UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Modulation of oxidative stress parameters in healthy volunteers by strenuous exercise

Yolanda de Haaij

Submitted in partial fulfillment of the requirements for the degree

***Magister Scientiae* (Biochemistry)**

in the

Faculty of Natural and Agricultural Sciences
Department of Biochemistry
University of Pretoria
Pretoria
South Africa

July 2006

I declare that the dissertation, which I hereby submit for the degree M.Sc. (Biochemistry) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: .... DATE: ....

"This world, after all our science and sciences, is still a miracle; wonderful, inscrutable, magical and more, to whosoever will think of it."

Thomas Dekker (1570-1632)
English writer and dramatist

ACKNOWLEDGEMENTS

I acknowledge with gratitude the following people and institutions for their valuable contributions towards this study:

The Assessment Unit of the High Performance Centre at the L.C. de Villiers Sports Grounds, University of Pretoria, for the use of their facilities for the First Exercise Study.

The Institute for Sports Research at the L.C. de Villiers Sports Grounds, University of Pretoria for the use of their facilities and in particular Mr. H. Nolte (biokineticist) for his expertise during the Second Exercise Study.

The Groenkloof Virgin Active Club and specifically Ms. Z. Olivier (biokineticist) for allowing us to use their facilities and expertise for the Third Exercise Study.

All the volunteers who participated in the three Exercise Studies.

Prof H.T. Groeneveld (statistician) and Dr. M. van der Linde (SAS programmer), Department of Statistics, University of Pretoria, for their assistance in the statistical analysis.

The University of Pretoria for educational grants.

The Department of Biochemistry of the University of Pretoria and Prof. J.A. Verschoor (Head of Department) for granting me the opportunity to undertake this study.

Mrs. S. van Wyngaardt for her all her technical help and suggestions during the experimental part of the study.

Dr. Z. Apostolides (my supervisor) for his valuable academic input and support during my studies. Thank you for granting me the opportunity to work in this interesting field in Biochemistry. All the extra effort you made to make this study an optimal learning and growth experience for me is much appreciated.

My family, friends and fellow students for their moral support.

My mother who taught me an appreciation for nature and science from childhood. This was the starting point of my interest and later my studies into the field of biological sciences.

My late father for all the effort he made to give his children the best opportunities he could and especially for making financial provision for our studies even after his passing away.

My dear husband, David, for his love, encouragement and endless patience during my studies. Without all your emotional, practical and financial support I could not have finished this dissertation.

Above all, my Heavenly Father for all His blessings during my time of study.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

TABLE OF CONTENTS

List of Tables .... *xii*
List of Figures .... *xiii*
List of Abbreviations .... *xvii*

CHAPTER 1: BACKGROUND

1 Free radicals, oxidative stress and antioxidant systems **1**
1.1 Free radicals and other reactive oxygen species as part of normal life 1
1.2 Antioxidant defense mechanisms 2
1.3 Oxidative stress 4
1.4 The plasma antioxidant system 5
1.5 Salivary antioxidants in relation to plasma antioxidants 7

2 Measurement of oxidative stress **9**
2.1 Direct measurement of free radical formation 9
2.2 The use of biomarkers to estimate levels of oxidative stress 9
2.3 The significance and measurement of total antioxidant capacity 12

3 The relationship between exercise and oxidative stress **14**
3.1 Strenuous physical exercise as a cause for oxidative stress 14
3.2 Antioxidant defense to exercise-induced oxidative stress 19
3.3 Exercise as a potential tool for bio-availability studies 20

4 Aims of study **23**

5 Hypotheses **24**

6 Null hypotheses **25**

CHAPTER 2: THE FIRST EXERCISE STUDY. THE USE OF INCREMENTAL TREADMILL RUNNING TO EXHAUSTION AS EXERCISE PROTOCOL

1 Introduction 26

2 Materials and methods 30

2.1 Sample collection and processing 30

2.2 Biochemical analysis 31

2.2.1 DPPH Assay 31

2.2.1.1 Materials 31

2.2.1.2 Assay principle 31

2.2.1.3 Preparation of DPPH stock and working solution 31

2.2.1.4 Preparation of plasma and saliva samples 32

2.2.1.5 Preparation of dilution series 32

2.2.1.6 Preparation of sample dilution series 32

2.2.1.7 Construction of a vitamin C standard curve 33

2.2.1.8 Assay procedure 33

2.2.1.9 Absorbance spectra for the reaction between DPPH and vitamin C 33

2.3 Subject selection 35

2.4 Experimental protocol 35

2.5 Statistical analysis 36

3 Results 36

4 Discussion 42

5 Conclusion 43

CHAPTER 3: THE SECOND EXERCISE STUDY. THE USE OF STRENUOUS TREADMILL RUNNING BASED ON MAXIMAL OXYGEN UPTAKE AS EXERCISE PROTOCOL

1 Introduction 44

2 Materials and methods 46
2.1 Sample collection and processing 46
2.2 Biochemical analyses 47
2.2.1 DPPH Assay 47
2.2.2 TBARS Assay 47
2.2.2.1 Materials 47
2.2.2.2 Assay principle 48
2.2.2.3 Hydrolysis of TEP to MDA 48
2.2.2.4 Construction of an MDA standard curve 49
2.2.2.5 Preparation of BHT 49
2.2.2.6 Preparation of TBA 49
2.2.2.7 Preparation of plasma samples 49
2.2.2.8 Assay procedure 49
2.2.3 The Oxidative Stress Ratio 50
2.3 Subject selection 50
2.4 Experimental protocol 51
2.5 Statistical analysis 52

3 Results 53

4 Discussion 59

5 Conclusion 60

CHAPTER 4: THE THIRD EXERCISE STUDY. THE USE OF STRENUOUS SPINNING EXERCISE BY A MALE POPULATION AS EXERCISE PROTOCOL

1 Introduction 62

2 Materials and methods 67
2.1 Sample collection and processing 67
2.2 Biochemical analyses 68
2.2.1 DPPH Assay 68
2.2.2 TBARS Assay 68
2.2.3 The Oxidative Stress Ratio 68
2.3 Subject selection 68
2.4 Ethical approval 69
2.5 Experimental setup 69
2.6 Experimental procedure 69
2.6.1 Exercise protocol 71
2.6.2 Leg press protocol 71
2.7 Statistical analysis 72

3 Results 73

4 Discussion 80

5 Conclusion 83

CHAPTER 5: METHODS FOR INVESTIGATING PROTEIN OXIDATION: THE SPECTROPHOTOMETRIC DNPH ASSAY AND THE OXYBLOT™ WESTERN BLOT

1 Introduction **85**

2 The DNPH Assay **89**
2.1 Approach 89
2.2 Materials and methods 89
2.2.1 Preparation of plasma 89
2.2.2 Protein concentration determination 90
2.2.3 Oxidation of samples by formaldehyde and malondialdehyde 90
2.2.4 Oxidation of samples by $FeCl_3$/ascorbate 91
2.2.5 Assay procedure 91
2.3 Results 92
2.4 Discussion 94

3 The Oxyblot™ method **98**
3.1 Materials and methods 98
3.1.1 Buffers and solutions 98
3.1.2 Preparation of plasma 101
3.1.3 Protein determination 101
3.1.4 Preparation of oxidized proteins for positive controls by oxidation of samples with $FeCl_3$/ascorbate 101
3.1.5 Derivatization of protein samples 101
3.1.6 Preparation of SDS-PAGE gel 103
3.1.7 Loading of samples on SDS-PAGE gel for Western blot 104
3.1.8 Electrophoresis of samples 104
3.1.9 Electroblot of protein samples to PVDF membrane 104
3.1.10 Incubation with 1°Ab and 2°Ab 105
3.1.11 Addition of chemiluminescent reagent 105
3.1.12 Visualization of carbonylated protein bands 106

3.1.13 Data interpretation 106
3.2 Expected results 106
3.2.1 Chemiluminescent low molecular weight marker 107
3.2.2 Carbonylated protein standard supplied with the Oxyblot™ kit 108
3.2.3 Plasma samples that were oxidized *in vitro* to yield *in vitro* carbonylated proteins 108
3.2.4 Plasma samples that were not oxidized *in vitro* to yield *in vitro* carbonylated proteins 109
3.3 Actual results obtained from Oxyblot™ method 110
3.4 Troubleshooting 111
3.4.1 Methods 111
3.4.1.1 Control experiment A: Coomassie staining of extra lanes of SDS-PAGE gel that was electrophoresed but not electroblotted 112
3.4.1.2 Control experiment B: Coomassie staining of SDS-PAGE gel following transfer and staining of extra lane of PVDF membrane to which proteins were transferred 112
3.4.1.3 Control experiment C: Dot blots of 2°Ab and chemiluminescent LMW markers directly onto PVDF membrane 112
3.4.1.4 Control experiment D: Dot blots of derivatized samples and protein standards directly onto PVDF membranes, followed by antibody incubations 113
3.4.2 Results 114
3.4.2.1 Control experiments A and B 114
3.4.2.2 Control experiment C 115
3.4.2.3 Control experiment D 115
3.4.3 Discussion 116

4 Discussion and conclusion 120

CHAPTER 6: CONCLUDING DISCUSSION

1 The Exercise Studies 123

1.1 Literature on changes in total antioxidant capacity and TBARS levels following exercise 123

1.2 Subject selection criteria and exercise protocol 124

1.3 Selection of biochemical assays 125

1.3.1 DPPH Assay 125

1.3.2 TBARS Assay 126

2 Possible explanations for results 127

3 Future recommendations 129

3.1 Subject screening 130

3.2 Timing of sample collection 131

3.3 Sample storage 131

3.4 Type of sample 131

3.5 Analysis of other biomarkers 132

3.6 Number of techniques 132

3.7 ORAC Assay for measuring total antioxidant capacity 133

3.8 Enzyme leakage as measure for lipid peroxidation 134

3.9 The use of an exogenous marker of oxidative stress 134

3.10 The use of serum γ-glutamyltransferase as marker of oxidative stress 135

3.11 The use of erythrocytes to study *in vivo* cellular antioxidant response 135

3.12 The use of albumin as general marker of protein oxidation in plasma 136

3.13 The use of glutamine synthetase activity as measure of protein oxidation 136

4 Conclusion 137

Summary *138*
Samevatting *139*
References *140*
Appendices.. *152*

LIST OF TABLES

Chapter 1:

Table 1.1 Oxidative stress factors (Moller *et al*, 1996). 2

Table 1.2 Antioxidants in human plasma (Adapted from Stocker and Frei, 1991). 6

Chapter 2:

Table 2.1 The dilution series of samples used for the DPPH Assay. 32

Table 2.2 Physical characteristics of volunteers. 41

Table 2.3 Heart rates, blood lactate concentrations and RPE ratings of volunteers. 41

Chapter 3:

Table 3.1 Physical characteristics of volunteers. 53

Table 3.2 Biokinetical data of volunteers. 54

Chapter 4:

Table 4.1 Summary of experimental procedure followed for the Third Exercise Study. 70

Table 4.2 Exercise protocol followed by volunteers in the Third Exercise Study. 72

Table 4.3 Physical characteristics of volunteers. 73

Table 4.4 Biokinetical data obtained from volunteers before, directly after or 2 days after exercise as indicated 74

Chapter 5:

Table 5.1 The variation on four sets of independent DNPH experiments on aliquots of the same plasma sample. 94

LIST OF FIGURES

Chapter 1:

Figure 1.1 Illustration of the relationship between oxidants and antioxidants in the determination of cellular redox balance (Powers *et al*, 2004).....4
Figure 1.2 Free radicals, their cellular targets and examples of by-products that may serve as biomarkers. 10
Figure 1.3 The steps involved in lipid peroxidation.....11
Figure 1.4 The electron leakage and subsequent formation of oxygen-centered free radicals (Karlsson, 1997).....14
Figure 1.5 The sites of electron leakage in the electron transport chain leading to the formation of reactive oxygen species (Radak, 2000).....17
Figure 1.6 Chemical structures of some of the common antioxidants.....19
Figure 1.7 Tocopherol recycling by ascorbate.....20

Chapter 2:

Figure 2.1 The effect of exercise duration and fitness levels of volunteers on rise in blood lactate levels. 29
Figure 2.2 The reduction of DPPH in the presence of a reducing equivalent. 34
Figure 2.3 The stepwise oxidation of ascorbic acid to dehydroascorbic acid with the release of two electron donors.....34
Figure 2.4 Absorbance graphs for (i) a set of plasma samples and (ii) a vitamin C standard 38
Figure 2.5 The absorbance spectra of DPPH titrated with vitamin C.....39
Figure 2.6 Decrease in absorbance of DPPH at increasing amounts of vitamin C.....40
Figure 2.7 Changes in plasma and salivary antioxidant levels following exhaustive exercises as determined by the DPPH Assay.....41

Chapter 3:

Figure 3.1 The reaction between thiobarbituric acid and malondialdehyde. 48
Figure 3.2 The acid hydrolysis of TEP to yield MDA. 48
Figure 3.3 A volunteer performing strenuous treadmill exercising while attached to the gas analyzer. 53
Figure 3.4 Increase in heart rate during the exercise protocol for maximum oxygen uptake determination. 55
Figure 3.5 Increase in oxygen uptake during the exercise protocol for maximum oxygen uptake determination. 55
Figure 3.6 Increase in the heart rates of the ten volunteers as exercise progressed. 56
Figure 3.7 Increase of oxygen uptake of the ten volunteers during the exercise protocol. 56
Figure 3.8 Relationship between the maximal heart rate and maximum oxygen uptake of the ten volunteers during the exercise protocol. 57
Figure 3.9 Changes in plasma and salivary antioxidant levels following exhaustive exercises as determined by the DPPH Assay. 58
Figure 3.10 Changes in oxidative stress markers following exhaustive treadmill running:
(i) Changes in plasma lipid peroxidation as determined with the TBARS Assay
(ii) Changes in the plasma Oxidative Stress Ratio. 58

Chapter 4:

Figure 4.1 Age-dependant decrease in maximal heart rate (100%) and different percentages (50, 70 and 90%) of maximal heart rate. 65
Figure 4.2 One of the groups of volunteers during a spinning class. 74
Figure 4.3 Heart rates of volunteers before and directly following spinning exercise. 75

Figure 4.4 Changes in maximum leg presses performed by volunteers following strenuous spinning exercises. 76

Figure 4.5 Changes in scores of the different components of the SF-MPQ following strenuous spinning exercises:
(i) Visual Analog Scale
(ii) Evaluative Overall Intensity
(iii) Pain Rating Index 77

Figure 4.6 Changes in plasma and salivary antioxidant levels following strenuous spinning exercises as determined by the DPPH Assay. 78

Figure 4.7 Changes in oxidative stress markers following strenuous spinning exercises:
(i) Changes in plasma lipid peroxidation as determined with the TBARS Assay
(ii) Changes in the plasma Oxidative Stress Ratio.................. 78

Figure 4.8 The percentage change in Oxidative Stress Ratio, TBARS and plasma DPPH results of the volunteers following exercise.. 79

Figure 4.9 The percentage change in plasma and salivary DPPH results of the volunteers following exercise. 79

Chapter 5:

Figure 5.1 The derivatization of carbonyl groups in protein side chains to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with DNPH (Levine *et al*, 1990). 86

Figure 5.2 The *in vitro* generation of carbonyls in BSA by reactive aldehydes. 92

Figure 5.3 Wavelength scan of DNPH Assay on untreated and *in vitro* oxidized ($FeCl_3$/ascorbate treated) plasma samples at the maximum absorbance region of the DNPH reagent. 93

Figure 5.4 Protein carbonylation of formaldehyde-treated BSA and plasma samples. 93

Figure 5.5 Basic outline of experimental protocol followed for performing Western blot on samples................................. 100

Figure 5.6 Representation of the theoretical results expected following the experimental protocol as outlined under Materials and Methods.. 107

Figure 5.7 Film following Oxyblot™ protocol..................................... 111

Figure 5.8 Results from control experiments A and B.
(i) Coomassie stain of SDS-PAGE gel following electroblot transfer
(ii) Coomassie stain of cut off of two extra lanes of derivatized positive control samples from SDS-PAGE gel shown in (i) following electrophoresis, but not electroblotted
(iii) PVDF membrane with cut off of extra lane of derivatized positive control sample after having been transferred from SDS-PAGE gel shown in (i) to PVDF membrane with electroblotting 114

Figure 5.9 Results from control experiment C. Chemiluminescence signals of dot blots of chemiluminescent marker (left) and for 2°Ab (right)... 115

Figure 5.10 Results from control experiment D. Chemiluminescence signal of dot blot of the protein standard........................... 115

LIST OF ABBREVIATIONS

1°Ab	Primary antibody
2°Ab	Secondary antibody
bpm	beats per minute
BHT	Butylated hydroxytoluene
BMI	Body mass index
BSA	Bovine serum albumin
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
dddH_2O	Triple distilled water
DNPH	2,4-Dinitrophenyl hydrazine
DNP-	2,4-Dinitrophenyl hydrazone
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenendiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESR	Electron-spin resonance
EtOH	Ethanol
FRAP	Ferric Reducing Antioxidant Power
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
HEPES	4-(2-Hydroxyethyl)piperazine-N'-(4-butanesulfonic acid)
HRP	Horseradish peroxidase
LBM	Lean body mass
LDL	Low-density lipoproteins
LLI	Low leg intensity
LMW	Low molecular weight
MDA	Malondialdehyde
2-ME	2-Mercapto-ethanol
MeOH	Methanol
MLI	Medium leg intensity
NaCl	Sodium chloride
ORAC	Oxygen Radical Absorbance Capacity

PPI	Present Pain Intensity
PVDF	Polyvinylidene fluoride
PUFA	Polyunsaturated fatty acids
RER	Respiratory Exchange Ratio
ROS	Reactive oxygen species
RPE	Rate of Perceived Exertion
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium-dodecyl sulphate polyacrylamide gel electrophoresis
SF-MPQ	Short-Form McGill Pain Questionnaire
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing 0.5% Tween®-20
TCA	Trichloro-acetic acid
TEAC	Trolox Equivalent Antioxidant Capacity
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TEP	Tetra-ethoxypropane
TRAP	Total Radical-Trapping Antioxidant Parameter
Tris	Tris(hydroxymethyl)aminomethane
Tween®-20	Polyoxyehylene sorbitan monolaurate
VAS	Visual Analog Scale
VCE	Vitamin C Equivalents
VO_{2max}	Maximal oxygen uptake
w/v	Weight per volume

CHAPTER 1

BACKGROUND

1 FREE RADICALS, OXIDATIVE STRESS AND ANTIOXIDANT SYSTEMS

1.1 Free radicals and other reactive oxygen species as part of normal life

In our daily lives our bodies are exposed to free radical attacks. The factors causing free radical attacks can be divided into those of endogenous or of exogenous origin and sometimes could lead to oxidative stress. Some common sources of free radical attack are listed in Table 1.1.

Oxidation and accompanying free radical formation are also an integral part of human metabolism. Oxygen is the ultimate electron acceptor in the mitochondrial electron transport chain where the flow of electrons ultimately produces energy in the form of ATP. Approximately 5% of the electrons passed in the electron transport chain leaks out of the chain and are exposed to oxygen leading to the formation of free radicals.

Free radicals are defined as molecules or ions containing an unpaired electron in its outer orbital (Halliwell and Gutteridge, 1999). This makes it very reactive towards various other molecules. The property of readily accepting electrons, combined with the abundance of oxygen in cells, explains in part why oxygen-centered free radicals are primary or secondary mediators of many free radical reactions. If free radicals and other reactive oxygen species (ROS) are not removed or neutralized, it can target cellular constituents like lipid membranes, proteins, DNA and RNA. These cellular targets are then

damaged by free radicals, often resulting in a loss of their form and function. In extreme cases, cell death is the ultimate consequence of free radical attack.

Table 1.1. Oxidative stress factors (Moller *et al*, 1996).

Endogenous factors	Exogenous factors
Physical exercise/sedentary life	Food consumption
Psychological stress	*Protein and/or lipid rich*
Inflammation	*Prooxidants (xenobiotics)*
Cancer	*Antioxidants (low dietary amount)*
Cell death	*Beverages (alcohol, coffee)*
	Airborne pollutants
	Cigarette smoke
	Air pollution
	Medical drugs
	Anticancer drugs
	Radiation
	Ionizing radiation
	Ultraviolet radiation
	Microwaves
	Skin absorbants
	Insecticides (eldrin, DDT, lindane)
	Drugs (lindane, psoralen derivatives)

1.2 Antioxidant defense mechanisms

In order to prevent the damaging reactions of free radicals to cellular constituents, our bodies have evolved complex antioxidant defense mechanisms. Antioxidants are defined as molecules that, when present at low concentrations, are able to react with and prevent damage of free radicals to cellular constituents. These antioxidant molecules can be intracellular or extracellular, enzymatic or non-enzymatic, lipid- or water soluble and endogenous or dietary derived. Based on these different properties, different classification systems exist for antioxidants.

There are different possible antioxidant mechanisms by which antioxidants may offer protection against free radical damage. These include the prevention of the formation of ROS, interception of ROS attack by scavenging the reactive metabolites and converting them to less reactive molecules, avoiding the transformation of less reactive ROS to more deleterious forms, facilitating the repair of damage caused by ROS and providing a favorable environment for the effective functioning of other antioxidants (Sen, 1995).

The mechanisms of antioxidant protection of extracellular fluids like plasma are different from intracellular mechanisms. The antioxidant capacity of the cell is mainly attributable to the enzyme system, whereas that of plasma is mostly accounted for by low molecular weight antioxidants (Halliwell and Gutteridge, 1999).

The low molecular weight non-enzymatic antioxidants present in the extracellular fluids exert its protective effect by two major mechanisms. The primary, preventative mechanisms involve sequestering of transition metals and thereby preventing free radical reactions they are involved in. The secondary mechanism includes the scavenging of free radicals, thereby intercepting the chain reactions they are involved in and so preventing them from reacting with and damaging cellular constituents.

The antioxidant defense system is very dynamic and responsive to any disturbances taking place in the redox balance of the body. Enzymatic and non-enzymatic antioxidants within cells, cell membranes and extracellular fluids can be up regulated and mobilized to neutralize inappropriate free radical formation that could take place due to oxidative stress caused by various factors.

Antioxidants are complex molecules as far as their function(s) are concerned. A specific antioxidant often has multiple antioxidant properties and functions, and they are often interrelated with each other in recycling reactions.

Antioxidants also often act together in a synergistic function, resulting in an antioxidant effect larger than the sum of the effects of the individual antioxidants. It is also generally accepted that all the antioxidant functions of the so far known antioxidants have not been elucidated yet and that yet unidentified antioxidants are present in foodstuffs and biological samples.

Because oxidative damage takes place initially and primarily in mitochondrial membranes and also in other cellular membranes, the lipid-soluble antioxidants are considered the first line of defense, and when this first line of defense is broken, the water-soluble antioxidants act as a second line of defense.

1.3 Oxidative stress

The body has a sophisticated antioxidant defense system to cope with free radical formation under normal conditions and thereby maintaining the redox balance. However, there is not an excess of antioxidant defenses and an overproduction of free radicals or a drop in the level of the antioxidant defenses will lead to an imbalance and cause deleterious effects, a situation known as oxidative stress. The relationship between oxidants, reductants (i.e. antioxidants) and oxidative stress is illustrated by Figure 1.1.

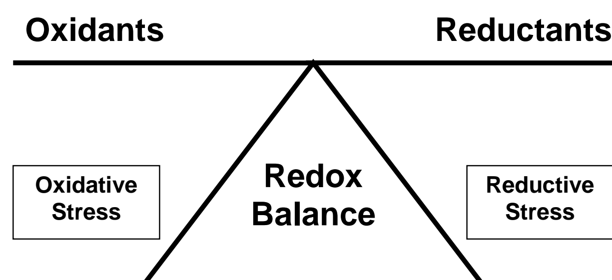


Figure 1.1. Illustration of the relationship between oxidants and antioxidants (reductants) in the determination of cellular redox balance. *Oxidative stress occurs when oxidants outnumber the available antioxidants. In contrast, reductive stress occurs when antioxidants outnumber the oxidants present in the cell (Powers et al, 2004).*

Oxidative stress can be the result of various conditions, including certain diseases and other factors as summarized in Table 1.1. Oxidative stress can also result when a healthy individual performs excessive exercise.

1.4 The plasma antioxidant system

The extracellular antioxidants are found in most body fluids such as plasma, saliva, lavage fluids and synovial fluids. Plasma antioxidants consist of both water-soluble and lipid soluble molecules. The water-soluble antioxidants are found in purely soluble conditions whereas lipid-soluble antioxidants are found incorporated into the lipoproteins present in plasma.

Blood plays an integral role within the strategy to maintain redox balance against oxidative stress conditions by transporting and redistributing antioxidants throughout the body. The concentration of antioxidants in human blood plasma is important in investigating and understanding the relationship among diet and oxidative stress since dietary antioxidants have a direct influence on plasma antioxidant levels (Alho and Leinonen, 1999). It may also help to identify conditions affecting oxidative status *in vivo* and provide information on the absorption and bio-availability of nutritional compounds (Ghiselli *et al*, 2000; Alho and Leinonen, 1999).

As mentioned earlier, low molecular weight antioxidants are major contributors of plasma antioxidants whereas enzymes contribute little to plasma antioxidant capacity. Many of these low molecular weight antioxidants are of dietary origin (Halliwell, 1996). These dietary-derived antioxidants are rapidly consumed during the scavenging of ROS and need to be regenerated or replaced continuously by new dietary-derived compounds. Thus, plasma antioxidant capacity is modulated either by oxidative stress or by the intake of dietary antioxidants and can be regarded as representative of the *in vivo* balance between the oxidizing species and antioxidant compounds (Ghiselli *et al*, 2000).

The antioxidants present in human plasma are summarized in Table 1.2, classified as water soluble and lipid soluble.

Table 1.2. Antioxidants in human plasma (Adapted from Stocker and Frei, 1991).

Antioxidant	Concentration (μmol/l)	Mechanism of action
Water soluble		
Ascorbate	30-150	Radical scavenger, regeneration of vitamin E
Urate	160-470	Radical scavenger, binds Cu^{2+} and Fe^{2+}
Glutathione	1-2	Radical scavenger and substrate for glutathione peroxidases
Bilirubin	5-20	Radical scavenger
Albumin	530-830	Binds Cu^{2+}, heme, scavenges HOCl
Caeruloplasmin		Binds Cu^{2+}
Transferrin		Binds Fe^{2+}
Lipid soluble		
Vitamin E	25-40	Peroxyl radical scavenger
Vitamin Q (Ubiquinone)	0.4-1.0	
Lycopene	0.5-1.0	
Β-carotene	0.3-0.6	

Two of the major water soluble antioxidants present in plasma, uric acid and ascorbic acid, are involved in both preventative and chain-breaking mechanisms: Uric acid is an inhibitor of lipid peroxidation by binding iron and copper in forms that do not stimulate free radical formation (primary defense) and by direct scavenging of free radicals when formed (secondary defense) (Maples and Mason, 1988). Ascorbic acid also acts as a free radical scavenger and is involved in the regeneration of vitamin E that became oxidized in plasma lipoproteins. Vitamin E is an important lipid-soluble, chain-breaking free radical scavenger. Its unique location in cellular membranes enhances its efficiency to quench free radicals originating from the mitochondrial inner membrane. The majority of antioxidants have, like uric acid and ascorbic acid, multiple antioxidant properties.

The Fenton reaction is a biologically important free radical reaction where a mixture of hydrogen peroxide and iron(II) salt cause the formation of hydroxyl radicals:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

The hydroxyl radical react with many organic molecules, including membrane lipids, leading to the initiation of lipid peroxidation.

Sequestering proteins like transferrin (iron binding) and caeruloplasmin and albumin (copper binding) play a major role in the antioxidant mechanism of plasma by binding metals and thereby preventing transition metal ions from accelerating damaging free radical reactions they are involved in such as lipid peroxidation. If this preventative antioxidant defense is overwhelmed, a secondary line of defense exists that scavenge peroxy radicals and therefore act as chain-breaking defense.

1.5 Salivary antioxidants in relation to plasma antioxidants

Since the early 1900s, saliva has proven to be a suitable medium from which to measure a wide range of hormones, pharmaceuticals and antibodies. There are many advantages to using saliva rather than blood for assays. It is non-invasive and unstressful to the donor and the risks for exposure to blood-borne pathogens for the laboratory technician is lower (Lac, 2001).

Because saliva is derived from blood, it is expected to have a free radical scavenging capacity comparable to that of blood. There are reports showing their similarity (Atsumi *et al* 1999, Chapple *et al* 1997, Satoh *et al* 1997). Atsumi and co-workers (1999) compared the changes in plasma and salivary antioxidant levels in young girls following exhaustive aerobic dancing using the DPPH Assay. They found a significant decrease in plasma and salivary antioxidant levels following exercise. Moreover, they compared the

antioxidant capacity in saliva and serum of 21 volunteers and found a significant correlation between them.

Uric acid, the major contributing antioxidant in saliva, is also the major contributing non-protein antioxidant in blood, contributing to as much as 60% and 85% of the total antioxidant capacity of serum and saliva respectively (Maxwell *et al*, 1997).

2 MEASUREMENT OF OXIDATIVE STRESS

2.1 Direct measurement of free radical formation

The direct measurement of ROS has been hampered due to a lack of suitable assays, its low steady state concentration and the transient nature of ROS and their products. For direct detection of free radicals, electron spin resonance (ESR) is the method of choice, since it is considered the most sensitive, specific and direct method of measuring free radicals. To date, only two reports regarding ESR and human serum post-exercise have been published (Ashton *et al*, 1998 and Groussard *et al*, 2003).

2.2 The use of biomarkers to estimate levels of oxidative stress

The free radicals that cause oxidative damage to biological molecules are short-lived and unstable. Therefore it is easier to measure biomarkers to get an indication of oxidative damage and the efficacy of antioxidants to protect molecules against free radical attack. In free radical biology, the term "biomarker" refers to a chemical change in a biological molecule that has arisen from attack by a free radical or other reactive species (Griffiths, 2002). Figure 1.2 shows a schematic diagram of free radicals, their major cellular targets and examples of by-products that may be produced and used as biomarkers.

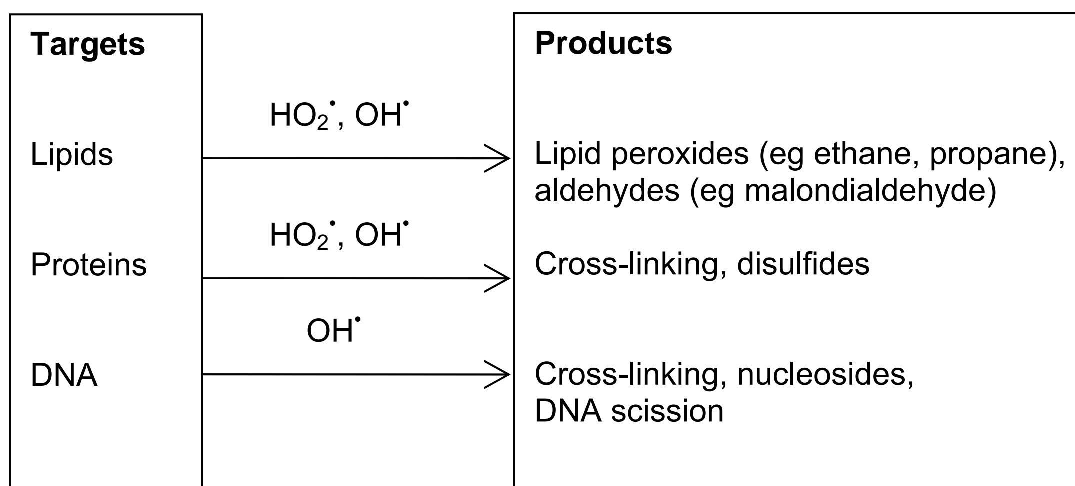


Figure 1.2. Free radicals, their cellular targets and examples of by-products that may serve as biomarkers.

The most well described consequence of free radical attack is lipid peroxidation. Lipid peroxidation of cell membranes results in decreased membrane fluidity, inability to maintain ionic gradients, cellular swelling, and tissue inflammation (Alessio, 1993). Lipid peroxidation is broadly defined as the oxidative deterioration of polyunsaturated lipids, i.e. lipids that contain more than two carbon-carbon double bonds. The membranes surrounding cells and cell organelles contain large amounts of polyunsaturated fatty acid (PUFA) side chains, making them susceptible to lipid peroxidation. Damage to PUFA tends to reduce membrane fluidity, which is known to be essential for the proper functioning of biological membranes (Halliwell and Gutteridge, 1999).

The mechanism by which lipid peroxidation takes place is exemplified by the formation of malondialdehyde (MDA) from a fatty acid with 3 double bonds (Figure 1.3).

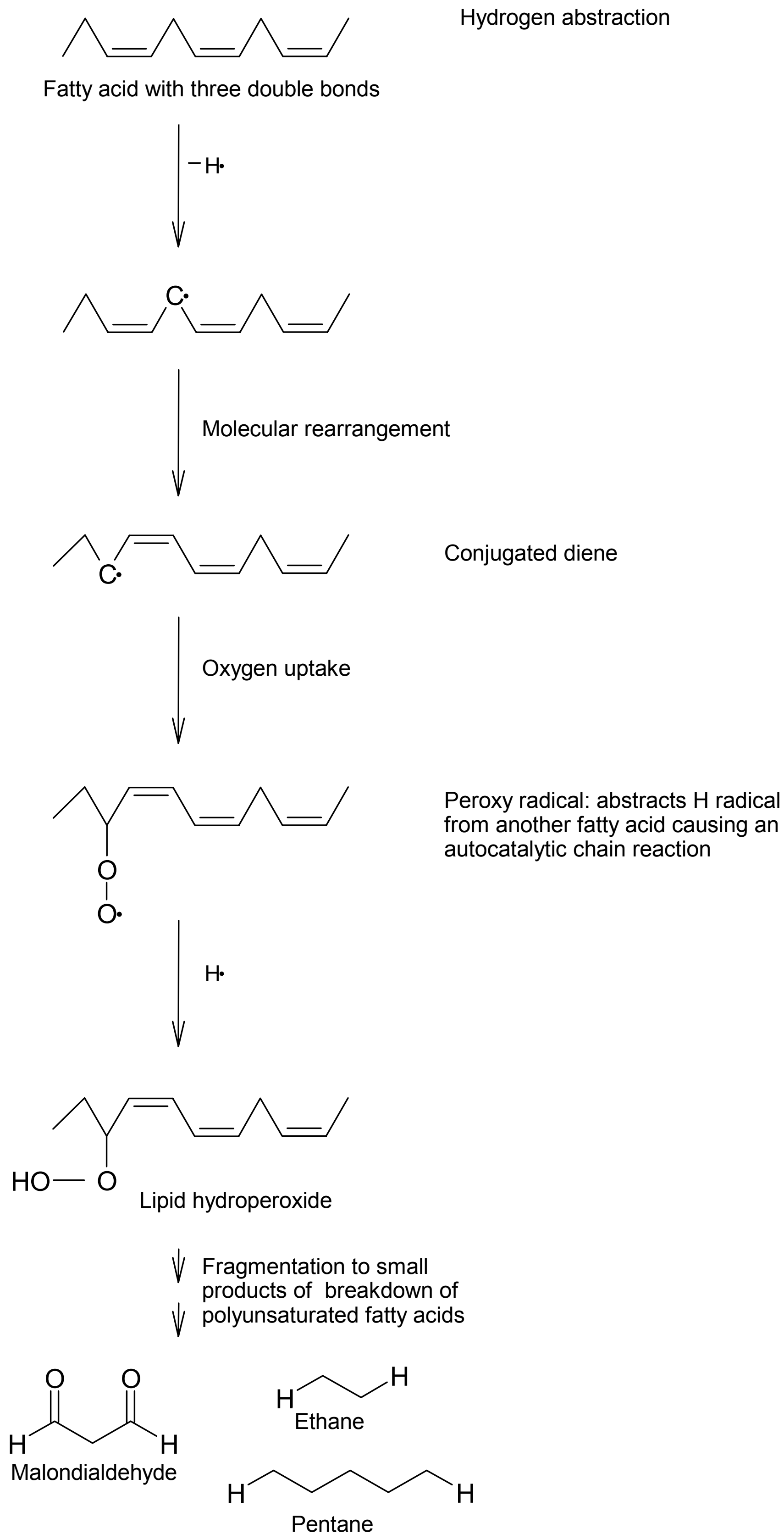


Figure 1.3. The steps involved in lipid peroxidation (Halliwell and Gutteridge, 1999).

Lipid peroxidation reactions are usually free radical-driven chain reactions in which one radical can induce the oxidation of PUFA that act as substrate molecules. Such a chain reaction is initiated by the abstraction of a hydrogen atom from a reactive methylene group of a PUFA residue. Molecular oxygen rapidly adds to the carbon-centered radical, yielding a peroxyl radical. This lipid peroxyl can abstract a hydrogen atom from another PUFA as in the first step. This reaction is termed propagation, implying that one initiating hit can result in the conversion of numerous PUFA to lipid peroxides. In the presence of transition metal ions, lipid hydroperoxides can give rise to the generation of radicals capable of (re-)initiating lipid peroxidation by redox-cycling of these metal ions. The extent of lipid peroxidation can be limited in a termination reaction where two lipid peroxyl radicals react with each other yielding a nonradical product. Alternatively, an endoperoxide can be formed, which is converted to a dialdehyde, for example malondialdehyde.

Proteins also appear to undergo modifications when exposed to free radicals, where the oxidation of amino acids results in physical changes such as fragmentation and aggregation of proteins themselves. Oxidative modifications of amino acid residues include derivatization of amino acid residues such as proline, arginine, and lysine to reactive carbonyl derivatives that can be determined by spectrophotometric and immunoblot methods, Although not as well described, free radicals can damage DNA resulting in products like 8-hydroxydeoxyguanosine which induces G-C to T-A transversion during DNA replication. The ratio of the reduced (GSH) to the oxidized (GSSG) glutathione can also be used as a biomarker of intracellular redox status.

2.3 The significance and measurement of total antioxidant capacity

Several methods for the assessment of the total antioxidant capacity in biological samples have been proposed. These tests are designed to measure

the sum of the contributions of all the antioxidants present in a biological fluid.

There are several advantages to determining the total antioxidant capacity rather than measuring the concentrations of individual antioxidants during studies to investigate the effect between diet and oxidative stress.

- The methods available to determine the levels of single antioxidants are tedious, especially if the concentrations of a large number of antioxidants are to be determined.
- The possible contribution of lesser-known compounds to antioxidant capacity are not taken into account when measuring only levels of more common antioxidants such as vitamin C.
- Changes in concentrations of single plasma antioxidants can give limited information on its antioxidant action because of the multiple properties of most antioxidants and the synergistic actions between them.

Various methods have been applied to estimate the antioxidant capacity of biological fluids. The methods based on spectrophotometric measurements seem to be the most widely applicable and the most frequently used. We have used the DPPH Assay in our laboratories previously for determination of the antioxidant properties of various teas *in vitro*. Since our ultimate aim is to measure the bio-availability of these teas and compare their *in vitro* and *in vivo* properties, the DPPH Assay was our method of choice for analyzing the biological samples.

3 THE RELATIONSHIP BETWEEN EXERCISE AND OXIDATIVE STRESS

3.1 Strenuous physical exercise as a cause for oxidative stress

Exercise is associated with oxidative stress in two ways. On the one hand, exercise increases oxidative metabolism and this induces oxidative stress, but on the other hand, the adaptations to regular exercise seem to have an antioxidant protective effect. We will focus on the oxidative stress effect caused by unaccustomed exercise.

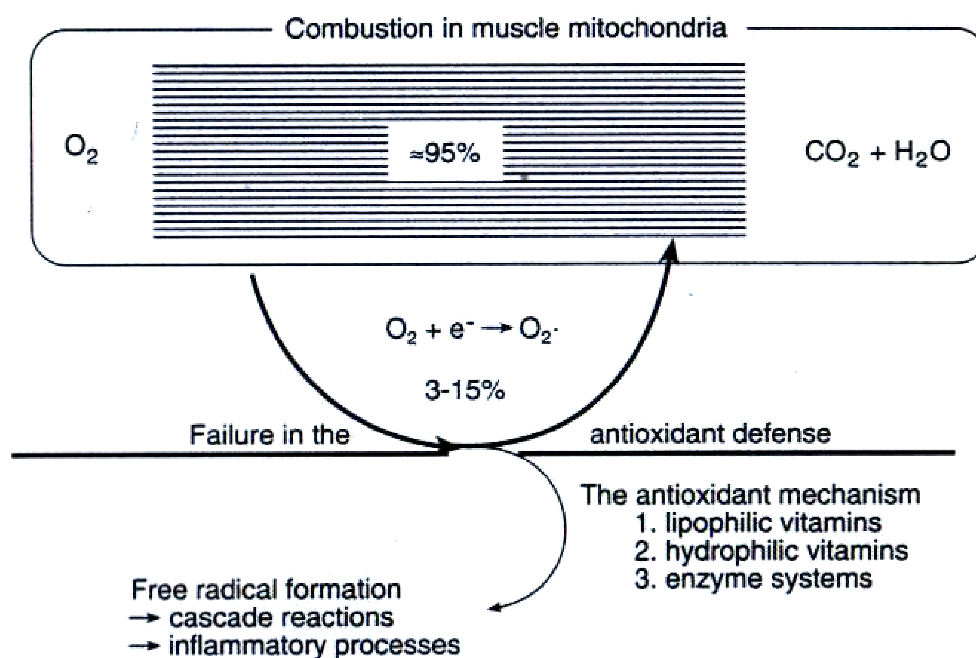


Figure 1.4. The electron leakage and subsequent formation of oxygen-centered free radicals, predominantly the superoxide radical, as a result of the electron transition from the citrate of the tricarboxylic acid cycle to the respiratory chain and the site of ubiquinol-cytochrome B. *Three to fifteen percent of the total turnover of molecular oxygen is assumed to pass through to the pool of oxygen-centered radicals (Karlsson, 1997).*

At rest, over 95% of mitochondrial oxygen consumption is reduced during normal respiration, forming energy and water. The remainder is leaked during passing in the electron transport chain and converted into ROS such as

superoxide, the hydroxyl radical and hydrogen peroxide as shown in Figure 1.4. Although the percentage of oxygen that is converted to these intermediates is less than 5%, cell components in the vicinity of the ROS are susceptible to a short-lived yet virulent attack.

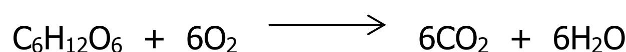
Theoretically, the amount of superoxide radicals that form during normal metabolism and the amount of antioxidants (vitamin C and glucose as examples) necessary to scavenge these radicals can be calculated in the following way:

The average man requires 2 000 kcal/day.

To meet this energy requirement, 500 g of glucose need to be broken down during glycolysis (assuming all these calories are obtained from glucose) since 1 g of carbohydrates release 4 kcal.

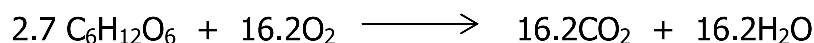
Five hundred grams of glucose is equivalent to 2.7 moles since the molecular mass of glucose is 180 $g.mol^{-1}$ (500 g / 180 $g.mol^{-1}$).

Taking this into account, the equation



$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

can be rewritten as (by multiplying by 2.7):



$$2.7\ C_6H_{12}O_6 + 16.2O_2 \longrightarrow 16.2CO_2 + 16.2H_2O$$

Therefore 16.2 moles of oxygen is consumed during the breakdown of the amount of glucose needed for the daily energy requirement of 2 000 kcal.

It is estimated that, under normal (resting) conditions, 5% of this oxygen escapes the electron transport chain and escapes as superoxide radicals.

This is equivalent to 0.81 moles (16.2 x 5%) of superoxide radicals.

In order to quench these free radicals, 0.81 moles of reducing equivalents are required. One mole of vitamin C releases two reducing equivalents (see Figure 2.4). Therefore, if vitamin C was used as only source of reducing equivalents, 0.405 moles of vitamin C would be required (0.81/2).

Since the molecular mass of vitamin C is 174 $g.mol^{-1}$ this means 70 g of vitamin C would be needed daily to quench the free radicals that form during metabolism (0.0405 x 174).

But the glucose involved in metabolism is also a source of reducing equivalents with each glucose molecule producing 6 H_2 during glycolysis and the Krebs cycle, which is the same as 12 H•.

Thus 0.0675 moles of glucose is necessary to neutralize all the radicals that form during metabolism per day (0.81/12). Taking the molecular mass of glucose of 180 $g.mol^{-1}$ into account this is the same as 12.2 g of glucose (0.0675 x 180).

This illustrates that the amount of free radicals that form during metabolism cannot be quenched solely by dietary intake of antioxidants like vitamin C. Other factors, such as the metabolism of substrates like glucose, are far more effective in coping with the free radical challenge. This also shows the importance of antioxidant enzymes in addition to the intake of dietary antioxidants in coping with free radical formation.

During exercise, whole body oxygen uptake is raised ten to twenty fold and oxygen flux in the active skeletal muscle fibers may increase by as much as 100- to 200-fold (Sen, 2001). Due to the significant increase in mitochondrial oxygen consumption and electron transport flux during exercise, more oxygen may undergo reduction, thus dramatically increasing net cellular levels of ROS

leading to oxidative stress. Superoxide production involves nicotinamide adenine dinucleotide-ubiquinone reductase (complex I) and ubiquinone-cytochrome c reductase (complex III) of the electron transport chain, the transition sites from two-electron to one-electron transfer (See Figure 1.5).

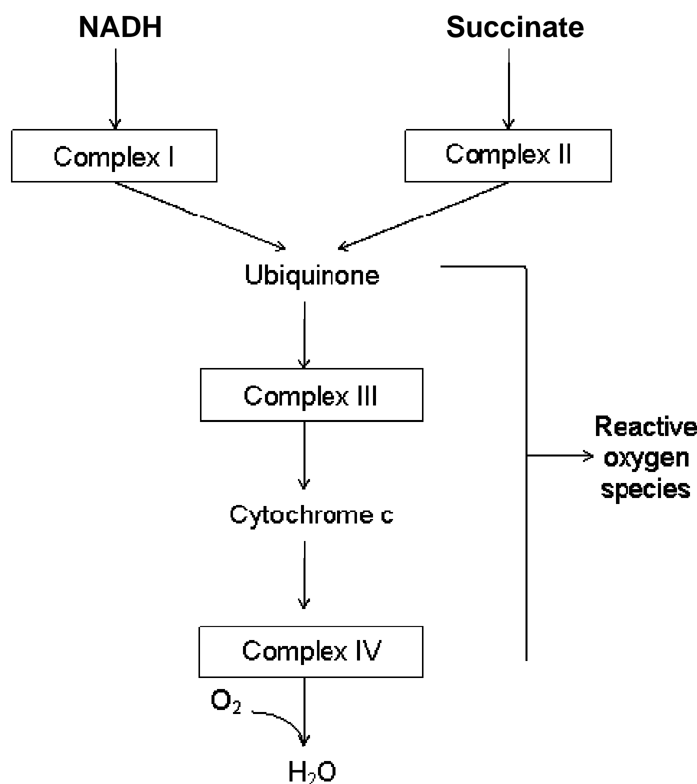
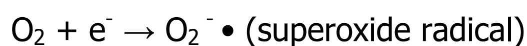
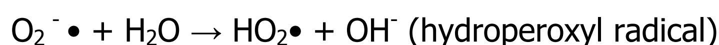


Figure 1.5. The sites of electron leakage in the electron transport chain leading to the formation of reactive oxygen species (Radak, 2000).

The formation of the superoxide radical due to the leakage of electrons to molecular oxygen and the subsequent formation of other ROS are shown below:



$O_2 + e^- \rightarrow O_2^{-}\bullet$ (superoxide radical)



$O_2^{-}\bullet + H_2O \rightarrow HO_2\bullet + OH^-$ (hydroperoxyl radical)

$HO_2\bullet + e^- + H \rightarrow H_2O_2$ (hydrogen peroxide)

$H_2O_2 + e^- \rightarrow \bullet OH + OH^-$ (hydroxyl radical)

Each of these oxygen-derived intermediates is considered highly reactive because its unstable configuration allows for the attraction of electrons from other molecules, resulting in another free radical that is capable of reacting with yet another molecule. This chain reaction is thought to contribute to lipid peroxidation, DNA damage, and protein degradation.

Heavy physical exercise enhances free radical production in skeletal muscle and other tissues mainly because of the increased oxygen flux through the mitochondrial electron transport chain. However, other pathways may also be involved in free radical formation during exercise under specific physiological conditions and in specific tissues. These mechanisms include

- increases in epinephrine and other catecholamines that can produce oxygen radicals when they are metabolically inactivated,
- production of lactic acid that can convert a weakly damaging free radical (superoxide) into a strongly damaging one (hydroxyl), and
- inflammatory responses to secondary muscle damage incurred with overexertion (Clarkson and Thompson, 2000).

These free radical mechanisms are not mutually exclusive; therefore, oxidative injury may be escalated during and after strenuous exercise.

The paradox exists that while exercise might induce ROS production, regular exercise is known to bring about significant benefits to health and to improve quality of life. Although strenuous, unaccustomed exercise leads to an increase in free radical attack and oxidative stress, regular training results in various adaptive responses. These adaptive responses involve upregulation of antioxidant enzymes and repair mechanisms resulting in a resistance of skeletal muscle to oxidative stress caused by exercise.

3.2 Antioxidant defense to exercise-induced oxidative stress

During exercise low-molecular weight antioxidants like antioxidant vitamins play an important role in the prevention of free radical formation and the breaking of free radical chain reactions, thereby keeping cellular homeostasis. Unlike antioxidant enzymes, levels of these antioxidants are not as tightly regulated, and thus could be heavily influenced by exercise.

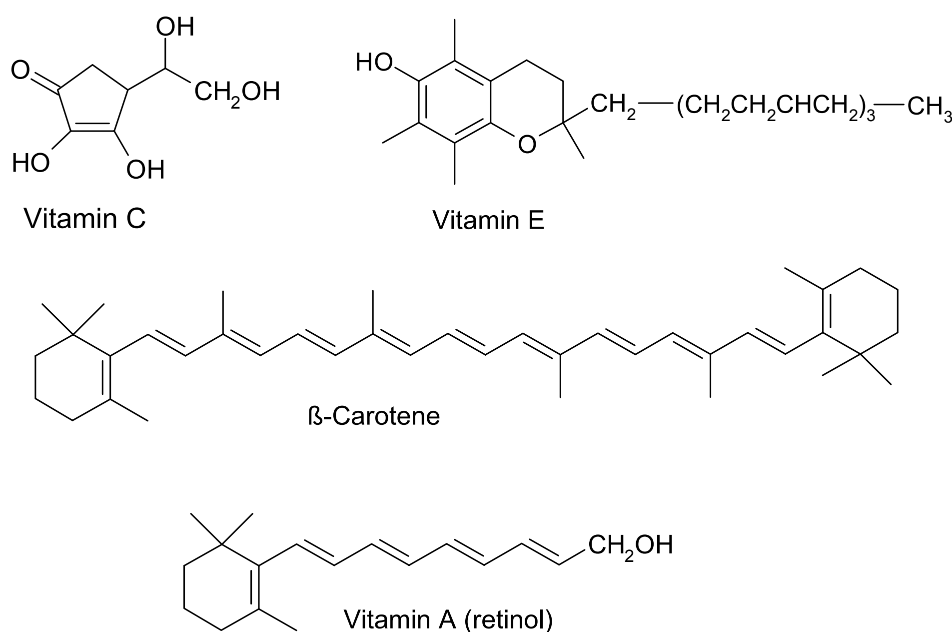


Figure 1.6. Chemical structures of some of the common antioxidant vitamins.

Vitamin E is the major lipid-soluble antioxidant in cell membranes. It protects against lipid peroxidation by acting directly with a variety of oxygen radicals, including singlet oxygen, lipid peroxide products, and the superoxide radicals, to form a relatively innocuous tocopherol radical. Vitamin C is water soluble and can directly react with superoxide, hydroxyl radicals, and singlet oxygen. Beta-carotene, the major carotenoid precursor of vitamin A, is the most efficient quencher of singlet oxygen (Clarkson and Thompson, 2000). The chemical structures of these antioxidant vitamins are shown in Figure 1.6.

Although antioxidants have antioxidant properties on their own, they are often involved in complex networks with each other in biological systems,

resulting in synergistic effects. An appropriate example is the interaction between glutathione, vitamin C and vitamin E during the recycling of vitamin E. In blood, vitamin C present in erythrocytes can protect tocopherol in LDL against oxidation by recycling the tocopherol radical back to the reduced tocopherol (Figure 1.7). During this recycling, glutathione is oxidized in order to reduce ascorbic acid back to its reduced form, ascorbate (vitamin C).

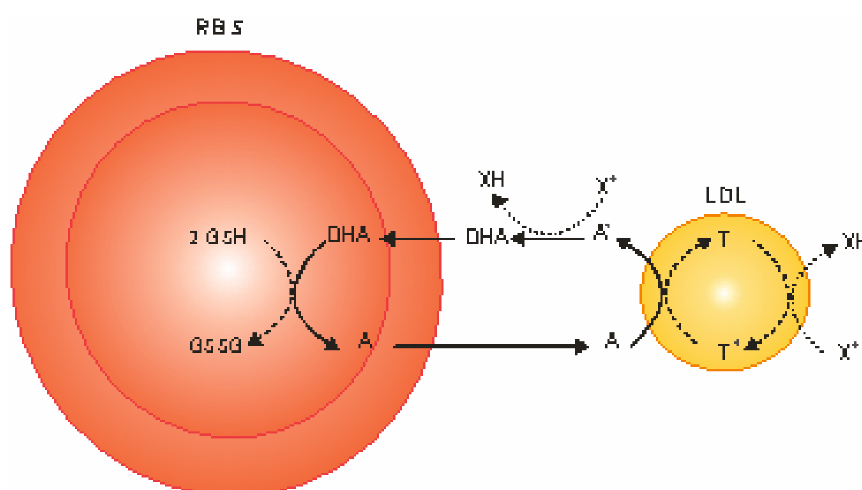


Figure 1.7. Tocopherol recycling by ascorbate. *Abbreviations: RBS, human erythrocyte; LDL, low density lipoprotein, DHA, dehydroascorbic acid, A, ascorbate; A•, ascorbate radical; T, tocopherol; T•, tocopherol radical, X•, free radical; XH, reduced free radical; GSH, reduced glutathione; GSSG, oxidized glutathione (Figure taken from May, 1998) .*

3.3 Exercise as a potential tool for bio-availability studies

In theory, the bio-availability of the antioxidants of a foodstuff can be determined by measuring the increase in blood antioxidant levels following consumption of the foodstuff and comparing this with the antioxidant content of the foodstuff itself. Reports in literature using this approach to measure the bio-availability of tea antioxidants (which is our research group's field of interest) are limited and inconsistent. While some groups observed an increase in blood antioxidant levels as expected following tea consumption (Benzie *et al*, 1999, Langley-Evans, 2000, Leenen *et al*, 2000, Serafini *et al*, 1996), others found no changes (Maxwell and Thorpe 1996, Van het Hoff *et*

al, 1997). These discrepancies are mainly attributed to the different methodologies used to measure antioxidant capacity. The time points of blood collection following tea consumption could also play a role.

Our attempts to detect increases in plasma antioxidant levels following ingestion of vitamin C by using the DPPH Assay were unsuccessful, even though blood was collected at the time of known peak plasma levels of this vitamin. This observation led us to believe that, under normal conditions in a healthy volunteer, the increase in blood antioxidant levels due to consumption of the vitamin C might be small in comparison with the already relative high baseline levels of blood antioxidant levels. This led us to consider possible ways of inducing oxidative stress in healthy volunteers, thereby lowering the baseline levels of antioxidants. Once the baseline level of antioxidants is lowered, changes in antioxidant levels due to the consumption of antioxidant-rich foods might be observed more clearly.

When considering the ways in which healthy people can be exposed to oxidative stress as set out in the beginning of this chapter, exercise is one of the few ethical means by which oxidative stress can be deliberately induced in healthy volunteers. The fact that exercise can cause a temporary increase in oxidative stress levels in healthy volunteers without causing any long term detrimental side effects give it the potential to be used for bio-availability studies. The ability of an antioxidant foodstuff consumed just before exercise to ameliorate the oxidative stress response due to the exercise gives a direct indication of the bio-availability of the antioxidants of the particular foodstuff. Foodstuffs with a higher content of bio-available antioxidants will be more able to protect against the oxidative stress induced by the exercising.

If an exercise protocol could be established that would induce oxidative stress in a reproducible manner, it would be of great value to investigate and compare the *in vivo* antioxidant properties of foodstuffs. Despite the wealth of literature on the effect of various types of exercises on oxidative stress

parameters, no single exercise protocol has been described yet that could be used for such bio-availability studies. The purpose of this study was to establish such an exercise protocol that could be used later for bio-availability studies.

4 AIMS OF STUDY

i) Determine whether there is a statistically significant increase in plasma TBARS levels of healthy volunteers following a strenuous exercise protocol.

ii) Determine whether there is a statistically significant decrease in plasma antioxidant levels as measured by the DPPH Assay of healthy volunteers following a strenuous exercise protocol.

iii) Determine whether there is a statistically significant decrease in salivary antioxidant levels as measured by the DPPH Assay of healthy volunteers following a strenuous exercise protocol.

iv) Determine whether there is a statistically significant increase in the Oxidative Stress Ratio of healthy volunteers following a strenuous exercise protocol.

v) Determine whether there is a significant positive correlation between plasma and salivary antioxidant levels in the healthy volunteers before and after exercise.

5 HYPOTHESES

i) Strenuous exercise will lead to a significant increase in plasma TBARS levels of the volunteers.

ii) Strenuous exercise protocol will lead to a significant decrease in plasma antioxidant levels as measured by the DPPH Assay of the volunteers.

iii) The strenuous exercise protocol will lead to a significant decrease in salivary antioxidant levels as measured by the DPPH Assay of the healthy male volunteers.

iv) Strenuous exercise will lead to a significant increase in plasma oxidative stress levels as measured by the Oxidative Stress Ratio of the volunteers.

v) There will be a statistically significant correlation between the plasma and salivary antioxidant levels of the volunteers before and after the strenuous exercises.

6 NULL HYPOTHESES

i) There will be no statistically significant difference in the plasma TBARS levels before and after strenuous exercise in the volunteers.

ii) There will be no statistically significant difference in plasma antioxidant levels as measured by the DPPH Assay following strenuous exercise in the volunteers.

iii) There will be no statistically significant difference in salivary antioxidant levels as measured by the DPPH Assay following strenuous exercise in the volunteers.

iv) There will be no significant difference in plasma oxidative stress levels as measured by the Oxidative Stress Ratio following strenuous exercise in the volunteers.

v) There will not be a statistically significant correlation between the plasma and salivary antioxidant levels of the volunteers before and after the strenuous exercise.

CHAPTER 2

THE FIRST EXERCISE STUDY

THE USE OF INCREMENTAL TREADMILL RUNNING TO EXHAUSTION AS EXERCISE PROTOCOL

1 INTRODUCTION

Exercise-induced oxidative stress has great potential in bio-availability studies as explained in the previous chapter: Healthy volunteers can be exposed to exercise-induced oxidative stress without suffering any long-term detrimental side effects. This makes exercise an ideal model to investigate the bio-availability of dietary antioxidants in healthy people as seen through their protective effect against exercise-induced oxidative stress.

Numerous studies on the effect of exercise on oxidative stress and antioxidant markers in humans have been described in the literature. A Medline search limited to English abstracts of articles on humans using the keywords "exercise" and "oxidative stress" published between 1965 and May 2006 showed up 557 hits. Despite the wealth of information available on this topic, generalizations and comparisons between studies are difficult because of the variation that exists between studies regarding the following factors.

- Volunteer selection (number of volunteers, age, gender, fitness level and health status)
- Exercise protocol (accustomed or unaccustomed exercise, isometric or eccentric exercise, muscle groups that are employed, aerobic or anaerobic exercise, intensity and duration of exercise and the type of standardization used for exercise intensity and duration)
- Sampling (type of sample(s) collected and time point(s) of collection)

- Target of free radical attack investigated (DNA, protein or membrane lipids)
- Type of antioxidants measured (total antioxidant capacity or levels of individual antioxidants)
- Specific types of assays used to measure these biomarkers

Taking all the above-mentioned factors that play a role on the effect of exercise on oxidative stress into consideration, it is not surprising that there is no unequivocal conclusion in the literature on the effect of exercise on oxidative stress parameters. This should also explain why, despite the wealth of literature on this topic, no standard exercise protocol has been established to date that is known to consistently induce oxidative stress in healthy volunteers and is therefore suitable for use in bio-availability studies.

In the past few years, our research group's interest has turned from tea analysis and tea quality improvement to the health properties of tea. Since most of the health benefits of teas made from *Camellia sinensis* (black, green and oolong tea) are attributed to their antioxidant content, our group compared the antioxidant capacities of these teas with each other as well as with other popular herbal infusions and spices using the DPPH Assay (Du Toit *et al*, 2001). This study confirmed the high antioxidant content of black, green and oolong tea *in vitro*. We thus decided to attempt more challenging *in vivo* work.

Our next aim was to investigate the effect of tea consumption on total plasma antioxidant levels thereby illuminating the bio-availability of tea antioxidants. Since the DPPH Assay was used in the study for the *in vitro* antioxidant of tea, it was the obvious assay of choice for the *in vivo* bio-availability study.

Exercise as a means to induce oxidative stress has been identified as a useful tool in such a bio-availability study. Since no standard exercise protocol for antioxidant bio-availability studies exist yet, our aim was to establish such an

exercise protocol. In our attempt to establish an exercise protocol suitable for bio-availability studies, we carried out three separate exercise studies, which will be discussed in this and the following two chapters.

We undertook the First Exercise Study as described in this chapter with a small number of volunteers, made some adjustments to the protocol as described later and then performed a Second Exercise Study as discussed in Chapter 3. Based on the results of the Second Exercise Study, we determined the number of volunteers that should be used to give the results enough statistical power and made some other improvements to the exercise protocol before performing the Third Exercise Study which is discussed in Chapter 4.

According to literature, there exist a strong relationship between the intensity of exercise performed and the oxidative stress response that results from it. An exercise protocol with high intensity results in high levels of oxidative stress on the volunteer's body. It is therefore expected to lead to a significant depletion in antioxidant defenses resulting in a great increase in oxidative stress.

We used the following standard parameters used in sport physiology to describe the intensity of the exercise as experienced by the volunteers during the First Exercise Study:

- Heart rate
- Lactate levels
- Borg scale of Ratings of Perceived Exertion (RPE)

Heart rate: Exercise causes an increase in heart rate in order to help supply the increasing need for oxygen in muscles. For this study the increase in heart rate was expressed as a percentage change of the resting heart rate value for each volunteer as an indication of exercise intensity.

Lactate levels: Lactate is one of the products of anaerobic glycolysis and is produced by the muscles. Its rate of production increases as the exercise rate increases and as more carbohydrates are broken down to pyruvic acid or lactic acid. During light and moderate exercise the energy demands are adequately met by aerobic oxidation through oxidative phosphorylation. Any lactic acid formed in the muscles during light exercise is rapidly transported to the liver where it is metabolized to pyruvic acid and ultimately glucose through the gluconeogenesis pathway. Therefore during light exercise the blood lactic acid levels remain fairly stable even though the oxygen consumption increases.

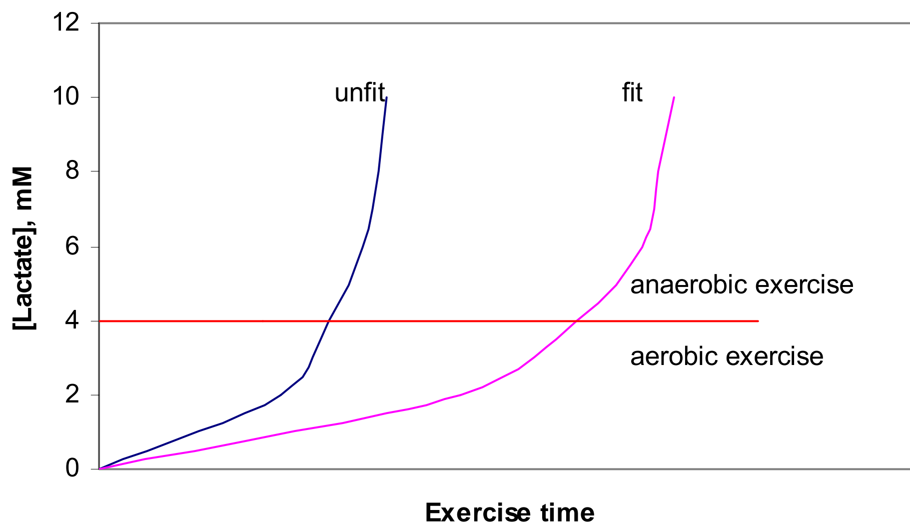


Figure 2.1. The effect of exercise duration and fitness levels of volunteers on rise in blood lactate levels. *Fit volunteers can rely on aerobic exercise for longer than unfit volunteers before passing the threshold of approximately 4 mM blood lactate concentration and starting to rely on anaerobic exercise.*

At a blood lactate level of about 4 mM, volunteers start relying upon anaerobic metabolism to meet the increased energy demand and the lactic acid levels start to accumulate and rise in an exponential fashion (Heck *et al*, 1985). In this way, the blood lactate levels can serve as an indication whether volunteers are relying on aerobic (blood lactate < 4 mM) or anaerobic (blood lactate levels > 4 mM) metabolism at different time points during an exercise protocol as shown in Figure 2.1.

Borg scale of Ratings of Perceived Exertion (RPE): The 15-point Ratings of Perceived Exertion (RPE) scale by Borg is the method used most frequently in sports literature to quantify perceived exertion (Borg, 1970 and Borg, 1985). The RPE scale ranges from 6 to 20, which is approximately analogous to a heart rate change of 60 to 200 bpm. For example, a rating of 13 corresponding to the description "somewhat hard" and is equivalent to a heart rate of about 130 bpm. A copy of the Borg Scale is included in Appendix F.

2 MATERIALS AND METHODS

2.1 Sample collection and processing

Plasma samples: Blood samples were obtained from the antecubetal veins of the volunteers by a registered nurse. EDTA-containing vacutainer tubes were used for collection of blood to be used for the DPPH Assay. Blood samples were obtained just before and immediately following exercise. The blood samples were kept on ice until arrival at the laboratory where they were processed. Plasma was obtained by centrifuging the blood at 3 000 g for 5 min in 10 ml centrifuge tubes. Before storing the samples were transferred to 1.5 ml microtubes and boiled at 95°C in a water bath for 10 min. The clear supernatant that was obtained after boiling, i.e. the non-protein fraction, was removed and diluted 1:4 (v/v) with HEPES buffer (0.9% NaCl, 25 mM HEPES, pH 7.4). The diluted deproteinized plasma samples were aliquoted into cryovials and stored at -80°C until analysis.

Saliva samples: Volunteers were requested to fast overnight and drink plenty of water on the morning of the exercise in order to improve saliva flow. Whole, non-stimulated saliva was obtained from the volunteers before and directly following exercise by tilting the head forward and allowing the saliva to flow freely from the side of the mouth into a sampling tube. The saliva samples were also kept on ice until arrival at the laboratory where each

sample was weighed and diluted 1:1 (w/v) with HEPES buffer. The diluted saliva was boiled at 95°C in a water bath for 10 min, transferred to microtubes and centrifuged at 3 000 g for 5 min to remove the insoluble fraction. The clear supernatant was transferred to cryovials and stored at -80°C.

2.2 Biochemical analysis

2.2.1 DPPH Assay

The method as described by Atsumi *et al* (1999) was used for the DPPH Assay.

2.2.1.1 Materials

K_3EDTA-containing vacutainer tubes were from BD Vacutainer (Becton, Dickinson and Co., UK). 2-[4-(2-Hydroxyethyl)]-(1)-ethanesulfonic acid (HEPES) and sodium chloride (NaCl) were from Merck (Merck NT Laboratory Supplies, Jhb, SA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid and ethanol (EtOH) were from Sigma-Aldrich (St. Louis, MO, USA). All the salts and reagents were of analytical grade. Triple distilled, deionized water (dddH_2O) (Millipore, Microsep, SA) was used for making up buffers.

2.2.1.2 Assay principle

The principle of the DPPH Assay is explained later in this section where the reaction between DPPH and vitamin C is described.

2.2.1.3 Preparation of DPPH stock and working solution

DPPH stock solution (0.5 mM DPPH in EtOH) was prepared freshly for each experiment and sonicated in a sonicator bath at room temperature in the dark for 30 min to enhance solubility. The stock solution was filtered before use to remove any undissolved DPPH particles. The DPPH working solution was prepared by adding DPPH stock solution to a HEPES buffer in 40% EtOH to a

final concentration of approximately 0.06 mM. The final absorbance of the DPPH working solution was adjusted to 0.3 ± 0.01 absorption units at 550 nm by addition of DPPH stock.

2.2.1.4 Preparation of plasma and saliva samples

Samples were removed from storage at -80˚C and thawed in water at room temperature just before use. The samples were centrifuged at 12 000 g for 30 s to remove any insoluble particles that formed during storage. The clear supernatant that was left was used for the assay.

2.2.1.5 Preparation of dilution series

For each sample, a dilution series was prepared in microtubes as shown in Table 2.1. HEPES buffer was used for the dilution of samples.

Table 2.1. The dilution series of samples used for the DPPH Assay.

Solution	**Volume (µl)**									
HEPES buffer	200	180	160	140	120	100	80	60	40	20
Plasma/saliva sample	0	20	40	60	80	100	120	140	160	180
DPPH working solution	800	800	800	800	800	800	800	800	800	800
Total volume	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

2.2.1.6 Preparation of sample dilution series

The presence of antioxidant in a sample leads to a decrease in the absorbance of DPPH at 550 nm. The absorbance value of the first sample in the dilution series (i.e. containing no plasma or saliva) was set as 100% and the absorbance of the rest of the samples were expressed in terms of the 100% absorbance. In this way a dilution curve was constructed from which an EC_{50} value (the amount of sample corresponding to a 50% decrease in

absorbance of DPPH at 550 nm) was read off and used as indication of the amount of antioxidant present in the sample.

2.2.1.7 Construction of a vitamin C standard curve

A vitamin C standard curve was constructed with each DPPH experiment. A 0.4 mM vitamin C working solution was prepared from a freshly prepared 20 mM stock solution. Water (dddH_2O) was used for preparation of both solutions. From the working solution a dilution series of 0 - 0.1 mM vitamin C was prepared and the DPPH Assay was carried out together with the plasma or saliva samples as described using the same DPPH working solution as for these samples.

2.2.1.8 Assay procedure

Each sample's dilution series was incubated in the dark for 30 min, vortexed and transferred to flat-bottomed 96-well mirotiter plates. Each microtube's sample was transferred to the plate using 200 µl per microtiter plate well. The samples were incubated for a further 30 min in the dark and the absorbance was read off at 550 nm using a microtiter plate reader. The vitamin C standards used for construction of the vitamin C standard curve were prepared in a similar way.

2.2.1.9 Absorbance spectra for the reaction between DPPH and vitamin C

In the presence of an antioxidant, DPPH in its oxidized, purple form can accept a reducing equivalent and be reduced to its colorless form. This color change of DPPH in the presence of an antioxidant forms the basis of the DPPH Assay. One molecule of DPPH can accept one reducing equivalent, as shown in Figure 2.2.

Vitamin C is a well known antioxidant present in various fruits and vegetables. It also plays an integral part in the *in vivo* antioxidant system of the body as has been briefly discussed in Chapter 1. For these reasons vitamin C is ideal

to use as an antioxidant standard for the DPPH Assay. During the complete oxidation of ascorbic acid to dehydroascorbic acid, two reducing equivalents are released (Figure 2.3).

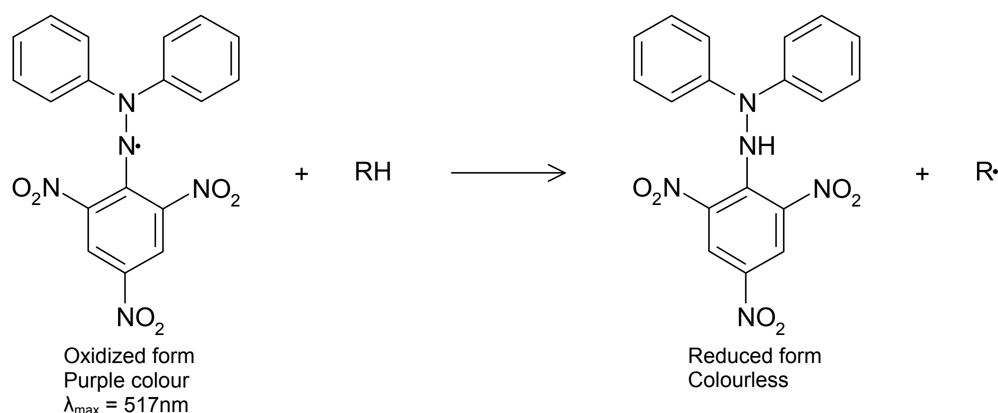


Figure 2.2. The reduction of DPPH in the presence of a reducing equivalent.

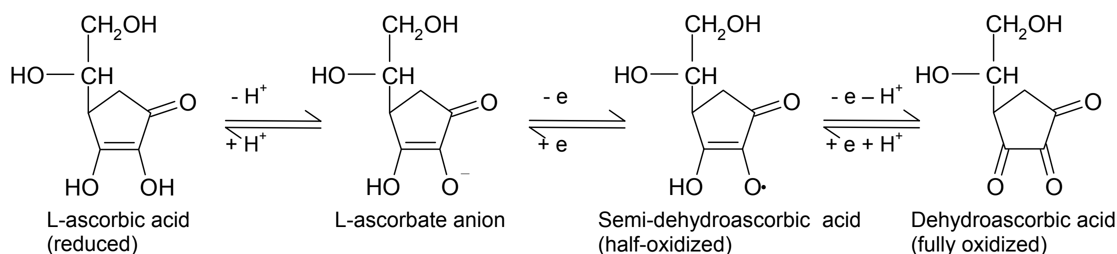


Figure 2.3. The stepwise oxidation of ascorbic acid to dehydroascorbic acid with the release of two electron donors (Adapted from Washko *et al*, 1992).

Based on the fact that one molecule of DPPH can accept one reducing equivalent and that one molecule of vitamin C can release two reducing equivalents, it is expected that DPPH reacts with vitamin C in a molar ratio of 2:1.

To confirm this, a series of titrations of DPPH with vitamin C in different molar ratios were carried out. A DPPH working solution of 0.23 mM in 40% EtOH was prepared together with a set of solutions containing the following concentrations of vitamin C: 0, 0.575, 0.115 and 0.23 mM. Each of these solutions was incubated with an equal volume of DPPH corresponding to

DPPH: vitamin C molar ratios of 1:0, 1:0.25, 1:0.5 and 1:1 respectively. After 1 hour of incubation in the dark at room temperature, the reaction samples were diluted 1:1 with dddH_2O and absorbance spectra were obtained.

2.3 Subject selection

Written, informed consent was obtained from six volunteers that were recruited from the Department of Biochemistry to participate in this study.

The volunteers were healthy and had not taken any vitamin supplements or antibiotics for at least one month before the study. They were requested not to engage in strenuous physical activity one day prior to the study. Cigarette smoke has been shown to cause a decrease in the antioxidant capacity of saliva (Nagler *et al*, 2000). Therefore smoking has been used as an exclusion criterion during subject selection for this and subsequent studies.

2.4 Experimental protocol

The exercise study took place at the Assessment Unit of the High Performance Centre at the L.C. de Villiers Sports Grounds of the University of Pretoria during June 2002.

The exercise protocol consisted of an incremental running exercise on a treadmill (Schiller STM-5S) under the supervision of a biokineticist. Volunteers were requested to continue exercising until they were exhausted and felt unable to continue. The total duration of the exercise protocol, depending on the level of fitness of the volunteer, was 5-10 min.

During the exercise protocol, heart rate, lactate concentrations and rate of perceived exertion (RPE) were monitored. Heart rates were recorded continuously during the exercise protocol using a heart rate monitor (Polar Accurex Plus). Blood lactate measurements were taken before and directly

after exercise using a lactate meter (Accurex BM lactate meter; Roche Diagnostics). This required a puncture of the fingertip to obtain a peripheral blood sample. The blood lactate values were reported in nmoles of lactate per µl of blood (i.e. mM). Each volunteer assigned a value from the Borg scale as an indication of his RPE at the end of the exercise protocol.

The body mass and length of the volunteers were measured before the exercise and the body mass indexes (BMI) were calculated from these values.

Blood and saliva samples were collected before and immediately after exercise and processed and analyzed as described in the previous section.

2.5 Statistical analysis

Results are expressed as means ± SEM.

DPPH values before and after exercise were compared using a paired, one-tailed Student's t-test. A p-value < 0.05 was considered statistically significant.

3 RESULTS

Typical absorbance graphs following the DPPH Assay on a set of plasma samples and a vitamin C standard curve are shown in Figures 2.4(i) and 2.4(ii), respectively. The absorbance graphs of the plasma shows graphs of blood samples obtained from the same volunteer before (blue line) and after (purple line) exercise. For the First Exercise Study, trend lines were fitted for the absorbance graphs using MS Excel. The equations of the trend lines were used to obtain the EC_{50} values of the samples. In this way, EC_{50} values could be obtained for samples whose dilution series did not cross the EC_{50} line. The EC_{50} values in Figure 2.4(i) were calculated as 173 µl/ml (blue line) and 197 µl/ml (purple line) of reaction mixture. It should be kept in mind that

these EC_{50} values indicate diluted samples (plasma samples were diluted 1:4 and saliva samples 1:1 with HEPES buffer before the assay) and a dilution factor should be taken into account when calculating the amount of antioxidants in terms of vitamin C equivalents.

The points of the vitamin C standard curve were chosen as such to ensure that it crosses the EC_{50} line. The EC_{50} value was determined graphically as shown in Figure 2.4(ii), where the EC_{50} is read off as 2.48 µg/ml reaction mixture.

A vitamin C standard curve was constructed under the same experimental conditions for each set (before and after exercise) of plasma (or saliva) samples of a volunteer. Since the experimental conditions are the same, the EC_{50} values of the set of samples and the vitamin C standard curve indicate the same antioxidant content. Based on this principle the antioxidant content present in a sample can be calculated and expressed in terms of vitamin C equivalents in the following way using the values obtained for Figures 2.4(i) and 2.4(ii) as example:

At EC_{50}

Antioxidant content of sample ≡ Antioxidant content of vitamin C

Therefore, the antioxidant content of 173 µl diluted plasma sample (blue line) ≡ the antioxidant content of 2.48 µg of vitamin C.

Plasma was diluted 1:4, therefore
the antioxidant content of 34.6 µl (173/5 = 34.6) undiluted plasma sample ≡ the antioxidant content of 2.48 µg of vitamin C.

Therefore, the antioxidant content of 1 µl undiluted plasma sample ≡ the antioxidant content of 0.072 µg (2.48/34.6) of vitamin C.

In other words,

The sample contains 0.072 µg Vitamin C Equivalents (VCE) / µl, which is the same as 72 µg VCE/ml.

Similarly, the antioxidant content of the plasma sample indicated by the purple line (EC_{50} = 197 µl/ml) is calculated to be 63 µg VCE/ml.

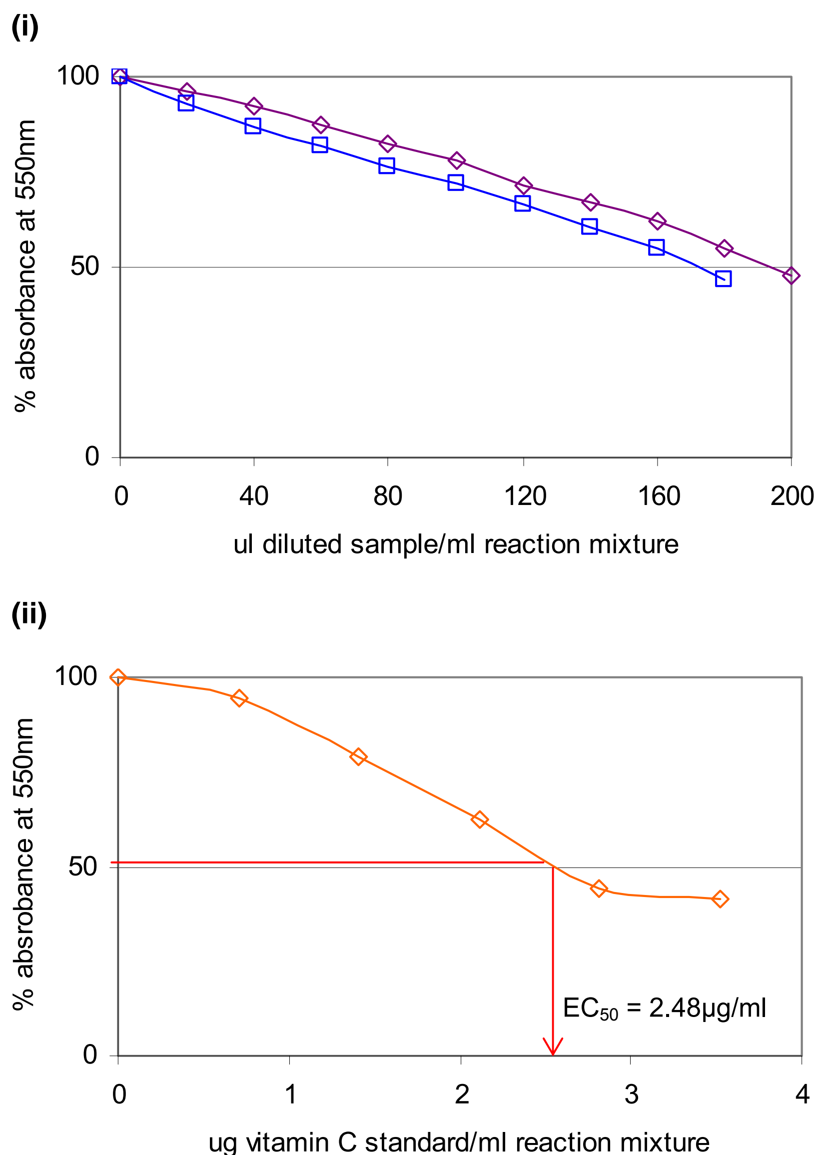


Figure 2.4. Absorbance graphs for (i) a set of plasma samples (plasma collected before exercise: blue line; plasma collected after exercise: purple line) and (ii) a vitamin C standard.

Based on the stochiometry of DPPH and vitamin C, it is expected that DPPH reacts with vitamin C in a molar ratio of 2:1. In the titration experiments to

confirm this, the reaction sample containing the DPPH: vitamin C ratio of 1:0 was used as reference sample. Figure 2.5 shows the absobance spectra that were obtained for the reaction between vitamin C and DPPH. In Figure 2.6 the absorbance values of the other ratios are expressed relative to the absorbance value of this sample at 520 nm.

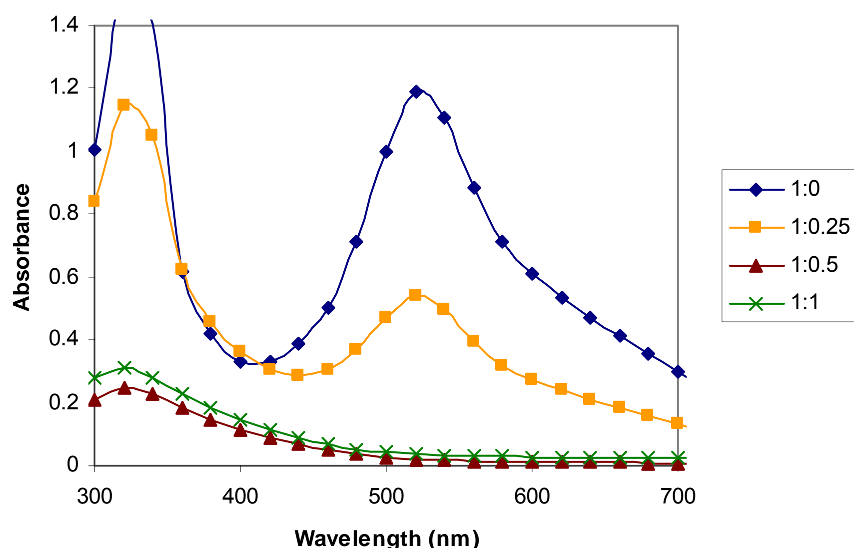


Figure 2.5. The absorbance spectra of DPPH titrated with vitamin C. *The molar ratio of DPPH to vitamin C is indicted in the legend.*

According to the reaction mechanisms of DPPH and vitamin C (Figures 2.2 and 2.3) it is expected that 0.5 moles of vitamin C should fully reduce 1 mole of DPPH, i.e. that a sample containing a molar ratio of DPPH: vitamin C of 1:0.5 should cause a 100% decrease in absorbance at the absorbance maximum of DPPH. Furthermore, it is expected that a molar ratio of DPPH: vitamin C of 1:0.25 should cause a 50% decrease in absorbance at the absorbance maximum of DPPH.

As indicated in Figure 2.6 the 1:0.5 ratio caused a 98% decrease in absorbance and the 1:0.25 ratio caused a 55% decrease in absorbance at 520 nm. This correlates well with the expected decreases.

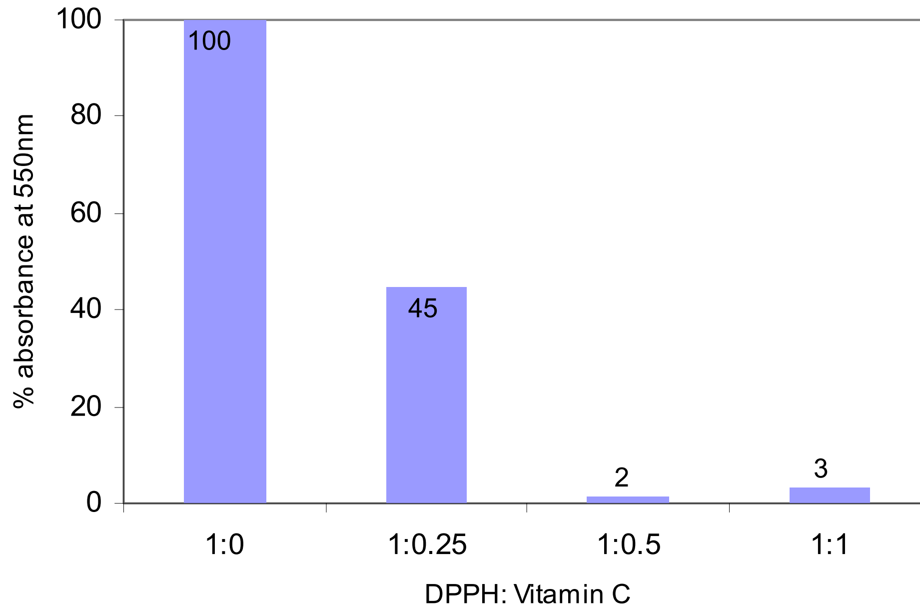
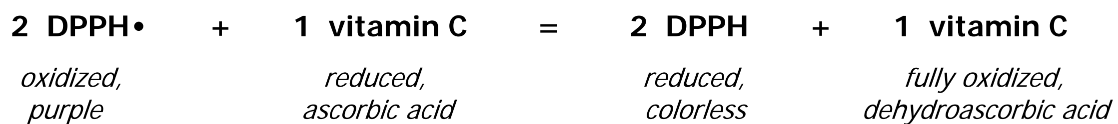


Figure 2.6. Decrease in absorbance of DPPH at increasing amounts of vitamin C.

These results confirm that DPPH reacts in a molar ratio of 1:0.5 with vitamin C, as is expected by studying the stochiometry of their reaction mechanisms:



The percentage change (% increase or decrease) of parameters following exercise was calculated according to the following equation:

$$\text{Percentage change} = \frac{(\text{value after exercise}) - (\text{value before exercise})}{(\text{value before exercise})} \times 100$$

The physical characteristics of the volunteers are shown in Table 2.2 and the heart rates, blood lactate concentrations and RPE scores are shown in Table 2.3.

Table 2.2. Physical characteristics of volunteers (*).

	Males	Females	Combined
N	2	4	6
Age (y)	36.5 ± 7.2	29.3 ± 5.2	31.7 ± 5.4
Height (cm)	173 ± 0.6	168 ± 1.8	169.5 ± 1.8
Body mass (kg)	73.5 ± 2.0	68.5 ± 6.1	70.2 ± 4.9
BMI (kg/m^2)	24.5 ± 0.5	24.2 ± 1.7	24.3 ± 1.3

** Results are expressed as averages ± SEM.*

Table 2.3. Heart rates, blood lactate concentrations and RPE ratings of volunteers.

	M ± SEM
Heart rate before exercise (bpm)	82 ± 1.2
Heart rate after exercise (bpm)	185 ± 1.7
Increase in heart rate (%)	126
Blood lactate concentration before exercise (mM)	2.2 ± 0.25
Blood lactate concentration after exercise (mM)	7.7 ± 0.55
Borg's RPE rating (out of 20)	16 ± 0.63

Figure 2.7 shows the results of the DPPH Assay on the plasma and saliva samples.

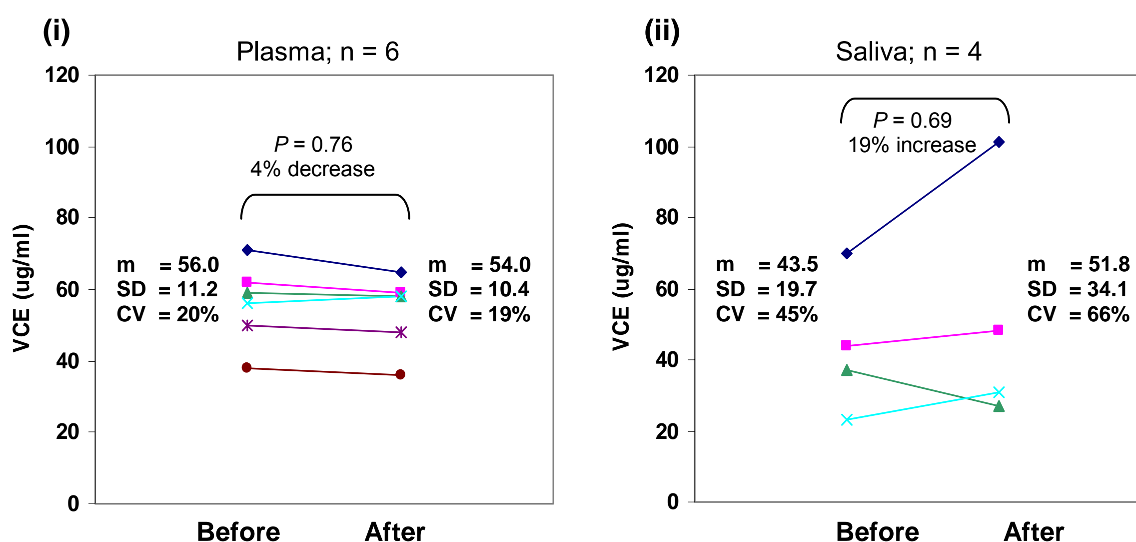


Figure 2.7. Changes in plasma (i) and salivary (ii) antioxidant levels following exhaustive exercises as determined by the DPPH Assay.

Plasma samples were analyzed in triplicate. Limited quantities of saliva were obtained and therefore saliva samples were analyzed only once or in duplicate. There were only sufficient saliva sample volumes available for analysis of 4 of the 6 volunteers' saliva samples for antioxidant activity.

4 DISCUSSION

The exercise protocol used for this exploratory study can be considered as an exhaustive exercise protocol, since the volunteers were requested to keep on running until they felt too exhausted to continue. The average Borg scale reading given by the volunteers directly after the exercise was 16 out of 20, which corresponds with the description "very hard" (See Appendix F for Borg scale).

The blood lactate levels increased from an average of 2.2 mM before exercise to an average of 7.7 mM at the end of the exercise protocol. This high post-exercise lactate level, which is much higher than 4 mM, indicates that the aerobic-anaerobic threshold has been exceeded (see Figure 2.1). This confirms that volunteers had to rely on anaerobic metabolism to meet energy requirements at the end of the exercise. Both the Borg scale readings and the increase in blood lactic acid levels indicate that the intensity of this exercise protocol was strenuous.

When comparing the baseline antioxidant levels ("Before") of the plasma and the saliva samples of the volunteers (see Figure 2.7), it can be seen that the saliva's average antioxidant capacity (43.5 µg/ml VCE) is approximately 75% of the plasma's average antioxidant capacity (56.0 µg/ml VCE). The values of the resting salivary antioxidant capacity relative to the resting plasma antioxidant capacity are in good agreement with the results obtained by Atsumi *et al*, 1999. He found that under normal (resting) conditions the salivary antioxidant capacity are about 70% that of the plasma antioxidant capacity.

Following the exhaustive exercising, it was expected that a decrease in antioxidant capacity in the plasma and saliva samples will be observed. Although a slight decrease (4%) in the antioxidant capacity of the plasma samples were observed, it was not statistically significant ($p = 0.76$). The saliva samples showed an unexpected increase in antioxidant capacity of 19%. This was also not statistically significant ($p = 0.69$). A possible explanation for this increase in salivary antioxidant levels might be that, during exercise, the volunteers' mouths dried and therefore the antioxidants present in the saliva became more concentrated per volume of saliva.

The antioxidant capacity of saliva showed a much bigger inter-individual variation than that of the plasma during rest, i.e. before exercise (the coefficient of variation was 45% for the saliva before exercise compared to a coefficient of variation of 20% for the plasma). This is probably because the oral environment is less buffered and less protected by antioxidant molecules than blood. The salivary free radical composition can also be affected by factors such as oral infections and food that is ingested which does not affect the blood antioxidant status.

5 CONCLUSION

Although a very strenuous exercise protocol was used for this study, it did not cause a significant decrease in the antioxidant capacity of either the plasma or saliva of the volunteers as was expected. This indicates a next attempt to establish an exercise protocol able to significantly and consistently induce oxidative stress in volunteers. In order to achieve this, a larger number of volunteers, smaller standard deviation and a larger difference between results before and after exercise were required. These shortcomings were addressed in the Second Exercise Study which will be discussed in Chapter 3.

CHAPTER 3

THE SECOND EXERCISE STUDY

THE USE OF STRENUOUS TREADMILL RUNNING BASED ON MAXIMAL OXYGEN UPTAKE AS EXERCISE PROTOCOL

1 INTRODUCTION

The purpose of the exercise studies, of which this is the Second Exercise Study, has been explained in detail in the Introduction of Chapter 2.

Based on the outcome of the First Exercise Study, which has been described in Chapter 2, it was decided to make some modifications on the experimental setup and exercise protocol when employing the Second Exercise Study.

For the Second Exercise Study the number of volunteers was increased from six to ten. This was done in order to increase the probability that the results obtained are representative of the general population.

Body fat percentage and lean body mass (LBM) were measured in addition to the physical characteristics measured during the First Exercise Study. This was also reported to describe the volunteer population that was used in this study more thoroughly.

Instead of using an exercise protocol where volunteers simply perform a high intensity exercise protocol until feeling exhausted as in the First Exercise Study, an exercise protocol standardized according to each volunteer's own for VO_{2max} values was used for the Second Exercise Study. The average duration of the exercise protocol used in the First Exercise Study was

relatively short (5-10 min), due to the low fitness levels of the volunteers and the high intensity of the treadmill running. It has been suggested in literature that a relatively high exercise intensity that is maintained for a sufficiently long duration is most likely to induce oxidative stress (Goldfarb, 1992, Quindry *et al*, 2003). Therefore, although the exercise intensity of the First Exercise Study might have been high enough, the duration might have been too short to elicit a measurable oxidative stress response.

For the Second Exercise Study, the same sport physiology parameters were determined for the volunteers as in the First Exercise Study (heart rate before and after exercise, lactate levels following exercise and RPE ratings). In addition to these variables the following two parameters were also measured:

- Maximal oxygen uptake (VO_{2max})
- Respiratory exchange ratio (RER)

Maximal oxygen uptake (VO_{2max}): VO_{2max} functionally represents the maximal amount of oxygen that can be removed from circulating blood and used by the working tissue during a specific period. Put in other words, it is the maximal rate at which oxygen can be consumed per minute during exercise of increasing intensity until exhaustion. The type of exercise should involve large-scale muscle group activity as is used for treadmill running. VO_{2max} is also referred to as maximal work rate.

Regular exercise training adapts the body's ability to remove oxygen from circulating blood by working tissue, i.e. it increases the VO_{2max} value. This makes VO_{2max} a good general measure to indicate fitness levels. Each volunteer's VO_{2max} level depends on his level of fitness and is therefore unique.

The VO_{2max} parameter has great value in exercise studies since it can be used to standardize an exercise protocol according to each volunteer's own level of fitness. Each volunteer's VO_{2max} can be determined during an exhaustive

exercise protocol. Once VO_{2max} is determined, oxygen uptake can be measured during subsequent protocols and exercise intensity can be adjusted to match a certain percentage of the VO_{2max} for a specific duration (e.g. at 80% of VO_{2max} for 10 min). Therefore, by using a protocol described in terms of VO_{2max}, results are not confounded by the fitness of the individual.

During the Second Exercise Study we attempted to employ an exercise protocol of sufficient intensity and duration to have a measurable effect on the oxidative stress levels of the volunteers. Using an exercise protocol based on the volunteer's VO_{2max} value is ideal for this purpose.

- It gives a good description of the volunteer's fitness level.
- It describes the exercise protocol accurately.
- The exercise protocol is standardized to the fitness level of each volunteer.

Respiratory exchange ratio (RER): The RER gives an indication of work intensity and is defined as the ratio of the rate of CO_2 exhaled to the rate of O_2 consumed. A RER value exceeding 1.15 is considered an indication that VO_{2max} has been reached during exercising (Ashton *et al*, 1998).

2 MATERIALS AND METHODS

In addition to the DPPH Assay, the TBARS (Thiobarbituric Acid Reactive Substances) Assay was also carried out in the Second Exercise Study. From the results of these assays, a combined oxidative stress parameter, the Oxidative Stress Ratio was calculated as described on p 57.

2.1 Sample collection and processing

Plasma samples: Blood samples to be used for the DPPH Assay were collected and processed as for the First Exercise Study (See Materials and Methods in Chapter 2 for description). For the TBARS Assay, blood was

collected in a similar way, but using heparinized vacutainer tubes instead of EDTA-containing tubes. Plasma samples were obtained from the heparinized blood samples for the TBARS Assay as described for the DPPH Assay. The plasma samples to be used for the TBARS Assay were not processed before storing as for the DPPH Assay but were stored directly at -80°C.

Saliva samples: Saliva samples to be used for the DPPH Assay in the Second Exercise Study were collected as for the First Exercise Study. (See Materials and Methods in Chapter 2 for description).

2.2 Biochemical analyses

2.2.1 DPPH Assay

The DPPH Assay was carried out as for the First Exercise Study as described under Materials and Methods in Chapter 2.

2.2.2 TBARS Assay

The method as described by Jentzsch *et al* (1996) was used for the TBARS Assay.

2.2.2.1 Materials

Heparinized vacutainer tubes were from from BD Vacutainer (Becton, Dickinson and Co., UK). Tetra-ethoxypropane (TEP), 2,6-di-tert-butyl-4-methylphenol or butylated hydroxytoluene (BHT), sodium hydroxide (NaOH) and HCl were from Sigma-Aldrich (St. Louis, MO, USA). NaCl and n-butanol were from Merck (Merck NT Laboratory Supplies, Jhb, SA). Trichloro acetic acid (TCA) and EtOH were from Saarchem (Merck NT Laboratory Supplies, Jhb, SA) and 2-thiobarbituric acid (TBA) was from Fluka. All the salts and reagents were of analytical grade. For making up buffers, dddH_2O (Millipore, Microsep, SA) was used.

2.2.2.2 Assay principle

The TBARS Assay is the most frequently used method for measuring lipid peroxidation. It is a relatively simple and affordable method that can be applied to crude biological systems such as plasma. During lipid peroxidation small amounts of MDA are produced that can react with TBA to generate a pink-colored product that absorbs light at 532 nm in acid solution by the reaction shown in Figure 3.1.

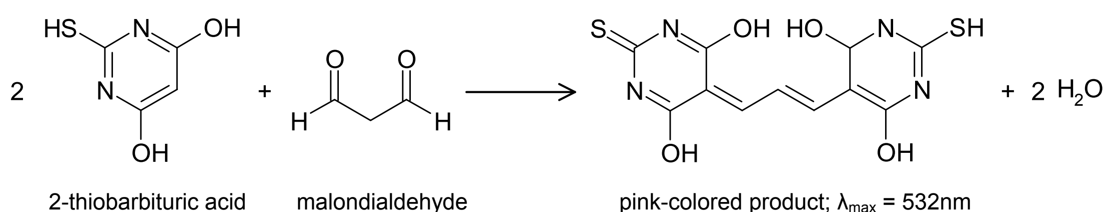


Figure 3.1. The reaction between thiobarbituric acid and malondialdehyde.

2.2.2.3 Hydrolysis of TEP to MDA

MDA is unstable and must therefore be freshly prepared. This is achieved by hydrolyzing one of its acetal derivatives just before use. We used 1,1,3,3-tetraethoxypropane (TEP) in the presence of hydrochloric acid as catalyst. The conversion of TEP to MDA in the presence of an acid catalyst is shown in Figure 3.2.

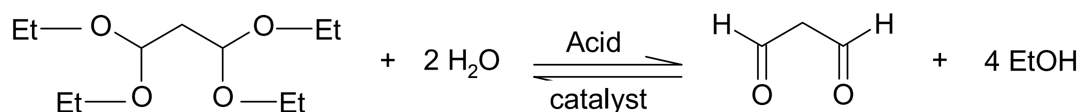


Figure 3.2. The acid hydrolysis of TEP to yield MDA.

A stock solution of 3.27 mM TEP in 0.1 N HCl was freshly prepared and left to stand in the dark for 30 min. During the acid hydrolysis reaction, TEP is fully converted to MDA. The MDA stock solution was then diluted 100 times with water to a final concentration of 32.7 µM. This solution was used as the standard with the highest concentration in the serial dilution for the construction of a standard curve.

2.2.2.4 Construction of an MDA standard curve

The 32.7 µM MDA standard prepared from the TEP stock solution was serially diluted (1:1) with $dddH_2O$ to obtain a dilution range of 32.7, 16.3, 8.18, 4.09, 2.04, 1.02 and 0.511 µM MDA. Water ($dddH_2O$) served as a blank.

2.2.2.5 Preparation of BHT

A stock solution of 50 mM BHT in 96% EtOH was prepared and diluted 1:9 with 96% EtOH to yield a 5 mM BHT working solution.

2.2.2.6 Preparation of TBA

A 1.6% TBA solution was prepared by dissolving 800 mg TBA in 50 ml 0.1 M NaOH. In order to improve the poor solubility of TBA in the NaOH, the solution was stirred with a magnetic stirrer. The solution was filtered before use in the assay to remove any insoluble TBA particles.

2.2.2.7 Preparation of plasma samples

Plasma samples were thawed in water at room temperature just before use. The samples were centrifuged at 12 000 g for 30 s to remove any insoluble matter. The clear supernatant was used for the assay.

2.2.2.8 Assay procedure

Twenty-five µl of the 5 mM BHT working solution was added to 200 µl of plasma sample and mixed thoroughly by vortexing to prevent formation of MDA during sample preparation. Two hundred µl of a 10% TCA solution was added and after proper vortexing for 1 min, 25 µl TBA was added. The samples were incubated in a water bath at 95°C for 1 hour and then brought to room temperature by putting them on ice. A phase separation was carried out by adding 500 µl n-butanol and 50 µl saturated NaCl, vortexing the sample for 2 min followed by centrifugation at 12 000 g for 1 min. The organic upper phase was removed and the absorbance was read at 535 nm and 572 nm on a spectrophotometer. Butanol was used as reference sample and the spectrophotometer was auto zeroed against the blank (0 µM MDA)

sample. The difference between the absorbance at 572 nm and the absorbance at 535 nm ($A_{535} - A_{572}$) was used as measure of the presence of lipid peroxides. The MDA standard curve was used to express the amount of lipid peroxides present in the sample in terms of MDA Equivalents (MDE). The unit for MDE is mM.

2.2.3 The Oxidative Stress Ratio

The TBARS Assay was optimized after the First Exercise Study to be used for the Second and Third Exercise Studies. Using a marker of oxidative stress (like the TBARS Assay that is used as an indicator of lipid peroxidation) together with a marker of antioxidant capacity (like the DPPH Assay) gives a better overall picture of oxidative stress status. It has been shown to be even more advantageous to combine an oxidative stress parameter with an antioxidant capacity parameter to create a new parameter, a so-called "Oxidative Stress Ratio" (Alessio *et al*, 1997). In this way, changes that took place in either oxidative stress or antioxidant capacity that might otherwise have gone unnoticed or be regarded as insignificant are amplified.

2.3 Subject selection

Students from the Department of Biochemistry were approached as possible volunteers for this study. Students interested in participating in this study received an information letter describing the nature of the study (see Appendix A). Written, informed consent was obtained from the selected volunteers (see Appendix B). For this study, the body fat percentage, lean body mass and VO_{2max} were determined in addition to the standard anthropometrical data described in the First Exercise Study.

2.4 Experimental protocol

This exercise study took place at the Institute for Sport Research at the L.C. de Villiers Sports Grounds of the University of Pretoria during November 2002.

For this study, heart rate, blood lactate values, VO_{2max} values and RPE ratings were used as indicators of fitness and exercise intensity of the volunteers. An example of a volunteer performing the treadmill running is shown in Figure 3.3. The exercise protocol was standardized for each volunteer based on his fitness level as indicated by his VO_{2max} determination. Heart rate and blood lactate values as well as RPE ratings were obtained as for the First Exercise Study.

The protocol involved two visits, three or four days apart, for each volunteer to the Institute for Sport Research.

On the first visit the VO_{2max} of each volunteer was determined while running on a motorized treadmill (Quintin model 24-72). The following treadmill protocol was followed: The protocol started at a running speed of 8 km/h. The speed was increased every two minutes by 2 km/h until the volunteer reached exhaustion.

The gas exchange of the volunteers was monitored during the exercising using a gas analyzer (Schiller CS-100). The gas exchange data was used to estimate the VO_{2max}. The following criteria were utilized to determine whether a subject has reached their VO_{2max} (Ashton *et al*, 1998):

- a RER exceeding 1.15,
- plateau or decrease in oxygen uptake,
- heart rate approximating the age-dependant maximal heart rate, and
- not being able to maintain running speed despite verbal encouragement.

The VO_{2max} parameter is a direct indication of the volunteer's fitness level and was used on the second visit to standardize each volunteer's exercise protocol with his own fitness level as follows: The volunteers performed a similar treadmill running exercise coupled to the gas analyzer, following a protocol based on the VO_{2max} values obtained during the first visit. This protocol consisted of 5 min of treadmill running at 65% of the volunteer's VO_{2max} directly followed by 5 min of treadmill running at 75% VO_{2max} and finally 5 min of running at 85% of the VO_{2max}.

Blood and saliva samples were obtained directly before and after performing the protocol by a qualified nurse. The samples were processed and stored as described previously. The DPPH Assay was carried out on the EDTA-plasma and saliva samples and the TBARS Assay was carried out on the heparinized plasma samples as described earlier. The Oxidative Stress Ratio was calculated for volunteers based on their DPPH and TBARS results.

Heart rate, lactate concentrations and RPE values were obtained during both exercise protocols. In addition, gas samples were sampled every 10 s of treadmill running during this study. Oxygen consumption (VO_2) and RER were determined. The VO_2 readings and RER values together with heart rate measurements obtained during the first visit were used to determine the VO_{2max} value for each volunteer.

2.5 Statistical analyses

Statistical analyses were carried out as for the First Exercise Study using MS Excel. The DPPH Assay, TBARS Assay and Oxidative Stress Ratio results were analyzed using the paired Student's t-test as described for the DPPH Assay in the First Exercise Study.

3 RESULTS

The physical characteristics of the volunteers are summarized in Table 3.1.

Table 3.1. Physical characteristics of volunteers (*).

	Males	Females	Combined
N	4	6	10
Age (y)	31 ± 6.1	23 ± 0.9	26.4 ± 2.6
Height (cm)	178 ± 3.6	170 ± 2.2	173 ± 2.4
Body mass (kg)	78.1 ± 4.7	74.5 ± 6.2	73.8 ± 4.3
BMI (kg/m^2)	24.4 ± 0.9	25.7 ± 1.8	24.6 ± 1.1
Body fat (%)	20.6 ± 0.7	34.4 ± 3.3	27.9 ± 2.7
LBM (kg)	61.8 ± 4.1	47.9 ± 2.2	52.8 ± 3.3

** Results are expressed as averages ± SEM.*

Figure 3.3 shows a volunteer performing strenuous treadmill running.

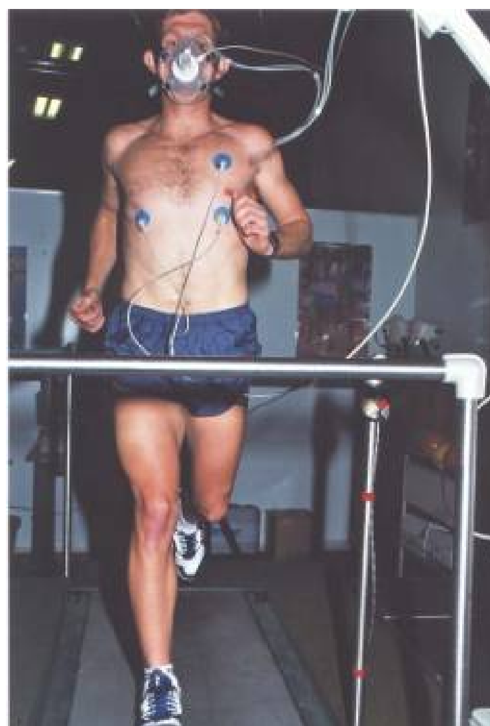


Figure 3.3. A volunteer performing strenuous treadmill exercising while attached to a gas analyzer and heart rate monitor.

The biokinetical data of the volunteers that participated in this study is shown in Table 3.2.

Table 3.2. Biokinetical data of volunteers (*).

	Males	Females	Combined
N	4	6	10
Heart rate before exercise (bpm)	86.3 ± 8.7	87.0 ± 3.6	86.7 ± 3.8
Heart rate after exercise (bpm)	188.5 ± 4.8	209.0 ± 15.6	200.8 ± 9.8
Increase in heart rate (%)	119	140	132
Blood lactate concentration after exercise (mM)	7.0 ± 1.0	6.4 ± 1.1	6.6 ± 0.7
Borg's RPE rating (out of 20)	16.3 ± 0.5	15.8 ± 0.7	16.0 ± 0.5
RER (dimensionless)	1.15 ± 0.02	1.16 ± 0.02	1.16 ± 0.01
VO_{2max} (ml/kg/min)	40.5 ± 3.9	32.7 ± 1.8	36.3 ± 2.1

** All data reported refer to the second visit, except for VO_{2max} values. Results are expressed as mean ± SEM.*

The heart rate and oxygen uptake of the volunteers during the first visit, when maximum oxygen uptake was determined, are shown in Figures 3.4 and 3.5, respectively.

The change in heart rate during the exercise protocol is shown in Figure 3.6, and the change in work rate (i.e. oxygen uptake) during the exercise protocol on the second visit is shown in Figure 3.7. The relationship between the change in heart rate and the change in oxygen uptake is shown in Figure 3.8.

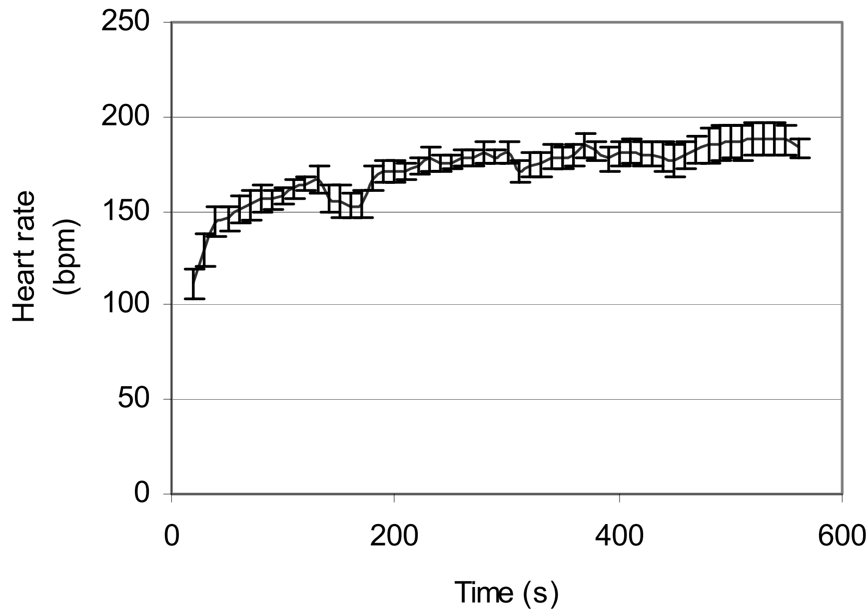


Figure 3.4. Increase in heart rate during the exercise protocol for maximum oxygen uptake determination. *The average of the heart rates of the ten volunteers is shown. The error bars indicate the standard error of the mean. Measurements were taken after a warm up session; therefore the starting heart rates are relatively high.*

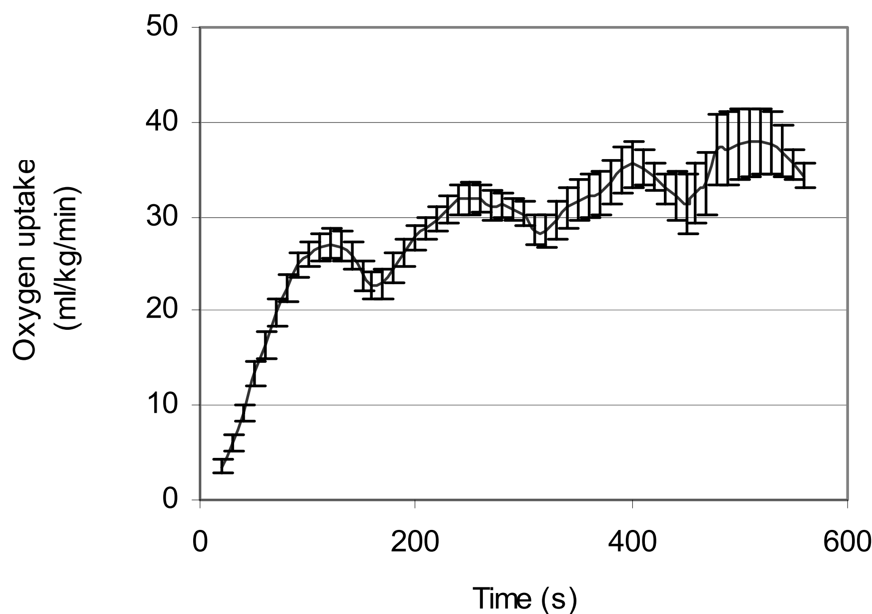


Figure 3.5. Increase in oxygen uptake during the exercise protocol for maximum oxygen uptake determination. *The average oxygen uptake of the ten volunteers is shown; the error bars indicate SEM. The exercise protocol was interrupted at three points to take finger pricks for lactate measurements, therefore the three dips in the curve.*

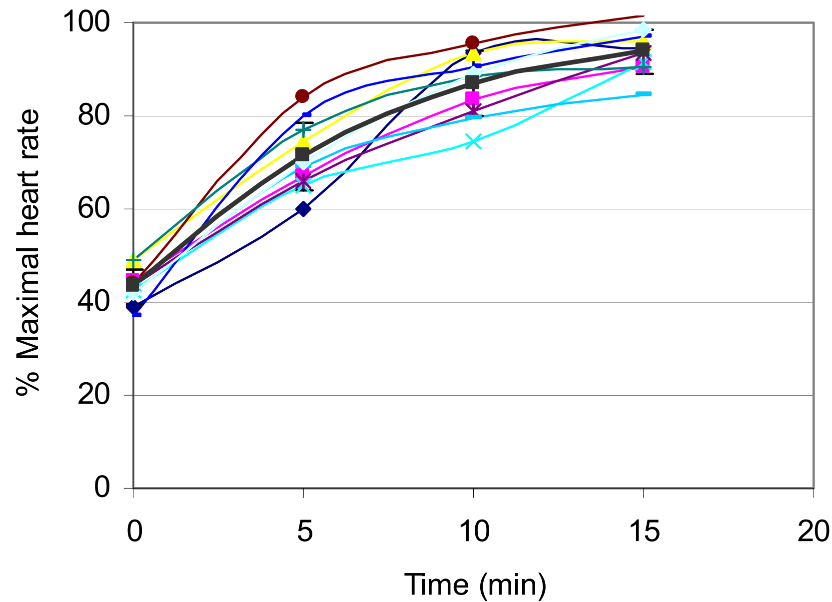


Figure 3.6. Increase in the heart rates of the ten volunteers as exercise progressed. *Heart rates are expressed relative to the age-predicted maximal heart rate. The average of the heart rates of the volunteers is indicated by the black line.*

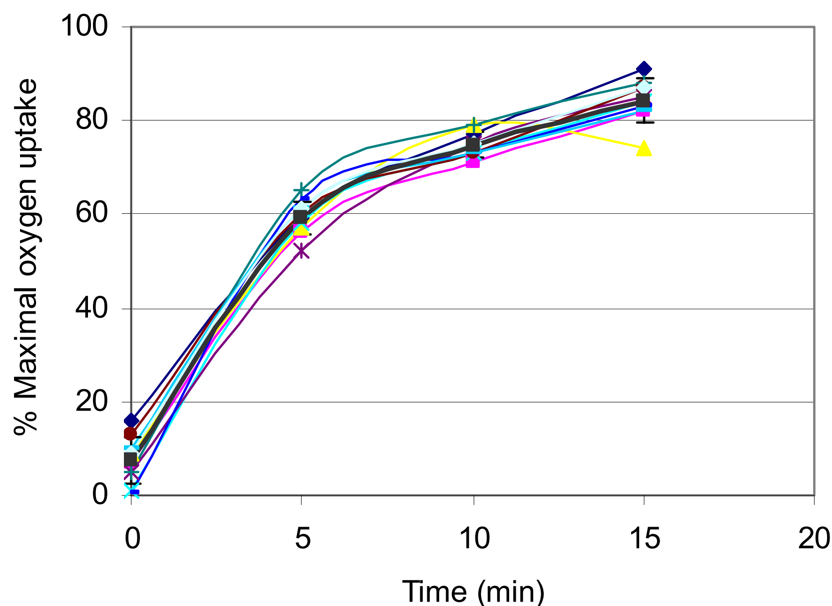


Figure 3.7. Increase in oxygen uptake of the ten volunteers during the exercise protocol. *Each volunteer's VO_{2max} was determined during a previous visit and the exercise protocol set up accordingly. The average oxygen uptake of the ten volunteers is shown with a black line.*

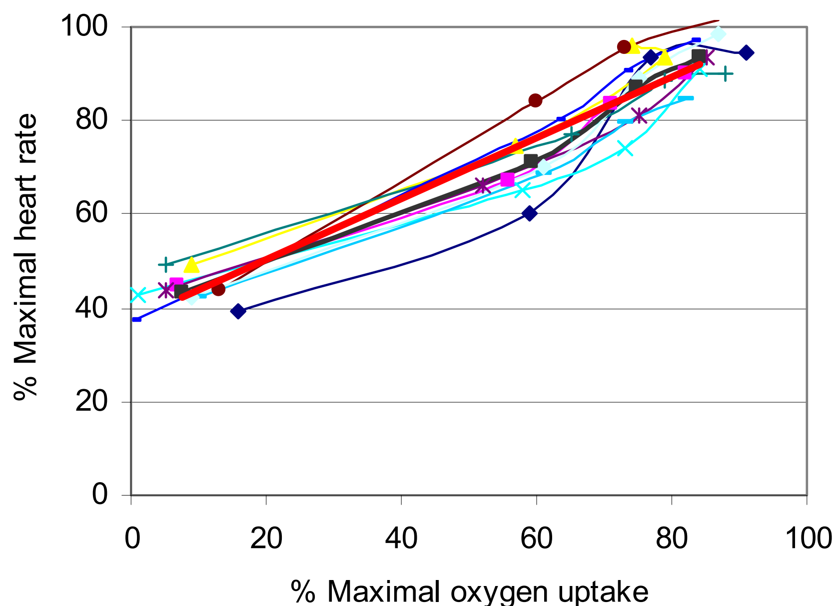


Figure 3.8. The relationship between the maximal heart rate and maximal oxygen uptake of the volunteers during the exercise protocol on the second visit. *The linear trend line is shown in red. The r-value for the straight line is 0.99.*

In addition to the DPPH Assay that was performed on plasma and saliva as described in the First Exercise Study (see results Figure 3.9) the TBARS Assay was performed on plasma as well. The results are shown in Figure 3.10(i). The results obtained from the plasma DPPH and TBARS Assays were combined in the Oxidative Stress Ratio (see results Figure 3.10(ii)) as explained under Section 2.2.3.

The Oxidative Stress Ratio is defined as follows:

$$\text{Oxidative Stress Ratio} = \frac{\text{Lipid peroxidation (MDE in mM)} \times 100}{\text{Total antioxidant capacity (VCE in µg/ml)}}$$

The levels of lipid peroxidation are expected to increase (an increase in the numerator value of the equation) and the total antioxidant capacity is expected to decrease after exercise (a decrease in the denominator value of the equation). Therefore it is expected that the Oxidative Stress Ratio will have an overall increase after exercise.

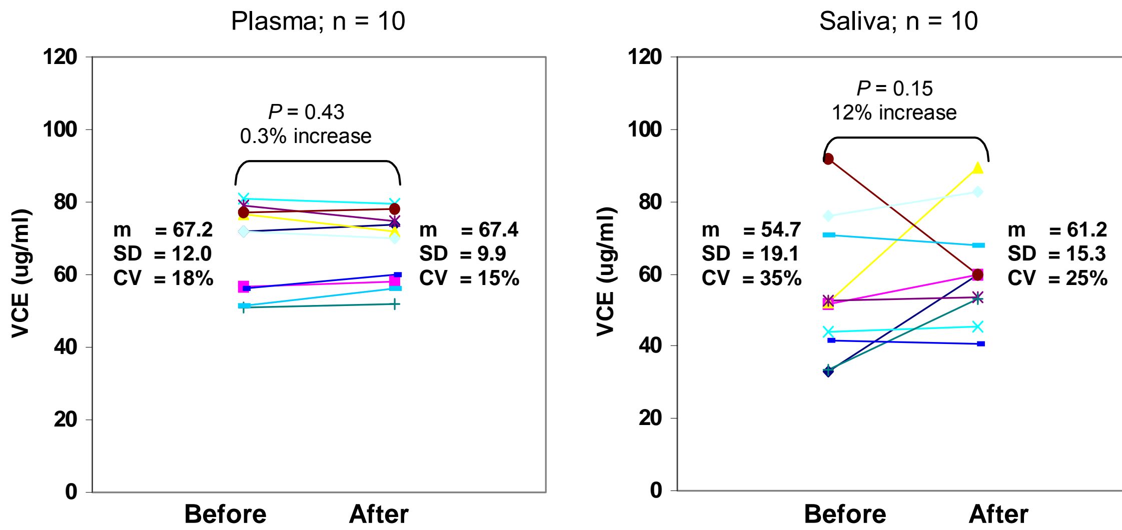


Figure 3.9. Changes in plasma and salivary antioxidant levels following exhaustive exercises as determined by the DPPH Assay.

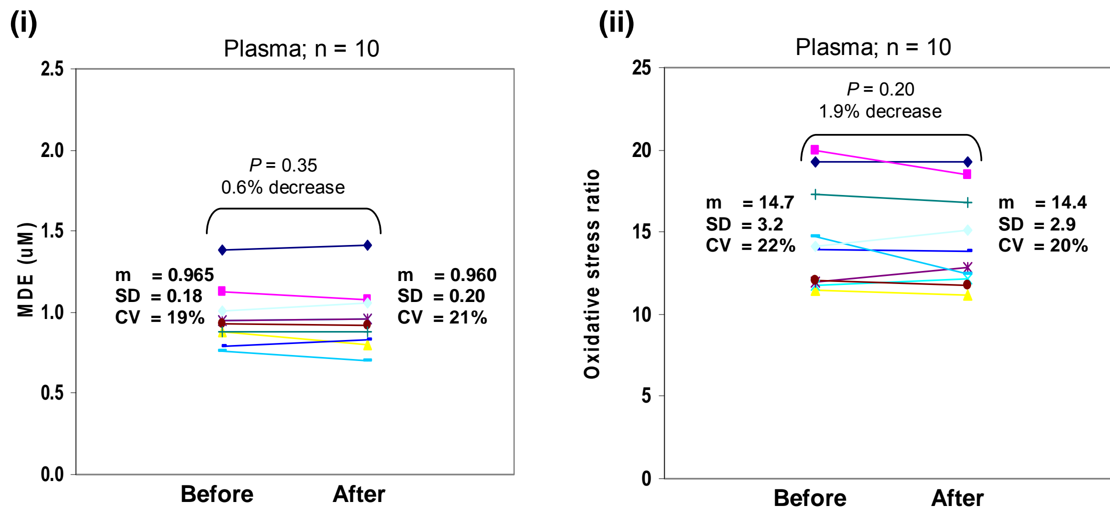


Figure 3.10. Changes in oxidative stress markers following exhaustive treadmill running:
(i) Changes in plasma lipid peroxidation as determined with the TBARS Assay
(ii) Changes in the plasma Oxidative Stress Ratio

4 DISCUSSION

The criteria used for determining VO_{2max} have been described under Methods. These criteria for achieving VO_{2max} have all been met during the first visit when each volunteer's VO_{2max} was determined. The average RER of the ten volunteers as determined by gas analyses were 1.15 and the average heart rate was 201 bpm which approximated the age-dependant maximal heart rate. Heart rate and oxygen uptake started reaching a plateau (Figures 3.4 and 3.5) and volunteers were unable to continue running despite verbal encouragement.

When performing the exercise protocol during the second visit based on the VO_{2max} determined during the first visit, volunteers reported an average Borg reading of 16 out of 20, indicating they experienced the exercising as "very hard" (see Appendix F for Borg scale). The average blood lactate concentration of 6.6 mM immediately after exercise confirms the high exercise intensity. The fact that the blood lactate concentration was higher than 4 mM is an indication that the aerobic-anaerobic threshold has been exceeded. The heart rates increased by 132% from rest following exercise. This is also an indication of the degree of intensity of this exercise protocol.

This exercise protocol caused a very slight increase of 0.3% in the total antioxidant capacity of the plasma as determined by the DPPH Assay as shown in Figure 3.9. This increase was not statistically significant ($p = 0.43$). The antioxidant capacity of the saliva increased by 12% which was also not statistically significant ($p = 0.15$). Both the change in plasma and salivary antioxidant levels were contradictory to the expected decrease in antioxidant capacity following strenuous exercise. The relationship between resting plasma and salivary antioxidants was as expected, with resting salivary antioxidants (54.7 μg/ml VCE) being approximately 80% that of resting plasma antioxidants (67.2 μg/ml VCE).

An interesting gender-related distribution of plasma antioxidant capacity was observed: There are two distinct populations that differ statistically significantly in both the VCE values before and after exercise ($p < 0.001$ for both). The group with the lower antioxidant capacity consists only of females, whereas the group with the higher antioxidant capacity consists of all the male volunteers ($n = 4$) as well as the remaining two females. Although this is an interesting observation, no explanation can be offered for this and it might simply be coincidental.

As can be seen from Figure 3.10, there was a very slight (0.6%) and not statistically significant ($p = 0.35$) decrease in lipid peroxidation following exercise as determined by the TBARS Assay. This is opposite to the increase in oxidative stress and therefore lipid peroxidation that is to be expected from such a strenuous exercise protocol.

The Oxidative Stress Ratio is a parameter that combines the results of the DPPH Assay and TBARS Assay of each volunteer to give a single value to indicate oxidative stress.

Due to the decrease in lipid peroxidation and increase in total plasma antioxidant capacity following exercise, this parameter showed a decrease in oxidative stress after exercise. This decrease of 1.9% was not statistically significant ($p = 0.20$).

5 CONCLUSION

Small changes in plasma DPPH, TBARS and Oxidative Stress Ratio results were observed following this exercise protocol. All these results were in the opposite direction as were expected, but none were statistically significant. The salivary DPPH results showed a more substantial change (12%) in the opposite direction as expected, but that also did not reach statistical significance.

Since the biokinetical data indicated that the heartbeats were 80% of the maximal heart rate and the blood lactate concentrations were more than 4 mM, it would be difficult to increase the difference (before and after exercise) any more. It was thus decided to increase the number of volunteers and reduce the standard deviation thereby increasing the statistical power of the experiment. This was included in the design of the Third Exercise Study that will be discussed in Chapter 4.

CHAPTER 4

THE THIRD EXERCISE STUDY

THE USE OF STRENUOUS SPINNING EXERCISE BY A MALE POPULATION OF TWENTY-THREE VOLUNTEERS AS EXERCISE PROTOCOL

1 INTRODUCTION

The aim of the three exercise studies, of which this chapter deals with the final study, the Third Exercise Study, has been described in detail in Chapter 2.

During the Second Exercise Study, which has been described in Chapter 3, only small, statistically non-significant changes in plasma DPPH, TBARS and Oxidative Stress Ratio results were observed following the exercise protocol. Based on this it was decided in consultation with a statistician to increase the number of volunteers for the Third Exercise Study to twenty three. This was done in order to increase the statistical power of the study and thereby increasing the probability to observe more significant changes in the antioxidant and oxidative stress measurements following exercise.

During subject selection for the Third Exercise Study it was aimed to keep the volunteer group as homogenous as possible. Therefore only male volunteers aged between 18 and 23 were selected for this study, excluding the possible variation that could result from differences in age and gender.

In order to accommodate the large number of volunteers with an exercise protocol, it was decided to let them participate in a spinning exercise class.

This allowed a total of three groups of seven or eight volunteers (7 + 8 + 8 = 23) to perform exercise simultaneously and therefore enabled a higher throughput of volunteers through the exercise protocol than for the First and Second Exercise Studies.

During the First and Second Exercise Studies, volunteers performed the treadmill running exercises individually. This made the collection of finger prick blood samples for lactate concentration determination possible. Because of the individual attention to each volunteer during the exercise protocol, it was practically possible to couple each volunteer on a gas analyzer for gas analyses data (VO_2 and RER measurements) during the Second Exercise Study. Since the group consisted of only ten volunteers in total for the Second Exercise Study, it was also possible from a financial point of view to obtain gas analyses data from them.

By taking the statistical advantages of using a larger number of volunteers into consideration, it was decided to use twenty three volunteers for the Third Exercise Study. However, the use of such a large group of volunteers in a spinning class setup led to some practical and financial limitations. Because of these limitations it was not possible to obtain blood lactate concentration and gas analyses data for the volunteers participating in the Third Exercise Study. Therefore only the following two parameters that were used during the First and Second Exercise Studies to describe the exercise intensity experienced by the volunteers could be used during this study:

- Heart rate
- Borg scale of Ratings of Perceived Exertion (RPE)

For the Third Exercise Study not only the percentage increase in heart rate of the volunteers following exercise was investigated as for the first two exercise studies, but also whether their age-dependant maximal heart rate has been reached.

There exists a direct relationship between maximal heart rate and VO_{2max}: During intense exercise, the metabolic need for oxygen in skeletal muscle increases many times over the resting value. One of the ways of meeting this increasing demand of oxygen is by increasing the cardiac output. This is achieved by increasing the heart's stroke volume and rate. At maximum work rate (VO_{2max}) a maximal heart rate is reached (Astrand and Rodahl, 1986). Therefore maximal heart rate can serve as a simple and affordable indicator of VO_{2max}. We exploited this relationship during the Third Exercise to circumvent the limitation of not being able to perform gas analyses to determine VO_{2max} values on the large group of volunteers.

Maximal heart rate (taking the age of the volunteer into consideration) can be estimated by the following formula (Gerstenblith *et al*, 1987):

$$\text{Maximal heart rate} = 220 - \text{age (years)}$$

From this equation, it can be seen that maximal heart rate decrease with aging. For the Third Exercise Study, we used a homogenous group of young male volunteers aged between 18 and 23. According to this formula, the maximal heart rate of a 21 year old subject (average age of volunteers for this study) is 199 bpm (220 – 21 = 199). This is however only an estimate and values can actually be 20 bpm higher or lower. Therefore the maximal heart rate for volunteers of this age group can be anything between approximately 179 and 219 bpm.

The relationship between volunteer age and maximal heart rate (100%) and different percentages thereof (50, 70 and 90%) is shown in Figure 4.1.

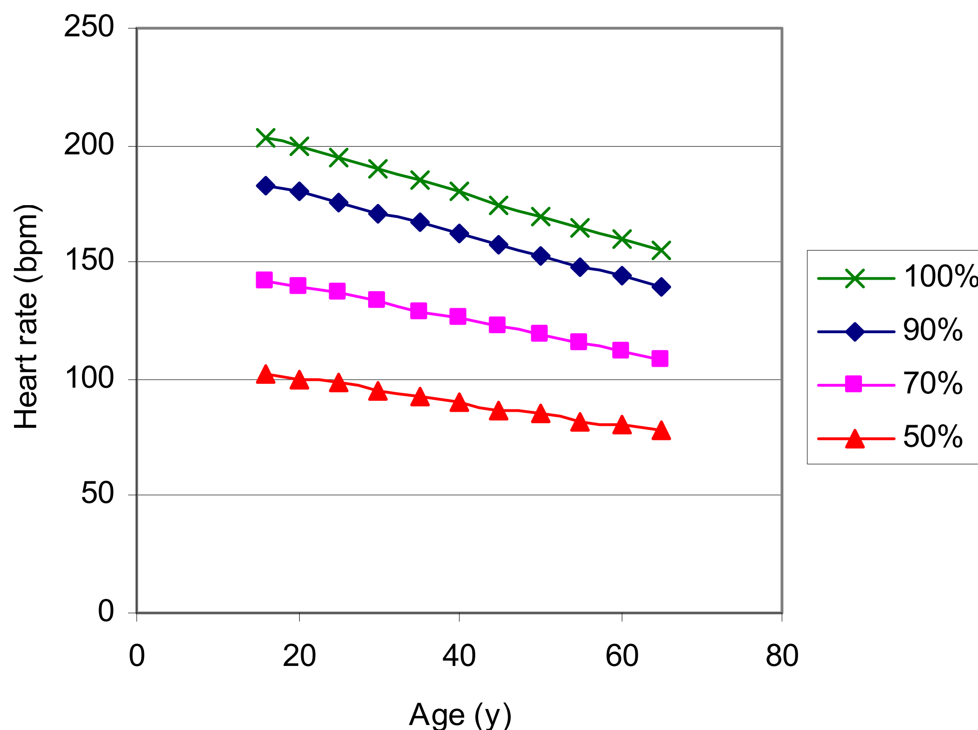


Figure 4.1. Age-dependant decrease in maximal heart rate (100%) and different percentages (50, 70 and 90%) of maximal heart rate.

In addition to the heart rate and RPE values, the measurement of muscle soreness was used as indication of exercise intensity during the Third Exercise Study. The two methods used for the measurement of muscle soreness were affordable and practically easy to be carried out by such a large group of volunteers making it suitable for this exercise study. These methods were:

- Leg presses
- The Short-Form McGill Pain Questionnaire (SF-MPQ)

Demanding or unaccustomed exercise often results in muscle soreness. It is widely believed that this soreness is a product of exercise-induced muscle damage, and that muscle damage may also explain the frequently observed impairment in muscle functioning following such exercise. This muscle damage is caused by both mechanical and metabolic factors. The adverse effects of free radicals are a major cause of muscle soreness, since the endogenous antioxidant system is overwhelmed following unaccustomed exercise leaving cell organelles susceptible to free radical attack. Post-exercise

muscle soreness develops gradually 24 to 48 hours after exercise and is associated with loss of strength and flexibility in the affected muscles (Newham *et al*, 1983).

Leg presses: An established indirect indicator of muscle damage is a decrease in dynamic strength (Chleboun *et al*, 1998, Donnely *et al*, 1992). This decrease in dynamic strength following leg exercises (for example running or spinning exercise) can be measured by a decrease in ability to perform leg presses. This approach can be used to obtain a quantifiable, objective indication of muscle soreness caused by this type of exercise.

Short-Form McGill Pain Questionnaire (SF-MPQ): The McGill Pain Questionnaire (MPQ) is a validated, reliable questionnaire that is often used to get a quantifiable indication of pain (Melzack, 1975). A shorter form of the McGill Pain Questionnaire, called the Short-Form McGill Pain Questionnaire (SF-MPQ) is also available and consists of two components. The SF-MPQ has been shown to correlate consistently and significantly with the standard MPQ to describe pain in terms of intensity (Melzack, 1987). For a copy of the SF-MPQ, see Appendix G.

The main component of the SF-MPQ is referred to as the Pain Rating Index (PRI) and consists of fifteen descriptors of pain, which are rated on an intensity scale as:

0 = none
1 = mild
2 = moderate
3 = severe

This component gives a score out of 45 (fifteen descriptors with a possible maximum of score of 3 for each on the intensity scale).

The second component of the SF-MPQ includes two Present Pain Intensity (PPI) indexes.

- The Visual Analog Scale (VAS)
- The Evaluative Overall Intensity Scale (EOI)

For the VAS scale, a mark is allocated on a 10 cm long line according to pain experienced between the two extremes on the ends of the line namely "no pain at all" and "worst possible pain". The distance of the mark from the beginning of the line is measured (in cm) to give a score out of 10.

The EOI is described on an integer scale of 0 - 5, with a maximum score of 5 referring to the worst possible pain.

2 MATERIALS AND METHODS

2.1 Sample collection and processing

Plasma samples: Blood samples to be used for the DPPH Assay were collected, processed and stored as described under Materials and Methods in Chapter 2 for the First Exercise Study. For the TBARS Assay, blood was collected, processed and stored as described under Materials and Methods in Chapter 3 as for the Second Exercise Study.

Saliva samples: Saliva samples were obtained and processed as described for the First Exercise Study under Materials and Methods in Chapter 2.

2.2 Biochemical analyses

2.2.1 DPPH Assay

The DPPH Assay was carried out as described for the First Exercise Study under Materials and Methods in Chapter 2.

2.2.2 TBARS Assay

The TBARS Assay was carried out as described for the Second Exercise Study under Materials and Methods in Chapter 3.

2.2.3 The Oxidative Stress Ratio

The Oxidative Stress Ratio was calculated as explained for the Second Exercise Study under Materials and Methods in Chapter 3.

2.3 Subject selection

Twenty-three male volunteers aged between 18 and 23 participated in this study. The volunteers were B.Sc. students that were recruited through advertising during lectures. Written informed consent was obtained from all the volunteers before commencement of the study (see Appendix D). Volunteers were all requested to fill out a questionnaire to obtain their physical data and to ensure that they meet all the inclusion criteria. To be eligible to participate in this study, they had to have a healthy weight and consider themselves generally healthy. Exclusion criteria for this study included: smoking, taking dietary supplements or prescribed medicine, suffering from periodontal disease, exercising regularly and being accustomed to spinning exercise. To ensure the volunteers were not fit for this study, they were also requested to provide the number of hours they spend exercising

per week. They were requested not to engage in strenuous physical activity during the week before exercise.

2.4 Ethical approval

The experimental protocol for this study was reviewed and found to be acceptable by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria. The Ethics Clearance Number S270/2003 was allocated to this study. For a copy of the letter of approval, see Appendix C.

2.5 Experimental setup

The exercise protocol was carried out at a local health club, the Groenkloof Virgin Active Gym in Pretoria. A personal trainer at the gym, who is a qualified biokineticist, assisted in the collection of anthropometric data as well as the design and performance of the exercise protocol. Because of practical limitations the volunteers were divided into three groups (two groups of eight volunteers each and one group of seven volunteers) that participated in this study separately between March and June 2004. They were all subjected to the same experimental procedure.

2.6 Experimental procedure

The experimental procedure is summarized in the Table 4.1. It consisted of two visits two the gym, two days apart.

The pain and exertion questionnaires used in this study were explained to the volunteers before the start of the study. RPE readings as indication of exertion were obtained directly after exercise, as for the First and Second Exercise Studies. In addition to this, volunteers were requested to fill in the SF-MPQ directly after the exercise session and again two days later.

The heart rates of the volunteers were read off from heart monitors before and immediately after exercise.

Leg presses were carried out by each volunteer before the spinning class and again two days after exercise.

More detail on the specific exercise protocols for the spinning exercise and leg presses are provided in the following sections.

Table 4.1. Summary of experimental procedure followed for the Third Exercise Study.

Day before first visit	
At 20h00:	Stop eating and drinking (except for water)
Day of first visit	
On waking:	No breakfast Drink plenty of water
From 8h00-9h00: (At the gym)	Height and weight measurements taken Resting heartbeat measured Blood and saliva samples obtained Leg presses carried out Pain questionnaires filled out
At 9h00:	**Spinning exercise performed**
After exercise:	Borg scale rating obtained Heartbeat measured Blood and saliva samples obtained
Day of second visit	
Sometime during day: (At the gym)	Leg presses repeated Pain questionnaires filled out

2.6.1 Exercise protocol

Each group of volunteers attended a high intensity spinning class at the gym under the instruction of the personal trainer. A spinning cycle (Johnny G Spinner Elite) and a heart monitor (Cardiosport System 122™) were allocated to each volunteer. The exercise intensity of the protocol was based on the heart rate of the volunteers and the cadence at which they pedaled during the spinning exercise protocol. Low leg intensity (LLI) was defined as 60-70 rpm and medium leg intensity (MLI) as 70-90 rpm. Spinning was carried out in either a seated or a standing position. The protocol consisted of a warming up session followed by two interval sessions, Interval 1 and Interval 2 as shown in Table 4.2.

2.6.2 Leg press protocol

The leg presses were carried out on a leg press machine at the fitness center just before the spinning exercises and again two days later. Volunteers started with five repetitions of their own body weight (rounded off to the nearest 5 kg). The weight was then increased in increments of 50 kg and volunteers had to perform 3 repetitions at each weight. Once a weight was reached where the volunteers were unable to perform 3 repetitions, the weight was increased in smaller increments (20–30 kg) and the weight where the volunteer was only able to perform one repetition was used as the maximum leg press value.

Table 4.2. Exercise protocol followed by volunteers in the Third Exercise Study.

	Position (seated or standing)	Time (min)	Cadence (LLI or MLI)	Heart rate (bpm)	Number of repetitions
Warming up	Seated	2	LLI	120	1
	Seated	2	MLI	130	1
	Seated	2	MLI	140	1
	Seated	3	MLI	150	1
	Seated	1	MLI	160	1
	Seated	1	MLI	180	1
Interval 1	Seated	3	LLI	150	1
	Seated	1	LLI	160	1
	Seated	2	LLI	170	1
	Standing	0.5	LLI	180	4
	Seated	1	LLI	180	
	Seated	3	MLI	150	1
Interval 2	Standing	0.5	LLI	150	6
	Seated	0.5	MLI	180	
	Seated	2	MLI	150	1

2.7 Statistical analysis

The number of volunteers for this study was calculated in consultation with the statistician. It was based on the following formula (Schlager, 1999):

$$n = 2t^2(s/d)^2$$

where n = number of volunteers

t = tabled t-value

s = standard deviation

d = difference between the before and after values

The s and d values were obtained from the Second Exercise Study.

The Windows software for dose effect analysis, Calcusyn (Biosoft, 1996), was used to obtain linear trendline equations from the absorbance values of the DPPH dilution series and obtain EC_{50} values for the Third Exercise Study. (For the First and Second Exercise Studies, MS Excel was used as described in Chapter 2 under the Results section.)

The changes in the various parameters of the volunteers measured before and after exercise were compared using the paired Student's t-test as for the First and Second Exercise Studies. For this study, the Statistical Analysis Software (SAS) package, version 8.2 (SAS Institute, 1996) was used instead of MS Excel as for the First and Second Exercise Study. Results were analyzed in consultation with a statistician and a SAS programmer. Before data analysis took place, all outliers in the dataset were identified by the SAS programmer using the procedure described in Appendix H. These outliers were removed before data analysis took place.

3 RESULTS

The physical characteristics of the volunteers are summarized in Table 4.3.

Table 4.3. Physical characteristics of volunteers

	M ± SEM
N	23
Age (y)	21.3 ± 0.3
Height (m)	1.82 ± 0.01
Body mass (kg)	78.3 ± 1.83
BMI (kg/m^2)	23.7 ± 0.43
Exercise (hours/week)	1.8 ± 0.41

One of the groups of volunteers participating in the spinning class is shown in Figure 4.2.

Figure 4.2. One of the groups of volunteers during a spinning class.

In this study, an indication of the exercise intensity was obtained by heart rate measures, Borg scale readings, leg presses and the SF-MPQ. These results are summarized in Table 4.4.

Table 4.4. Biokinetical data obtained from volunteers before, directly after or two days after exercise as indicated.

	N	Before exercise	Directly after exercise	Two days after exercise	p-value	Increase or decrease (%)
Heart rate (bpm)	18	88 ± 3.4	188 ± 2.5	—	< 0.005 **	115↑
Borg's RPE (out of 20)	23	—	15.6 ± 0.4	—	—	—
Leg presses (kg)	13	223 ± 18.2	—	217 ± 15.6	0.37	2.6↓
SF-MPQ; PRI (0-45)	20	7.4 ± 0.8	—	17.4 ± 3.2	0.003 **	136↑
SF-MPQ; PPI *VAS (0-10)*	19	4.8 ± 0.4	—	5.4 ± 0.6	0.15	13↑
SF-MPQ; PPI *EOI (0-5)*	19	1.8 ± 0.16	—	2.6 ± 0.31	0.016 *	44↑

Results are expressed as means ± SEM.
** Significant at $p < 0.05$*
*** Highly significant at $p < 0.01$*

The heart rates of the volunteers before exercise and directly after exercise are shown in Figure 4.3. Since age-dependant maximal heart rate is an indicator of VO_{2max} the upper and lower brackets of this parameter are also indicated.

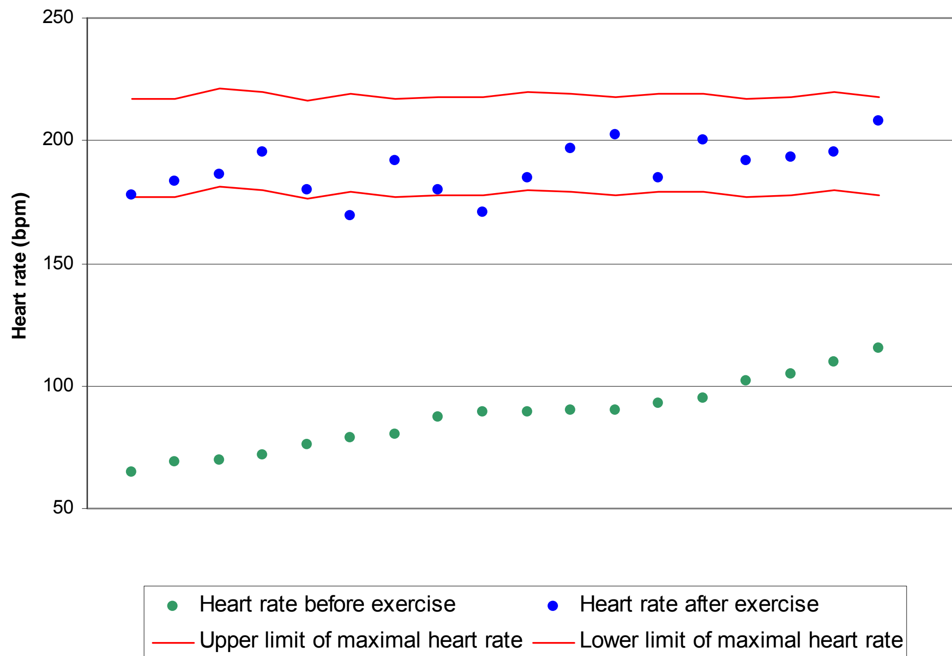


Figure 4.3. Heart rates of volunteers before and directly following spinning exercise. *Volunteers are ranked by their heart rate before exercise. The interval (upper and lower limits) for maximal heart rate based on volunteer age is also indicated. Upper limit = 220 – age(years) + 20. Lower limit = 220 – age(years) – 20.*

Leg press results were obtained before the spinning exercises as well as two days following the spinning class. Due to a technical problem, leg press results were not obtained correctly for the first group of volunteers two days after their spinning class. The leg press results presented here therefore refers to the last two groups of 13 volunteers in total. There was a 3% decrease in the maximum weight that the volunteers were able to push with the leg presses two days after the spinning exercise (See Figure 4.4).

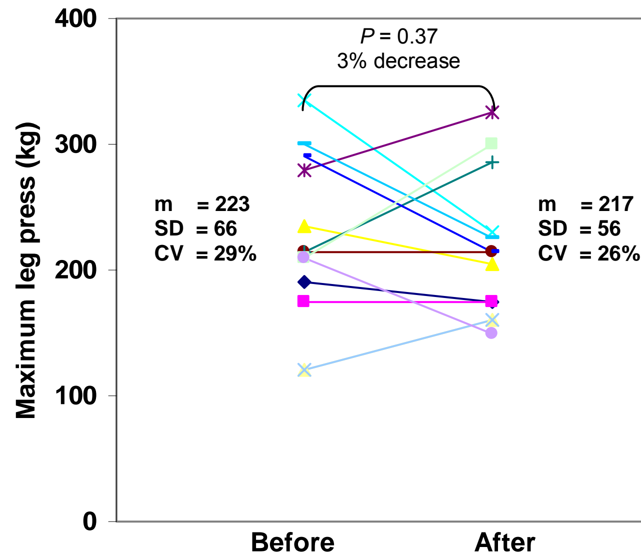


Figure 4.4. Changes in maximum leg presses performed by volunteers following strenuous spinning exercises; N = 13.

Volunteers completed the SF-MPQ directly following exercise as well as two days later. For all the components increases in pain readings were reported as shown in Figure 4.5. The pain readings according to the Pain Rating Index increased by 133% and reached statistical significance (p = 0.0003). The two subcomponents of the Present Pain Intensity Index, the Visual Analog Scale and the Evaluative Overall Intensity, increased by 13 and 44%, respectively. Only the Evaluative Overall Intensity reading reached statistical significance (p = 0.016).

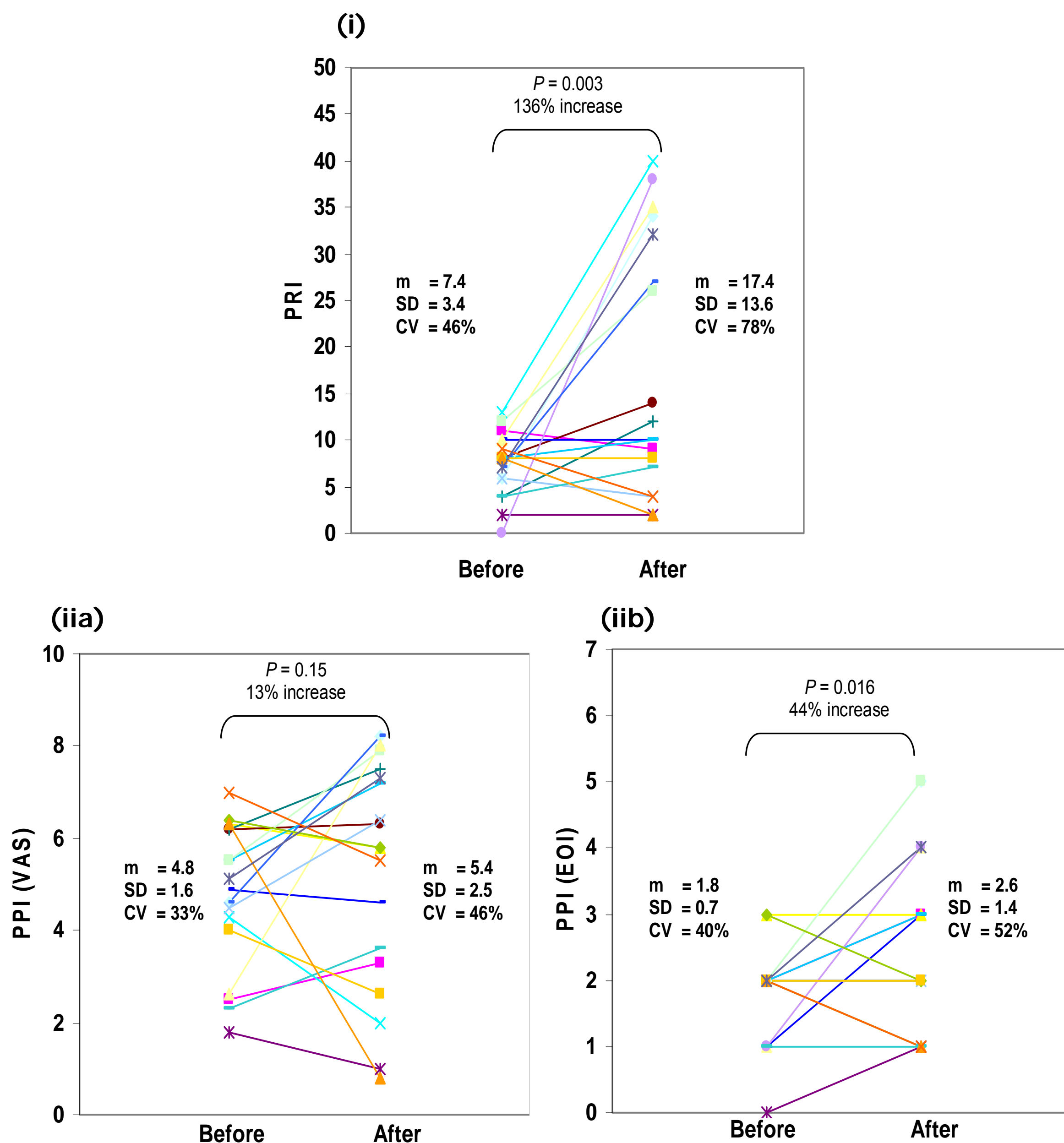


Figure 4.5. Changes in the scores of the different components of the SF-MPQ following strenuous spinning exercises:
(i) The Pain Rating Index (N = 20)
(ii) The Present Pain Intensity Indexes
(iia) The Visual Analog Scale (N = 19)
(iib) The Evaluative Overall Intensity of Pain (N = 19)

The plasma antioxidant levels increased by 9% following exercise from 71.6 to 77.9 µg/ml VCE as shown in Figure 4.6. This was statistically significant (p = 0.03). After removal of the eight outliers, there was only sufficient data to report the changes in salivary DPPH results of fifteen volunteers. The

salivary antioxidant levels of these fifteen volunteers increased by 20% from 63.8 to 76.8 μg/ml VCE. This was also statistically significant (p = 0.0045).

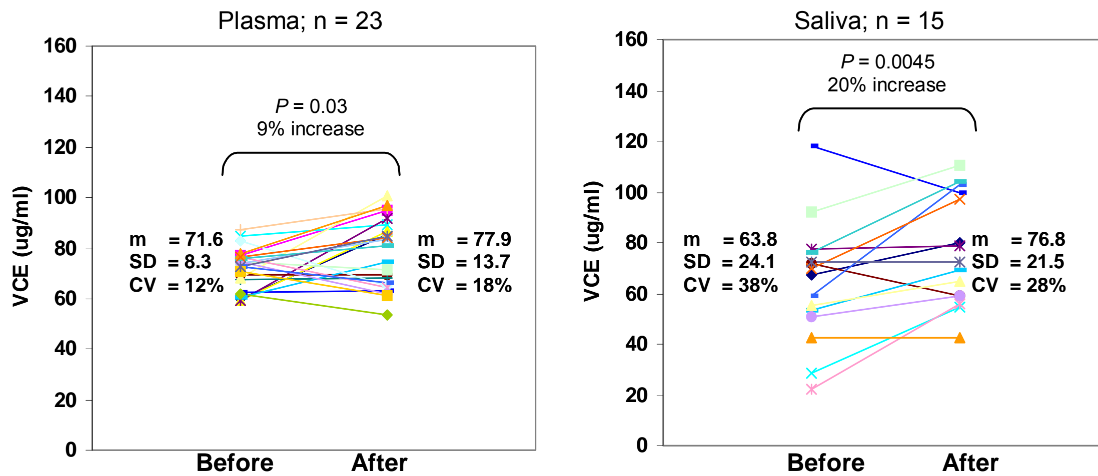


Figure 4.6. Changes in plasma and salivary antioxidant levels following strenuous spinning exercises as determined by the DPPH Assay.

There was a 2% increase in plasma lipid peroxidation as determined by the TBARS Assay. TBARS levels increased from a resting value of 1.180 μM to 1.208 μM directly after exercise. This increase was not statistically significant as shown in Figure 4.7(i). The Oxidative Stress Ratio decreased by 3% from 8.3 to 8.1. This decrease was also not significant.

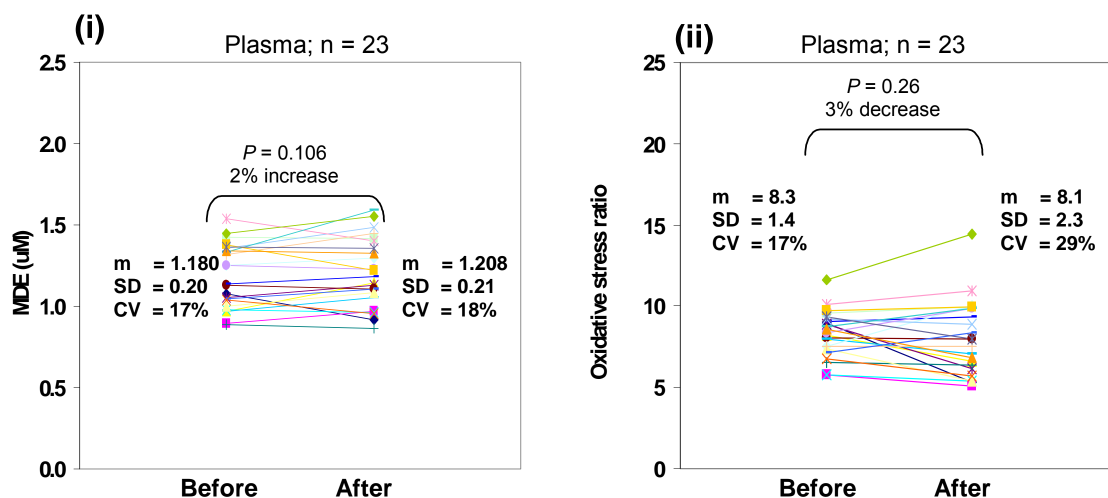


Figure 4.7. Changes in plasma oxidative stress markers following strenuous spinning exercises:
(i) Changes in lipid peroxidation as determined with the TBARS Assay
(ii) Changes in the Oxidative Stress Ratio.

The changes in the Oxidative Stress Ratio, TBARS and plasma DPPH for the volunteers following exercise are shown in Figure 4.8. In Figure 4.9, the changes in plasma and saliva antioxidant levels for the volunteers are shown for comparison.

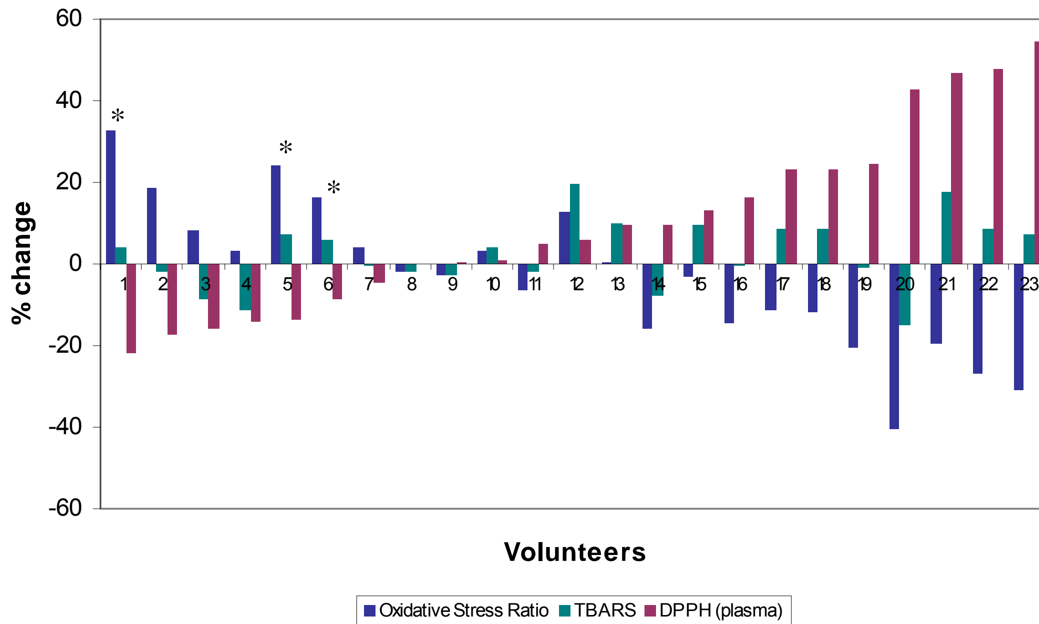


Figure 4.8. The percentage change in the Oxidative Stress Ratio, TBARS and plasma DPPH results of the volunteers following exercise. *Volunteers are ranked by DPPH results. Volunteers showing the expected percentage changes for these three parameters are indicated by an (*), N = 23.*

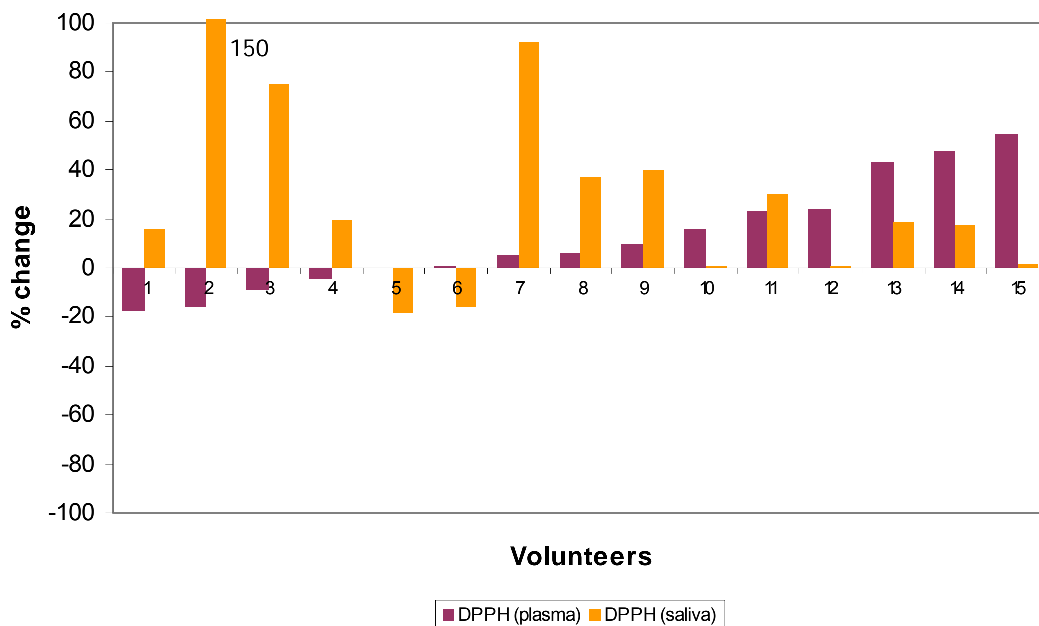


Figure 4.9. The percentage change in plasma and salivary DPPH results of the volunteers following exercise. *Volunteers are ranked by plasma DPPH results as for Figure 4.8, N = 15.*

4 DISCUSSION

Spinning exercise was the exercise of choice for this study, since it can be carried out groupwise making it ideal for such a large group of volunteers. (The volunteers still had to be divided into three groups of seven or eight to make the collection of the blood and saliva samples more manageable). Spinning is also a very intense type of exercise employing the same group of leg muscles used for treadmill running. One of the exclusion criteria for subject selection for this study was being accustomed to spinning exercise. This ensured that all volunteers participating were subjected to very intense and unaccustomed exercise. The spinning protocol was designed in consultation with a biokineticist and aimed at maintaining a high exercise intensity for a long period. The exercise protocol alternated between high and lower cadence spinning speed and sitting and standing positions, ensuring the volunteers were able to maintain exercising at a relatively high intensity for a long period. The exercise duration of this protocol was about 25 min which is longer than the protocols used for the First and Second Exercise Studies.

Due to the large number of volunteers used for this study, it was not practically possible to perform finger pricks for lactate measurements or perform gas analyses as for the previous study. Therefore the exercise protocol could not be standardized with the VO_{2max} values of the volunteers as for the Second Exercise Study. However, the spinning exercise protocol used for this study was standardized in terms of cadence and heartbeat ensuring that all volunteers performed exercise at the same intensity.

Various parameters were measured during this study to monitor the exercise intensity, including the heart rates and Borg scale readings as for the previous studies. Muscle soreness, as an indirect indication of exercise intensity, was measured by requesting volunteers to fill out SF-MPQ and perform leg presses two days following exercise.

As can be seen from Table 4.4, the average increase in heart rate of the volunteers following exercise was 115% with a heart rate maximum after exercise of 188 bpm. This compares well with the heart rates of the volunteers of the Second Exercise Study (their heart rates increased by an average of 132% to a maximum value of 200 bpm). Based on this, it can be assumed that the exercise intensity of this study was comparable to that of the Second Exercise Study.

As can be seen from Figure 4.3, most of the volunteer heart rate readings were within the maximal heart rate interval which is one marker that indicated that the exercise intensity was close to 100% of VO_{2max}. This is confirmed by the Borg scale readings that, as with the First and Second Exercise Studies, corresponded with the description "very hard".

Since muscle soreness develops gradually 24 to 48 hours after exercise (Newham *et al*, 1983), the volunteers performed their leg presses two days after the spinning protocol. Baseline leg press results were obtained from the volunteers before participating in the spinning class. The weight that the volunteers were able to push with a leg press two days after the exercise was 3% less than the weight they were able to push before the exercises.

The pain readings were given by the volunteers directly following exercise and two days later. There was an increase in pain readings for all three components of the SF-MPQ two days later, with two of the three components showing a statistically significant increase. The Pain Rating Index, which is the major component of the SF-MPQ and consists of fifteen descriptors of pain that had to be rated by the volunteers, showed a drastic increase of 136%. This was statistically highly significant ($p < 0.01$). This indicates that the exercise protocol was strenuous enough to induce significant muscle soreness in the volunteers, pointing towards a high exercise intensity.

The same markers of oxidative stress that were used in the Second Exercise Study were also measured in this study, namely plasma and salivary DPPH Assay, TBARS Assay and Oxidative Stress Ratio values.

The resting plasma DPPH values obtained for this study (71.6 µg/ml VCE) is comparable with the resting plasma DPPH values of the Second Exercise Study (67.2 µg/ml VCE). The resting salivary DPPH values were higher than that obtained for the Second Exercise Study (68.8 µg/ml VCE vs 54.7 µg/ml VCE). The resting salivary antioxidant levels in this study was also high relative to the plasma DPPH values, being about 90% of the plasma values instead of the 70% reported in literature (Atsumi *et al*, 1999).

The increase of 9% in plasma antioxidant levels as measured by the DPPH Assay, which was statistically significant, was opposite to the expected decrease in antioxidant levels following strenuous exercise. The increase in salivary antioxidants following exercise was even higher, rising by 20% which was also statistically significant. The same tendency, of an increase instead of a decrease in total antioxidants, was also observed during the Second Exercise Study.

There was an increase in lipid peroxidation as determined by the TBARS Assay. This is expected following strenuous exercise that should cause an increase in oxidative stress. This increase was however small (2%) and did not reach statistical significance.

The Oxidative Stress Ratio was also expected to increase following the strenuous exercise protocol that should cause an increase in oxidative stress. Despite the increase in lipid peroxidation as determined by the TBARS Assay, there was a decrease in the Oxidative Stress Ratio. This was due to the increase in the plasma antioxidants as determined by the DPPH Assay which is the denominator in the equation of the Oxidative Stress Ratio.

There was no statistically significant correlation between the volunteers' changes in plasma and salivary DPPH values, TBARS values and Oxidative Stress Ratio values caused by the exercise protocol.

Figure 4.8 shows the changes in oxidative stress parameters of the volunteers after exercise. An increase is indicated by a positive value for percentage change whereas a decrease is indicated by a negative value. The volunteers were ranked by their change in plasma DPPH values which were expected to decrease after exercise (negative percentage change). The TBARS values were expected to increase, therefore showing a positive percentage change. The Oxidative Stress Ratio was also expected to increase and show a positive percentage change as for the TBARS. As can be seen from Figure 4.8, the responses of the volunteers varied and only three volunteers (indicated by asterisks) responded in the expected way with all three parameters.

Figure 4.9 is similar to Figure 4.8, but here the change in plasma and salivary antioxidant levels are compared. All the saliva samples responded in the opposite direction as expected (showing increases instead of decreases following exercise). The responses of the saliva samples varied more in terms of size of response than that of the plasma samples. No correlation was seen between changes in plasma and salivary antioxidant levels following exercise.

5 CONCLUSION

There was a statistically significant increase in the plasma DPPH results following this exercise protocol. This is contradictory to the theoretical decrease in antioxidant levels that should be observed following strenuous exercise and which have been found in some reports in literature (Atsumi *et al*, 1999, Child *et al*, 1999, Ginsburg *et al*, 2001, Santos-Silva *et al*, 2001). Small changes in TBARS and Oxidative Stress Ratio results were observed following this exercise protocol. The TBARS showed an expected increase following exercise, but that was not significant. The results of the Oxidative

Stress Ratio were in the opposite direction as were expected, and was also not statistically significant. The salivary DPPH results showed a more substantial change (20%) in the opposite direction as expected, and reached statistical significance.

Possible explanations for these unexpected results and recommendations for future studies on the modulation of oxidative stress levels by exercise are discussed in Chapter 6.

CHAPTER 5

METHODS FOR INVESTIGATING PROTEIN OXIDATION: THE SPECTROPHOTOMETRIC DNPH ASSAY AND THE OXYBLOT™ WESTERN BLOT

1 INTRODUCTION

It is well known that intense exercise is associated with elevated levels of free radicals which could lead to oxidative stress. Oxidative stress often leads to lipid, nucleic acid, carbohydrate and protein modification. The primary cellular target of oxidative stress depends upon the cell type, the nature of the stress imposed (radical or non-radical oxidant), the site of generation (intra-or extra cellular), the proximity of ROS to a specific target, and the severity of the stress (Halliwell and Gutteridge, 1999).

Oxidative damage to lipids, DNA or protein may all be seriously deleterious and may be concomitant. However, proteins are possibly the most immediate target for inflicting oxidative damage on cells because they are often catalysts rather than stoichiometric mediators; hence, the effect of damage to one molecule is greater than stoichiometric (Dalle-Donne *et al*, 2003a).

In addition to the loss of catalytic function, oxidation of proteins can also result in the loss of DNA binding activities of DNA transcription factors, increased sensitivity to denaturation, and increased susceptibility to proteolytic degradation by proteinases (Stadtman *et al*, 1992).

Oxidation of proteins can lead to the formation of oxidized amino acids, such as dityrosine, 3-nitrotyrosine, 3-chlorotyrosine and oxohistidine (Dean *et al*, 1997). Proline, arginine and lysine can also be derivatized to carbonyl

derivatives. Fragmentation of polypeptide chains and formation of protein-protein cross linkages are also possible outcomes of oxidation reactions (Fagan *et al*, 1999).

Highly reactive hydroxyl radicals are thought to be generated *in vivo* by catalytic action of transition metals such as iron and copper that bind to appropriate sites of proteins and can modify nearby amino acid residues (Stadtman, 1990).

The accumulation of oxidized proteins is often measured by the content of reactive carbonyls (Stadtman *et al*, 1992). Spectrophotometric assays for the appearance of carbonyl groups on proteins have been developed to quantify oxidative protein damage using 2,4-dinitrophenyl hydrazine (DNPH) as shown in Figure 5.1. This method includes a washing procedure and quantitates the stable hydrazone derivatives formed by the reaction of carbonyls with the carbonyl reagent DNPH (Levine *et al*, 1992). These spectrophotometric assays remain one of the primary ways to identify reactive carbonyl groups on proteins since it is convenient, fast and inexpensive.

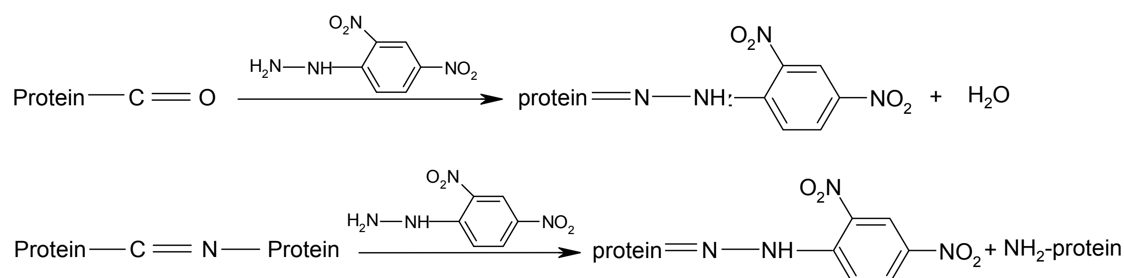


Figure 5.1. The derivatization of carbonyl groups in protein side chains to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with DNPH (Levine *et al*, 1990).

Protein carbonyls can also be measured by antibodies to DNPH by employing immunoblot analysis (Nakamura and Goto, 1996). This type of immuno/Western blot for the measurement of oxidized proteins is commonly b referred to as "Oxyblot". With the Oxyblot™ method, oxidized proteins are derivatized with DNPH reagent as for the spectrophotometric method. The DNP-derivatized protein samples are then separated by SDS-PAGE followed by

Western blotting. Alternatively, the derivatized protein samples can be directly dot blotted onto a membrane. After either method of preparation, the membranes are incubated with primary antibody (1°Ab) specific to the DNP moiety of the protein (rabbit anti-DNP antibody). This step is followed by incubation with a horseradish peroxidase-antibody directed against the 1°Ab. This secondary antibody (2°Ab) is a goat anti-rabbit IgG. The membranes are then treated with luminol chemiluminescent reagents. The luminol is converted to a light-emitting form at 428 nm by the antigen/1°Ab/2°Ab/peroxidase complex (bound to the membrane) in an H_2O_2 catalyzed oxidation reaction. This light is detected by exposure to photographic films.

More quantitative and qualitative information may be obtained with this method regarding oxidative status of individual tissue proteins compared to the spectrophotometric DNPH Assay. In addition to being a more sensitive method, the Oxyblot™ method also has the advantages over the spectrophotometric DNPH Assay that its reliability is not affected by the purity of the protein sample analyzed and that unwashed DNPH reagent does not contribute to variability of the method.

Most studies of *in vivo* protein oxidation following exercise have been limited to animal studies (Griffiths, 2000). Reznick *et al* (1992a) showed for the first time an increase in protein oxidation in skeletal muscle of rats after a single bout of exercise, related to an exercise-induced decrease in lipophilic antioxidants. Later Goto *et al* (1999) used an immunoblot method to show that exercise at high altitude caused higher carbonylation of skeletal muscle proteins of rats (most notably a protein likely to be actin) than at sea levels but no significant difference was observed in lipid peroxidation. This suggests that increased oxidation of skeletal muscle proteins are independent of lipid peroxidation in high-altitude training. These findings were in agreement with Radak *et al*, (1997). They found that high altitude training increased reactive

carbonyl derivatives but not lipid peroxidation in the skeletal muscle of rats, showing that lipid peroxidation and protein oxidation are not inseparable reactions as they have different mechanisms. On the other hand, Liu *et al* (2000) found a positive correlation between MDA, a measure of lipid peroxidation and protein carbonylation as measured by the spectrophotometric DNPH Assay in the slow muscle in chronic exercise rats and in the brain and the liver in acutely exercised rats. This suggests that oxidants produced during exercise attack both lipids and protein. Exhaustive running of rats have been shown to induce a 40% increase of protein carbonyls in the lung as determined by quantitation of Western blot signals (Radak *et al*, 1998). An increase in blood protein carbonyls in one of the few human studies on this topic has been reported after aerobic, but not isometric exercise (Alessio *et al*, 2000).

In the present study we attempted to employ both the spectrophotometric DNPH Assay as well as an Oxyblot™ method for use in the Exercise Studies as a marker of oxidative stress to proteins. The spectrophotometric DNPH Assay will subsequently be discussed first, followed by the Oxyblot™ method.

2 THE DNPH ASSAY

2.1 Approach

The first aim was to optimize the spectrophotometric DNPH Assay in order for repeatability to be high enough for measuring *in vivo* oxidative damage in plasma samples. Then, since the DNPH Assay is a relatively easy and inexpensive assay, the idea was to use the DNPH Assay to screen all the plasma samples of the volunteers of the Third Exercise Study for increases in protein carbonylation following exercise. Once the volunteers whose protein carbonylation levels have increased have been identified with the spectrophotometric DNPH Assay, the Oxyblot™ was to be performed on only the samples of those volunteers that displayed an increase in protein carbonylation with the DNPH Assay. In this way, it can be determined which specific plasma proteins have been oxidized. Once they have been identified, it can be speculated what specific consequences the exercise-induced oxidative stress might have due to free radical attack on certain specific proteins.

2.2 Materials and methods

2.2.1 Preparation of plasma

Blood samples were obtained from the antecubetal vein of a healthy volunteer. It was collected into EDTA-containing vacutainer tubes and kept on ice. In order to separate the plasma, it was centrifuged at 3 000 g for 5 min. The plasma was removed, aliquoted and stored in cryovials at -70°C. Before use, it was thawed in water at room temperature.

2.2.2 Protein concentration determination

Protein concentration determination of the plasma samples was carried out using Coomassie reagent.

For construction of the standard curve, a standard range of bovine serum albumin (BSA) diluted with saline (0.9% NaCl) was used. A 1 mg/ml stock solution of BSA in saline was prepared and diluted to 0, 2.5, 5, 10, 15, 20 and 25 µg/ml.

The plasma samples were diluted in tenfolds up to a 100 000x dilution.

A volume of 100 µl of each of the BSA dilutions and the plasma samples were transferred to a 96-well microtiter plate in duplicate and 100 µl Coomassie reagent was added to each of the wells. After 15 min of incubation at room temperature, the absorbance was read on a microtiter plate reader using a filter at 595 nm.

A standard curve was constructed from the BSA dilution series absorbance readings and used to determine the samples' protein concentrations.

2.2.3 Oxidation of samples by formaldehyde and MDA

Formaldehyde oxidation of proteins: Protein samples to be oxidized (plasma and BSA) were diluted to a final protein concentration of 1 mg/ml in a series of formaldehyde oxidation buffers (varying concentrations of formaldehyde — 0, 5, 10 20, 30 mM — in 50 mM phosphate buffer, pH 7.4).

MDA oxidation of proteins: BSA samples to be oxidized were diluted to a final protein concentration of 1 mg/ml in a series of MDA oxidation buffers (varying concentrations of MDA — 0, 6.25, 12.5, 25, 50, 100 mM — in 50 mM phosphate buffer, pH 7.4).

The samples were incubated overnight at 37°C. The reaction was stopped by adding TCA (trichloro-acetic acid) to a final concentration of 10% w/v. The sample was centrifuged at 11 000 g for 3 min and washed with TBS. The protein content was determined with the Coomassie reagent and the samples stored at 4°C.

2.2.4 Oxidation of samples by $FeCl_3$/ascorbate

Some of the samples were oxidized with $FeCl_3$/ascorbate instead of aldehydes. For this oxidation method, 5-10 mg/ml protein sample was added to $FeCl_3$/ascorbate oxidation buffer (25 mM HEPES, pH 7.2, 25 mM ascorbate, 100 mM $FeCl_3$). The sample was incubated at 37°C for 5 hours. Following incubation, the samples were dialyzed overnight against 50 mM HEPES containing 1 mM EDTA. The protein content was determined and the samples stored at 4°C.

2.2.5 Assay procedure

The protocol for the DNPH as described by Reznick *et al* (1994) was followed. Protein samples to be assayed (oxidized and non-oxidized samples) were diluted to a final protein concentration of 1 mg/ml. Proteins were precipitated with ice-cold trichloro-acetic acid (TCA), with a final concentration of 10% w/v. The samples were left on ice for 10 min and the precipitated proteins removed by centrifugation at 11 000 g for 3 min. The protein pellet was resuspended in 10 mM DNPH/2 M HCl and vortexed continuously at room temperature for 1 hour. The proteins were precipitated again with 10% w/v TCA, incubated on ice for 10 min and centrifuged at 11 000 g for 3 min. The pellet was washed with ethanol:ethylacetate (1:1), using a glass rod to break up the pellet. The pellet was left to stand for 10 min before being centrifuged at 11 000 g for 5 min. The washing steps were repeated twice. After the final washing step, the pellet was resuspended in potassium phosphate buffer (50 mM, pH 7.4) containing 6M guanidine. The sample was incubated at 37°C for

30 min followed by centrifugation to remove any insoluble material. The maximum absorbance of the sample between 355-390 nm was obtained using the guanidine containing phosphate buffer as reference sample. The protein content was also determined spectrophotometrically at 280 nm using a BSA standard curve. The carbonyl content (in nmol carbonyls/mg protein) was determined from these absorbance readings using Beer Lambert's law and the extinction coefficient (ε = 22 000 $M^{-1}.cm^{-1}$).

2.3 Results

The concentration-dependant generation of carbonylated proteins by formaldehyde and MDA is shown in Figure 5.2.

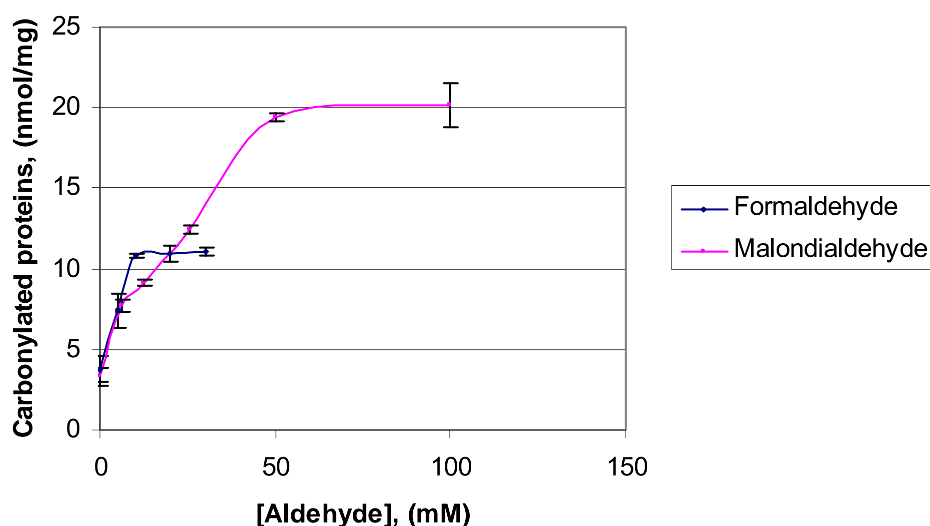


Figure 5.2. The *in vitro* generation of carbonyls in BSA by reactive aldehydes. *Error bars indicate standard deviation of three sets of experiments.*

A wavelength scan of the DNPH Assay at its maximum absorbance region on untreated and in vitro oxidized plasma is shown in Figure 5.3. It can be seen that the oxidized plasma samples have an almost three-fold higher level of protein carbonylation.

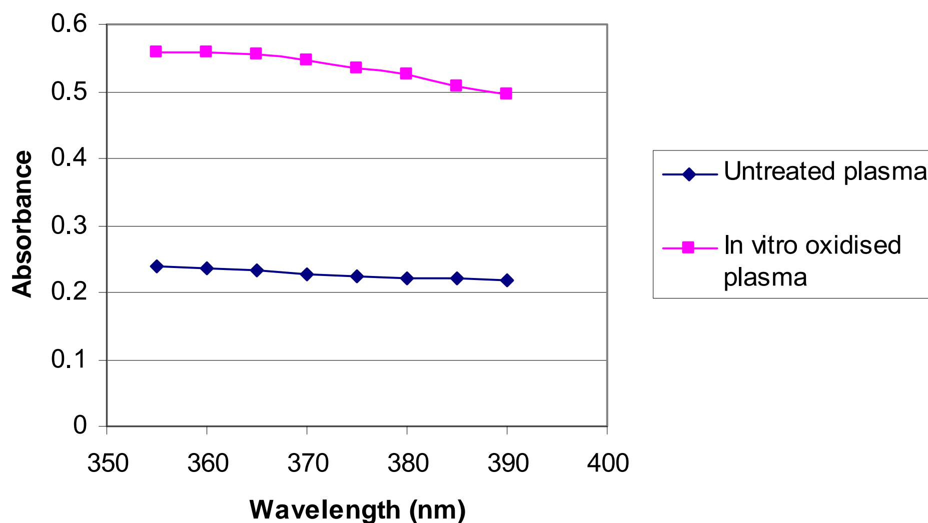


Figure 5.3. Wavelength scan of DNPH Assay on untreated and *in vitro* oxidized ($FeCl_3$/ascorbate treated) plasma samples at the maximum absorbance region of the DNPH reagent.

The increase in levels of carbonylation of BSA and plasma samples in the presence of increasing amounts of formaldehyde is shown in Figure 5.4.

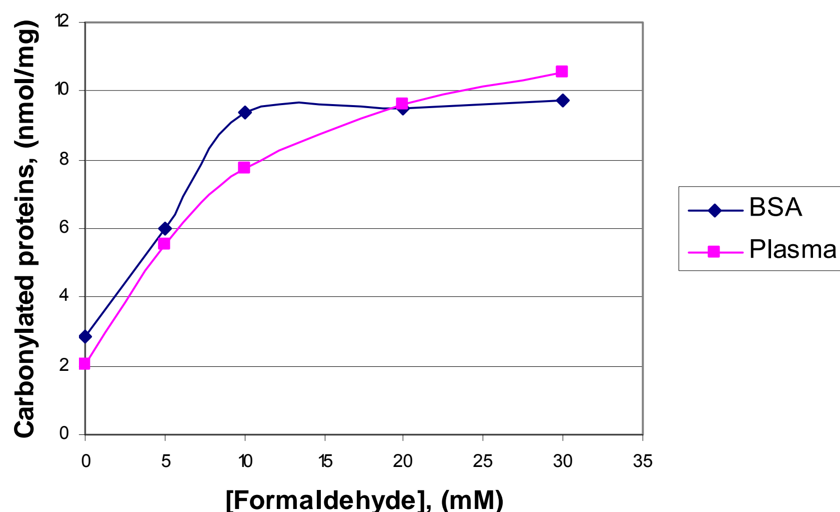


Figure 5.4. Protein carbonylation of formaldehyde-treated BSA and plasma samples.

The difference in carbonyl content as determined by the DNPH Assay on different aliquots of the same plasma sample is shown in Table 5.1.

Table 5.1. The variation on four sets of independent DNPH experiments on aliquots of the same plasma sample.

Set	[Protein carbonyl] nmol/mg
1	1.6
2	0.04
3	0.63
4	1.1
Ave	0.84
SD	0.67
CV	79%

2.4 Discussion

The purpose of the DNPH Assay was to screen plasma samples of the volunteers of the Third Exercise Study and determine which volunteers showed elevated levels of protein carbonylation following exercise. Only the plasma samples of those volunteers with increased protein carbonylation levels as indicated by the DNPH Assay would then be subjected to the Oxyblot™ to obtain more specific information regarding the pattern of carbonylation of the different plasma proteins.

Although the DNPH Assay was to be used to measure *in vivo* protein carbonylation levels in human plasma (induced by oxidative stress caused by strenuous exercise), it was first attempted to obtain reproducible results using a purified protein sample (BSA) that was subjected to *in vitro* oxidation, since this was expected to give higher values of carbonylation and lower variation between samples.

Based on the work of Adams *et al* (2001), BSA was subjected to increasing amounts of the aldehydes formaldehyde and MDA to induce *in vitro* protein carbonylation. As expected, protein carbonylation increased with increasing amounts of aldehyde, until a plateau of maximum carbonylation is reached (Figure 5.2). The formaldehyde-treated BSA reached a plateau at a

concentration of 10 mM formaldehyde, with a maximum of 10 nmol/mg protein carbonyls formed. The MDA-treated BSA reached a plateau at a much higher concentration, approximately 60 mM, with the maximum protein carbonyls being formed at 20 nmol/mg. Adams *et al* (2001) found that formaldehyde reached a plateau at a much lower aldehyde concentration of about 1 mM formaldehyde with a maximum of about 20 nmol/mg protein carbonyls being formed. MDA reached a plateau at a concentration of about 10 mM with a maximum of about 25 nmol/mg protein carbonyls being formed. These results suggest that the formaldehyde used in our experiments might have been faulty, since it required a tenfold higher concentration to reach a plateau in comparison with the results by Adams *et al* (2001) and the maximum carbonyls measured at this high concentration was about half of the maximum obtained by Adams *et al* (2001). Although we required a higher concentration of MDA compared to Adams *et al* (2001) (60 mM vs. 10 mM) to reach a plateau in carbonyl formation, our maximum levels of protein carbonyls induced with MDA compared well with Adams *et al* (2001), being between 20 and 25 nmol/mg for both. Our baseline level of protein carbonylation in the BSA (with no aldehyde added) of 3.54 nmol/mg compared very well with the baseline obtained by Adams *et al* (2001) of 3.50 nmol/mg.

The carbonyl reagent has a maximum absorption in the region of 355-390 nm. We decided to do a wavelength scan of an untreated plasma sample and a plasma sample subjected to $FeCl_3$/ascorbate oxidation in that region to decide what exact wavelength to use for the spectrophotometric determination of protein carbonylation. This is shown in Figure 5.3. With this scan, the success of the *in vitro* oxidation of plasma by $FeCl_3$/ascorbate could also be investigated. The oxidative modification of the plasma led to an increase of approximately 2.4 fold in protein carbonylation. No literature could be found to compare these results to.

Next, the *in vitro* oxidation of plasma by formaldehyde was compared to that of BSA as shown in Figure 5.4. As expected, the plasma protein carbonylation levels increased with increasing amounts of formaldehyde. The response of the plasma sample was similar to that of the BSA, although it did not seem as if a plateau in carbonylation of the plasma was reached in this concentration range. The observation that the plasma required a formaldehyde concentration similar to that of the BSA for comparable carbonylation supports the previous speculation that the formaldehyde used might have been deteriorated (see discussion on Figure 5.2). An interesting observation is that the baseline level of protein carbonylation is higher for BSA than it is for plasma. The baseline level of protein carbonylation for plasma observed here, 2.0 nmol/mg compares well with that obtained in literature of 1.83 nmol/mg (Adams *et al*, 2001). A similar finding of commercial albumin having a higher baseline of protein carbonylation than plasma has been reported previously (Reznick *et al*, 1992b). According to Reznick *et al* (1992b) possible reasons for this include the fact that plasma contains other agents that are able to protect albumin and also that commercial albumin is probably contaminated with transition metals such as iron and copper leading to inherently higher levels of metal-catalyzed oxidation during purification.

Since the ultimate aim of the DNPH Assay is to measure *in vivo* protein carbonylation levels in plasma, an important question that needs to be addressed is what the variability of the assay is on plasma samples that were not subjected to *in vitro* oxidation. In order to answer this question, a plasma sample was collected, aliquoted and the different aliquots were subjected to the DNPH Assay on 4 different days (thus independent experiments) as shown in Table 5.1. The variation between the results of the four different experiments was very high (coefficient of variation was 79%). Interestingly, the average value of protein carbonylation of plasma we obtained, despite the high variation, of 0.84 nmol/mg was in good agreement with literature, reporting values of 0.4-1.0 nmol/mg for untreated plasma (Halliwell, 1996).

Further investigation into literature led to the questioning of the suitability of the spectrophotometric DNPH Assay for measuring *in vivo* oxidation of proteins of a crude extract such as plasma. The spectrophotometric carbonyl assay has been criticized as being non-specific and unreliable, especially in human studies (Urso and Clarkson, 2003). The spectrophotometric carbonyl assay measures reactive carbonyls in certain isolated (purified) proteins that have been oxidized *in vitro* very well; however, the carbonyl assays may not accurately measure carbonyl levels due to protein oxidation *in vivo*. This method has been criticized for its high variability when analyzing biological samples (crude protein extracts), due to the contribution of certain proteins such as heme-containing proteins to the background absorbance (Adams *et al*, 2001). The nucleic acids in crude extracts and the unwashed free DNPH have also been identified as two major problems in this assay. By using streptomycin sulphate treatment one can remove most nucleic acids from a crude tissue extract, but then the reactive carbonyl levels are too low to be reliably quantified (Cao and Cutler, 1995).

Based on the above-mentioned literature and our own experience (see Table 5.1), the conclusion was made that the spectrophotometric DNPH Assay was not suitable for measuring *in vivo* protein oxidation in plasma samples. It was decided to use the Oxyblot™ determination of protein oxidation.

3 THE OXYBLOT™ METHOD

3.1 Materials and methods

The reagents and protocol provided with the Oxyblot™ Protein Oxidation Detection Kit (catalogue no S7150) supplied by Intergen Company (NY, USA) were used.

3.1.1 Buffers and solutions

The following buffers and solutions were prepared for use in the Oxyblot™ method:

- Electrophoresis buffer (0.025M Tris, 0.192M glycine, 3.5mM SDS, pH ±8.3, not to be adjusted)
- Acrylamide stock solution (30%w/v acrylamide, 0.8%w/v bisacrylamide)
- 1.5M Tris-HCl, pH 8.8 (1.5M Tris-base, pH adjusted to pH 8.8 using 4N HCl)
- 0.5M Tris-HCl, pH 6.8 (0.5M Tris-base, pH adjusted to pH 6.8 using 4N HCl)
- Electroblot transfer buffer (12mM Tris, 96mM glycine, 20% methanol)
- 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 9.0 (10mM CAPS, pH adjusted to pH 9.0 using 0.1M NaOH)
- Laemmli gel stain (40% MeOH, 10% acetic acid, 0.1% Coomassie Blue R-250)
- TBS, pH 7.4 (1mM Tris, 150mM NaCl)
- TBS-T (TBS containing 0.05% Tween-20)
- Blocking buffer (TBS-T containing 1% ELITE fat free milk powder, pH adjusted to 7.2-7.4)
- Gel loading buffer (To prepare 8ml: Add 1.0ml 0.5M Tris-HCl, 0.8ml glycerol, 0.1ml 14.3M 2-mercaptoethanol (2-ME), 1.6ml 10% SDS, 0.32ml 0.05% bromophenol blue to 4.18ml distilled water)

- Coomassie reagent (0.025% Coomassie, 40% MeOH, 7% glacial acetic acid)
- Destain solution (40% MeOH, 7% glacial acetic acid)

The following reagents were supplied in the kit:

- DNPH derivatization solution
- Derivatization control solution
- Neutralization solution
- Mixture of standard proteins with attached DNP residues
- 1° Antibody: Rabbit anti-DNP antibody
- 2° Antibody: Goat anti-rabbit IgG (HRP-conjugated)

Upon receiving the kit, the reagents were prepared and stored as recommended in the accompanying user's manual.

A basic outline of the experimental protocol that was followed is shown in Figure 5.5.

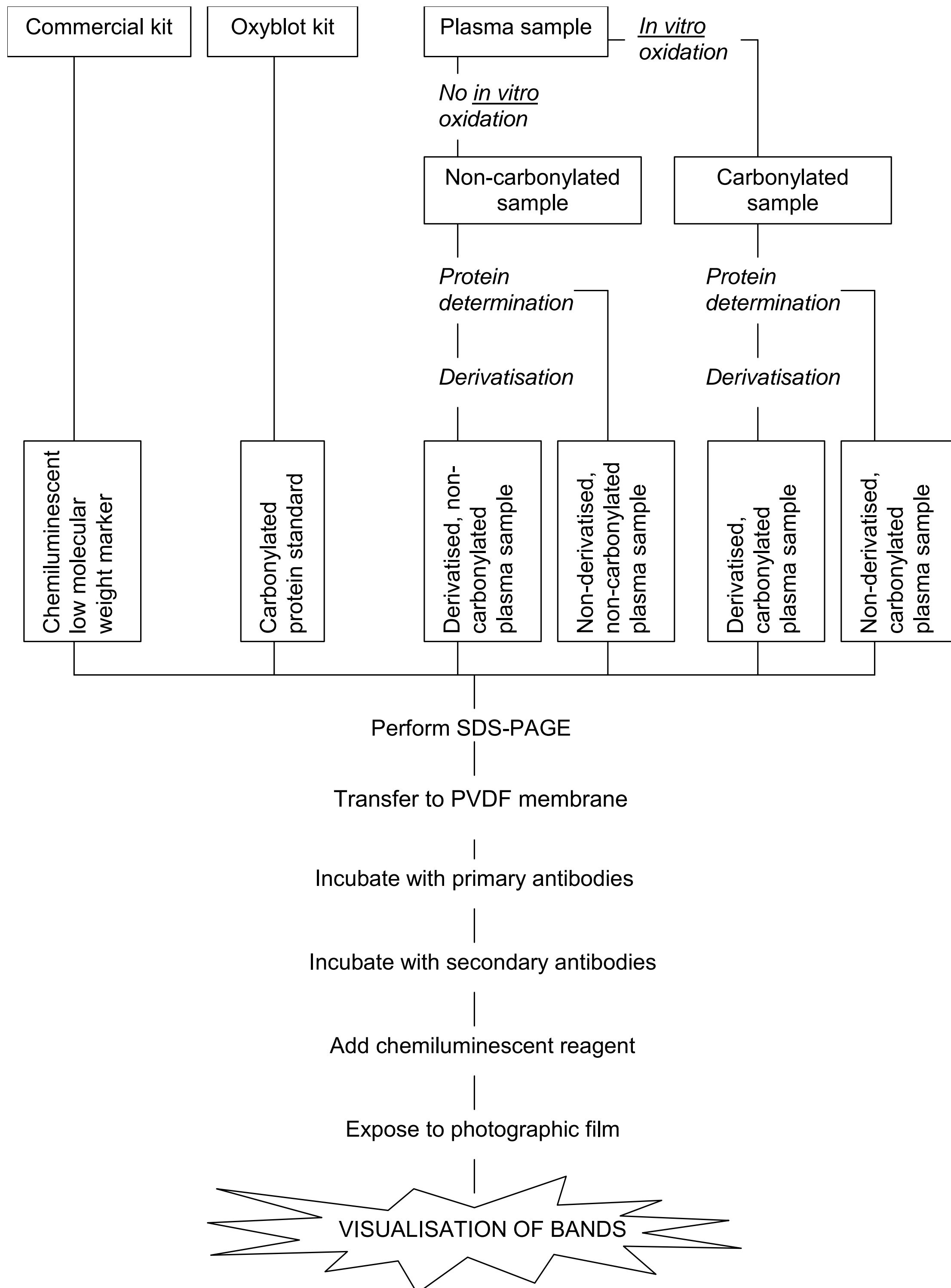


Figure 5.5. Basic outline of experimental protocol followed for performing Western blot on samples.

3.1.2 Preparation of plasma

Blood samples were obtained from the antecubetal vein of a healthy volunteer. It was collected into EDTA-containing vacutainer tubes and kept on ice. In order to separate the plasma, it was centrifuged at 3 000 g for 5 min. The plasma was removed, aliquoted and stored in cryovials at -70°C. Before use, it was thawed in a water bath at room temperature.

3.1.3 Protein determination

Protein determination was carried out using Coomassie reagent as described for the spectrophotometric DNPH Assay.

3.1.4 Preparation of oxidized proteins for positive controls by oxidation of samples with $FeCl_3$/ascorbate

For this oxidation method, 5-10 mg/ml protein sample was added to $FeCl_3$/ascorbate oxidation buffer (25 mM HEPES, pH 7.2, 25mM ascorbate, 100 mM $FeCl_3$). The sample was incubated at 37°C for 5 hours. Following incubation, the samples were dialyzed overnight against 50 mM HEPES containing 1 mM EDTA. The protein content was determined and the samples stored at 4°C.

3.1.5 Derivatization of protein samples

The derivatization reaction (see Figure 5.1) was carried out according to the protocol described in the manual provided with the kit.

Two aliquots of both the plasma sample (which was not oxidized *in vitro*) and the oxidized positive control (which was oxidized *in vitro*) were used for the derivatization reaction. One aliquot was subjected to the derivatization reaction using the DNPH solution provided in the kit. The other aliquot served

as negative control, by substituting the DNPH solution with the derivatization–control solution.

A protein concentration determination using the Coomassie reagent was performed on the plasma sample and oxidized control. The samples were then diluted to a protein concentration of 4 mg/ml with saline for the derivatization reaction. An amount of 5 µl of plasma sample was transferred into each of two microtubes. Each of the aliquots was denatured by addition of 5 µl of 12% SDS for a final concentration of 6% SDS.

One sample was derivatized by adding 10 µl of DNPH solution to the tube. To the aliquot designated as the negative control, 10 µl of the derivatization-control was added instead of the DNPH solution.

Both tubes were incubated at room temperature for 15 min. The reaction was stopped by adding 7.5 µl neutralization solution to each of the tubes.

The derivatization reaction was carried out the same way for the oxidized positive control sample and its negative control.

Mixture of standard proteins with attached DNP residues: The proteins of molecular weight protein standard mix already have DNP residues and were therefore not derivatized. Before loading onto the polyacrylamide, 20 µl gel loading buffer was added to 2.5 µl protein standard. In order to maintain consistency in reducing conditions, 2-mercapto-ethanol (2-ME) was added to the sample mixture to a final concentration of 0.76M.

Chemiluminescent LMW marker: Chemiluminescent-BlueRanger® Prestained Peroxidase-Labeled Protein Molecular Weight Marker Mix (Pierce Biotechnology, USA) was used as chemiluminescent LMW marker. This marker consists of a stabilized and lyophilized mixture of seven proteins with a molecular weight ranging from 220K to 18K. The proteins are prestained

with a covalently bound dye that yields uniform blue color band intensities when electrophoresed on a gel. This enables the visual detection of the marker during electrophoresis as well as its transfer to a membrane. The marker also has a chemiluminescent feature due to peroxidase enzyme covalently linked to the proteins. Upon incubation with peroxidase chemiluminescent substrates, the marker proteins generate light at 425 nm that can be detected by CCD camera or on photographic film.

The marker is available in single dose microtubes. The content of a microtube was dissolved in 10 µl distilled water and 5 µl was loaded onto the gel. Since the marker is already reduced, there was no need for further addition of a reducing agent.

3.1.6 Preparation of SDS-PAGE gel

A 12% separating gel was prepared by gently mixing 3.4 ml distilled water, 2.5 ml 1.5M Tris-HCl (pH 8.8), 4 ml bisacrylamide stock solution and 100 µl 10% SDS in a vacuum flask. The sample was deaerated for 15 min using a vacuum pump. Directly following degassing, 50 µl freshly prepared ammonium persulfate (10% w/v in distilled water) and 5 µl TEMED were added to the gel solution and after gentle mixing the gel was poured between two electrophoresis plates, layered with water and allowed to polymerize in the 37°C incubator for at least 30 min.

A 4% stacking gel was prepared by gently mixing 6.1 ml distilled water, 2.5 ml 0.5M Tris-HCl (pH 6.8), 1.3 ml bisacrylamide stock solution and 100 µl 10% SDS in a vacuum flask. After degassing for 15min, ammonium persulfate and TEMED were added as described above. A comb was inserted, and the gel was allowed to solidify. The gel was stored under electrophoresis buffer at 4°C.

3.1.7 Loading of samples on SDS-PAGE gel for Western blot

Chemiluminescent LMW marker: Five microliters of the chemiluminescent LMW marker was loaded directly onto gel after dilution with distilled water. The proteins are pre-reduced and therefore addition of SDS was not necessary.

Mixture of standard proteins with attached DNP residues: Gel loading buffer was added to the protein standards prior to loading the gel (2.5µl of protein standard plus 20µl of gel loading buffer). 2-ME to a final concentration of 0.74M was also added as reducing agent since the derivatized protein samples were reduced and consistency should be maintained.

Derivatized protein samples: Twenty microliters of gel loading buffer was added to 20 µl of each of the derivatized protein samples. 2-ME to a final concentration of 0.74M was added as a reducing agent.

Samples were not heated prior to loading into gel, as instructed in the user's manual of the kit.

3.1.8 Electrophoresis of samples

Electrophoresis was carried out at 40 V for the stacking gel and 100 V for the separating gel using electrophoresis buffer.

3.1.9 Electroblot of protein samples to PVDF membrane

Following electrophoresis, the gel was removed and equilibrated in electroblot transfer buffer for 15 min. PVDF (Polyvinylidine-difluoride, Merck) membrane was activated by wetting with methanol for 15 min followed by wetting with electroblot transfer buffer.

Ten layers of filter paper were equilibrated in electroblot transfer buffer. Five layers of filter paper were then layered, followed by the PVDF membrane and the gel. The wells were marked with a pencil on the PVDF membrane. Five layers of filter paper were then layered again.

The blot was carried out using a Trans Blot Semi-Dry Transfer Cell (Bio-Rad) at 25 V for 30 min, with the current being constant between 15 and 25 mA.

3.1.10 Incubation with 1˚Ab and 2˚Ab

Following the electroblot, the membrane was removed and kept in blocking buffer at 4°C overnight. The 1°Ab was diluted as instructed in the user's manual in blocking buffer just before use. The membrane was incubated in the 1°Ab solution for approximately 3 hours at room temperature with gentle shaking. The membrane was rinsed twice with TBS-T. It was then washed once for 15 min and twice for 5 min each with TBS-T at room temperature. The 2°Ab was then diluted in blocking buffer and the membrane was incubated in the 2°Ab solution for approximately 1 hour at room temperature with gentle shaking. The washing steps as for the 1°Ab were repeated.

3.1.11 Addition of chemiluminescent reagent

Excess buffer was drained from the membrane and freshly prepared chemiluminescent reagent was added. The chemiluminescent reagent (Supersignal West Pico chemiluminescent reagent, Pierce, USA) needs to be made up 1:1 just before use with the enhancer provided to produce the active chemiluminescent reagent. Enough solution was made up to cover the membrane. The membrane was incubated for 1 min with the chemiluminescent reagent at room temperature, after which the excess buffer was drained off. The membrane was then placed in a plastic bag, the air bubbles were removed and the edges were sealed off.

3.1.12 Visualization of carbonylated protein bands

The membrane was placed in a film cassette (Sigma Chemical Company) with the protein side up. In a darkroom, a photographic film was placed on top of the membrane and exposed for 3 min. It was then visualized by developing the film (Kodak Scientific Imaging Film from Biomax™ Eastman Kodak Company), for 3 min and fixing for 3 min. For this, Ilford PQ Universal Paper Developer and Ilford Rapid Fixer from Ilford were used. If no signal was observed, the exposure period was increased before developing and fixing.

3.1.13 Data interpretation

Proteins that have undergone oxidative modification could be identified as appearing as a band only in the lane containing the derivatized sample, but not in the lane containing the negative control.

3.2 Expected results

Following the experimental protocol as outlined in Figure 5.5, a representation of the theoretical results expected following visualization of the bands on photographic film is shown in Figure 5.6. The observations of the expected results are explained below.

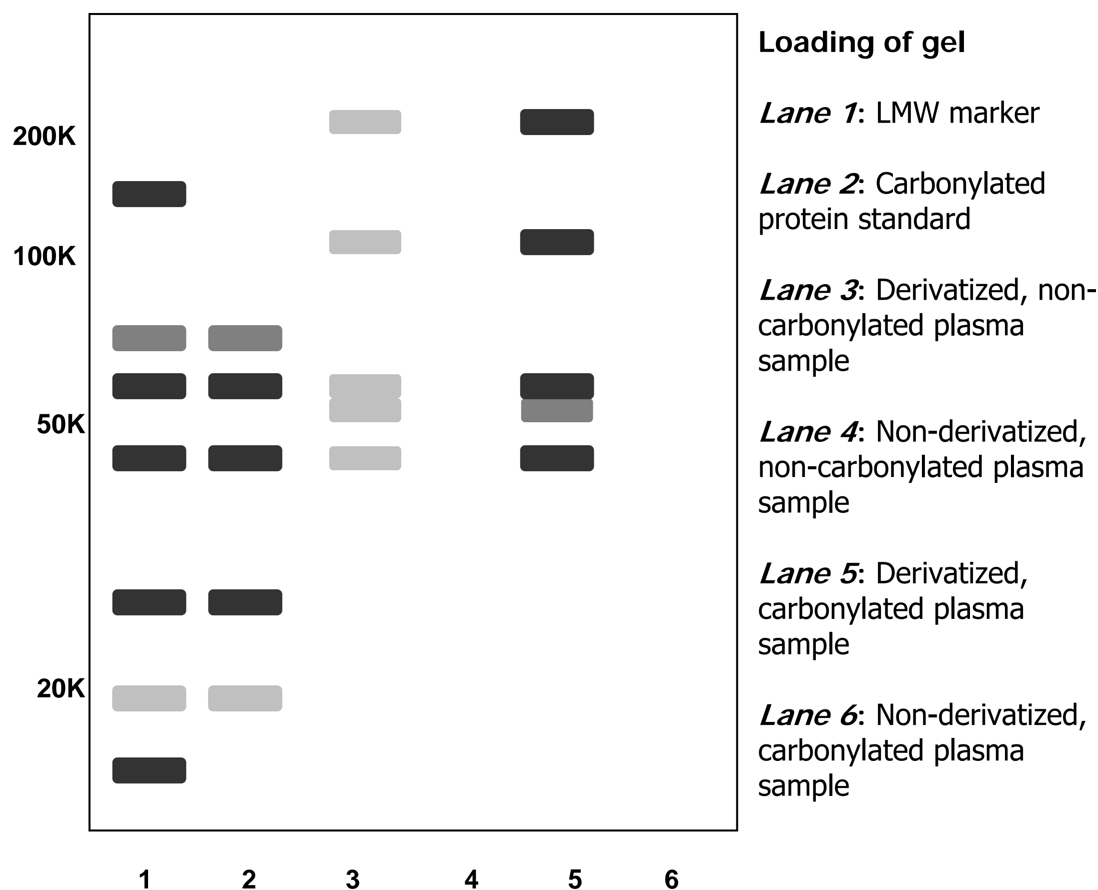


Figure 5.6. Representation of the theoretical results expected following the experimental protocol as outlined under Materials and Methods in Figure 5.5. *The intensity of the expected bands is indicated by the darkness of the grey color used to indicate the band.*

3.2.1 Chemiluminescent low molecular weight marker (lane 1, Figure 5.6)

The markers have a peroxidase enzyme covalently linked to the proteins and are therefore not dependant on the attachment of antibodies in order to be visualized in the presence of a peroxidase substrate such as the chemiluminescent reagent used in this protocol.

If bands are visualized in the lane of the chemiluminescent low molecular weight marker, as is expected, the following conclusions can be made.

- The electrophoresis was carried out successfully.
- The transfer of the proteins from the gel to the membrane was successful.

- The film was exposed for a sufficient time period to the membrane.
- The reagents (i.e. the developer and fixer) used for visualization of bands were working properly.

This marker also serves indirectly as a control for the attachment of the antibodies to the protein samples on the membrane: if the chemiluminescent LMW marker gives a signal but not the molecular weight protein standards, then there is a strong possibility that the incubation with the antibodies was unsuccessful.

3.2.2 Carbonylated protein standard supplied with the Oxyblot™ kit (lane 2, Figure 5.6)

These proteins already have DNP residues attached to them and therefore serve as an internal control for the electrophoresis, western transfer and immunodetection steps of the Oxyblot™ procedure. Bands are expected in this lane and if bands are observed here, the conclusions as for the presence of bands in chemiluminescent marker lane can be confirmed. The following conclusions can also be made by the presence of these lanes.

- The 1°Ab is recognizing the DNP-sites of the derivatized proteins;
- The 2°Ab is recognizing and binding to the 1°Ab;
- The chemiluminescent reagent is in working order.

3.2.3 Plasma samples that were oxidized *in vitro* to yield *in vitro* carbonylated proteins (lanes 5 and 6, Figure 5.6)

The proteins of these samples were subjected to $FeCl_3$/ascorbate oxidation, resulting in induced high levels of carbonylation. Due to the *in vitro* oxidative treatment, these samples have "unnaturally" high levels of protein carbonylation in comparison with similar samples that were not subjected to the oxidative treatment. These samples were divided into two groups that were treated as follows:

Derivatized samples (lane 5, Figure 5.6): These samples were treated with the DNP-reagent before being loaded onto the gel. The DNP-reagent attaches to the carbonylated groups on the proteins, enabling the 1°, anti-DNP antibody to recognize the carbonylated groups on the proteins and ultimately being visualized. Therefore, in these lanes, strong bands are expected to appear. If bands appear here, all the conclusions can be made as for carbonylated protein standard supplied with the kit, as well as the following conclusions.

- The *in vitro* carbonylation was carried out successfully.
- The DNPH reagent recognizes the carbonylated sites successfully.

Non-derivatized samples (lane 6, Figure 5.6): Although these samples were also subjected to the *in vitro* carbonylation, no bands are expected to appear here, since no DNP-residues are attached to the carbonyl groups and therefore the 1°, anti-DNP antibody is not able to recognize and bind to the carbonylated protein groups. If bands appear here after visualization, it is an indication of non-specific binding of the 1° antibodies and therefore bands present in the corresponding derivatized sample lane does not necessarily indicate the presence of carbonylated proteins.

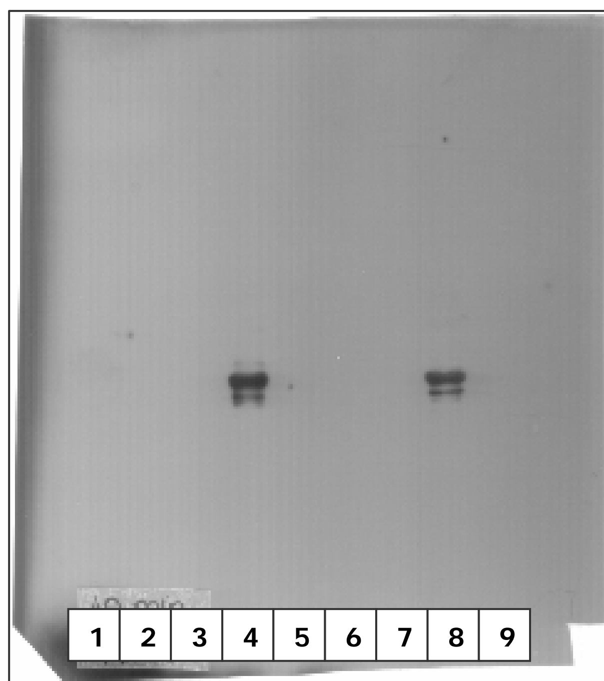
3.2.4 Plasma samples that were not oxidized *in vitro* to yield *in vitro* carbonylated proteins (lanes 3 and 4, Figure 5.6)

These samples were not subjected to $FeCl_3$/ascorbate oxidation and are not expected to have as high levels of carbonylation as those that were oxidized. However, they are expected to have some levels of carbonylation, since proteins are exposed to oxidation processes *in vivo* by various "natural" processes taking place in the body. The same conclusions can be made for these samples as for the plasma samples that were oxidized *in vitro* (above), i.e. bands are expected to appear for the derivatized samples (lane 3) but not for the non-derivatized samples (lane 4). These bands are expected to be of lower intensity since they have lower levels of carbonylation. By comparing

the derivatized non- (*in vitro*) carbonylated lane (lane 3) with the (derivatized) *in vitro* carbonylated lane (lane 5), it can be seen which bands are expected to show carbonylation. The *in vitro* carbonylated protein serves as a positive control of what the presence of carbonyl groups would look like when visualized.

3.3 Actual results obtained from Oxyblot™ method

Protein bands in some lanes were obtained only in one of the first Oxyblot™ experiments (Figure 5.7). This experiment was performed before the chemiluminescent LMW marker was incorporated as a control. The protein samples used included plasma protein samples that were not oxidized *in vitro* as well as samples that were oxidized *in vitro*. Each derivatized samples of these were loaded together with a non-derivatized negative control. All the samples were loaded in two different concentrations, namely 10 µl and 15 µl. The protein standard provided with the kit was also loaded (volume of 20 µl). After 3 min exposure of the photographic film to the PVDF membrane, a slight band started to appear in the lane where 15 µl of the derivatized *in vitro* oxidized plasma sample. After 20 min and 40 min of exposure, clear bands were visible in this lane and the 10 µl loaded with the same sample. No other bands were observed, even after 40 min of exposure.



Loading of gel

Lane 1: Protein standard, 20µl
Lane 2: Plasma, *in vitro* oxidized, non-derivatized, 15µl
Lane 3: Plasma, not *in vitro* oxidized, non-derivatized, 15µl
Lane 4*: Plasma, *in vitro* oxidized, derivatized, 15µl
Lane 5: Plasma, not *in vitro* oxidized, non-derivatized, 15µl
***Lane* 6**: Plasma, *in vitro* oxidized, non-derivatized, 10µl
Lane 7: Plasma, not *in vitro* oxidized, non-derivatized, 10µl
Lane 8*: Plasma, *in vitro* oxidized, derivatized, 10µl
Lane 9: Plasma, not *in vitro* oxidized, derivatized, 10µl

Figure 5.7. Film following Oxyblot™ protocol and 40 min of developing. *Lanes loaded as described in the legend. Lanes where bands appeared indicated with (*).*

3.4 Troubleshooting

3.4.1 Methods

Several attempts were made to obtain results with the Oxyblot™ using the standard protocol as described under the Methods section. The effect of slight variations, for example the blotting buffers and conditions and the type of PVDF membrane and photographic film, on the success of the Oxyblot™ were also investigated.

Since bands of carbonylated proteins were only observed in the one Oxyblot™ experiment (and even in this experiment bands were not observed in all lanes where expected) as shown in Figure 5.7, a series of control experiments were performed in an attempt to find the source of error.

3.4.1.1 Control experiment A: Coomassie staining of extra lanes of SDS-PAGE gel that was electrophoresed but not electroblotted

Purpose: Verifying the success of the SDS-PAGE

Two extra samples of derivatized positive control protein sample were loaded on the side of the SDS-PAGE gel. These lanes were cut off before the electroblot was carried out and stained with Coomassie reagent. The bands obtained here served as a control specifically for the electrophoresis.

3.4.1.2 Control experiment B: Coomassie staining of SDS-PAGE gel following transfer and staining of extra lane of PVDF membrane to which proteins were transferred

Purpose: Determining the success of transfer of proteins samples from SDS-PAGE gel to PVDF during electroblot transfer.

As an indication of the success of transfer of the protein samples from the gel onto the PVDF membrane, the gel was Coomassie stained after the electroblot. The intensity of the protein bands was then compared with the intensity of the protein bands on the gel that was excised before the electroblot (i.e. the original amount of proteins that were electrophoresed). Normally some of the proteins of higher molecular weight are not transferred to the membrane but remain stuck in the gel. However, usually sufficient amounts of these protein molecules are still transferred to the membrane to be detected with the Western blot.

3.4.1.3 Control experiment C: Dot blots of 2˚Ab and chemiluminescent LMW markers directly onto PVDF membrane

Purpose: Verifying whether the 2°Ab and chemiluminescent LMW markers as well as chemiluminescent reagents are in working order.

The PVDF membrane was activated by allowing it to stand in methanol for 15 min and then rinsing it with CAPS buffer. Concentrated 2°Ab and chemiluminescent LMW marker (1 µl of each) were spotted onto the membrane and allowed to air dry. Chemiluminescent reagent (10 µl) was added and incubated for 4 min. The photographic film was exposed to the membrane for 5 min and was then developed and fixed.

3.4.1.4 Control experiment D: Dot blots of derivatized samples and protein standards directly onto PVDF membranes, followed by antibody incubations

Purpose: Verifying whether the 1°Ab binds properly to the DNP-lated proteins and is recognized by the 2°Ab.

The PVDF membrane was activated by allowing it to stand in methanol for 15 min and then rinsing it with CAPS buffer. The PVDF membrane was then cut into four squares of ± 10 mm^2. Two of the membrane squares were spotted with 1 µl of oxidized derivatized plasma protein samples. (These samples were oxidized using the $FeCl_3$/ascorbic acid system). The other two squares were spotted with 1 µl of the mixture of standard proteins with attached DNP residues provided with the kit as internal control. The spots were allowed to dry while being kept moist to prevent them from becoming flaky. Each of the spotted membrane squares were then transferred to a 1.5 ml microtube and blocked with blocking buffer for 30 min while being rotated on a rotavamp. After blocking, the blocking buffer of one of the squares containing the oxidized derivatized plasma protein sample and one of the squares containing the standard proteins was replaced with freshly prepared 1°Ab. The other squares were kept in blocking buffer. The spotted membrane squares were incubated for 3 hours while being rotated. The 1°Ab solution was then removed and the spotted membrane squares washed 3 times for 20 min each with TBS-T. After washing, all four of the spotted membrane squares were incubated with 2°Ab for 1 hour. Both the 1°Ab and

the 2°Ab were diluted in the same way it is diluted for the Western blot. The washing steps as after incubation with the 1°Ab was repeated. Excess buffer was removed; chemiluminescent reagent (10 µl) was added and the samples on the membrane squares incubated for 4 min. The photographic film was exposed to the membrane for 5 min and was then developed and fixed.

3.4.2 Results

3.4.2.1 Control experiments A and B

(Controls for verifying success of electrophoresis and transfer of proteins samples from SDS-PAGE gel to PVDF during electroblot transfer)

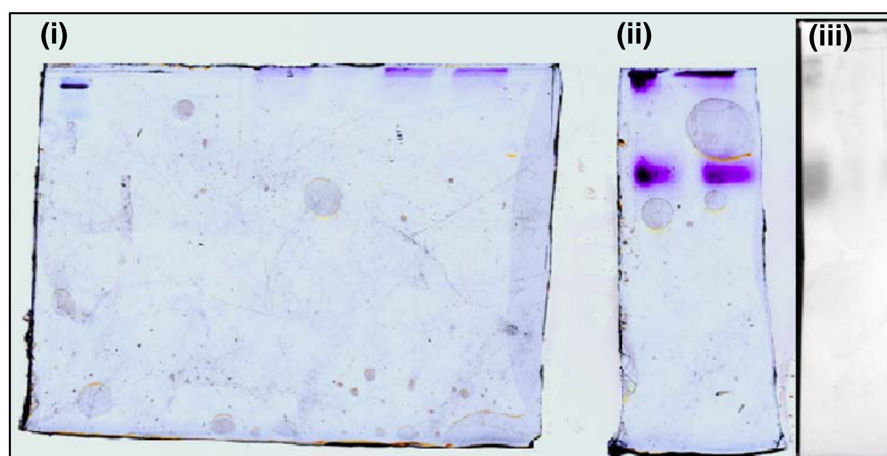


Figure 5.8. Results from control experiments A and B.
(i) Coomassie stain of SDS-PAGE gel following electroblot transfer *(Loading of gel: chemiluminescent marker, lane open, protein standard, lane open, derivatized plasma, lane open, derivatized positive control x 2)*
(ii) Coomassie stain of cut off of two extra lanes of derivatized positive control samples from SDS-PAGE gel shown in (i) following electrophoresis, but not electroblotted
(iii) PVDF membrane with cut off of extra lane of derivatized positive control sample after having been transferred from SDS-PAGE gel shown in (i) to PVDF membrane with electroblotting.

3.4.2.2 Control experiment C

(Dot blots of 2°Ab and chemiluminescent LMW markers directly onto PVDF membrane)

A chemiluminescent signal was obtained for both the 2°Ab and chemiluminescent LMW following incubation with the chemiluminescent substrate (Figure 5.9).

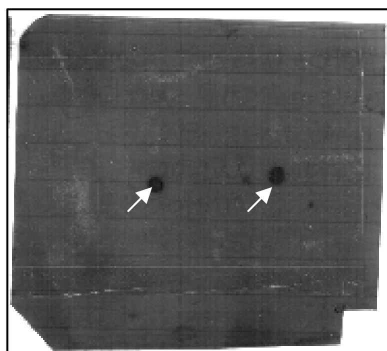


Figure 5.9. Results from control experiment C. Chemiluminescence signals of dot blots of chemiluminescent marker (left) and for 2°Ab (right).

3.4.2.3 Control experiment D

(Dot blots of derivatized samples and protein standards directly onto PVDF membranes, followed by antibody incubations)

A chemiluminescence signal was obtained for the protein standard blot that was incubated by both 1°Ab and 2°Ab. No signals were obtained for either of the oxidized, derivatized control samples or for the protein standard blot that was incubated by the 2°Ab only.

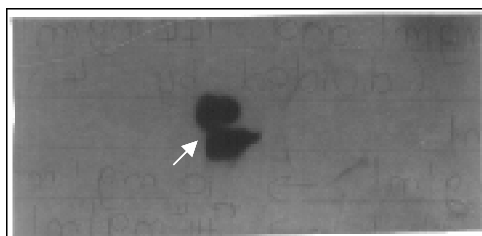


Figure 5.10. Results from control experiment D. Chemiluminescence signal of dot blot of the protein standard.

3.4.3 Discussion

The oxidation of plasma proteins by ROS is a probable consequence of strenuous exercise. This leads to the introduction of carbonyl groups into protein side chains by a site-specific mechanism. The specialized type of Western blot using the Oxyblot™ protocol and reagents enable the immunodetection of these carbonyl groups, which gives an indication of the oxidation status of specific proteins. The main advantage offered by the Oxyblot™ method for detection of protein oxidation relative to spectrophotometric methods for detection of protein oxidation is the fact that proteins are separated and individual proteins can be analyzed quantitatively by comparison of signal intensity of the same protein in different lanes.

Although this Oxyblot™ technique has the potential to be very powerful, it consists of various successive steps, increasing the probability of making errors while carrying out the experiment. Therefore the use of controls plays a particularly significant role in this technique.

The challenge we faced during this experiment was that we didn't observe any protein bands on the photographic film after the experiment has been carried out (except for the one assay that was reported in the results). Some of the possible reasons for this include:

- The addition of DNPH groups during derivatization was unsuccessful.
- No transfer of protein sample during electroblot took place. (This could be caused by poor electroblot conditions, orientation of the membrane and gel with respect to the electrodes of the transfer apparatus, improper membrane hydration, failure of the power supply or the electroblot transfer apparatus)
- Either of the antibodies does not bind properly to their substrates.
- The 2°Ab has lost its peroxidase activity.
- The chemiluminescent reagent is not recognized by the 2°Ab's peroxidase enzyme, i.e. has deteriorated.

- The photographic film, developer or fixer is not working.

From control experiment A it can be seen that the electrophoresis was carried out successfully and that the protein sample separated into bands (Figure 5.8; ii).

From the results of the controls for evaluating the success of transfer of proteins samples from SDS-PAGE gel to PVDF during electroblot transfer (control experiment B), it can be seen that a sufficient amount of protein has been transferred from the SDS-PAGE gel onto the PVDF membrane (Figure 5.8; iii) and almost no protein remained left on the electroblot gel (Figure 5.8; i).

A chemiluminescent signal was obtained for both the 2°Ab and chemiluminescent LMW dot blots following incubation with the chemiluminescent substrate (control experiment C). These results show that the peroxidase activity of the 2°Ab and chemiluminescent LMW are still working. It also shows that the chemiluminescent substrate used for detecting the peroxidase activity is working. The fact that a signal was obtained by these dot blot samples implies that the photographic film, developer and fixer used for this experiment are also working.

A signal was obtained for the protein standard dot blotted onto the PVDF membrane and incubated by both 1°Ab and 2°Ab (control experiment D; Figure 5.10). This proves that both antibodies are still working. No signals were obtained for either of the samples that were incubated by the 2°Ab alone, as expected. These are negative controls to ensure that non-specific binding is not taking place. The fact that no signal was obtained for the oxidized, derivatized control sample (which had to be derivatized to attach a DNP residue for recognition by the 1°Ab) while a signal was obtained for the protein standard (which is already pre-DNP-lated) under the same

experimental conditions raises the question of whether the derivatization procedure followed to attach DNP groups to the protein sample was effective.

The results obtained from the Western blot that showed bands at the oxidized, derivatized control sample lane but not in the protein standard lane (Figure 5.7) is contradictory to the above-mentioned results. Both these experiments prove that the 1°Ab and 2°Ab are working. However, the Oxyblot™ results showed that the derivatization reaction carried out was successful, since DNP groups of the derivatized samples could be recognized by the 1°Ab, eventually leading to a signal. For some unknown reason the protein standard did not give a signal during this experiment.

By combining the results from all the controls carried out, the following conclusions can be drawn.

- The electrophoresis carried out was successful (control experiment A).
- The electroblotting conditions are satisfactory (control experiment B).
- Both 1°Ab and 2°Ab are working (control experiments C and D).
- The chemiluminescent substrate is working (control experiments C and D).
- The chemiluminescent LMW is working (control experiment C).
- The photographic film, developer and fixer used are working (control experiments C and D).
- The protein standard is working (control experiment D).

The conditions as described in the protocol of the user's manual for the Oxyblot™ kit was used for carrying out the experiments. Therefore no explanation can be found as to why the protein standards don't give a signal following the Oxyblotting procedure, although it gives a signal following direct dot blotting and antibody incubations.

From the dot blot experiments, the derivatization reaction of the protein samples have been identified as a probable source of error during the dot blot

experiments since a signal was obtained when the proteins standard was dot blotted but not for do blotted derivatized protein sample.

4 DISCUSSION AND CONCLUSION

During exercise, different types of ROS are generated. These different ROS don't attack cellular targets in a random manner. Some are more effective in inducing oxidative DNA damage or lipid peroxidation than they are at modifying proteins and vice versa (Dalle-Donne *et al*, 2003b). It has even been shown that protein oxidation can result without lipid peroxidation being induced when fresh human plasma is exposed to cigarette smoke (Cross *et al*, 1993).

Therefore, to obtain a representative impression of the effect of exercise-induced oxidative stress on the cellular milieu, it is advisable to employ a variety of biomarkers. For this reason, it was attempted to use the spectrophotometric DNPH Assay as well as the Oxyblot™ method in addition to the TBARS Assay and DPPH Assay as a biomarker for measuring oxidative stress on proteins following strenuous exercise.

Carbonylated proteins have a major advantage over lipid peroxidation products as markers of oxidative stress: oxidized proteins are generally more stable. They form early and circulate in the blood for longer periods compared with other parameters of oxidative stress such as MDA (Dalle-Donne *et al*, 2003b). The usage of protein carbonyl groups as biomarkers of oxidative stress has some advantages in comparison with the measurements of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins (Dalle–Donne *et al*, 2003b).

Although both the spectrophotometric DNPH Assay and the Oxyblot™ method measures carbonylated proteins by using the DNPH reagent, they are distinctly different techniques each with its own advantages and disadvantages. It was thought that by using them complementary to each other, the benefits of each of these assays could be appreciated. The spectrophotometric DNPH Assay was one of our assays of choice, because of

its popularity as marker for oxidized proteins. Because of its relative ease, low cost and high throughput, it was thought that the spectrophotometric assay would be a suitable assay for screening the plasma samples of the exercised volunteers and determine whose plasma samples showed an increase in protein carbonylation following exercise. Then, once this has been determined by the spectrophotometric DNPH Assay, the Oxyblot™ method would only be implemented on those samples that showed an increase in carbonylation following exercise. By using the Oxyblot™ method, more specific information can be obtained on the pattern of carbonylation of plasma proteins, which is not possible to determine with the spectrophotometric DNPH Assay. For example, using the western blot immunoassay, Schachter and co-workers found that plasma fibrinogen was more susceptible to oxidative modification compared to the other major plasma proteins, albumin, immunoglobulins and transferrin (Schachter *et al*, 1994). This type of information can not be provided by the spectrophotometric DNPH Assay.

The Oxyblot™ method also offers the advantage that it is more sensitive than the spectrophotometric DNPH Assay and that changes in proteins that are present in smaller amounts than major plasma proteins such as albumin are more likely to be picked up. By using the spectrophotometric DNPH Assay for initial screening instead of subjecting all the samples of the volunteers directly to the Oxyblot™ method, the disadvantages of the Oxyblot™ method such as its expense, labor intensity, time demands and low throughput relative to the spectrophotometric DNPH Assay are bypassed.

Although the spectrophotometric DNPH Assay seemed like a suitable assay for our purpose at first, and assured by its popularity, this ended up not being the case. As explained in detail in the Discussion of the DNPH Assay, our own experience that it gives rise to too high variability when employed for measuring *in vivo* oxidation in plasma samples was confirmed by criticism of this assay in literature. Therefore, the spectrophotometric DNPH Assay could

not be used for the initial screening of plasma samples for the Third Exercise Study as planned.

Despite the great potential of the Oxyblot™ method, we experienced problems with the commercial kit we used. After repeated attempts to get this method working and extensive troubleshooting as described in detail under the Results of the Oxyblot™ method, we were still unable to obtain results. The troubleshooting that we did indicated that the reagents in the kit for the first step, namely the derivatization of the carbonylated proteins, were faulty. After having invested a large amount of time and money in this method, we finally decided not to continue with it.

CHAPTER 6

CONCLUDING DISCUSSION

Exercise provides a suitable marker to study the dynamic balance between oxidative stress and antioxidants. The purpose of this study was to establish an exercise protocol able to consistently induce oxidative stress. Such an exercise protocol would have been useful to evaluate the *in vivo* antioxidant properties of dietary antioxidants thereby providing insight into the bio-availability of such antioxidants.

1 THE EXERCISE STUDIES

1.1 Literature on changes in total antioxidant capacity and TBARS levels following exercise

The reports in literature regarding the effect of exercise on various oxidative stress parameters, including total antioxidant capacity and lipid peroxidation as estimated by the TBARS Assay are inconsistent. The major reasons for these inconsistencies in results have been listed in the Introduction of Chapter 2.

Several studies measuring changes in plasma total antioxidant capacity following exercise found increases in oxidative stress levels (Child *et al*, 1999, Ginsburg *et al*, 2001, Santos-Silva *et al*, 2001). However, Alessio *et al* (1997) found no increase in plasma total antioxidant capacity in response to 30 min of exercise.

The most commonly used marker to estimate oxidative stress imposed by exercise is lipid peroxidation. For this purpose the TBARS Assay is the most popular method.

Several studies report that single bouts of exercise increase blood levels of lipid peroxidation (Davies *et al*, 1982, Hartmann *et al*, 1995, Koska *et al*, 2000, Miyazaki *et al*, 2001). There are also some reports indicating no change in plasma lipid peroxidation following exercise (Viinikka *et al*, 1984, Niess *et al*, 1996, Dufaux *et al*, 1997, Duthie *et al*, 1990). Moreover, decreased levels of plasma lipid peroxidation in response to exercise have also been reported (Rokitzki *et al*, 1994, Hubner-Wozniak *et al*, 1994, Child *et al*, 1999). For a comprehensive literature review of studies on the effect of exercise on markers of lipid peroxidation, refer to Radak (2000).

1.2 Subject selection criteria and exercise protocol

We selected a group of young, healthy but not fit, male volunteers to participate in this study. The ideal subject for this study would be one that responds to the exercise protocol with a dramatic increase in oxidative stress. It has been shown that females tolerate exercise-induced oxidative stress more effectively and recover better following exercise (Ilhan *et al*, 2004). This makes females less suitable volunteers for this study. Furthermore, regular training exercise stimulates various adaptive responses making trained volunteers more fit for responding against free radical attack caused by exercise (Leaf *et al*, 1999 and Powers *et al*, 1999). Because of the up-regulation of antioxidant defense systems due to training and thereby the blood's increased resistance to oxidative stress, trained volunteers are not expected to show a strong oxidative stress response following exercise. This makes fit volunteers not suitable for our study. Although younger volunteers have better antioxidant defenses than older volunteers (Kostka *et al*, 2000), we did not want to subject older volunteers to such a strenuous exercise protocol. It was thought that young, healthy volunteers would cope best with the physically demanding exercise protocol. By using volunteers of similar age, gender and fitness level, the inter-individual variations caused by these factors could be minimized.

The exercise protocol was optimized after the First and Second Exercise Studies. The exercise protocol for the Third Exercise Study was carefully selected in consultation with a biokineticist. It has been shown that high intensity aerobic exercise for a moderate amount of time is most likely to induce oxidative stress in volunteers (Goldfarb, 1992 and Quindry *et al*, 2003). Since our volunteers were not fit, we considered the spinning exercise class of 30 min as an ideal protocol as far as exercise intensity and duration is concerned.

The increase in heart rate of the volunteers following the exercise protocol, as well as their RPE and pain questionnaire scores confirm that the exercise protocol was very intensive and, based on this, we expected to observe an increase in oxidative stress levels as measured by the biochemical assays.

1.3 Selection of biochemical assays

1.3.1 DPPH Assay

The DPPH Assay was the method of choice for measuring the antioxidant capacity of the low molecular weight antioxidants in the blood. This assay was used to assess the *in vitro* antioxidant capacity of various teas by our laboratory (Du Toit *et al*, 2001). Since the ultimate aim of our study was to establish an exercise to use to determine the *in vivo* antioxidant capacity of these teas, the DPPH Assay seemed to be the logical choice. By using the same assay, direct comparisons can be made between the *in vitro* and *in vivo* antioxidant capacity of the various teas. Furthermore, it has previously been shown that strenuous aerobic dancing exercise caused a decrease in antioxidant capacity in serum and saliva using the DPPH Assay (Atsumi *et al*, 1999).

However, it should always be kept in mind that the DPPH Assay uses deproteinized plasma and therefore the non-protein, low molecular weight

antioxidants are measured. The antioxidant contribution of the thiol groups of proteins that act as chain breaking antioxidants are not taken into account. The low molecular weight antioxidants like glutathione and uric acid are easily influenced by exercise (Atsumi *et al*, 1999) and dietary intake (Papas, 1996) making it a suitable marker for bio-availability studies. A further advantage of the DPPH Assay for use in our exercise study is that the reduction of the DPPH signal is comparable with the scavenging of the superoxide anion, which is the main contributor to oxidative stress during exercise (Atsumi *et al*, 1999). Plasma proteins contribute significantly to the total antioxidant capacity of blood, with albumin, the major plasma protein, being estimated to contribute to about 30% of the total antioxidant capacity according to the ORAC and TEAC Assays (Cao and Prior, 1998). This contribution to the total antioxidant capacity is not taken into account with the DPPH Assay and therefore caution should be exercised when interpreting the antioxidant capacity results of the DPPH Assay. The DPPH Assay has also been criticized for the fact that DPPH activity is sterically hindered by bulky molecules and that it might therefore give an underestimation of the total antioxidant activity (Atsumi *et al*, 1999). Furthermore, the DPPH radicals not only interact with antioxidants but also with alkyl radicals (Frankel and Meyer, 2000).

1.3.2 TBARS Assay

The TBARS Assay is the most popular assay to measure lipid peroxidation caused by exercise. It has the advantages of being a simple and inexpensive technique. By using this assay, increases, decreases and no changes in lipid peroxidation levels following exercise have been reported in literature as discussed previously. Although this assay has been criticized for being a nonspecific technique when applied to plasma it is able to offer empirical insight into the complex processes of lipid peroxidation. Since lipid peroxidation is the main cellular consequence of oxidative stress, it can be considered a suitable biomarker for measuring oxidative stress.

2 POSSIBLE EXPLANATIONS FOR RESULTS

It was expected that our exercise protocol would induce oxidative stress in the volunteers, leading to an increase in lipid peroxidation as measured by the TBARS Assay and a decrease in antioxidant capacity as measured by the DPPH Assay. We found a 2% increase in the TBARS values of the volunteers following exercise but this did not reach statistical significance ($p = 0.106$). We also found a paradoxical increase in plasma (9%, $p = 0.03$) and saliva (20%, $p = 0.005$) antioxidant values as measured by the DPPH Assay indicating a significant decrease in oxidative stress following exercise.

There are many possible explanations for these unexpected observations:

It might be possible that during the exercise the body already adapts to the induced stress. The exercise duration was an average of 30 min, which might give the body sufficient opportunity to start reacting to the stress by starting to up regulate antioxidant defenses. If this were the case, it might explain the increase in plasma antioxidant levels, which indicates an increase in antioxidant defenses.

The antioxidant response following an oxidative stress challenge such as strenuous exercise is a dynamic response changing rapidly with time (Mikami *et al*, 2000). Exercise studies usually measure TBARS levels directly following exercise. However, from the literature it is unclear when TBARS levels reach a peak following exercise. Work by Kanter *et al* (1988) showed evidence that oxidative stress induced by exercise occurs mainly in the early post-exercise phase following an 80 km race. However, a peak level of serum TBARS following downhill running has been reported 6 hours following exercise by Maughan *et al* (1989). For a study performed by McAnulty *et al* (2004) plasma samples were collected before, directly after and 15 and 30 min after strenuous running exercise. They found a significant increase in lipid peroxides 15 min following exercise, but no significant increase directly

following exercise or 30 min later. As plasma TBARS levels and peak values subsequent to exercise may vary according to exercise intensity, we cannot exclude the possibility that we missed the maximal plasma levels of TBARS.

It has been shown that during high intensity exercise intracellular uric acid is rapidly replenished by uptake from plasma after exercise (Hellsten *et al*, 2001). If other low molecular weight antioxidants are mobilized from the blood to the muscles following exercise, a decreased level of blood antioxidants might imply a higher level of antioxidants in muscle tissue where protection is needed. In this case, an apparent decrease in total antioxidant capacity in plasma might in fact be indicative of an increase in total antioxidant capacity in muscle cells, with the total antioxidant capacity in muscle cells being of physiological relevance. On the other hand, a rise in plasma antioxidants following exercise, similar to what we have observed, might also reflect a rapid redistribution (Meydani *et al*, 1989).

Some plasma antioxidants, like ascorbic acid, uric acid and ubiquinol, have a short lifetime and the effect of storage of the plasma samples should be taken into account. It has been shown that the plasma antioxidant capacity as measured by the TRAP (Total Radical-Trapping Antioxidant Parameter) Assay shows a modest drop during the first 4 hours of storage at -80°C, followed by a significant and rapid loss within the first 3 days of storage (Ghiselli *et al*, 2000). On this basis, it is recommended that antioxidant capacity assays are run immediately after blood collection and plasma separation. When laboratory conditions do not allow this procedure, quick plasma separation should be followed by storage at -80°C and samples should be analyzed for antioxidant capacity within 3 days. Another study demonstrated that the antioxidant capacity of human serum as measured by the ORAC (Oxgen Radical Absorbance Capacity), FRAP (Ferric Reducing Antioxidant Power) and TEAC (Trolox Equivalent Antioxidant Capacity) Assays are stable over an 8-week time period (Cao and Prior, 1998). The effect of storage of plasma samples on the antioxidant capacity as measured by the DPPH Assay has not

yet been investigated. Atsumi *et al* (1999) analyzed the plasma and saliva samples of 86 volunteers using the DPPH Assay and although it is not stated for what period they stored the samples, it was probably for a long period due to the number of volunteers and therefore the number of samples. Due to the high throughput of samples for assays in our exercise studies, it was necessary to store samples up to two months at -80°C before analysis. During this period, the antioxidants in the samples for the DPPH Assay might have been oxidized.

Groussard *et al* (2003) found a paradoxical decrease in plasma TBARS of volunteers following a single anaerobic sprint, suggesting that this exercise stimulates the elimination of MDA. Their other markers confirmed that oxidative stress was induced by the exercise protocol and they therefore concluded that the plasma TBARS level is not a suitable marker during this type of exercise. Other authors have also reported a post-exercise decrease in plasma MDA or TBARS in humans following maximal aerobic exercise (Margaritis *et al*, 1997 and Rokitzki *et al*, 1994). Moreover, Leaf and his group (Leaf *et al*, 1997) observed no changes in plasma MDA after a maximal exercise test to exhaustion, whereas pentane and ethane, two suitable markers of lipid peroxidation, were shown to increase in expired gas. A likely explanation for this observation was that MDA was removed from plasma during exercise recovery. If this is true, plasma MDA might not be a suitable marker for oxidative stress following exercise.

3 FUTURE RECOMMENDATIONS

The ultimate aim of our studies is to develop a standardized exercise protocol known to be able to consistently induce oxidative stress in healthy volunteers. The effect of consumption of various antioxidants before participating in the exercise protocol on the changes in the oxidative stress-antioxidant parameters will then give an indication of the bio-availability of the antioxidants.

Based on the work that we performed, many recommendations for future studies in this challenging field can be made. These recommendations range from minor modifications in the methodology we used to major changes in the choice of biomarker and even choice of the model to induce oxidative stress. These recommendations for future studies will be discussed below. It should be stressed that these recommendations are suggestions in an ideal setup, and that the implementation of these suggestions might not always be feasible with the limited resources available in practice.

3.1 Subject screening

In this study, we selected a subject population homogenous in terms of age, gender, fitness level, BMI and general health. Even so, great inter-individual variation existed in their response to the exercise protocol. Major causes for this variation probably include lifestyle differences and genetic differences over which an investigator has limited control when using human subjects.

Subjects that respond to exercise by having dramatic increases in oxidative stress will clearly be more suitable subjects for the bio-availability studies. This led to the suggestion that volunteers to be used for bio-availability studies be pre-screened to determine their response to exercise on oxidative stress and only those who responded favorably to be ultimately used for the bio-availability studies.

The possibility of performing such a prescreening procedure was investigated based on the resting heart rates of the volunteers in the Third Exercise Study and their antioxidant responses. It would be expected that volunteers with a higher resting heart rate would be more unfit and therefore more susceptible to oxidative stress induced by strenuous exercise. However, no correlation could be found between resting heart rate values and changes in oxidative stress parameters in these volunteers. Future investigations using other parameters (including anthropometrical data such as BMI as well as pain and

exertion ratings following exercise) to predict oxidative stress responses to exercise in volunteers are warranted.

3.2 Timing of sample collection

To reduce the possibility of missing maximal levels of oxidative stress markers due to collection samples at the non-optimal time point after exercise, it would be advisable to collect samples at different time points following exercise. Based on work in literature, 0, 15 and 30 min following exercise might be suitable time points to consider (McAnulty *et al*, 2004).

3.3 Sample storage

Based on the literature, there is uncertainty as to the period of time plasma antioxidants remain stable *in vitro* (Ghiselli *et al*, 2000 and Cao and Prior, 1998). It is therefore recommended that, when repeating a study similar to ours, that samples be analyzed rapidly. Since no information is available in literature on the effect of sample storage on antioxidant capacity as measured with the DPPH Assay, it will also be worthwhile to do a time experiment to investigate the relationship between total antioxidant capacity and storage time.

3.4 Type of sample

In studies on the effect of exercise on oxidative stress levels in humans blood samples are usually analyzed. This is probably because of the ease with which blood samples are available. However, it is sometimes difficult to interpret the physiological relevance of changes in blood oxidative stress levels. For example, an increase in blood antioxidant capacity could be interpreted as either an overall increase in antioxidants in muscles and subsequently in blood, or it could be interpreted as a mobilization of antioxidants from the muscles to the blood, so that an increase in blood antioxidants could be

indicative of a decrease in muscle antioxidants. The changes that take place in the muscle tissue are of physiological relevance, since it is the primary site of free radical formation during exercise. Although it is not always practically possible, the ideal would be to analyze muscle biopsies to obtain an insight in the oxidative stress processes taking place in the muscle itself during exercise. The chances of finding evidence for oxidative damage during exercise are more likely to be seen in muscle than in blood (Witt *et al*, 1992).

It should also be taken into account that MDA, the most commonly used marker of oxidative stress, diffuses from its source, is metabolized and quickly removed to the urine. Jenkins *et al* (1993) found that TBARS are rapidly cleared from electrically stimulated muscle and that rats fatigued by running showed a significant increase in urinary TBARS. It may therefore be advantageous to analyze urine samples for markers.

3.5 Analysis of other biomarkers

Lipid peroxidation is just one of the consequences of free radical attack due to oxidative stress. Other macromolecules like proteins and DNA are also exposed to free radical damage following exercise. Assays that measure damage to these macromolecules are available making it possible to use them as biomarkers. By measuring for oxidative stress in a variety of different cellular constituents might give a better indication of the processes involved and specific targets of oxidative stress.

3.6 Number of techniques

In order to verify the changes in a certain biomarker, it is advisable to measure the same biomarker with more than one technique. Because of its limited specificity, the TBARS Assay needs to be supported by other assays of lipid peroxidation. Various assays exist for measuring lipid hydroperoxides and

exhaled hydrocarbons (ethane and pentane). It is expected that these assays show the same patterns of change since they measure the same biomarker.

It would also be advisable to support the DPPH Assay with at least one other assay that measures the total antioxidant capacity. The total antioxidant capacity is not a fixed value but should rather be viewed as a concept. The measured antioxidant capacity of a sample depends on which technology and which oxidant is used in the measurement. A study comparing the total antioxidant capacity of human serum as measured by different total antioxidant capacity assays did not find correlations between all the total antioxidant capacities as measured with different assays (Cao and Prior 1998). Different methods to measure total antioxidant capacity cannot replace one another, but should rather be viewed as complementing each other. For this reason it is recommended that different techniques for measuring total antioxidant capacity be used so that a reliable interpretation of the total antioxidant capacity concept can be offered.

3.7 ORAC Assay for measuring total antioxidant capacity

The ORAC Assay seems to be a particularly advantageous measure for total antioxidant capacity; by utilizing different extraction techniques, one can remove plasma proteins and also make some gross differentiations between aqueous and lipid soluble antioxidants (Cao and Prior, 1998). In this way, information can be obtained from not only changes in the total antioxidant capacity in the plasma as a whole, but also from changes taking place specifically for protein antioxidants, lipid soluble antioxidants and water soluble antioxidants. This can provide more insight in the mobilization of the different antioxidant fractions following exercise.

3.8 Enzyme leakage as measure for lipid peroxidation

True oxidative stress should lead to cellular damage. Several authors as listed (Lovlin *et al*, 1987) have indicated that lipid peroxidation is the event most likely to be responsible for increased permeability of and damage to the plasma membrane. Therefore a marker indicating damage to the plasma membrane will be a direct marker of lipid peroxidation caused by oxidative stress. Sumida *et al* (1989) measured the activities of the cytosolic enzyme β-glucuronidase and the mitochondrial isozyme glutamic-oxaloacetic transaminase (m-GOT) in serum following exercise. They found leakage of these enzymes from the muscle tissue to the blood following exercise, concluding that membrane damage due to lipid peroxidation took place. Similarly, Chevion *et al* (2003) found elevated levels of plasma creatine phosphokinase, also reflecting damage to the muscles resulting in a loss of cell membrane integrity and consequent leakage of proteins into the blood. This approach to measuring lipid peroxidation might be of more physiological relevance than measuring TBARS, since it directly proves that membrane damage as a consequence of lipid peroxidation took place, meeting the criterion for true oxidative stress.

3.9 The use of an exogenous marker of oxidative stress

Studies in humans have relied on endogenous markers for measuring exercise-induced oxidative stress. Meijer *et al* (2001) successfully used antipyrine as an exogenous marker for assessing oxidative stress in elderly following exercise. After oral ingestion, antipyrine is completely absorbed and uniformly distributed in the total body water. Antipyrine reacts with hydroxyl radicals, leading to the formation of hydroxylated products of antipyrine, which can be measured by HPLC-MS. Thus, the ratio of the hydroxylated products of antipyrine to the plasma concentration of antipyrine reflects the oxidative stress *in vivo*. The use of an exogenous marker such as antipyrine

has the advantages over the TBARS Assay that it is an extremely sensitive method and does not lack specificity when applied to human plasma.

3.10 The use of serum γ-glutamyltransferase as marker of oxidative stress

Following the recent analysis of serum samples of more than 9000 participants, γ-glutamyltransferase (GGT) was suggested as a novel possible marker of oxidative stress (Lim *et al*, 2004). GGT was shown to be inversely associated with various serum antioxidants including α-carotene, β-carotene, β-cryptoxanthin, zeaxanthin/lutein, lycopene and vitamin C. This suggests that GGT might possibly be a suitable functional biomarker of oxidative stress status since it integrates the effects of exposure of oxidants coupled with a range of antioxidant protective mechanisms *in vivo*. As measurement of GGT is accurate, reliable, easy and inexpensive, it seems to be a promising tool in oxidative stress studies in the future.

3.11 The use of erythrocytes to study *in vivo* cellular antioxidant response

In our study we investigated the extra cellular antioxidant response following exercise by analyzing plasma (and saliva) samples. However, the cellular antioxidant response play an important role during exercise-induced stress, since the primary site of free radical formation is at the mitochondria within the cell. Furthermore, the cellular and extra cellular antioxidant systems are interdependent on each other, and therefore a true insight into the antioxidant response following exercise could only be obtained by investigating the responses of both antioxidant systems. Therefore it is recommended to analyze samples containing cellular antioxidants. For this purpose, erythrocytes might be suitable since they contain antioxidants and are easy to remove from the body.

3.12 The use of albumin as general marker of protein oxidative damage in plasma

The use of albumin as a possible general marker of protein oxidative damage in plasma has been suggested (Jana *et al*, 2002). The antioxidant properties of albumin lie in its ability to act as a sink for radicals by reacting with a variety of ROS (Bourdon and Blache, 2001). Oxidative modifications of albumin have been shown to attenuate its antioxidant capacity (Jana *et al*, 2002).

By using albumin as marker of protein oxidation in plasma instead of using all plasma proteins, the disadvantages of using a crude protein extract as discussed previously can be circumvented making the spectrophotometric DNPH Assay more suitable for determination of protein carbonylation.

3.13 The use of glutamine synthetase activity as measure of protein oxidation

As an alternative to the assays that measure protein carbonylation as marker of oxidative stress, the activity of the enzyme glutamine synthetase can also be used. This enzyme is easily modified by attack of free radicals and therefore serves as a possible marker of the effect of oxidative stress on proteins (Oliver *et al*, 1990). Since the suitability of carbonyls as marker of protein oxidation *in vivo* is questioned by some authors (Urso and Clarkson, 2003), it is advisable to use another marker, such as the glutamine synthetase activity, in addition to carbonyl-based markers for a more reliable indication of oxidative stress in proteins.

4 CONCLUSION

Based on the results of the First, Second and Third Exercise Studies, it can be concluded that the null-hypotheses stated in Chapter 1 can be accepted, namely:

i) There was no statistically significant difference in the plasma TBARS levels before and after strenuous exercise in healthy male volunteers.
ii) There was no statistically significant decrease in plasma antioxidant levels as measured by the DPPH Assay following strenuous exercise in healthy male volunteers.
iii) There was no statistically significant decrease in salivary antioxidant levels as measured by the DPPH Assay following strenuous exercise in healthy male volunteers.
iv) There was no statistically significant difference in plasma oxidative stress levels as measured by the Oxidative Stress Ratio following strenuous exercise in healthy male volunteers.
v) There was no statistically significant correlation between the plasma and salivary antioxidant levels of the volunteers before and after the strenuous exercise in healthy male volunteers.

It should be recognized and appreciated that the body's antioxidant system and its response to exercise is very complex. Although oversimplifying the antioxidant system and its response to exercise is a good starting point for investigation in this field, a more involved approach in future is necessary to gain an in depth insight into the system and the processes involved.

SUMMARY

Title of dissertation: Modulation of oxidative stress parameters in healthy volunteers by strenuous exercise

Student: Yolanda de Haaij

Supervisor: Dr. Z. Apostolides

Department: Biochemistry

Degree: *Magister Scientiae*

Antioxidant additives to foods and beverages are important marketing tools. However, little evidence exists on the bio-availability of such antioxidants. Strenuous exercise is expected to induce reversible oxidative stress in healthy volunteers. This is expected to lead to a decrease in antioxidant levels and an increase in lipid peroxidation levels and the Oxidative Stress Ratio. By measuring the ability of an ingested antioxidant to decrease the exercise-induced oxidative stress, information of its bio-availability can be obtained. Therefore an exercise protocol able to consistently induce oxidative stress can be a valuable tool in antioxidant efficacy and bio-availability studies.

For this dissertation three different exercise studies, the First, Second and Third Exercise Study were undertaken to investigate the effect of strenuous exercise on plasma and salivary oxidative stress parameters in healthy volunteers. The DPPH Assay was employed as a measure for total antioxidants in plasma and saliva and the TBARS Assay was used as a measure of plasma lipid peroxidation. The Oxidative Stress Ratio, a combined value for the DPPH and TBARS markers was calculated from these values. Some sports physiology parameters were also measured.

The possible use of two markers of protein carbonylation as an indication of oxidative stress following exercise was also investigated. They were a spectrophotometric assay, the DNPH Assay and a Western blot method, the Oxyblot™ method.

Not one of the three exercise studies succeeded in modulating the oxidative stress parameters in the expected direction to a statistically significant level. Possible improvements for future studies are discussed and include the prescreening of volunteers to exclude exercise tolerant volunteers.

SAMEVATTING

Die teenwoordigheid van antioksidante in voedsel en vloeistowwe speel ’n belangrike rol in die bemarking daarvan uit ’n gesondheidsoogpunt. Tog is inligting rakende die biobeskikbaarheid van sulke antioksidante beperk. Daar word verwag dat strawwe oefening omkeerbare oksidatiewe stres in gesonde vrywilligers sal veroorsaak. Dit behoort ’n afname in antioksidantvlakke en ’n toename in lipiedperoksidasie tot gevolg te hê. Deur die vermoë van ’n dieetafkomstige antioksidant om die afname in oefening-geïnduseerde oksidatiewe stres te bepaal, kan gevolgtrekkings oor die biobeskikbaarheid van die antioksidant verkry word. ’n Oefenprotokol wat herhaalbaar oksidatiewe stres kan induseer kan op hierdie wyse ’n waardevolle hulpmiddel wees in studies oor die effektiwiteit en biobeskikbaarheid van antioksidante.

Vir hierdie dissertasie was drie verskillende oefenstudies uitgevoer om die effek van strawwe oefening op plasma en speeksel oksidatiewe stres parameters in gesonde vrywiligers te ondersoek. Die DPPH Toets was gebruik om die totale antioksidantvlakke in plasma en speeksel te bepaal en die TBARS Toets is gebruik as maatstaf van lipiedperoksidasie. Die Oksidatiewe Stres Verhouding, a gekombineerde waarde van die DPPH en TBARS Toetse, was uit hierdie waardes bereken. Sommige sportfisiologie parameters was ook gemeet.

Die moontlike gebruik van twee merkers van proteïenkarbonilering as aanduiding van oksidatiewe stres as gevolg van oefening was ook ondersoek. Dit het ’n spektrofotometriese toets, die DNPH Toets, sowel as ’n Westerse klad, die Oxyblot™ metode, ingesluit.

Geen van die drie oefenstudies het daarin geslaag om die oksidatiewe stres parameters in die verwagte rigting en op ’n statisties betekenisvolle manier te verander nie. Moontlike verbeteringe vir toekomstige studies word bespreek. Dit sluit die vooraf uitsifting van vrywilligers wat verwag word om nie met verhoogde oksidatiewe stresvlakke op oefening te reageer nie in.

REFERENCES

ADAMS, S., Green, P., Claxton, R., Simcox, S., Williams, M.V., Walsh, K., Leeuwenburgh, C. (2001). Reactive carbonyl formation by oxidative and non-oxidative pathways. *Frontiers in Bioscience* **6**: a17-24.

ALESSIO, H.M. (1993). Exercise-induced oxidative stress. *Medicine and Science in Sports and Exercise* **25**(2): 218-224.

ALESSIO, H.M., Goldfarb, A.H., Cao, G. (1997). Exercise-induced oxidative stress before and after vitamin C supplementation. *International Journal of Sports Nutrition* **7**: 1-9.

ALESSIO, H.M., Hagerman, A.E., Fulkerson, B.K., Ambrose, J., Rice, R.E., Wiley, R.L. (2000). Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. *Medicine and Science in Sports and Exercise* **32**: 1576-1581.

ALHO, H., Leinonen, J. (1999). Total antioxidant activity measured by chemiluminescence methods. *Methods in Enzymology* **299**: 3-15.

ASHTON, T., Rowlands, C.C., Jones, E., Young, I.S., Jackson, S.K., Davies, B., Peters, J.R. (1998). Electron spin resonance detection of oxygen-centred radicals in human serum following exhaustive exercise. *European Journal of Applied Physiology and Occupational Physiology* **77**(6): 498-502.

ASTRAND, P.-O., Rodahl, K. (1986). *Textbook of work physiology physiological bases of exercise*, 3rd Edition, New York: McGraw-Hill Companies.

ATSUMI, T., Iwakura, I., Kashiwagi, Y., Fujisawa, S., Ueha, T. (1999). Free radical scavenging activity in the nonenzymatic fraction of human saliva: a simple DPPH assay showing the effects of physical exercise. *Antioxidants and Redox Signaling* **1**(4): 537-541.

BENZIE, I.F.F., Szeto, Y.T., Strain, J.J., Thomlinson, B. (1999). Consumption of green tea causes rapid increase in plasma antioxidant power in humans. *Nutrition and Cancer* **34**: 83-87.

BORG, G.A. (1970). Perceived exertion as an indicator of somatic stress. *Scandinavian Journal of Rehabilitation Medicine* **2**: 92-98.

BORG, G.A. (1985). General and differential aspects of perceived exertion and loudness assessed by two new methods. *Reports form the Department of Psychiatry of the University of Stockholm* **636**: 13.

BOURDON, E., Blache, D. (2001). The importance of proteins in defense against oxidation. *Antioxidants and Redox Signaling* **3**: 293-311.

CALCUSYN (1996). Chou, T.-C., Hayball, M.P. *Windows software for dose effect analysis*, Biosoft, UK.

CAO, G., Cutler, R.G. (1995). Protein oxidation and aging. I. Difficulties in measuring reactive protein carbonyls in tissues using 2,4-dinitrophenylhydrazine. *Archives of Biochemistry and Biophysics* **320**(1): 106-114.

CAO, G., Prior, R.L. (1998). Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clinical Chemistry* **44**(6): 1309-1315.

CHAPPLE, I.L., Mason, G.I., Garner, I., Matthews, J.B., Thorpe, G.H., Maxwell, S.R., Whitehead, T.P. (1997). Enhanced chemiluminescent assay for measuring the total antioxidant capacity of serum, saliva and crevicular fluid. *Annals of Clinical Biochemistry* **34**(4): 412-21.

CHEVION, S., Moran, D.S., Heled, Y., Shani, Y., Regev, G., Abbou, B., Berenshtein, E., Stadtman, E.R., Epstein, Y. (2003). Plasma antioxidant status and cell injury after severe physical exercise. *Proceedings of the National Academy of Sciences* **100**(9): 5119-5123.

CHILD, R., Brown, S., Day, S., Donnely, H., Roper, H., Saxton, J. (1999). Changes in indices of antioxidant status, lipid peroxidation and inflammation in human skeletal muscle after eccentric muscle actions. *Clinical Science* **96**: 105-115.

CHLEBOUN, G.S., Howell, J.N., Conatser, R.R., Giesey, J.J. (1998). Relationship between muscle swelling and stiffness after eccentric exercise. *Medicine and Science in Sports and Exercise* **30**: 529-535.

CLARKSON, P.M., Thompson, H.S. (2000). Antioxidants: what role do they play in physical activity and health? *American Journal of Clinical Nutrition* **72**(suppl): 637-646.

CROSS, C.E., O'Neill, C.A., Reznick, A.Z., Hu, M.L., Marcocci, L., Packer, L. (1993). Cigarette smoke oxidation of human plasma constituents. *Annals of the New York Academy of Sciences* **686**: 72-89.

DALLE-DONNE, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R. (2003a). Protein carbonyl groups as biomarkers of oxidative stress. *Clinical Chimica Acta* **329**: 23-38.

DALLE-DONNE, I., Giustarini, D., Colombo, R., Rossi, R., Milzani, A. (2003b). Protein carbonylation in human diseases. *Trends in Molecular Medicine* **9**(4): 169-176.

DAVIES, K.J., Quintanilha, A.T., Brooks, G.A., Packer, L. (1982). Free radicals and tissue damage produced by exercise. *Biochemical and Biophysical Research Communications* **31** [107(4)]: 1198-1205.

DEAN, R.T., Fu, S., Stocker, R., Davies, M.J. (1997). Biochemistry and pathology of radical-mediated protein oxidation. *Biochemical Journal* **324**: 1-18.

DONNELY, A.E., Clarkson, P.M., Maughan, R.J. (1992). Exercise-induced muscle ddamage: Effects of light exercise on damaged muscle. *European Journal of Applied Physiology* **64**: 350-353.

DUFAUX, B., Heine, O., Kothe, A., Prinz, U., Rost, R. (1997). Blood glutathione status following distance running. *International Journal of Sports Medicine* **18**: 89-93.

DUTHIE, G.G., Robertson, J.D., Maughan, R.J., Morrice, P.C. (1990). Blood antioxidant status and erythrocyte lipid peroxidation following distance running. *Archives of Biochemistry and Biophysics* **282**(1): 78-83.

DU TOIT, R., Volsteedt, Y., Apostolides, Z. (2001). Comparison of the antioxidant content of fruits vegetables and teas measured as vitamin C equivalents. *Toxicology* **166**: 63-69.

FAGAN, J.M., Sleczka, B.G. Sohar, I. (1999). Quantitation of oxidative damage to tissue proteins. *The International Journal of Biochemistry and Cell Biology* **31**: 751-757.

FRANKEL, E.N., Meyer, A.S. (2000). The problems using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture* **80**: 1925-1941.

GERSTENBLITH, G., Renlund, D.G., Lakatta, E.G. (1987). Cardiovascular response to exercise in younger and older men. *Federation Proceeding, Federation of American Societies for Experimental Biology* **46**(5, suppl): 1834-1839.

GHISELLI, A., Serafini, M., Natella, F., Scaccini, C. (2000). Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. *Free Radicals in Biology and Medicine* **29**(11): 1106-1114.

GINSBURG, G.S., O'Toole, M., Rimm, E., Douglas, P.S., Rifai, N. (2001). Gender differences in exercise-induced changes in sex hormone levels and lipid peroxidation in athletes participating in the Hawaii Ironman Triathlon. *Clinica Chimica Acta* **305**: 131-139.

GOLDFARB, A.H. (1992). Antioxidants: role of supplementation to prevent exercise-induced oxidative stress. *Medicine and Science in Sports and Exercise* **25**(2): 232-236.

GOTO, S., Nakamura, A., Radak, Z., Nakamoto, H., Takahashi, R., Yasuda, K., Sakurai, Y., Ishii, N (1999). Carbonylated proteins in aging and exercise: immunoblot approaches. *Mechanisms of Ageing and Development* **107**: 245-253.

GRIFFITHS, H.R. (2000). Antioxidants and protein oxidation. *Free Radical Research* **33**(suppl): 47-58.

GRIFFITHS, H.R. (2002). Chapter 3: Biomarkers. *Molecular Aspects of Medicine* **23**: 101-208.

GROUSSARD, C., Rannou-Bekono, F., Machefer, G., Chevanne, M., Vincent, S., Sergent, O., Cillard, J., Gratas-Delamarche, A. (2003). Changes in lipid peroxidation markers and antioxidants after a single sprint anaerobic exercise. *European Journal of Applied Physiology* **89**(1): 14-20.

HALLIWELL, B. (1996). Oxidative stress, nutrition and health. Experimental strategies for optimisation of nutritional antioxidant intake in humans. *Free Radical Research* **25**(1): 57-74.

HARTMANN, A., Niess, A.M., Grunert-Fuchs, M., Poch, B., Speit, G. (1995). Vitamin E prevents exercise-induced DNA damage. *Mutation Research* **346**(4): 195-202.

HALLIWELL, B., Gutteridge, J. (1999). *Free Radicals in Biology and Medicine*, 3rd Edition, Oxford University Press, England.

HECK, H., Mader, A., Hess, G., Mucke, S., Muller, R., Hollman, W. (1985). Justification of the 4 mmol/l lactate threshold. *International Journal of Sports Medicine* **6**: 117-130.

HELLSTEN, Y., Svensson, M., Sjodin, B. (2001). Allantoin formation and urate and glutathione exchange in human muscle during submaximal exercise. *Free Radicals in Biology and Medicine* **31**: 1313-1322.

HUBNER-WOZNIAK, E., Panczenko-Kresowka, B., Lerczak, K., Posnik, J. (1994). Effects of graded treadmill exercise on the activity of blood antioxidant enzymes, lipid peroxides and nonenzymatic antioxidants in long distance skiers. *Biology of Sport* **11**(4): 217-226.

ILHAN, N., Kamanli, A., Ozmerdivenli, R., Ilhan, N. (2004). Variable effects of exercise intensity on reduced glutathione, thiobarbituric acid reactive substance levels and glucose concentration. *Archives of Medical Research* **35**: 294-300.

JANA, C.K., Das, N., Sohal, R.S. (2002). Specificity of age-related carbonylation of plasma proteins in the mouse and rat. *Archives of Biochemistry and Biophysics* **397**(2): 433-439.

JENKINS, R.R., Krause, K., Schofield, L.S. (1993). Influence of exercise on clearance of oxidant stress products and loosely bound iron. *Medicine and Science in Sports and Exercise* **25**: 213-217.

JENTZSCH, A.M., Bachmann, H., Furst, P., Biesalski, H.K. (1996). Improved analysis of malondialdehyde in human body fluids. *Free Radicals in Biology and Medicine* **20**(2): 251-6.

KANTER, M.M., Lesmes, G.R., Kaminsky, L.A., Laham-Saeger, J., Nequin, N.D. (1988). Serum creatine kinase and lactate dehydrogenase changes following an eighty kilometers race. *European Journal of Applied Physiology* **57**: 60-63.

KARLSSON, J. (1997). *Antioxidants and exercise*, 1st Edition, Human Kinetics Europe Ltd., Europe.

KOSKA, J., Blazicek, P., Marko, M., Grna, J.D., Kvetnansky, R., Vigas, M. (2000). Insulin, catcholamines, glucose and antioxidant enzymes in oxidative damage during different loads in healthy humans. *Physiology Research* **49**(suppl): 95-100.

KOSTKA, T., Drai, J., Berthouze, S.E., Lacour, J.-R., Bonnefoy, M. (2000). Physical activity, aerobic capacity and selected markers of oxidative stress and the antioxidant defence system in healthy active elderly men. *Clinical Physiology* **20**(3): 185-190.

LAC, G. (2001). Saliva assays in clinical and research biology. *Pathologie Biologie* **49**(8): 660-667.

LANGLEY-EVANS, S.C. (2000). Consumption of black tea elicits an increase in plasma antioxidant potential in humans. *International Journal of Food Sciences and Nutrition* **51**: 309-315.

LEAF, D.A., Kleinman, M.T., Hamilton, M., Barstow, T.J. (1997). The effect of exercise intensity on lipid peroxidation. *Medicine and Science in Sports and Exercise* **29**: 1036-1039.

LEAF, D.A., Kleinman, M.T., Hamilton, M., Deitrick, R.W. (1999). The exercise-induced oxidative stress paradox: the effects of physical exercise training. *American Journal of Medical Sciences* **317**: 295-300.

LEENEN, R., Roodenburg, A.J., Tijburg, L.B., Wiseman, S.A. (2000). A single dose of tea with or without milk increases plasma antioxidant activity in humans. *European Journal of Clinical Nutrition* **54**: 87-92.

LEVINE, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B., Shaltiel, S., Stadtman, E.R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology* **186**: 464-478.

LEVINE, R.L., Williams, J.A., Stadtman, E.R., Schachter, E. (1992). Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology* **233**: 346-57.

LIM, J.-S., Yag, J.-H., Chun, B.-Y., Kam, S., Jacobs, D.R. Lee, D.H. (2004). Is serum γ-glutamyltransferase inversely associated with serum antioxidants as marker of oxidative stress? *Free Radicals in Biology and Medicine* **37**(7): 1018-1023.

LIU, J., Yeo, H.C., Overvik-Douki, E., Hagen, T., Doniger, S.J., Chu, D.W., Brooks, G.A., Ames, B.N. (2000). Chronically and acutely exercised rats: biomarkers of oxidative stress and endogenous antioxidants. *Journal of Applied Physiology* **89**: 21-28.

LOVLIN, R., Cottle, W., Pyke, I., Kavanagh, M., Belcastro, A.N. (1987). Are indices of free radical damage related to exercise intensity. *European Journal of Applied Physiology* **56**: 313-316.

MAPLES, K.R., Mason, R.P. (1988). Free radical metabolite of uric acid. *Journal of Biological Chemistry* **263**(4): 1709-1712.

MAY, J.M. (1998). Ascorbate function and metabolism in the human erythrocyte. *Frontiers in Bioscience* **2**: d1-10.

MARGARITIS, I., Tessier, F., Richard, M.J., Marconnet, P. (1997). No evidence of oxidative stress after a triathlon race in highly trained competitors. *International Journal of Sports Medicine* **18**: 186-190.

MAUGHAN, R.J., Donnely, A.E., Gleeson, M., Whiting, P.H., Walker, K.A. Clough, P.J. (1989). Delayed-onset muscle damage and lipid peroxidation in man after a downhill run. *Muscle Nerve* **12**: 332-336.

MAXWELL, S.R.J., Thorpe, P. (1996). Tea flavonoids have little short-term impact on serum antioxidant activity. *British Medical Journal* **313**: 329.

MAXWELL, S.R., Thomason, H., Sandler, D., LeGuen, C., Baxter, M.A., Thorpe, G.H., Jones, A.F., Barnett, A.H. (1997). Poor glycaemic control is associated with reduced serum free radical scavenging (antioxidant) activity in non-insulin-dependent diabetes mellitus. *Annals of Clinical Biochemistry* **34**(6): 638-644.

MCANULTY, S.R., McAnulty, L.S., Nieman, D.C., Dumke, C.L., Morrow, J.D., Utter, A.C., Henson, D.A., Proulx, W.R., George, G.L. (2004). Consumption of blueberry polyphenols reduces exercise-induced oxidative stress compared to vitamin C. *Nutrition Research* **24**: 209-221.

MEIJER, E.P., Coolen, S.A.J., Bast, A., Westerterp, K.R. (2001). Exercise training and oxidative stress in the elderly as measured by antipyrine hydroxylation products. *Free Radical Research* **35**: 435-443.

MELZACK, R. (1975). The McGill Pain Questionnaire: major properties and scoring methods. *Pain* **1**: 277-299.

MELZACK, R. (1987). The short-form McGill Pain Questionnaire. *Pain* **30**: 191-197.

MEYDANI, M., Cohn, M.J.S., Macauley, J.B., McNamara, J.R., Blumberg, J.B., Schaefer, E.J. (1989). Postprandial changes in the plasma concentration of α- and γ-tocopherol

in human subjects fed a fat-rich meal supplemented with fat soluble vitamins. *Journal of Nutrition* **114**: 1252-1258.

MIKAMI, T., Yoshino, Y., Ito, A. (2000). Does a relationship exist between the urate pool in the body and lipid peroxidation during exercise? *Free Radical Research* **32**: 31-39.

MIYAZAKI, H., Oh-Ishi, S., Ookawara, T., Kizaki, T., Toshinai, K., Ha, S., Haga, S., Ji, L.L., Ohno, H. (2001). Strenuous endurance training in humans reduces oxidative stress following exhausting exercise. *European Journal of Applied Physiology* **84**(1-2): 1-6.

MOLLER, P., Wallin, H., Knudsen, L.E. (1996). Oxidative stress associated with exercise, psychological stress and lifestyle factors. *Chemico-Biological Interactions* **102**: 17-36.

NAGLER, R., Lischinsky, S., Diamond, E., Drigues, N., Klein, I., Reznick, A.Z. (2000). Effect of cigarette smoke on salivary proteins and enzyme activities. *Archives of Biochemistry and Biophysics* **379**(2): 229-236.

NAKAMURA, A., Goto, S. (1996). Analysis of protein carbonyls with 2,4-dinitrophenyl hydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis. *Journal of Biochemistry* **119**: 768-774.

NEWHAM, D.J., McPhail, G., Mills, K.R., Edwards, R.H. (1983). Ultrastructural changes after concentric and eccentric contractions of human muscle. *Journal of the Neurological Sciences* **61**: 109-122.

NIESS, A.M., Hartmann, A., Fuchs-Grunert, M., Poch, B., Speit, G. (1996). DNA damage after exhaustive treadmill running in trained and untrained men. *International Journal of Sports Medicine* **17**: 394-403.

OLIVER, C.N., Starke-Reed, P.E., Stadtman, E.R., Liu, G.J., Carney, J.M. Floyd, R.A. (1990). Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. *Proceedings of the National Academy of Sciences* **87**: 5144-5147.

PAPAS, A.M. (1996). Determinants of antioxidant status in humans. *Lipids* **31**(suppl): 77-82.

POWERS, S.K., Ji, L.L., Leeuwenburgh, C. (1999). Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. *Medicine and Science in Sports and Exercise* **31**: 987-997.

POWERS, S.K., DeRuisseau, K.C., Quindry, J., Hamilton, K.L. (2004). Dietary antioxidants and exercise. *Journal of Sports Sciences* **22**: 81-94.

RADAK, Z., Asano, K., Lee, K., Ohno, H., Nakamura, A., Nakamoto, H., Goto, S. (1997). High altitude training increases reactive carbonyl derivatives but not lipid peroxidation in skeletal muscle of rats. *Free Radicals in Biology and Medicine* **22**: 1109-1114.

RADAK, Z., Nakamura, A., Nakamoto, H., Asano, K., Ohno, H., Goto, S. (1998). A period of anaerobic exercise increases the accumulation of reactive carbonyl derivatives in the lungs of rats. *European Journal of Physiology* **435**: 439-441.

RADAK, Z. (Editor) (2000). *Free radicals in exercise and aging*, Human Kinetics Publishing Champaign, Italy.

REZNICK, A.Z., Witt, E., Matsumoto, M., Packer, L. (1992a). Vitamin E inhibits protein oxidation in skeletal muscle of resting and exercised rats. *Biochemical and Biophysical Research Communications* **189**(2): 801-806.

REZNICK, A.Z., Cross, C.E., Hu, M.-L., Suzuki, Y.J., Khwaja, S., Safadi, A., Motchnik, P.A., Packer, L., Halliwell, B. (1992b). Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. *Biochemical Journal* **286**: 601-611.

REZNICK, A.Z., Packer, L. (1994). Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods in Enzymology* **233**: 357-363.

ROKITZKI, L., Logemann, E., Sagredos, A.N., Murphy, M., Wetzel-Roth, W., Keul, J. (1994). Lipid peroxidation and antioxidative vitamins under extreme endurance stress. *Acta Physiology Scandinavia* **151**: 149-158.

QUINDRY, J.C., Stone, W.L., King, J., Broeder, C.E. (2003). The effects of acute exercise on neutrophils and plasma oxidative stress. *Medicine and Science in Sports and Exercise* **35**(7): 1139-1145.

SANTOS-SILVA, A., Rebelo, M.I., Castro, E.M., Belo, L., Guearra, A., Rego, C., Quitanhilla, A. (2001). Leukocyte activation, erythrocyte damage, lipid profile and antioxidantive stress imposed by high competition physical exercise in adolescents. *Clinica Chimica Acta* **306**: 119-126.

SAS INSTITUTE (1996). *SAS User's Guide: Statistics*. SAS Institute, Cary, NC, USA.

SATOH, K., Kadofuku, T., Sakagami, H. (1997). Copper, but not iron, enhances apoptosis-inducing activity of antioxidants. *Anticancer Research* **17**(4A): 2487-2490.

SCHACHTER, E., Williams, J.A., Lim, M., Levine, R.L. (1994). Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay. *Free Radicals in Biology and Medicine* **17**(5): 429-437.

SCHLAGER, G. (1999). *Statistics for biochemists: A primer*. Library of Congress Cataloging-in-Publication Data, USA.

SEN, C.K. (1995). Oxidants and antioxidants in exercise. *Journal of Applied Physiology* **79**(3): 675-686.

SEN, C.K. (2001). Antioxidants in exercise nutrition. *Sports Medicine* **31**(13): 891-908.

SERAFINI, M., Ghiselli, A., Ferro-Luzzi, A. (1996). In vivo antioxidant effect of green and black tea in man. *European Journal of Clinical Nutrition* **50**: 28-32.

STADTMAN, E.R. (1990). Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radicals in Biology and Medicine* **9**: 315-325.

STADTMAN, E.R., Starke-Reed, P.E., Oliver, C.N., Carney, J.M., Floyd, R.A. (1992). Protein modification in aging. *Exercise* **62**: 64-72.

STOCKER, R., Frei, B. (1991). Endogenous Antioxidant Defenses in Human Blood Plasma. In: *Oxidative Stress: Oxidants and Antioxidants*, H. Sies (Editor), Academic Press, Florida, 213-243.

SUMIDA, S., Tanaka, K., Kitao, H., Nakadomo, F. (1989). Exercise-induced lipid peroxidation and leakage of enzymes before and after vitamin E supplementation. *International Journal of Biochemistry* **21**(8): 835-838.

URSO, M.L., Clarkson, P.M. (2003). Oxidative stress, exercise, and antioxidant supplementation. *Toxicology* **189**(1-2): 41-54.

VIINIKKA, L., Vuori, J., Ylikorkala, O. (1984). Lipid peroxides, prostacyclin, and thromboxane A2 in runners during acute exercise. *Medicine and Science in Sports and Exercise* **16**(3): 275-277.

VAN HET HOFF, K., De Boer, H.S., Wiseman, S.A., Lien, N., Westrate, J.A., Tijburg, L.B. (1997). Consumption of green or black tea does not increase the resistance of LDL to oxidation in humans. *American Journal of Clinical Nutrition* **60**: 1125-1132.

WASHKO, P.W., Welch, R.W., Dhariwal, K.R., Wang, Y., Levine, M. (1992). Ascorbic acid and dehydroascorbic acid analyses in biological samples. *Analytical Biochemistry* **204**(1): 1-14.

WITT, E.H., Reznick, A.Z., Viguie, C.A., Starke-Reed, P., Packer, L. (1992). Exercise, oxidative damage and effects of antioxidant manipulation. *Journal of Nutrition* **122**: 766-773.

LIST OF APPENDICES

Appendix A Volunteer information letter: Second Exercise Study

Appendix B Volunteer informed consent: Second Exercise Study

Appendix C Letter of Ethical Approval: Third Exercise Study

Appendix D Volunteer informed consent: Third Exercise Study

Appendix E Volunteer questionnaire: Third Exercise Study

Appendix F Borg's Ratings of Perceived Exertion (RPE)

Appendix G Short Form McGill Pain Questionnaire (SF-MPQ)

Appendix H Method used for removal of outliers: Third Exercise Study

APPENDIX A

VOLUNTEER INFORMATION LETTER: SECOND EXERCISE STUDY

Effect of exhaustive exercise on plasma and salivary antioxidant levels

Dear Volunteer

Thank you for your interest in the above-mentioned trial. According to literature, strenuous exercise induces oxidative stress and therefore reduces antioxidant levels in volunteers. Since this is one of the few ethical ways of inducing oxidative stress in healthy, non-smoking volunteers, it is a useful tool in oxidant-antioxidant studies.

The aim of this study is to establish an exercise protocol that will deplete the antioxidant levels of the volunteers. Once this is achieved, the exercise protocol will be used for further studies on the bio-availability of tea antioxidants.

1 Experimental outline

This trial will be divided into two parts:

(i) Determination of the VO_{2max} levels of the volunteers
(ii) Performing exhaustive exercise

These parts will take place on two different days within the same week and should not take longer than a morning. The study will be carried out at the Sports Research Centre of the University of Pretoria on the L.C. de Villiers Sports Grounds. All the exercise studies will be carried out under the guidance of a biokineticist.

1.1 Determination of the VO_{2max} levels of the volunteers

(Monday, 11 November; 8h00 or 10h00)

For this determination, volunteers will be performing treadmill running while being coupled to a gas analyzer and electrodes to monitor heart rate. The volunteers' maximum oxygen uptake (VO_{2max}) will be determined. This is an indication of the volunteer's fitness level and will determine of the intensity at which he will be

performing the exhaustive exercise later. This is a way of standardizing each volunteer's exercise intensity to his own level of fitness.

1.2 Performing exhaustive exercise

(Thursday, 14 November or Friday, 15 November)

Once the volunteer's VO_{2max} levels have been determined, the volunteers will return to perform exhaustive exercise. The exercise protocol will last for 15 min and the volunteer will perform treadmill running with incremental increases in speed:

65% of VO_{2max} for 5 min
75% of VO_{2max} for 5 min
85% of VO_{2max} for 5 min

The volunteer will be coupled to a gas analyzer and electrodes. The biokineticist will program the treadmill with the exercise protocol and it will only be expected from the volunteer to keep running.

During this part of the experiment, the volunteers' anthropometric data will also be obtained, including BMI (body-mass index) and fat- and muscle percentage. These results will be made available to the volunteers.

Before and after performing the exhaustive exercise, the volunteers will donate saliva samples. Blood will also be drawn before and after exercise by a registered nurse.

2 Criteria

Since various factors can affect the outcome of this study, volunteers will be requested to meet certain criteria:

2.1 Determination of the VO_{2max} levels

- *For this part, the only thing that will be expected from the volunteers is not to engage in sports of strenuous physical activity on the previous day.*

2.2 Performing exhaustive exercise

- *For this part, volunteers should also not engage in exercise on the previous day.*

- *Volunteers should fast overnight, i.e. not eat after 20h00 the previous night and should not eat breakfast or drink anything but water.*
- *Volunteers should rinse their mouths thoroughly after having brushed their teeth.*
- *Volunteers are advised to drink plenty of water during the morning of the exercise trial since it promotes saliva secretion.*

3 Compensation

In order to compensate for the overnight fasting, all volunteers are invited to a breakfast at the restaurant of the High Performance Centre at the L.C. de Villiers Sports Grounds on Monday, 18th November.

4 Other points

- *Volunteers should dress comfortably for the treadmill running*
- *Shower facilities are available, so volunteers can shower and change after the exercises*
- *Each volunteer should sign an informed consent letter.*

Please feel free to ask me about anything that seems unclear.

Regards

Yolanda de Haaij

APPENDIX B

VOLUNTEER INFORMED CONSENT: SECOND EXERCISE STUDY

Effect of exhaustive exercise on plasma and salivary antioxidant levels

(11, 14 and 15 November 2002)

Hereby I, ________________________________ (name of volunteer), give consent to participate in the above-mentioned trial. The procedures have been explained to me and I was given the opportunity to ask questions. I realize participation is voluntarily and that I am allowed to withdraw from this trial at any stage.

Volunteer's signature *Date*

______________________________ ____________________

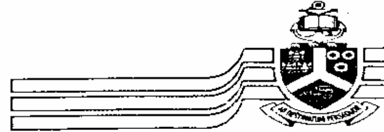
Witnesses' signatures

____

____

APPENDIX C

LETTER OF ETHICAL APPROVAL: THIRD EXERCISE STUDY



University of Pretoria

Faculty of Health Sciences Research Ethics Committee
University of Pretoria
Tel (012) 339 8619 Fax (012) 339 8587
E Mail dbehari@med.up.ac.za
Soutpansberg Road Private Bag x 385
MRC-Building Pretoria
Level 2, Room 20 0001

Date: 19/11/2003

Number	:	S270/2003
Title	:	The effect of strenuous spinning exercise on the plasma and salivary antioxidant levels of healthy male volunteers
Investigator	:	Mrs Y de Haaij, Dept of Biochemistry, University of Pretoria (SUPERVISOR DR Z APOSTOLIDES)
Sponsor	:	None

This Student Protocol has been considered by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria on 18/11/2003 and found to be acceptable.

Prof P Carstens	BLC LLB LLD (Pret) Faculty of Law
Prof S.V. Grey	(female) BSc (Hons); MSc; DSc: Deputy Dean
Prof C B Ijsselmuiden	MD; DTM & H; DPH ;FFCH (CM); MPH; School of Health Systems and Public Health
Prof V.O.L. Karusseit	MBChB; MFGP (SA); M.Med (Chir); FCS (SA): Surgeon
Dr M E Kenoshi	MB,CHB; DTM & H (Wits); C.E.O. of the Pretoria Academic Hospital
Prof M Kruger	(female) MB.ChB.(Pret); Mmed.Paed.(Pret); PhDd. (Leuven)
Dr N K Likibi	MB.BCh.; Med.Adviser (Gauteng Dept.of Health)
Miss B Mullins	(female) BscHons; Teachers Diploma;
Snr Sr J. Phatoli	(female) BCur (Et.Al) Senior Nursing-Sister
Prof H.W. Pretorius	MBChB; M.Med (Psych) MD: Psychiatrist
Prof P. Rheeder	MBChB; M.Med (Int); LKI (SA); MSc (CLIN.EPI): Specialist Physician
Reverend P Richards	B.Th. (UNISA), M.Sc. (Applied Biology) (Knights), M.Sc (Med) (Wits), TechRMS, DipRMS
Dr L Schoeman	(female) Bpharm, BA Hons (Psy), PhD
Dr C F Slabber	BSc (Med) MB BCh, FCP (SA) Acting Head; Dept Medical Oncology
Prof J.R. Snyman	MBChB, M.Pharm.Med: MD: Pharmacologist
Dr R Sommers	(female) MBChB; M.Med (Int); MPhar.Med
Dr TJP Swart	BChD, MSc (Odont), MChD (Oral Path) Senior Specialist; Oral Pathology
Dr S.J.Christa v/d Walt	(female) D. Cur, M.Ed, Department of Nursing
	Student Ethics Sub-Committee
Mrs E Ahrens	(female)B.Cur
Prof S Meij	(female) DScTHED;MSc
Prof P. Rheeder	MBChB;M.Med(Int);LKI(SA);MSc (CLIN.EPI): Specialist Physician
Dr R Sommers	SECRETARIAT (female)MBChB; M.Med (Int); MPharMed
Dr S.J.Christa v/d Walt	(female) D. Cur, M.Ed, Department of Nursing
Mrs N Lizamore	(female) BSc(Stell), BSc (Hons) (Pret),MSc (Pret) DHETP (Pret)
Prof R S K Apatu	MBChB(Legon); PhD(Cambridge)
Adv. Annelize G Nienaber	(female) BA(Hons) (Wits); LLB; LLM (UP); Dipl Datametrics (UNISA)
Dr S I Cronje	DD (UP) – Old Testament Theology
Dr M M (Mimi) Geyser	(female) BSc; MBChB; BSc HONS (Pharm); Dip PEC; MpraxMed

PROF J R SNYMAN
MBChB, M.Pharm.Med MD Pharmacologist
CHAIRPERSON of the Faculty of Health Sciences Research
Main Ethics Committee - University of Pretoria

PROF P. RHEEDER
MBChB; M Med (Int); LKI (SA); MSc (CLIN.EPI): Specialist Physician
CHAIRPERSON of the Faculty of Health Sciences Research
Students Ethics Committee – University of Pretoria

APPENDIX D

VOLUNTEER INFORMED CONSENT: THIRD EXERCISE STUDY

***Title of study**: The effect of strenuous spinning exercise on plasma and salivary antioxidant levels of healthy male volunteers*

Dear Mr. ………………………………………………… Date ……../……../…….

1 The nature and purpose of this study

You are invited to take part in a research study investigating the effect of spinning exercise on the plasma and salivary antioxidant levels of healthy male volunteers.

Performance of strenuous exercise, for example spinning exercise, is one of the few ethical ways of inducing oxidative stress in healthy humans. The oxidative damage caused to cellular constituents by exercise is reversible and the body has different mechanisms of repairing this damage. Therefore, exhaustive exercise has no known detrimental effect on the volunteer.

2 Explanation of procedures to be followed

This study involves performing strenuous spinning exercise carried out at a local gym. To be suitable to participate in this study, you should be male, aged between 19 and 25, generally healthy, not overweight (BMI>25) and not taking any supplements or prescribed medication. You should also not be exercising regularly (more than twice a week) since favorable results are more likely to be obtained from unfit (but generally healthy) volunteers.

Your length, weight and resting heart rate will be measured before the spinning exercise. Blood samples will be taken by a registered nurse from your antecubetal vein (i.e. from your arm) before and directly after the spinning exercise. You will also donate saliva samples before and directly after exercise (the procedure will be explained to you beforehand).

The spinning exercise will be performed in a group of 11 volunteers under the guidance of a spinning instructor. You should try to spin for as long as possible. The total duration of your spinning will be measured. Your heart rate and level of exhaustion (as a number on a scale) will be measured directly after the exercise. This will give an indication of your level of exhaustion. You will also be asked to fill in pain questionnaires 3 days after the exercise as an indication of muscle soreness.

3 Risk and discomfort involved

The only discomfort expected for this trial is the drawing of blood from the vein and muscle soreness associated with unaccustomed exercise.

However unlikely, there is always some risk associated with heavy exercising. The criteria for volunteer selection are such to minimize this risk.

Subject participation in this study is voluntarily. Volunteers themselves therefore accept full responsibility for any unfortunate complication that may occur during the active physical involvement in this experiment.

4 Possible benefits of this study

If this study finds that spinning exercise is able to reduce antioxidant levels in the body, it will have great potential in future investigations of uptake of antioxidants from foodstuffs and supplements into the bloodstream: a foodstuff/supplement whose antioxidants are indeed taken up into the bloodstream after consumption will, if taken before the spinning exercise, prevent or at least mitigate the decrease in antioxidant levels caused by the spinning exercise.

Not only will the scientific community benefit from such a model to investigate the uptake (i.e. bio-availability) and quality of antioxidants ingested. It can also be applied by the industries involved in developing antioxidant supplements for athletes to determine the efficacy of their products.

5 Incentive

Since subjects participate in this study voluntarily, no incentives will be awarded for their participation. Where applicable, the necessary transport costs will be paid to enable volunteers to participate in this study.

6 Withdrawal from study

You have the right to withdraw from this study at any time.

7 Contact persons

If you have any questions concerning this study, you are welcome to contact:

i) Principal investigator:

Dr. Apostolides — Tel (012) 420 2486

ii) Sub-investigator:

Yolanda de Haaij — Tel (012) 420 2011

8 Confidentiality

All data obtained from this study will be regarded as confidential. Results will be published in such a fashion that volunteers remain unidentifiable.

9 Consent to participate in this study

I ………………………………………………………………… (name of volunteer) have read this form and understand the above information. The content and meaning of this information have been explained to me. I have been given opportunity to ask questions and am satisfied that they have been answered satisfactorily. I hereby volunteer to take part in this study.

(Upon signing on the allocated space on the following page, you will receive a copy of this informed consent agreement).

Volunteer:

Signature .... *Date* ....

Name in print ....

I.D. number ☐☐☐☐☐☐☐☐☐☐☐☐☐

Person obtaining informed consent:

Signature .... *Date* ....

Name in print ....

Witness:

Signature .... *Date* ....

Name in print ....

APPENDIX E

VOLUNTEER QUESTIONNAIRE: THIRD EXERCISE STUDY

Volunteer name:
Allocated volunteer number:

NAME AND SURNAME	
Student number	
ID number	
Contact number	
Age	
Gender	
Generally healthy? (yes/no)	
Taking prescribed medication at the moment? (yes/no)	
Smoking? (yes/no)	
Taking dietary supplements? (yes/no)	
How many hours do you exercise per week?	
What type of exercise do you do?	
Height	
Weight	
BMI (kg^2/cm)	
Leg press before exercise	
Leg press 2 days after exercise	
Resting heart rate (before exercise)	
Heart rate after exercise	
Exercise duration	
Borg scale reading	
McGill pain reading (just after exercise)	
McGill pain reading (2 days later)	

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

APPENDIX F

BORG'S RATINGS OF PERCEIVED EXERTION (RPE)

Volunteer name:
Allocated volunteer number:

The ratings below describe the level of exertion you experience during exercise. Please mark the rating that best describes your intensity of pain with a cross (X):

Rating	***Description***	***Your rating (pick only one)***
6	*No exertion at all*	
7	*Extremely light*	
8	*Extremely light*	
9	*Very light*	
10	*Very light*	
11	*Light*	
12	*Moderate*	
13	*Moderate*	
14	*Hard*	
15	*Hard*	
16	*Very hard*	
17	*Very hard*	
18	*Extremely hard*	
19	*Extremely hard*	
20	*Maximum effort*	

APPENDIX G

SHORT FORM McGILL PAIN QUESTIONNAIRE (SF-MPQ)

Volunteer name:
Allocated volunteer number:

1 Pain Rating Index (PRI)

The words below describe average pain. Place a cross (X) in the column that represents the degree to which you feel that type of pain. Please limit yourself to a description of the muscle pain in your legs only.

		None		***Mild***		***Moderate***		***Severe***
Throbbing	*0*		*1*		*2*		*3*	
Shooting	*0*		*1*		*2*		*3*	
Stabbing	*0*		*1*		*2*		*3*	
Sharp	*0*		*1*		*2*		*3*	
Cramping	*0*		*1*		*2*		*3*	
Gnawing	*0*		*1*		*2*		*3*	
Hot-burning	*0*		*1*		*2*		*3*	
Aching	*0*		*1*		*2*		*3*	
Heavy	*0*		*1*		*2*		*3*	
Tender	*0*		*1*		*2*		*3*	
Splitting	*0*		*1*		*2*		*3*	
Tiring-Exhausting	*0*		*1*		*2*		*3*	
Sickening	*0*		*1*		*2*		*3*	
Fearful	*0*		*1*		*2*		*3*	
Punishing-Cruel	*0*		*1*		*2*		*3*	

2 Present Pain Intensity Index (PPI)

2.1 Visual Analog Scale (VAS)

Tick along the scale below for leg muscle pain.

No pain |____| *Worst pain possible*

2.2 Evaluative overall intensity of total pain experience (EOI)

Please limit yourself to a description of the pain in your leg muscles only. Place a cross (X) next to the single most appropriate description:

Evaluative		
0	*No pain*	
1	*Mild*	
2	*Discomforting*	
3	*Distressing*	
4	*Horrible*	
5	*Excruciating*	

3 Scoring

(For office use only)

		Score
I-a	*S-RPI (Sensory Pain Rating Index*	
I-b	*A-PRI (Affective Pain Rating Index*	
I-a+b	*T-PRI (Total Pain Rating Index)*	
II	*PPI-VAS (Present Pain Intensity-Visual Analog Scale)*	
III	*Evaluative overall intensity of total pain experience*	

APPENDIX H

Method used by Dr. Van der Linde (February 2005) for removal of outliers: Third Exercise Study

PROC GLM together with the Output Delivery System of SAS V8.2 was used to determine two statistics namely

(i) the Root Mean Square Error of the dependant variable in an appropriate model using the "FitStatics" variable as supplied by the Output Delivery System and

(ii) the value of the Residual of the dependant variable from the same model using the "OUTPUT" statement.

The Standard Error of the appropriate residuals was determined by dividing the Residual value with the Root Mean Square Error for the group of Residuals.

The "PROBNORM" function of SAS was then applied.

*If the Standardized Residual is found to be < 0 then the Probability associated with the Standardized Residual was determined as 2*PROBNORM(Standardized Residual value). Where the Standardized Residual was found to be >=0 the Probability associated with such residual was determined as the value of PROBNORM(Standardized Residual value).*

The Standard Errors were investigated and as a guide any dependant variable for any respondent for which the Standardized Residual was >= 3 or <= -3, tested values of such a variable were considered for discarding.

A plot indicating the Root Mean Square Error vs. discard determination was performed to assist visually in making the decision of what data to discard as being considered to be an outlier.

Following the conservative discarding of data, appropriate statistics was applied to selected variables or derived variables that remained after appropriate discards were performed.