is possible with regards to the potential mechanism(s) by which chronic *Oxiprovin*™ supplementation or NAC may protect against muscle damage. Although it has previously been shown in an animal model that chronic *Oxiprovin*™ supplementation limited neutrophil infiltration, it was necessary to show that this finding is equally valid in a human model. A possible mechanism by which neutrophil invasion may have been limited lies in the interference with leukocyte extravasation. The finding that the circulating neutrophil count remains elevated at 24 h post suggests that neither selectin- nor integrin-mediated binding takes place. Thus, in all

likelihood, the inability for neutrophils to enter the damaged tissue area is associated with abolished chemotaxis or a diminished expression of adhesion factors on either of the leukocyte or endothelial cells surfaces, rather than tight binding accompanied by oppressed diapedisis or transmigration. The ORAC results further support the notion that *Oxiprovin*™'s ability to prevent oxidative stress is not due to an improved ability to scavenge free radicals.

We therefore fail to reject the possibility that the protective effect results from a diminished expression of factors responsible for cellular adhesion. However, this warrants further investigation particularly when considering that NAC presented with a similar trend towards a reduced sVCAM-1 expression without preventing the CK response.

## 6.3 Conclusion

Based on these findings and in the context of the literature available, we conclude that *Oxiprovin*™ supplementation (210mg/d) which includes a seven day loading period may diminish plyometric EIMD by limiting (but not completely inhibiting) the neutrophil response. Furthermore, we suggest that secondary muscle damage may be prevented by the blunted neutrophil infiltration, rather than only the quenching of free radicals released by the oxidative burst. Following on from this, we have presented reason that the blunted neutrophil response may be as result of deterred (selectin- or integrin-mediated) binding or other interferences with the neutrophil extravasation cascade. We propose several directions for future research which will be discussed at the end of this chapter.

## 6.4 Implications and Clinical Applications of the Current Findings

The findings of this study may have several applications. Chronic *Oxiprovin*™ (and not NAC treatment) may have beneficial effects for preventing CK accumulation. At this point it cannot be confirmed that muscle damage is moderated while recovery is promoted, or that practice may occur more frequently, thereby allowing athletic skill and performance to improve. However, considering that chronic *Oxiprovin*™ supplementation presented with no side-effects, this form of supplementation need not at this point be excluded from the supplement regimens of rugby players. This finding may also have clinical implications for rhabdomyolysis patients or those presenting with chronically elevated CK activity levels. Clinical applications may thus include the recommendation or prescription of *Oxiprovin*™ as preventative measure for statins-induced rhabdomyolysis.

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## 6.5 Directions for Future Research

The expression of adhesion factors on neutrophil cell surfaces should be researched, for example by means of cell culture models which include the incubation of control-group and *Oxiprovin*™ treated neutrophils with and without an endothelial cell layer to concomitantly evaluate surface molecule and chemokine expression. Thus, one may quantify and compare the expression of factors including VCAM-1, ICAM-1, PECAM-1, the Mac-1 integrin as well as the chemokines (CXC and CC chemokines), their receptors and the selectins involved with neutrophil activation (E-selectin, P-selectin).

Most relevant to a sporting paradigm, it would be interesting to evaluate changes in muscle girth, strength, or the loss in muscle force generating capacity and power out-put, following a plyometric exercise protocol with or without *Oxiprovin*™ supplementation. Unpublished research (214) has recently shown that an isometric force testing chair provides a reliable method of quantifying skeletal muscle force generating capacity. Thus, it may be possible to associate chronic *Oxiprovin*™ or NAC supplementation with maintained or improved muscle force generating capacity at several time-points before and after a plyometric exercise intervention. Such findings would mark chronic *Oxiprovin*™ supplementation as a very appealing option for union rugby players and other athletes alike. However, it may also be valuable to include a no-exercise group, to control for possible gains in strength following participation in maximal force-testing.

Finally, the finding that NAC supplementation improves serum ORAC only after exercise may provide added benefit when administered in combination with *Oxiprovin*™. Thus, the antioxidant benefit associated with NAC supplementation combined with specific polyphenol effects may prove to be synergistically more effective.

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# APPENDICES

## 8.1 APPENDIX A: *OXIPROVIN*™ PACKAGE ANSERT

Anti-Oxidant

**Scheduling status:**
Not scheduled

**Composition:**
Each capsule contains:
100% Grape Seed Extract
Proanthocyanidin 70 mg
Microcrystalline cellulose

**Pharmacological classification:**
Western herbal
Dietary supplement

**Pharmacological action:**
Anti-oxidant

**Dosage:**
Adults: 1 Capsule twice a day with water
Children: 1 Capsule daily with water
or dissolved in water

**Side effects:** None known

**Storage instructions:**
Store below 25°C in a cool dry place.
Contains no preservatives, colourants
or flavourants.

**Pure Grape Seed Extract**

**Product may be beneficial for:**
Arthritis and inflammation • Strengthen Immune System • Improve Blood Circulation
Strengthen Blood Vessels • Decrease Allergies
Eliminates Free Radicals • Retard Ageing

**Manufacturer:**
BRENN-O-KEM (Pty) Ltd
P.O. Box 71
Wolseley 6830
South Africa
Tel +27 (0) 23 231 1060
Fax +27 (0) 23 231 1977

www.oxiprovin.com

**Date of publication:** May 2005

**Disclaimer:** Oxiprovin does not claim to cure diseases. It's a natural supplement & anti-oxidant.

Stellenbosch University http://scholar.sun.ac.za

## 8.2 Appendix B: *Warren Chem Specialites (Pty) Ltd* ISO 9001 Registration Form

# Registration Certificate

***This document certifies that the administration systems of***

*Warren Chem Specialities (Pty) Ltd*

*11 Mansell Road, Killarney Gardens, Cape Town, South Africa*

***have been assessed and approved by QAS International***
***to the following management systems, standards and guidelines:***

*ISO 9001 : 2008*

*With the permitted exclusion of clause 7.3 Design and Development*

***The approved administration systems apply to the following:***

*The distribution of fine chemicals for various applications including food beverages, pharmaceuticals, complimentary health products and the wine industry.*

Original Approval ........25th June 1997........................
Current Certificate ........25th June 2012........................
Certificate Expiry ........25th June 2013........................
Certificate Number ........A1433SA........................

**On behalf of QAS International**

www.qas-international.com

*This certificate remains valid while the holder maintains their quality administration systems in accordance with the standards and guidelines stated above, which will be audited annually by QAS International.*
*The holder is entitled to display the above registration mark for the duration of this certificate.*
*This certificate must be returned to QAS International on reasonable request.*
*Issuing Office: QAS International, The Gig House, Oxford Street, Malmesbury, Wiltshire, SN16 9AX*

Stellenbosch University http://scholar.sun.ac.za

## 8.3 Appendix C: HREC Response to Ethics Application

UNIVERSITEIT•STELLENBOSCH•UNIVERSITY

24 November 2011

**MAILED**

Prof KH Myburgh
Dept of Physiological Sciences
c/o Merriman and Bosman Rd
Mike de Vries Building
Stellenbosch University
7600

Dear Prof Myburgh

**Intramuscular antioxidant status, immune and satellite cell activity following an acute bout of plyometric exercise**

**ETHICS REFERENCE NO: N10/02/060**

**RE : AMENDMENT: MODIFICATIONS REQUIRED**

Your letter received 21 October 2011 refers.

The Chairperson of the Health Research Ethics Committee reveiewed the amended documentation in accordance with the authority given to him by the Committee.

The following modifications are required:

1. NAC is being used outside of its licensed indication - notification to the MCC is needed.

2. Safety is not a significant concern with this product but no potential adverse events are discussed at any point and it should be.

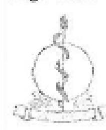
3. No justification of the dose is produced and studies seem to suggest that 150mg/kg is conventionally used. Please include a justification.

4. Participant informed consent form:
4.1 No mention is made of side -effects of either ACC or oxyprovin. Risks of taking either, including possible anaphylaxis must be elucidated.
4.2 Please check for spelling errors especially on page 5 of 9.

On receipt of the additional information/corrected document(s) the application will be reconsidered. Please HIGHLIGHT or use the TRACK CHANGES function to indicate ALL the corrections/amendments clearly in order to allow rapid scrutiny and appraisal.

01 December 2011 11:55

Page 1 of 2



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## 8.4 Appendix D: Letter Indicating Amendments to Application for Ethical Approval

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jou kennisvennoot • your knowledge partner

**DELIVERED/MAILED**

*22 March 2012*

To the chairperson, *Health Research Ethics Committee*

**RE: AMENDMENT: MODIFICATIONS REQUIRED (ETHICS REFERENCE NO: N/10/02/060)**

Dear Sir, the chairperson,

Your correspondence regarding the application amendment refers (received December 2011).

We have made the following modifications as requested by the HREC:

1) Glutathione is produced naturally by all cells in the body, and is commercially available as an anti-oxidant supplement. Its properties therefore make it ideal for use as an antioxidant control. However, because of the hydrolysis of glutathione by intestinal and hepatic glutamyltransferase, dietary glutathione is not a major determinant of circulating glutathione, and it is unfortunately not possible to increase circulating glutathione concentration to a clinically beneficial extent by oral administration. However glutathione concentrations can be raised by administration of certain supplements that serve as precursors. N-acetylcysteine, commonly referred to as NAC, is the most bioavailable precursor of glutathione, and the most feasible one for the purposes of human studies, particularly for the inclusion of a control group for the effect of antioxidants.

   We have consulted with the MCC and were advised that we would have to register a clinical trial with them. However, we are not testing for a new indication, but simply using NAC as a precursor for glutathione as a control group for it's well known antioxidant effects. We have included all relevant literature in our proposal. There is no protocol to our knowledge whereby the MCC receives or acknowledges the use of the supplement as a control group. In addition, due to time constraints, we do not find applying for a clinical trial a feasible option.

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2) As acknowledged, safety is not a significant concern with this product. However possible adverse effects have now been elaborated. This information is now found in the full protocol, the protocol synopsis and the participant information leaflet and consent form.

3) Regarding justification of the dose, recent studies have shown that chronic supplementation produces better results that acute supplementation. However, although these trends could be detected, they were of poor significance, likely due to insufficient amounts of supplementation. As Oxiprovin™ has no RDA, a higher dose than has been used in previous studies may be required to yield a significant treatment effect. Furthermore it has no contra-indications and no side-effects have been reported. This is now clearly indicated in the protocol.

4) Participant information leaflet and consent form:

4.1) Mention is made of all known adverse reactions possible with ACC™ and Oxyprovin™. The risks of taking either are elucidated, including the risk of Anaphylaxis.

4.2) Spelling errors have been corrected, with particular attention paid to page 5 of 9.

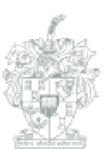
Thank you for reconsidering our application. All corrections/amendments have been clearly highlighted, in order to allow rapid scrutiny and appraisal.

Kind regards,

KH Myburgh

***Professor KH Myburgh***

PhD, Fellow of the American College of Sports Medicine.
*Department of Physiological Sciences, Faculty of Natural Sciences, Stellenbosch University, RSA*



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## 8.5 Appendix E: Approval of Amendment to Application for Ethical Approval

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09 May 2012 **MAILED**

Prof KH Myburgh
Dept of Physiological Sciences
c/o Merriman and Bosman Rd
Mike de Vries Building
Stellenbosch University
7600

Dear Prof Myburgh

**Intramuscular antioxidant status, immune and satellite cell activity following an acute bout of plyometric exercise**

**ETHICS REFERENCE NO: N10/02/060**

**RE : AMENDMENT**

Your letter dated 22 March 2012 refers.

The Chairperson of the Health Research Ethics Committee approved the amended documentation in accordance with the authority given to him by the Committee.

The submitted amendment was approved.

Yours faithfully

**MRS MERTRUDE DAVIDS**
**RESEARCH DEVELOPMENT AND SUPPORT**
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352

09 May 2012 14:45 Page 1 of 1

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## 8.6 Appendix F: Participant Recruitment Letter

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**DELIVERED/MAILED**

*10 May 2012*

Valued squad member,

The Department of Physiological Sciences, Stellenbosch University is conducting a research study aimed at determining the effect of an oral grape seed extract supplement in preventing muscle damage and the array of symptoms associated with plyometric exercise, a fundamental component of various rugby-union training regimes. Some examples of the parameters we would like to measure include fatigue, delayed onset of muscle soreness and time to recovery.

As part of a group of 24 participants, you have been selected to take part in a research study because you have met the following inclusion and exclusion criteria:

Inclusion Criteria:

- You are aged between 18 and 25 years.
- You are a local or regional rugby player in pre-season training phase.
- You are willing and have signed informed consent form after fully understanding it.
- You acknowledge that you may leave the study at any point, for whatever reason, at no detriment to yourself, baring the benefits of completing the study.
- You are willing to comply with the requirements of the study.

Exclusion Criteria:

- You do not smoke.
- You do not have Asthma.
- You are not suffering from Hay fever.
- You do not have a history of Anaphylaxis.
- You are not currently taking dietary antioxidant supplementation in any form.
- You have not had a muscle or sport related injury within the previous 60 days.
- You have no history of gastrointestinal disorders or disturbances such as peptic ulcerations.
- You are currently not on treatment and are not using anti-inflammatory drugs, NSAIDS or corticosteroid-containing medication (including inhaled forms), and have not done so within the previous 60 days.

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To compensate for your time and inconvenience, you will be rewarded R500 upon satisfactory completion of the study. This amount is determined pro-rata for your investment in the study. Upon completion you will also receive a *Maties* or Stellenbosch University branded sports towel from the Department of Physiological Sciences, as gift.

Furthermore, you will receive all information regarding the full outcome of the study, and receive the product as supplement for personal training. What's more, you will be eligible for free sports performance testing, body composition testing, fitness and $VO_2$ max testing, and various other blood sample analyses should you complete the study. Other more specific tests are also possible.

The study will take place over a 2 week period and will involve compliance to certain dietary and treatment criteria, 7 visits to the Department of Physiological Sciences in Merriman street, 8 blood draws (5ml tubes), completion of a plyometric exercise protocol, and completion of various questionnaires. Please take note that your health and safety, and good clinical practice is the first level of priority in this study, and that if you feel unsafe or violated in anyway, you have the fullest right to discontinue your participation. Depending on subject availability the study will likely take place during April, commencing with the resuming of the 2nd academic term.

If you would like to take part, or would require further information, please contact me on the details below before 15 May 2012. You will receive and be required to read and sign the participant information leaflet and consent form, to indicate that you understand clearly the reasoning behind the study, the requirements of the protocol and your willingness to participate.

I look forward to working together on this exciting and likely high impact South African research study with you.

Kind Regards,

Chris Delport *BSc, BMedSci, MSc II (Stel)*
**Department of Physiological Sciences**

*e-mail:* *chrisdelport@sun.ac.za*
*cell:* *076 683 8863*

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## 8.7 Appendix G: Participant Information Leaflet and Consent Form

***PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM***

**TITLE OF THE RESEARCH PROJECT:**

Antioxidant status and immune cell activity following an acute bout of plyometric exercise. *Subtitle*: An in vivo study in humans with or without grape seed extract, N-Acetylcysteine or a placebo supplementation

**REFERENCE NUMBER:** N10/02/060

**PRINCIPAL INVESTIGATOR:** Prof. K. H. Myburgh (PhD)

**SUB INVESTIGATORS:**

Dr. C Smith (PhD)
J. T. Viljoen (BSc Physiotherapy)
C. J. Delport (BSc Hons)

**ADDRESS:**

Department of Physiological Sciences
c/o Merriman & Bosman Road
Mike de Vries Building
Stellenbosch University
Stellenbosch
7600

**CONTACT NUMBER:** 021-808 3149

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

HREC General ICF Version 2, July 2009 Page 1 of 10

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**What is this research study all about?**

Plyometric exercise of the lower body involves a series of explosive jumping activities aimed at increasing maximum power in the leg muscles. This type of training now forms part of many athletes and rugby teams' training regimens and results in improved athletic performance in all sports involving running, jumping and cycling. Unaccustomed eccentric exercise such as plyometric exercise can result in microscopic muscle damage and associated swelling and inflammation. Symptoms of this type of exercise-induced muscle damage include delayed onset of muscle soreness (DOMS) as well as decreased muscle strength and power.

Anti-oxidants are naturally occurring substances which are found in diets rich in fruit and vegetables, e.g. vitamin C, vitamin E, and β-carotene. They have been shown to be beneficial in various situations. In this study we will observe the effects which anti-oxidant supplementation has on recovery after plyometric exercise. Proanthocyanidin is a potent antioxidant naturally found in the extract of grape seeds and marketed at Oxiprovin™, while NAC or N-Acetylcysteine is a pharmaceutically produced antioxidant and nutritional supplement, available in the market in various products including well known ACC™.

We aim to determine how well each of the antioxidants work to aid muscle recovery, and to see what effects each has on the immune system, and preventing muscle damage in the first place.

The study will be conducted in the *Exercise Physiology Laboratory* at the *Mike de Vries building, Department of Physiological Sciences, at Stellenbosch University*. A total number of 24 subjects will be recruited for the purposes of this study. You will be randomly divided into one of three treatment groups. All three groups will undergo exactly the same testing procedure. During this study you will be given an oral supplement to take in the form of a capsule which serves as the treatment. This will be filled with one of the two antioxidant supplements or a placebo powder. You will be unaware as to which of the three treatments you are receiving. We will only be able to tell you once all the samples have been analyzed. But we will tell you at the same time that we give you your own results.

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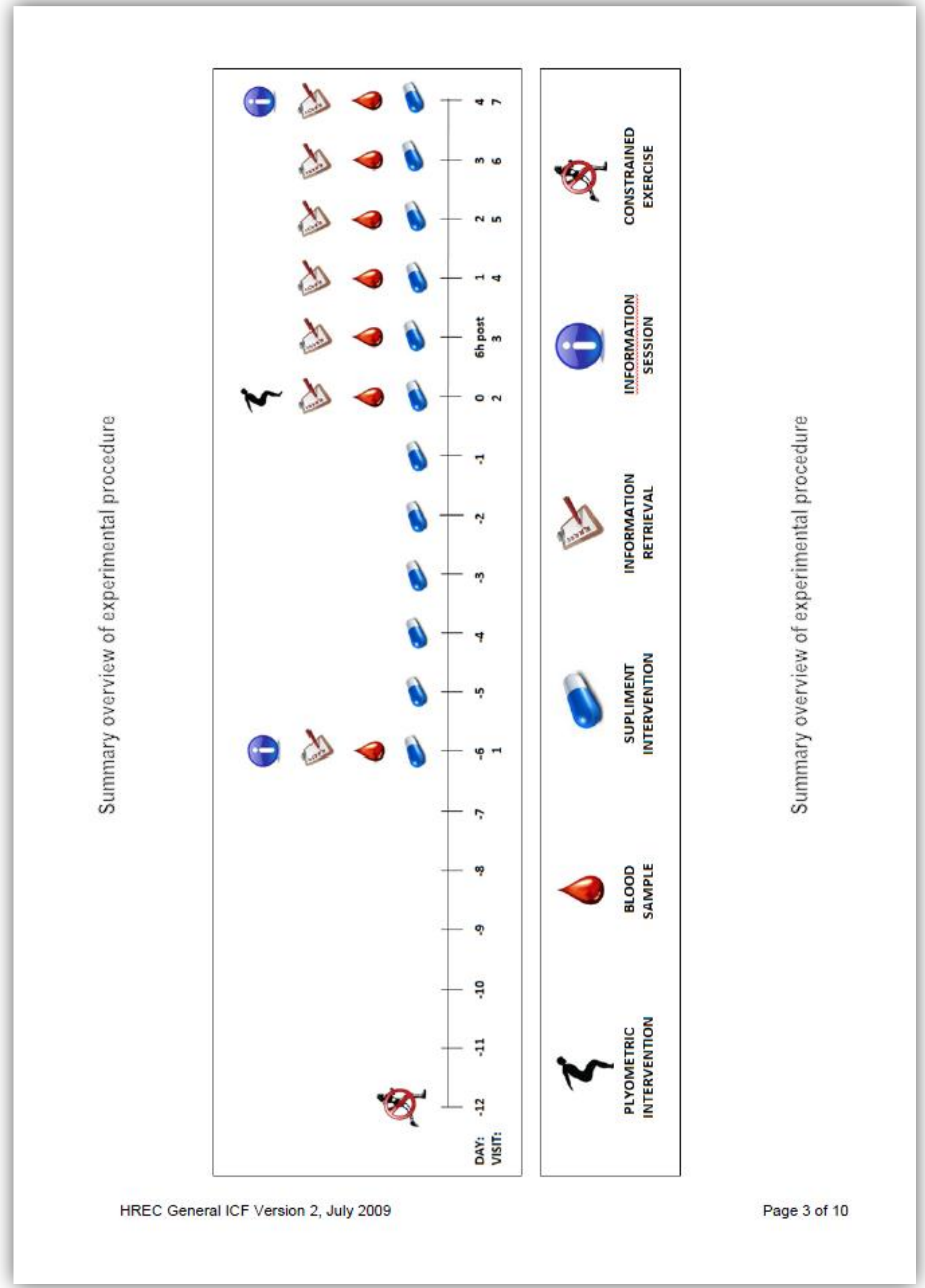

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**The Study Protocol**

Each subject is expected to come into the laboratory a total of 7 times on 6 different **days (on the day of exercise you will come in twice).** Day 0 indicates the day that you will do the plyometric jumping exercise. The only visit before that is a baseline assessment and information session. The study ends 4 days after the plyometric exercise.

***During the visits, the following will happen:***

**Visit 1:** During your first visit you will be asked to sign the informed consent form which is based on the information in this leaflet. A blood sample will be collected from a vein in the arm near the elbow. All blood samples will be equal to 20ml (approximately equal to 4 tablespoons). We will then divide you into your supplement groups randomly, and you will be issued with your treatment regime (capsules) which you should start on the same day that your baseline blood sample has been taken.

**Visit 2:** A blood sample will be collected from the arm. You will perform the plyometric exercise regime: a 10 minute warm–up, followed by the exercise intervention after which a second blood sample will be collected. You will also be asked to rate you immediate perceived level of discomfort following the plyometric exercise and again after 15 minutes.

**Visit 3:** Six hours after the exercise intervention, a blood sample will be taken followed by the perceived level of discomfort questionnaire.

**Visit 4, 5, 6, 7:** These visits will take place on day 1 – 4 of the study. You will be asked to rate your perceived level of discomfort in response to your general daily activity. A single blood sample will also be collected from a vein in the arm near the elbow during each of these visits. On the last day, you will be interviewed by an experienced dietician to determine your dietary intake over the past 7 days, be debriefed and receive your compensation.

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**The Plyometric Exercise Regimen**

To induce transient (small scale) muscle damage, you will be asked to perform 15 sets of 10 maximal vertical jumps, separated with a 60 second recovery time between sets. Prior to starting the exercise, you will perform a maximal vertical jump with a chalk mark made by the fingertips at the highest point of the jump. This mark will then be used as a target height that you will attempt to maintain for each jump. On the landing, you will be instructed to adopt a knee joint angle of approximately 90° in order to promote transient (micro) muscle damage as described in scientific literature. This plyometric exercise protocol will induce non-severe muscle damage, which should not significantly reduce your mobility after the protocol is done, if at all.

**Muscle Soreness Assessment Protocol**

Muscle soreness will be assessed before the study, immediately after the plyometric exercise regime, 6 hours post, as well as on days 1, 2, 3, and 4. In each instance you will be asked to indicate your perceived muscle soreness, pain, discomfort and fatigue using a rating scale on a template provided, in response to a squat test (slowly adopting the squat position from a standing position, focusing on each of the relevant muscle areas).
This will be done in order to rate the level of discomfort associated with your daily activity in contrast with the squat test.

**Blood Collection**

Each time, a qualified person (phlebotomist) will draw 20ml of blood from the brachial vein just left of the elbow, on alternating arms.

**Why have you been invited to participate?**

You are invited to participate in this study because your physical characteristics fit the expected criteria needed for this study (e.g. you are of appropriate age and physical activity level).

**Will you benefit from taking part in this research?**

The results of this study will allow you to assess your body's inflammatory response to a standardized injury in comparison to the whole group. Furthermore, your ability to recover from the injury will also be placed into the context of the group. In addition the use of this supplement may prove to speed up recovery from the muscle injury, allowing for a better means of assisting yourself and your team in the treatment of future subsequent injuries.

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**What will your responsibilities be?**

- You will be expected to participate in the study by doing the activities described above for visits 1 – 7, and complying with the stated requirements.

- You will be expected to comply with your treatment requirements; taking 3 capsules with your 1st meal every day from the day of the 1st visit until the end of the study.

- You will be expected to attend all meetings at the appointed times.

- You will be expected to keep record of all dietary intake and breaches in the disallowed section (eg. Exercise)

- You will be expected to keep your copy of the informed consent form.

- You will be expected to contact the investigators if anything unforeseen (e.g. additional accidental injury or excessive pain) occurs which could influence the outcome of the study.

NOTE: Your participation is **entirely voluntary** and you are free to decline to participate or withdraw from the study at any time. If you choose to say "no" or withdraw, this will have no consequences for you in any way.

Any further questions that you may have relating o the experiment will be answered in full by the co-ordinators: Prof K. H. Myburgh (021-808 3149) or Mr. C. J. Delport (076 683 8863).

**What is not allowed while participating in this research?**

- No medicine is allowed to be taken during this study (e.g. anti-inflammatories or hay-fever medication).

- The use of ice of physiotherapy is not allowed.

- No complementary health supplements (e.g. vitamins, creatine or anti-oxidants) should be taken from within 1 week prior to the study until completion.

- Smoking or any kind, the consumption of alcohol is to be avoided for the full duration of the study.

- No caffeine is to be consumed within the 8 hours prior to a blood draw.

NOTE: If you are in doubt or have questions, about any of these, or would like more specific details, please contact one the investigators.

**Are there in risks involved in your taking part in this research?**

The risk of having blood drawn includes soreness and bruising at the puncture site as well as the minimal risk of developing a haematoma of resultant infection. This will be minimized by using sterilized equipment. All the procedures will be done by experience personnel with the sufficient relevant training. The amount of blood to be taken is not considered to be a significant amount, and is therefore not expected to have any significant risk for you. If you experience too much discomfort as a result of your participation, please contact the principal investigator, at the number provided at the top of this form. No biopsies will be taken. The risks of side-effects of the treatments are discussed below.

**Are side-effects or other risks possible?**

As with many other pharmaceuticals, side-effects are a possibility when using NAC supplementation. The side-effects of NAC may include bronchospasm, nausea, vomiting, stomatitis, rhinorrhoea, headache, tinnitus, urticaria, chills and fever. Anaphylaxis has been less frequently been reported.
Oxiprovin™ (the grape seed extract) treatment has no reported symptoms, side-effects or contra-indications. If ***any*** negative effect should occur, as result of use of either supplement, treatment should cease immediately. The study doctor will be informed and the participant will leave the study at no detriment to the participant.
The placebo powder has no negligible effect and no nutritional value.

**If you do not agree to take part, what alternatives do you have?**

Your participation is entirely voluntary and you are free to decline to participate, even after you expressed interest in finding out more information. If you say no, this will not affect you negatively in any way whatsoever.

**Who will have access to your medical records?**

All information will be kept strictly confidential and protected. Experimentation will not commence without your consent. Your identity will remain anonymous. Only Chris Delport will have access to your personal information and he will assign you a study subject number. This number will be used when your experimental data is assessed by Prof. Myburgh and Prof. Smith. The Health Research Ethics Committee members, who approved this study, will also have the right to inspect research records if they request.

**What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?**

You will be taken to a sports medicine doctor for an assessment and any medication required, for example anti-inflammatories will be supplied free of charge for up to 8 days. Your data will be used up to that point and you may decide if you wish to stop.
In the unlikely event that you sustain any other form of injury as a result of your participation due to negligence on our side, the University has insurance to cover medical expenses.

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**Will you be paid to take part in this study and are there any costs involved?**

No you will not be paid to take part in the study buy your transport and meal costs will be covered for each study visit. There will be no costs involved for you, if you do take part.

As participant you will be compensated with an amount of R500.00 for your time commitment while participating in the study. This compensation is provided on a pro-rata basis for the time you invest in the study and if you withdraw from the study at any point after you agreed to participate, you will only be compensated pro rata for the time that you did participate. There will be no additional costs involved for you, if you do take part.

**Is there anything else that you should know or do?**

- You should inform your family practitioner or usual doctor that you are taking part in a research study.
- You should also inform your medical insurance company that you are participating in a research study.
- You can contact Prof. K. H. Myburgh or Dr. Smith (tel 021 808 3149) if you have any further queries or encounter any problems. Alternatively you can also contact Mr. Chris Delport (cell: 076 683 8863) or Dr. Brink, the study doctor.
- You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.
- The researchers involved in this study have no financial or non-financial interests, which may inappropriately influence us in the conduct of this research study.
- The study will take place during May and June 2012. The 24 participants will be divided into two groups whom will conduct the protocol on different dates according to their availability, the university exams and the requirements of the coach and the league.

The dates are as follows:

- GROUP1 Testing period: **17 May – 2 June 2012**
- GROUP2 Testing period: **22 May – 7 June 2012**

| Group 1 | | |
|---|---|---|
| **Date:** | **Time** | **Event:** |
| 17-May-2012 | 08:00 | Stop all exercise, alcohol, smoking and other treatment or supplementation till end of study |
| 23-May-2012 | 08:00-09:00 | Study Explanation and Baseline Measurements, receive supplements |
| ***29-May-2012*** | ***06:00-08:00*** | ***Plyometric Jump Exercise (15 x 10 max height squat jumps) with pre and post blood draws*** |
| ***29-May-2012*** | ***14:00-14:30*** | ***Blood draw*** |
| 30-May-2012 | 08:00-08:30 | Blood draw |
| 31-May-2012 | 08:00-08:30 | Blood draw |
| 01-Jun-2012 | 08:00-08:30 | Blood draw |
| 02-Jun-2012 | 08:00-08:45 | Blood draw, study debrief and receive compensation |

| Group 2 | | |
|---|---|---|
| **Date:** | **Time** | **Event:** |
| 22-May-2012 | 08:00 | Stop all exercise, alcohol, smoking and other treatment or supplementation till end of study |
| 28-May-2012 | 08:00-09:00 | Study Explanation and Baseline Measurements, receive supplements |
| ***03-Jun-2012*** | ***06:00-08:00*** | ***Plyometric Jump Exercise (15 x 10 max height squat jumps) with pre and post blood draws*** |
| ***03-Jun-2012*** | ***14:00-14:30*** | ***Blood draw*** |
| 04-Jun-2012 | 08:00-08:30 | Blood draw |
| 05-Jun-2012 | 08:00-08:30 | Blood draw |
| 06-Jun-2012 | 08:00-08:30 | Blood draw |
| 07-Jun-2012 | 08:00-08:45 | Blood draw, study debrief and receive compensation |

*If you have any questions, or for more details, please contact Chris Delport on 0766838863 or email: chrisdelport@sun.ac.za.*

Stellenbosch University http://scholar.sun.ac.za

**Declaration by participant**

By signing below, I ………………………………………………… agree to take part in a research study entitled:

***Antioxidant status and immune cell activity following an acute bout of plyometric exercise. Subtitle: An in vivo study in humans with or without grape seed extract, N-Acetylcysteine or a placebo supplementation***

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ……………………………………………… on (*date*) …………………………………………………………

……………………………………………………………………… ………………………………………………………………
**Signature of participant** **Signature of witness**

**Declaration by investigator**

I *(name)* ……………………………………………………… declare that:

- I explained the information in this document to ………………………………………………………
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above.
- I did not use a interpreter.

Signed at (*place*) ……………………………………………… on (*date*) …………………………………………………………

……………………………………………………………………… ………………………………………………………………
**Signature of investigator** **Signature of witness**

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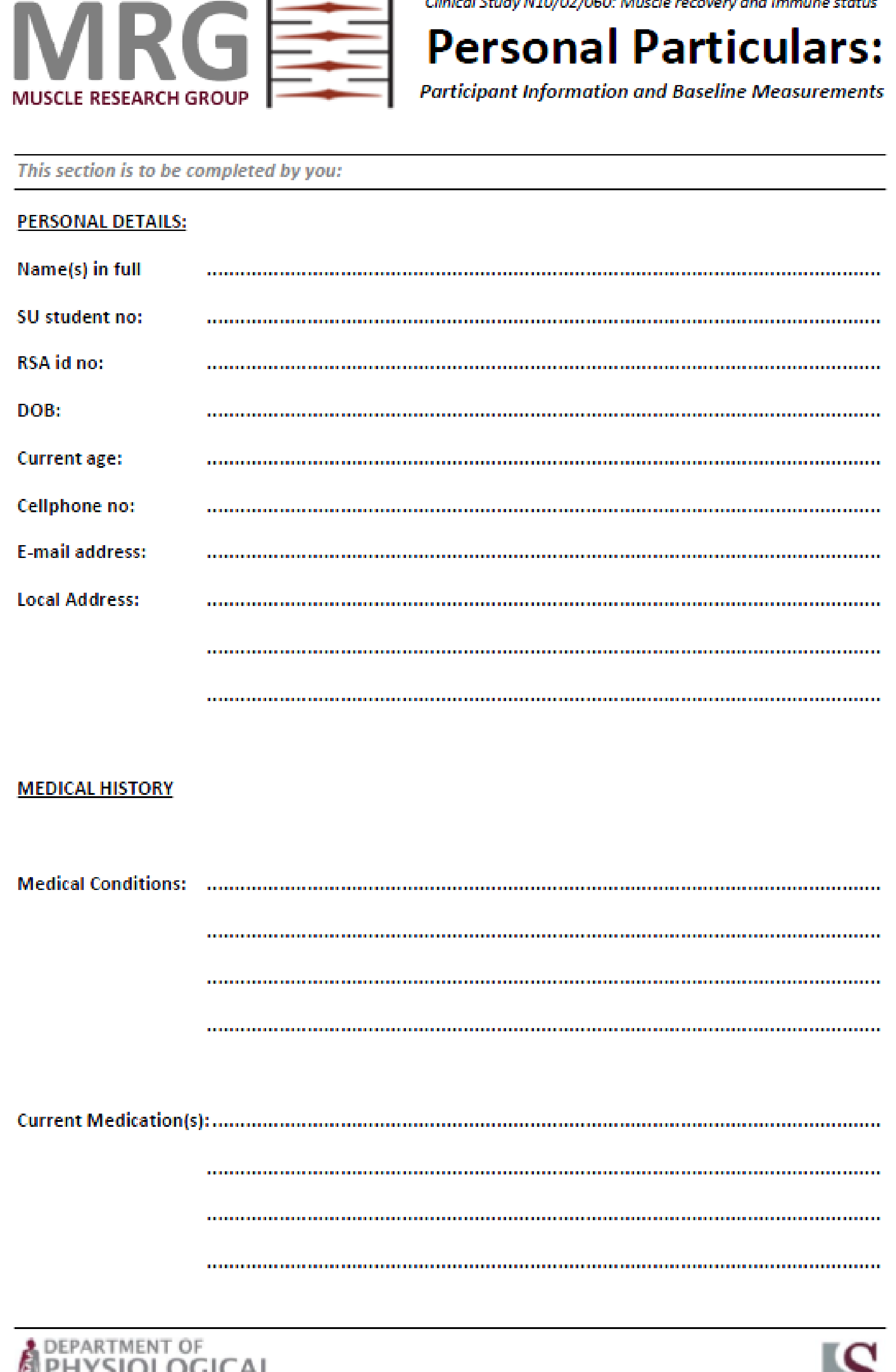
## 8.8 APPENDIX H: PARTICIPANT PERSONAL INFORMATION FORMS



MRG MUSCLE RESEARCH GROUP

*Clinical Study N10/02/060: Muscle recovery and immune status*

**Personal Particulars:**

***Participant Information and Baseline Measurements***

*This section is to be completed by you:*

PERSONAL DETAILS:

Name(s) in full ..........................................................................................

SU student no: ..........................................................................................

RSA id no: ..........................................................................................

DOB: ..........................................................................................

Current age: ..........................................................................................

Cellphone no: ..........................................................................................

E-mail address: ..........................................................................................

Local Address: ..........................................................................................

..........................................................................................

..........................................................................................

MEDICAL HISTORY

Medical Conditions: ..........................................................................................

..........................................................................................

..........................................................................................

..........................................................................................

Current Medication(s): ..........................................................................................

..........................................................................................

..........................................................................................

..........................................................................................

DEPARTMENT OF PHYSIOLOGICAL SCIENCES

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**INJURY STATUS**

Please only indicate the area(s) where you are currently injured, or have experienced injury within the previous 12 months.

| Region | Left/ Right | Current | Within past 12 months | Resolved | Injury Details |
|---|---|---|---|---|---|
| | *L/R* | *Yes/No* | *Yes/No* | *Yes/No* | Specify |
| ***Shoulder*** | | | | | |
| ***Elbow*** | | | | | |
| ***Wrist*** | | | | | |
| ***Hand/Finger*** | | | | | |
| ***Neck*** | | | | | |
| ***Thoracic Spine*** | | | | | |
| ***Lower Back*** | | | | | |
| ***Sacroiliac-joint*** | | | | | |
| ***Hip/Groin*** | | | | | |
| ***Quadriceps*** | | | | | |
| ***Hamstring*** | | | | | |
| ***Knee*** | | | | | |
| ***Shin/Lower Leg*** | | | | | |
| ***Ankle*** | | | | | |
| ***Achilles' Tendon*** | | | | | |
| ***Foot*** | | | | | |
| ***Other** (specify):* ______________ | | | | | |

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RUGBY HISTORY

Years of competitive rugby: ..........................................................................................................

Highest level achieved: ..........................................................................................................

Position: ..........................................................................................................

OTHER SPORTS AND TRAINING HABITS

Please only complete the relevant areas for the past 6 months.

| Sport | X per week | Duration per session |
|---|---|---|
| Running | | |
| Swimming | | |
| Hiking | | |
| Cycling | | |
| Stretching | | |
| Hockey | | |
| Tennis | | |
| Gym / Cardiovascular | | |
| Gym / Weight Training (resistance) | | |
| Other: ___________________________ | | |
| Other: ___________________________ | | |
| Other: ___________________________ | | |

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*This section is to be completed by the investigators:*

***Weight (kg):*** ...........................................................................................

***Height (m):*** ...........................................................................................

***Heart Rate (BPM):*** ...........................................................................................

***Blood Pressure:*** ...........................................................................................

***Participant id:*** ...........................................................................................

***Supplement id:*** ...........................................................................................

***Other:*** ...........................................................................................

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## 8.9 APPENDIX I: PARTICIPANT DIET AND EXERCISE LOG

MRG MUSCLE RESEARCH GROUP

Clinical Study N10/02/060: Muscle recovery and immune status

**Daily Diet and Exercise Log:**

*meals / drinks / supplements / activity / exercise*

*Name of Participant:* ______________________________

Because nutrition and activity influence your blood parameters, performance and so the outcome of this study, you have been required and kindly agreed to abstain from exercise, medication, physio-therapy, supplements, smoking and alcohol for the full study duration.

The space below will provide an outline for you to *briefly* document your daily nutrition & activity:

17 May 2012 Intake:..........................................
Exercise: ..........................................

18 May 2012 Intake:..........................................
Exercise: ..........................................

19 May 2012 Intake:..........................................
Exercise: ..........................................

20 May 2012 Intake:..........................................
Exercise: ..........................................

21 May 2012 Intake:..........................................
Exercise: ..........................................

22 May 2012 Intake:..........................................
Exercise: ..........................................

23 May 2012 Intake:..........................................
Exercise: ..........................................

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## 8.10 Appendix J: Retrospective Supplement Log

MRG MUSCLE RESEARCH GROUP

Clinical Study N10/02/060: Muscle recovery and immune status

**Retrospective Supplement Log:**

*Vitamins / Medication / Supplementation*

*Name of Participant:* ______________________________ *Date:* ________________

**Performance/Recovery Supplements** *Weight:* ______________

Are you currently taking, or have you taken any of the following supplements within the last 2 weeks? If so, please circle either yes/no and give details where possible. If there are any not mentioned, please specify.

| Supplement type | | Product Name | Frequency | |
|---|---|---|---|---|
| | | | If daily, how many times per day? | Total in last 2 weeks? |
| Coffee | Yes / No | | | |
| Tea | Yes / No | | | |
| Protein powder | Yes / No | | | |
| Glutamine | Yes / No | | | |
| Creatine | Yes / No | | | |
| Amino acids | Yes / No | | | |
| Smoke | Yes / No | | | |
| Alcohol | Yes / No | | | |
| Other | Yes / No | | | |

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**Vitamins and Minerals**

Are you currently taking, or have you taken any of the following vitamins within the last 2 weeks? If so, please circle either yes/no where applicable. If there are any not mentioned please specify.

| Type | Yes/No | Product Name | Frequency | |
|---|---|---|---|---|
| | | | If daily, how many times per day? | Total in last 2 weeks? |
| Vitamin C | Yes / No | | | |
| Multivitamin | Yes / No | | | |
| Vit D | Yes / No | | | |
| Vit B | Yes / No | | | |
| Other | Yes / No | | | |
| Other | Yes / No | | | |

**Medication**

Are you currently taking, or have you taken any of the following medications within the last 2 weeks? If so, please circle either yes/no where applicable. If there are any not mentioned please specify.

| Name | Yes/No | If daily, how many times per day? | Total in last 2 weeks? |
|---|---|---|---|
| Ibubrofen | Yes / No | | |
| Panado | Yes / No | | |
| Disprin | Yes / No | | |
| Anti-inflammatories | Yes / No | | |
| Voltaren | Yes / No | | |
| Transact patches | Yes / No | | |
| Other | Yes / No | | |
| Other | Yes / No | | |

***I hereby give permission for footage collected during the protocol period to be used by the investigators for academic and possibly publication purposes.***

*Signed:*______________________ *Place:*_____________________ *Date:*___________________

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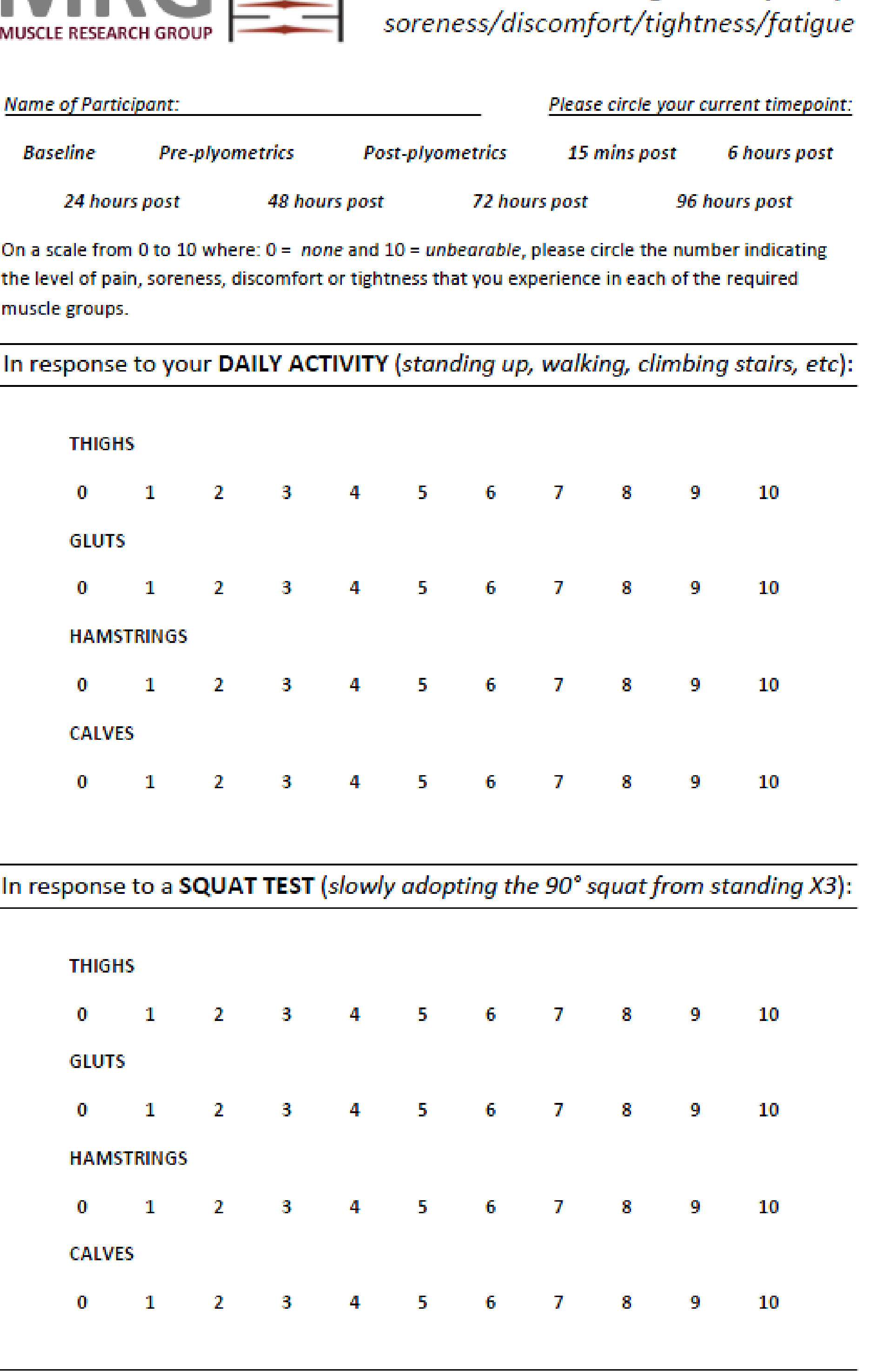
## 8.11 Appendix K: Visual Analog Scale (VAS) for Pain Assessment



MRG MUSCLE RESEARCH GROUP

Clinical Study N10/02/060: Muscle recovery and immune status

**Pain Visual Analog Scale (VAS):**
*soreness/discomfort/tightness/fatigue*

*Name of Participant:* ______________________

*Please circle your current timepoint:*

*Baseline* *Pre-plyometrics* *Post-plyometrics* *15 mins post* *6 hours post*

*24 hours post* *48 hours post* *72 hours post* *96 hours post*

On a scale from 0 to 10 where: 0 = *none* and 10 = *unbearable*, please circle the number indicating the level of pain, soreness, discomfort or tightness that you experience in each of the required muscle groups.

In response to your **DAILY ACTIVITY** (*standing up, walking, climbing stairs, etc*):

THIGHS

0 1 2 3 4 5 6 7 8 9 10

GLUTS

0 1 2 3 4 5 6 7 8 9 10

HAMSTRINGS

0 1 2 3 4 5 6 7 8 9 10

CALVES

0 1 2 3 4 5 6 7 8 9 10

In response to a **SQUAT TEST** (*slowly adopting the 90° squat from standing X3*):

THIGHS

0 1 2 3 4 5 6 7 8 9 10

GLUTS

0 1 2 3 4 5 6 7 8 9 10

HAMSTRINGS

0 1 2 3 4 5 6 7 8 9 10

CALVES

0 1 2 3 4 5 6 7 8 9 10

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## 8.12 Appendix L: Protocol Used for the ORAC Assay

**Reagents**

Reagents used for the ORAC assay are tabulated below:

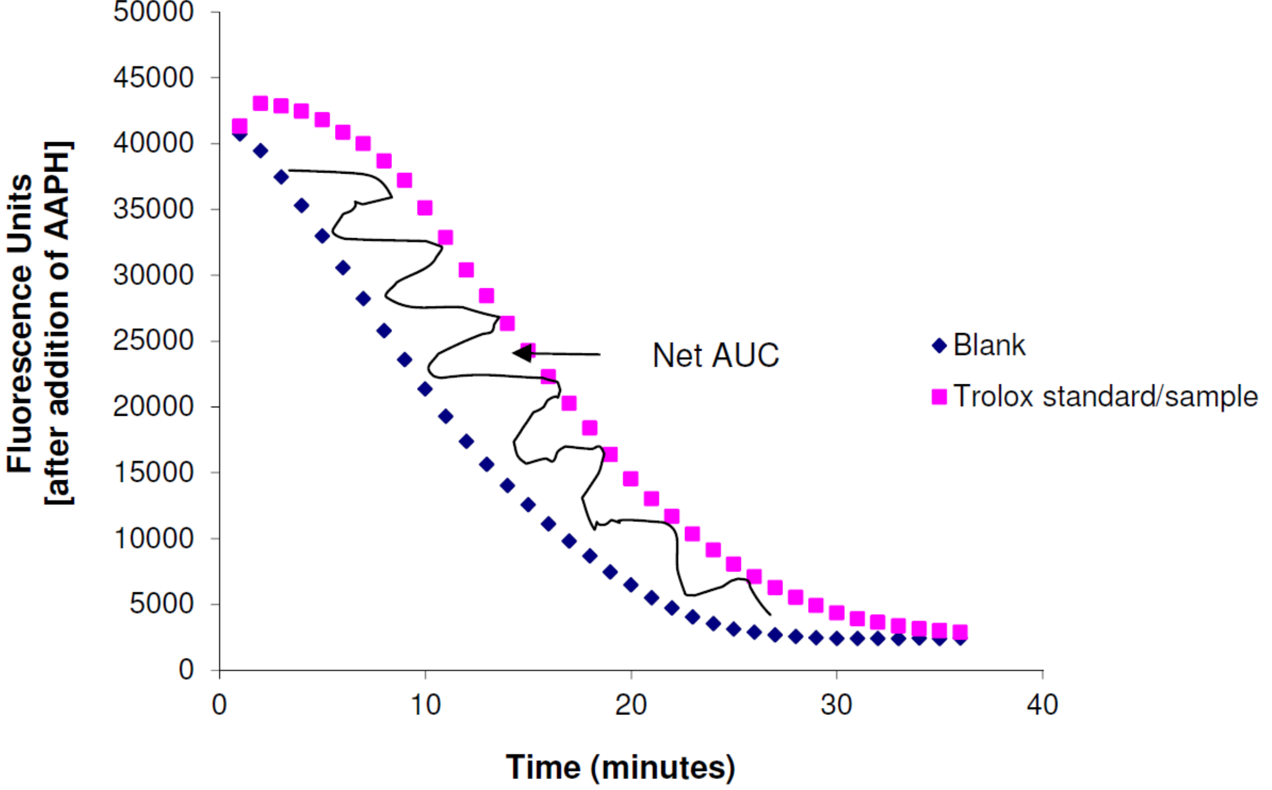
| **Reagent** | **[Stock]** | **[Working]** | **Supplier** |
|---|---|---|---|
| AAPH* (2,2'-Azobis-(2-methylpropionamidine)-dihydrochloride) | | 153 mM<br>0.414 g in 10 ml Phosphate buffer<br>pH 7.4 | Sigma-Aldrich<br>44,091-4 |
| Fluorescein (light sensitive sodium salt bioreagent) | $5 \times 10^{-3}$ mM<br>(stored at 4°C) | $8.16 \times 10^{-5}$ mM<br>Made up in phosphate buffer, pH 7.4 | Sigma-Aldrich<br>46960-25G-F |
| Potassium Phosphate buffer | | 75 mM (pH 7.4) (4 °C)<br>6.5g $K_2HPO4$ in 500 ml dist. $H_2O$<br>5.1g $KH_2PO4$ in 500 ml dist. $H_2O$ | Sigma-Aldrich<br>60353 (Fluka)<br>60218 (Fluka) |
| Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) | 0.02 M<br>(stored at -20 °C) | 6.25 µM; 12.5 µM; 25 µM; 50 µM<br>in phosphate buffer, pH 7.4 | Sigma-Aldrich<br>56510 (Fluka) |

**Method**

1. Defrost serum samples on ice.
2. Dilute serum samples 25x with phosphate buffer.
3. Add 25 µl TROLOX standards and serum in triplicate to the respective wells.
4. Add 150µl fluorescein working solution to each well of a black ORAC plate.

| | BUFFER | TROLOX | PLASMA | AAPH | FLUORESCEIN |
|---|---|---|---|---|---|
| **BLANK** | 25 µl | ----- | ----- | | 150 µl |
| **STD** | ----- | 25 µl | ----- | | 150 µl |
| **SAMPLE** | ----- | ----- | 25 µl | | 150 µl |

5. Cover plate with lid and incubate in preheated reader (37°C) for 10 min with 3 min shaking.
6. Rapidly add 25µl AAPH to all wells.
7. Read for 45 minutes and 20 seconds with fluorescence measured every 80s. (Excitation wavelength = 485 ± 20 nm; emission wavelength = 530 ± 25 nm).
8. The net area under the curve (AUC) of all standards and samples are calculated from a graph of time (in minutes) against relative fluorescence intensity, as illustrated below:



The AUC was calculated using *Microsoft Excel XP* as

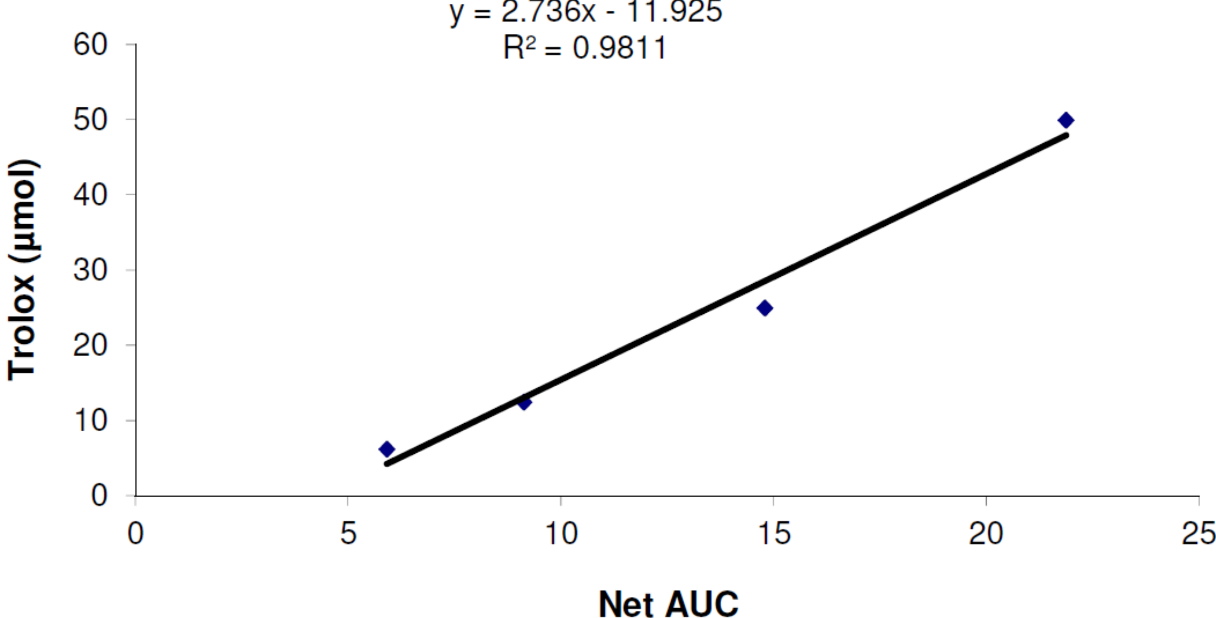
$0.5 + f_1/f_0 + \ldots f_i/f_0 + \ldots + f_{34}/f_0 + f_{35}/f_0$ (1)

where $f_0$ = initial fluorescence reading at 0 minutes and $f_i$ = fluorescence reading at time i.

The net AUC is obtained for all the samples, by subtracting the AUC of the blank from that of a sample or a Trolox standard.

Net AUC = [($AUC_{sample}$ or Trolox – $AUC_{blank}$) (2)

9. The standard curve is obtained by plotting the Trolox standard concentrations against the average net AUC of these standards:




10. The final ORAC values are calculated using the regression equation between Trolox concentration and the net AUC. Results have been corrected for variance between plates by expressing the µmol Trolox equivalents per liter for liquid samples (serum) values as a percentage of the controls. All baseline values were converted to 100, allowing for comparable results.

**Note:** The dilution factor (25x in this study) needs to be included for final ORAC value calculation.

## 8.13 Appendix M: Serum TBARS Method Protocol

# Antioxidant Research Unit

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**SPECTOPHOTOMETRY**

**Serum TBARS Method**

1. 50µL serum + 6.25 µL 4mM cold BHT/$C_2H_5OH$ (Ethanol [EtOH]) + 50µL 0.2M ortho-phosphoric acid
2. Vortex for 10 seconds
3. Add 6.25µL TBA reagent (0.11M in 0.1M NaOH)
4. Heat at 90°C for 45 minutes
5. Place on ice for 2 minutes
6. Leave at room temperature for 5 minutes
7. Add 500µL n-butanol and 50 µL saturated NaCl (for better separation of phases)
8. Vortex for 10 seconds
9. Microfuge 12k RMP for 2 minutes at 4°C
10. 300 µL top butanol phase into wells
11. Read $A_{532} – A_{572}$

Extinction Coefficient (Ɛ) = 1.54 x $10^5$ $M^{-1}$ $CM^{-1}$

Inter-assay CV = <8%

$$c = \frac{A}{\varepsilon}$$

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