THE EFFECTS OF HIGH INTENSITY EXERCISE ON LYMPHOCYTE DNA AND ANTIOXIDANT STATUS IN TRAINED ATHLETES

By

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BSc (Hons) (University of Natal)

Submitted in partial fulfilment of the requirements

for the degree of

M Med Sc

in the

Department of Physiology, Faculty of Medicine

University of Natal

Durban

1998

The hunger of the mind can only be appeased by the acquisition of wisdom

...Bhagawan Sri Sathya Sai Baba

ABSTRACT

Apoptosis (programmed cell death) and exercise immunology have been the focus of research for the past five years. Trained athletes are particularly susceptible to a wide variety of viral and bacterial infections and this has been related to oxidative damage which is a mediator of apoptosis. Apoptosis, a normal physiological mechanism has also been implicated in the pathogenesis of a wide variety of diseases. To date, the link between apoptosis and exercise has not been shown by established methods or ultrastructurally. The objective of the study was to determine the effects of a single bout of high intensity exercise on lymphocyte DNA and antioxidant status in trained athletes. The study was carried out in two phases.

In the first phase, 11 trained athletes were subjected to a treadmill run to exhaustion using a ramp protocol to determine their maximum oxygen uptake ($VO_{2\ max.}$). Fifteen millimetres of blood was collected before exercise, immediately after exercise, 24 hours and 48 hours after exercise. Whole blood (4 µl) was used in the determination of DNA damage in lymphocytes using the single cell gel electrophoresis (SCGE) assay. The remaining blood was centrifuged and used for the following: Vitamin C concentration was determined by the 2,4 dinitrophenylhydrazine method, vitamin E concentration was determined by the High Pressure Liquid Chromatography (HPLC) method and lipid peroxides were determined by the measurement of hydroperoxides.

In the second phase, 3 trained athletes who had participated in phase 1, were subjected to a $VO_{2\ max.}$ test. Blood samples (10 ml) were collected before and immediately after exercise, 24 hours and 48 hours later. Lymphocytes were isolated using Histopaque 1077. An *in situ* cell death detection kit, Fluorescein was used for the detection and quantification of apoptosis in lymphocytes at a single cell level, based on labelling of DNA strand breaks. Analysis was carried out using flow cytometry. Lymphocytes were also prepared for Transmission Electron Microscopy (TEM) using conventional techniques.

The results showed that immediately after exercise there was a non-significant decrease in vitamin C concentrations (p=0,16), and a non-significant increase in vitamin E (p=0,82) and lipid peroxide concentrations (p=0,21). There was no significant difference in all 3 levels over the 48 hour period, when compared to the pre-exercise values. The SCGE assay revealed that the immediate post exercise samples showed DNA damage in lymphocytes of all subjects as evidenced by fluorescent strands of DNA outside the cell while DNA damage was observed in only one subsequent sample. In the pre-exercise samples, DNA was visualised as a central core, whereas in all samples taken after exercise, DNA was located at the periphery or confined to one pole of the cell. The pattern of DNA distribution seen in the SCGE assay over the 48 hour period were characteristic features of apoptosis. Flow cytometric analysis showed an increase in apoptosis in lymphocytes immediately after exercise with a further increase after 24 hours. After 48 hours the numbers decreased to control levels. TEM showed that majority of cells were normal before exercise while other lymphocytes were smaller with indented nuclei. Immediately after exercise the lymphocytes displayed features of indented nuclei and microsegregation, cell shrinkage, swelling of the endoplasmic reticulum, mitochondria and Golgi. These changes persisted after 24 hours but were not observed after 48 hours when most of the cells showed normal morphology. The ultrastructural changes observed were also characteristic features of apoptosis.

These results suggest that high intensity exercise may cause an increase in apoptosis as evidenced by DNA damage in the SCGE assay and fully supported by the results achieved during flow cytometry and by the ultrastructural changes observed.

AUTHOR'S DECLARATION

This study represents original work by the author and has not been submitted in any form to another university or institution. Where use was made of work of others it has been duly acknowledged in the text.

The research described in this thesis was performed in the Department of Physiology, University of Natal, under the supervision of Mr A. Chuturgoon and Professor M. Mars.

SN Govender

DEDICATION

I dedicate this work to the greatest teachers in my life. I place this thesis at the Lotus Feet of my beloved teacher Bhagawan Sri Sathya Sai Baba and my parents. They have guided me through my studies and have always showered their choicest Blessings upon me.

PRESENTATIONS AND PUBLICATIONS

PAPERS DELIVERED AT CONFERENCES

1. Effects of high intensity exercise on lymphocytes. **Govender SN,** Bux S, Weston A, Mars M and Chuturgoon AA. Electron Microscope Society of South Africa. December 4-6, 1996. Durban.

2. Effects of high intensity exercise on lymphocyte DNA and vitamin C levels. **Govender SN,** Bux S, Weston A, Mars M and Chuturgoon AA. 14 th Conference of the Society of Biochemistry and Molecular Biology. January 20-22, 1997. Grahamstown.

3. Effects of high intensity exercise on lymphocyte DNA and plasma vitamin C. **Govender SN,** Bux S, Weston A, Chuturgoon AA and Mars M. 7th South African Sports Medicine Association Congress. March 24-26, 1997. Sun City.

4. Evaluation of High Intensity Exercise on Lymphocytes using Electrophoresis, Flow Cytometry and Transmission Electron Microscopy. **Govender SN,** Bux S, Naicker V, Annamalai K, Mars M and Chuturgoon AA. Faculty Research Day, University of Natal Medical School. September 10, 1997. Durban.

5. High intensity short duration exercise - a cause of apoptosis in lymphocytes. **Govender SN,** Bux S, Naicker V, Annamalai K, Mars M and Chuturgoon AA. The 25th Annual Congress of the Physiological Society of Southern Africa and the 2nd Congress of the African Association of Physiological Sciences. September 21-24, 1997. Durban.

6. Flow Cytometric and Ultrastructural Evaluation of High Intensity Exercise on Lymphocytes. **Govender SN**, Bux S, Mars M and Chuturgoon AA. Joint Kwa-Zulu Natal Biochemistry and Microbiology Symposium October 15-17, 1997. Durban.

7. Flow Cytometric and Ultrastructural Evaluation of High Intensity Exercise on Lymphocytes. **Govender SN**, Bux S, Naicker V, Annamalai K, Mars M and Chuturgoon AA. Electron Microscope Society of South Africa. December 2-6, 1997. Cape Town.

ABSTRACTS IN REFEREED JOURNALS

1. **Govender SN,** Bux S, Weston A, Mars M, and Chuturgoon AA. Effects of high intensity exercise on lymphocytes. *Proc. Electron Microsc. Soc. of S.A.*, **26,** 1996.

2. **Govender SN,** Bux S, Weston A, Mars M, and Chuturgoon AA. Effects of high intensity exercise on lymphocyte DNA and plasma vitamin C. *S A Sports Med.*, **4 (1)**, March 1997.

3. **Govender SN,** Bux S, Naicker V, Mars M and Chuturgoon AA. Flow cytometric and Ultrastructural evaluation of high intensity exercise on lymphocytes. *Proc. Electron Microsc. Soc. of S.A.*, **27**, 1997.

PRIZES

1. Gold medal for the Best Junior Basic Science Presentation at Faculty Research Day, University of Natal Medical School. September 10, 1997. Durban.

2. Award for Best Student Life Sciences Presentation at Electron Microscope Society of South Africa Conference. December 2-6, 1997. Cape Town.

ACKNOWLEDGEMENTS

My sincere thanks go to:

Mr A Chuturgoon, supervisor of this study, for his assistance and guidance throughout my Masters degree.

Professor M Mars, co-supervisor of this study for his guidance, constructive criticism and assistance in preparation of this manuscript.

Mrs S Bux for teaching me the microscopy techniques and assisting me in the interpretation of the microscopy results.

Dr A Weston for her assistance and guidance in the exercise laboratory.

Department of Anatomical Pathology for the use of the fluorescent microscope.

James Wesley-Smith and **Priscilla Martins** from the Electron Microscope Unit, University of Natal. Their assistance with regards to the use of electron microscope is greatly appreciated.

Dr V Naicker and **Mr K Annamalai** from the Department of Haematology, University of Natal, Durban for their assistance and guidance in the use of the flow cytometer.

Mrs I Elson and **Mr M. Wagner** from the Analytical Unit, University of Natal, Durban for guidance on HPLC work.

Mrs A Naicker from the Optical and Imaging Unit for the use of the dark room for the processing of photographs.

Mrs M Hurley and **Mrs N Perumal** for making my stay in the Physiology Department a pleasant one.

All the subjects in the study for making themselves available during the competitive season.

Mrs E Gouws of the Medical Research Council for her invaluable advice on the statistical analysis

The **Masters students** of the Physiology Department, University of Natal, Durban for their encouragement and assistance during the study.

My close friends, **Mrs S Mudaly** and **Miss Nikki Coumi** for their friendship and tolerance during my brief moments of insanity. Their willingness to read this manuscript repeatedly bears testimony to the friendship that we have developed during the past two years. Their assistance and guidance is greatly appreciated.

Miss Thesla Palanee, my best friend, for her support, patience, encouragement and faith in my ability. She was always there in my time of need and always brought a smile to my face during the times of adversity. Her wonderful sense humour, her willingness to sacrifice her work to help me and her wonderful character will always be cherished forever.

My **parents**, for always believing in me and encouraging ~~me~~ to be the best that I can be.

My beloved teacher, **Bhagawan Sri Sathya Sai Baba** for being there for me all the time. His guidance and Blessings assisted me in overcoming all my obstacles that I encountered during these difficult two years.

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ABBREVIATIONS

AFCs	antigen forming cells
AIDS	Acquired Immune Deficiency Virus
AS_2O_3	arsenic oxide
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
Ca^{2+}	calcium
CO_2	carbon dioxide
Cu	cupper
$CuSO_4.5H_2O$	cupric sulphate
DAD	diodo array detector
dd	double distilled
DHLA	dhydrolipioic acid
DMSO	dimethylsulfoxide
2,4 DNPH	2,4 dintrophenylhydrazine
DNA	deoxyribonucleic acid
DNAse I	deoxyribonuclease I
EDTA	ethylenediaminetetraacetic acid
ER	endplasmac reticulum
FCM	flow cytometry
Fe	iron
fl	ficolitres
GSH	glutathione
H^+	hydrogen ion
H_2O	water
H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
HBSS	Hanks balance salt solution
HIV	Human Immunodeficiency Virus
HPLC	high pressure liquid chromatography
KCl	potassium chloride
KI	potassium iodide

$KMnO_4$	potassium permangante
$L^{\cdot}$	lipid radical
LDL	low density lipoprotein
LMPA	low melting point agarose
$LO^{\cdot}$	alkoxy radical
$LOO^{\cdot}$	hydroperoxy radical
MDA	malondialdehyde
Mg	magnesium
MHC	major histocompatibility complex
MHR	maximum heart rate
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	sodium dihydrogen orthophosphate
NaN_3	sodium azide
NH_4SO_4	ammonium sulphate
NK	natural killer cells
NP-HPLC	normal phase high pressure liquid chromatography
O_2	oxygen
$O_2^{\cdot-}$	superoxide radical
$OH^{\bullet}$	hydroxyl radical
PBS	phosphate buffered saline
PCD	programmed cell death
P_{O2}	partial pressure of oxygen
PUFA	polyunsaturated fatty acids
rER	rough endoplasmic reticulum
RNA	ribonucleic acid
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
RP-HPLC	reverse phase high pressure liquid chromatography
rpm	revs per minute
SCGE	single cell gel electrophoresis assay
SD	standard deviation
SOD	superoxide dismutase

TBARS	thiobarbiturate
T_C	cytotoxic T cells
TCA	trichloroacetic acid
TCRs	T cell antigen receptors
T_D	delayed type hypersensitivity cells
TdT	terminal deoxynucleotidyl transferase
TEM	transmission electron microscopy
T_H	T helper cells
T_S	suppressor T cells
TUNEL	dUTP nick end labelling
URTI	upper respiratory tract infection
UV	ultraviolet
V'e	pulmonary ventilation
VLDL	very low density lipoprotein
$VO_{2\ max.}$	maximum oxygen uptake test
WBC	white blood cells

CHAPTER ONE

INTRODUCTION

"Activity is the law of life, and without it, any degree of health worthy of the name is an absolute impossibility. A certain degree of moderation and self-restraint is one of the first essentials for success in running for health....never carry your efforts beyond the point at which they impede your health and well-being"

Bernard Macfadden, 1911

Acute respiratory infections have long plagued coaches and athletes during the preparations for major competitions, and military commanders who must ask their units to sustain physical exertion for several days under stressful conditions. The possible relationship between physical exercise and immune function has attracted the attention of the coach who is seeking an early indication of illness and the clinician who is concerned in the prevention of transplant rejections, cancer and autoimmune infections (Shephard *et al.*, 1991). Some athletes believe that while regular training promotes resistance to upper respiratory tract infections (URTI), the actual competition event increases the risk (Nieman, 1994). The Centre for Disease Control has estimated that over 425 million colds and influenza related illnesses occur annually in the United States, resulting in $ 2,5 billion in lost school days and work days. The National Centre for Health Statistics reports that there is an annual incidence of 90 per 100 persons that are afflicted with acute respiratory conditions (Nieman and Nehlsen-Cannarella, 1994). Understanding the relationship between exercise and infection and immunity has potential public health implications, and for the athlete, may mean the difference between being able to compete or missing an event due to illness.

The relationship between physical activity and infection may be modelled in the form of the J curve (Fig 1.1). This model suggests that while the risk of URTI may decrease below that of a sedentary individual when one engages in moderate exercise training, risk may increase above average during periods of excessive amounts of high-intensity exercise.

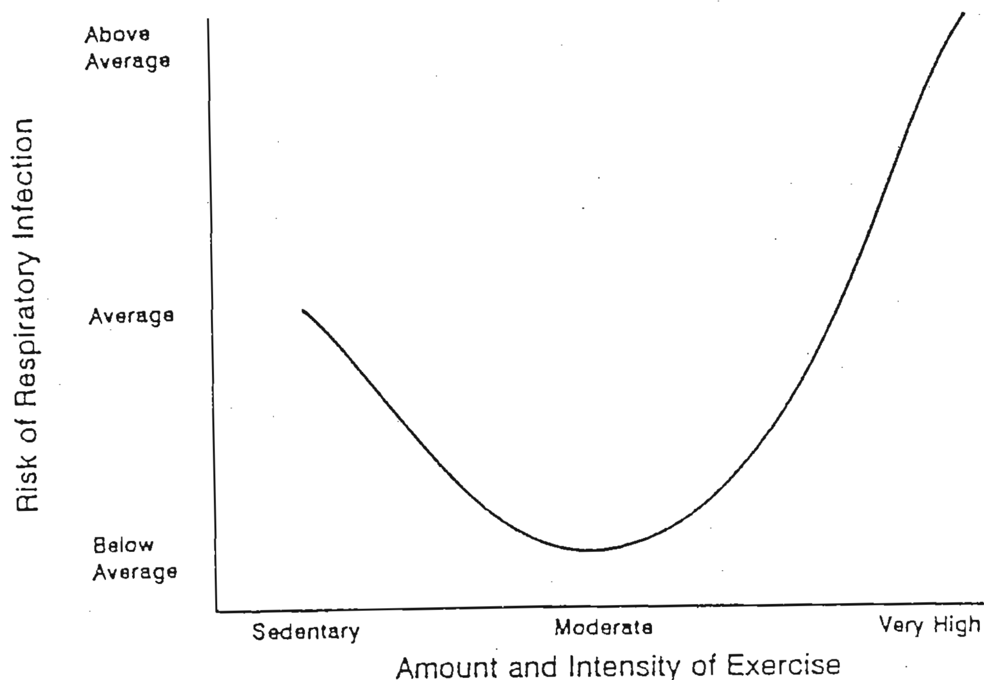


Fig 1.1 J-shaped model of relationship between varying amounts of exercise and the risk of URTI. This model suggests that moderate exercise may lower risk of respiratory infection while excessive amounts may increase the risk (Nieman and Nehlsen-Cannarella, 1994).

The immune system may be a limiting factor in human performance (Fitzgerald, 1988). The immune system is comprised two functional divisions: the innate, which acts as a first line of defence against infectious agents and the adaptive, which produces a specific reaction and immunological memory to each infectious agent when activated (Male, 1991). The innate division is comprised of natural killer (NK) cells and phagocytes including neutrophils, eosinophils and macrophages. The adaptive immune system is comprised of cells (T and B lymphocytes) and immunoglobulins. Exercise, a form of stress has been known to cause alterations in the numbers and function of these cells (Nash, 1994). The earliest and most consistent observation of the exercise-immune interaction has been with regard to the so-called 'leucocytosis of exercise' (Hoffman-Goetz and Pederson, 1994). Although there is a leucocytosis following severe exercise, the blood lymphocyte levels fall below normal and the duration of suppression depends on the intensity and duration of the exercise (Hoffman-Goetz and Pederson, 1994; Haq *et al.*, 1993; Weight *et al.*, 1991; Kendall *et al.*, 1990). Lymphocytes, which are a subset of leucocytes (white blood cells) are the most important cells in defending the body against invasion from foreign material. To date there is a paucity of studies that have investigated the effects of high intensity exercise on leucocyte deoxyribonucleic acid (DNA) damage. Studies by Hartmann and

co-workers (1994,1995) have described evidence of DNA damage of white blood cells (WBC) after high intensity exercise. The reasons for their findings have not been elucidated. However, oxidative stress has been proposed as the cause of damage. There are to date no published reports regarding the effects of exercise on lymphocyte DNA after high intensity exercise.

To date the emphasis in exercise immunology has been based largely on two situations of exercise stress: the situation of acute time-limited exercise stress, such as experienced by the occasional 'weekend exerciser'; and the situation of chronic exercise stress with superimposed acute episodes, observed during high performance athletic competition (Hoffman-Goetz and Pederson, 1994). Far less is known about chronic intermittent exercise stress, experienced by individuals engaging in regular episodic exercise (Hoffman-Goetz and Pederson, 1994).

With the above in mind and considering the minimal amount of literature regarding the effects of exercise on lymphocytes and lymphocyte DNA and the reasons for the decrease in lymphocyte numbers after high intensity exercise, the objectives of this study were to examine the effects of high intensity maximal exercise to exhaustion on:

(1) lymphocytes and lymphocyte DNA in trained athletes; and

(2) antioxidant status which forms a defence against oxidative stress.

CHAPTER TWO

LITERATURE REVIEW

2.1 An overview of the problem

The increasing popularity of regular exercise is probably due to the public awareness of the beneficial effects of exercise on physical and emotional well-being. Many fitness fanatics claim that exercise influences resistance to common infections (Fitzgerald, 1988). On the other hand there have also been anecdotal reports from athletes and their coaches that intense training is associated with increased respiratory infections (Pederson and Bruunsgaard, 1995; Fitzgerald, 1988).

At the 1988 Olympic Games some of the world's best athletes were unable to compete due to illness or injury through overtraining: for example, the respiratory infection that struck UK Olympic gold medallist Sebastian Coe during the UK Olympic trials in August 1987 so affected his running that he failed to qualify for the team to go to Seoul (Fitzgerald, 1988). The immune system may be the limiting factor in human performance. While some individuals can withstand rigorous training and competition schedules without missing a day, others are very susceptible to infections. A robust immune system will allow an athlete to stay free of infection in the same way that a robust physique will allow him to stay free of injury. Overtraining is considered by both top athletes and their coaches to be the prime cause of illnesses that afflict them with increasing frequency as the competitive season progresses. These illnesses range from frequent persistent colds, sore throats and flu-like illnesses to severe debilitating states resembling post viral fatigue syndrome, which can cause an athlete to miss an entire season, or even force him to give up competitive sport completely. The incidence of opportunistic and other infections among top athletes raises the question: can too much exercise have a damaging effect on the immune system? (Fitzgerald, 1988).

2.2 The Immune System

The body's immune system comprises all specific and non-specific defence mechanisms for dealing with foreign materials. It is a highly complex, yet precisely ordered array of cells, hormones and soluble immunomodulators that inhabit the bone marrow, lymphoid tissues and ducts, mucous membranes and peripheral circulation. The overall function of the system is to regulate susceptibility to, severity of, and recovery from infection and neoplastic illness. These functions are normally realised through recognition and/or entrapment of micro-organisms and neoplasia followed by their orderly neutralisation, or by protection provided by the cutaneous and mucosal barriers (Nash, 1994).

The defensive function is performed by leucocytes and a number of accessory cells, which are distributed throughout the body, but are found particularly in the lymphoid organs, including the bone marrow, thymus, spleen and the lymph nodes. Large accumulations of these cells are also found at sites where pathogens may enter the body, such as the mucosa of the gut and the lung. Cells migrate via the blood stream and lymphatic system and as they do so, they interact with each other to generate a co-ordinated immune response aimed at eliminating pathogens or minimising the damage they cause (Male, 1991).

Lymphocytes are the key cells controlling the immune response. They function specifically by recognising foreign material and distinguishing it from the body's other components. They generally react to foreign material but not against the body's tissue. Lymphocytes are of two main types:

B cells which produce antibodies and T cells which have a number of functions including:

(1) helping B cells to make antibody;

(2) recognising and destroying cells infected with viruses;

(3) activating phagocytes to destroy pathogens they have taken up; and

(4) controlling the level and quality of the immune response.

Lymphocytes recognise foreign material by specific cell-surface antigen receptor molecules. To specifically recognise the enormous variety of different molecules, the antigen receptors must be equally diverse. Each lymphocyte makes only one type of antigen receptor, and thus can only recognise a very limited number of antigens. But since the receptors differ on each clone of cells, the lymphocyte population as a whole has a great diversity of specific antigen receptors. Lymphocytes are further subdivided into a variety of subsets (Male, 1991).

2.2.1 Subsets of Lymphocytes

2.2.1.1 B cells

B cells are lymphocytes which develop in the foetal liver and subsequently in the bone marrow. They are distributed throughout the secondary lymphoid tissues, particularly in the follicles of the lymph nodes and the spleen. They respond to antigenic stimuli by dividing and differentiating into plasma cells, under the control of cytokines released by the T cells. Plasma cells or antibody forming cells (AFCs) are terminally differentiated B cells. They have an expanded cytoplasm with characteristic parallel arrays of rough endoplasmic reticulum (rER) and are entirely devoted to the production of secretory antibody. Plasma cells are seen in the red pulp of the spleen, the medulla of the lymph nodes, and in small numbers at the site of inflammation (Male, 1991).

2.2.1.2 T cells

These are lymphocytes which develop in the thymus. This organ is seeded by lymphocytic stem cells from the bone marrow during embryonic development. These cells then develop their T cell antigen receptors (TCRs) and differentiate into the two major peripheral T cell subsets, one which expresses the CD4 marker and the other CD8. T cells can also be differentiated into two populations depending on whether they use an α/β or a γ/δ type of antigen receptor. The essential role of the T cells is to recognise antigens originating from within cells of the host. Different populations of T cells have differentiated functions:

(1) T helper (T_H) cells:

These T lymphocytes help B cells to divide, differentiate and produce antibody. They also release cytokines which control the development of leucocyte lines from haemopoietic stem cells. Other cytokines are required for the development of cytotoxic T cells and cause activation of macrophages, allowing them to destroy the pathogens they have taken up. The majority of the T_H cells are $CD4^+$ and recognise antigen presented on the surface of antigen presenting cells in association with class II molecules encoded by the major histocompatability complex (MHC).

(2) T_H1/T_H2 cells:

These cells are subsets of T helper cells differentiated *in vitro* according to the blends of cytokines they produce. T_H1 cells may promote delayed hypersensitivity reactions while T_H2 cells release cytokines which are particularly required for B cell differentiation. Both types can promote development of cytotoxic T cells.

(3) T delayed hypersensitivity (T_D) cells:

These cells are responsible for bringing macrophages and other inflammatory cells to areas where delayed hypersensitivity reactions occur. These appear to be a particular functional group of T_H cells.

(4) T cytotoxic (T_C) cells:

These cells are capable of destroying virally infected target cells, or allogenic cells. The majority of T_C cells are $CD8^+$ and recognise antigen on the target cell surface associated with MHC class I molecules.

(5) T suppressor (T_S) cells:

These are functionally defined T cells which down regulate the action of other T cells and B cells (Male, 1991).

2.3 Exercise and the Immune System

The sudden temporary changes in the immune system caused by a bout of exercise are called acute responses to exercise, and disappear shortly after the cessation of exercise. The persistent changes in the structure and function of the immune system after regular exercise training are called chronic adaptations to exercise. Cardiorespiratory exercise can be categorised according to the intensity and duration. Moderate and high intensity exercise may be defined as 40%-60% and 60%-100% maximum oxygen uptake ($VO_{2\,max.}$) (McArdle *et al.*, 1996). For most adults, a brisk walk, recreational cycling and swimming, or informal sports play are included in the definition of moderate intensity exercise (Nieman and Nehlsen-Cannarella, 1994).

Running, intensive cycling or swimming, or competitive sports involving continuous movement are defined as high intensity exercise (Nieman and Nehlsen-Cannarella, 1994).

The desirable and undesirable influences of exercise on selected elements of the immune system function have been studied since the first report of violent leucocytosis experienced during the Boston marathon in 1902 was published by Larrabee in 1902.

Since its first description, the relationship between exercise and the immune system has revealed the following:

(1) Exercise alters the distribution and trafficking of peripheral mononuclear cells (McCarthy and Dale, 1988), augments the immune surveillance capacity through heightened natural immunity and stimulates other acute transitory alterations of host defence (Keast *et al.*, 1988).

(2) Several longitudinal endurance training studies and cross-sectional comparisons of trained and untrained cohorts suggest a desirable effect of conditioning on resting immune function (Nehlsen-Canarella *et al.*, 1991).

(3) Exercise training may buffer psychic and nonpsychic stress such as anxiety and depression, thus sparing their commonly suppressing influence on immune competence (Nash, 1994).

(4) Important information has emerged that exercise performed immoderately may hasten immune dysfunction and increase illness susceptibility (Nash, 1994).

Based on the testimonials of fit individuals who report less frequent infections than their untrained counterparts, it has been suggested that improved fitness levels can positively influence the immune response (Fitzgerald, 1988). Additionally the general sense of well-being and euphoria which may accompany chronic exercise may suggest to the participant an overall "healthy" feeling, irrespective of any impact on the types of acute illnesses. Conversely, overtrained athletes often report a deterioration in their health, with increased infections, fatigue and injury (Katz, 1994). Numerous investigations have dealt with the effect of physical activity on the immune response. Difficulties exist in the interpretation of these studies because of a number of variables viz. differences in type of activity, duration of exercise, intensity of exercise, timing of immunological assessment in relation to exertion, fitness level of study subjects and effects of hormonal changes that accompany exercise.

The type of exercise studied for effects on the immune response is inconsistent. It is unclear whether different forms of exercise might induce differences in immunity, for example, do running and rowing similarly alter immune function? The duration and intensity of the exercise also appears to be an important confounding factor. It is difficult to imagine that *in vitro* lymphocyte studies performed on an individual immediately after a 100 m sprint would provide similar results to those obtained following the completion of a marathon.

Interpretation of data is further confounded by differences in fitness levels of the study participants. Many studies include healthy and untrained subjects, while others study the immune function of highly trained athletes. It is difficult to determine whether alterations in immunity are related solely to the type of exercise, the fitness levels of the participant or a combination of these two factors (Katz, 1994).

2.3.1 Changes in leucocyte subpopulations

Numerous studies have established that high intensity, cardiorespiratory exercise is associated with a unique biphasic perturbation of the circulating leucocyte count (Field *et al.*, 1991; Gabriel *et al.*, 1991; Hansen *et al.*, 1991). Acute exercise provokes an increase of peripheral venous leukocyte count roughly proportional to the intensity and duration of activity (McCarthy *et al.*, 1987). Brisk exercise increases the leucocyte count in proportion to the effort. During exercise, cycling an ergometer for 60 minutes at 60% of maximal aerobic capacity, the rise in leucocytes consists mainly of lymphocytes, polymorphonuclear neutrophils and monocytes (Shinkai *et al.*, 1992). The same general pattern of increase occurs during even brief, heavy exertion, such as maximal bicycle ergometry for 30 or 60 seconds. Reports and clinical studies have linked excessive physical activity to an increased risk of infection (Fitzgerald, 1988). Results have shown that while a moderate dose of endurance exercise has a beneficial effect upon immune responses, more intense and more stressful exercise has adverse effects (Shephard *et al.*, 1991). The changes are short lived but there is growing evidence that they have a measurable clinical impact by altering the resistance to viral infections (Shephard *et al.*, 1991). The threshold for adverse reactions appears to depend upon the relative intensity of effort, and regular training can thus shift the threshold for adverse reactions upwards (Shephard *et al.*, 1991).

The neutrophil concentration increases during and after high intensity exercise (McCarthy and Dale, 1988). During exercise, natural killer (NK) cells which are capable of killing a variety of

virally infected and transformed target cells (Male, 1991), and B and T cells are also recruited to the blood, resulting in an elevated lymphocyte count (Katz, 1994). After intense exercise, the lymphocyte level decreases below pre-exercise values and the duration of this suppression depends on the intensity and duration of the exercise (Hoffman-Goetz and Pederson, 1994).

At rest, trained subjects have a slightly elevated non-specific immunity. During exercise, leucocytes are recruited to the blood and if muscle damage occurs the cytokine level is enhanced. Thus, short term, time limited exercise stress induces an inflammatory response. Following long term intense exercise the number of lymphocytes in the blood is suppressed, and the function of NK and B cells is inhibited (Pederson and Bruunsgaard, 1995).

2.3.2 Exercise and lymphocytes

Concentrations of peripheral blood leucocytes, especially lymphocytes and their subpopulations can change in response to many circumstances such as disease, stress, smoking, drugs, age and physical exercise (Westermann and Pabst, 1990). Approximately 2% of the total lymphocyte pool in the normal adult is present in the peripheral blood (Gabriel *et al.*, 1991) and the mean transit time under resting conditions amounts to 30 minutes (Schick *et al.*, 1975). Approximately 5×10^{11} lymphocytes travel under normal conditions in the blood each day, which is comparable to the total number of lymphocytes in the human body. Most of lymphoid and nonlymphoid organs are included in the migration route of lymphocytes (Pabst, 1988). About 50% of the travelling lymphocytes pass through the spleen. Some organs contain many lymphocytes, such as lymph node, spleen and nonlymphoid organs such as the lung. Small alterations in these organs can cause major changes of lymphocytes in the blood (Gabriel *et al.*, 1991).

Acute exercise increases the number of lymphocytes during exercise and this is particularly marked where cycling has been used as the exercise mode, suggesting that results depend on the type of exercise performed (Pederson *et al.*, 1988). Stauber *et al.* (1988) have further suggested that lymphocytes enter the muscle tissue to facilitate repair processes. Migration of lymphocytes to lymphoid tissue increases their potential for phagocytosis and break down my macrophages. During high intensity exercise the lymphocyte concentration increases, however a drop in lymphocyte levels of 30-50% below the pre-exercise levels is observed, remaining at this level for 3-6 hours (Weight, 1996; Nieman, 1994). The increased lymphocyte concentration is due to the recruitment of all lymphocyte subpopulations to the blood. Thus both $CD3^{+}CD4^{+}$ T cells,

$CD3^+CD8^+$ T cells, $CD19^+$ B cells and NK cells increasing during exercise. Simultaneously, the CD4/CD8 ratio decreases, because the CD8 count increases more than the CD4 count. The percentage $CD4^+$ cells decreases mainly because the number of NK cells increase more than any other lymphocyte subpopulation. Accordingly, the relative fraction of lymphocyte subpopulations change (Pederson *et al.*, 1996).

The extent and duration of the alterations are very much dependent on the exercise induced changes in epinephrine and cortisol levels. The concentrations of epinephrine and cortisol begin to increase strongly when the exercise intensity increases to above 60% of VO_{2max}.(Hoffman-Goetz and Pederson, 1994) and their highest levels are reached after near maximal intensity exercise sessions. Immediately post-exercise, the blood concentration of epinephrine decreases rapidly to pre-exercise levels in contrast to that of cortisol that can remain elevated for 2 or more hours (Nieman and Nehlsen-Cannarella, 1994).

Exercise of high intensity is known to induce a rapid increase in the density of lymphocytic β_2-adrenergic receptors (Maisel *et al.*, 1990). Epinephrine is a potent β_2-adrenergic agonist, and it has been associated with substantial increases in the number of circulating lymphocytes that are supplied by several storage sites (primarily the spleen), with some contributions from other lymphoid organs, the lungs and the walls of endothelial venules. Increases in serum cortisol concentrations have been shown to inhibit the entry of lymphocytes into the circulation and facilitate the egress from the blood to other lymphoid compartments (Cupps and Fauci, 1982; Tonnesen *et al.*, 1987). Following high intensity exercise, epinephrine causes a transient increase in the circulating lymphocyte count, whereas longer acting cortisol quickly dominates events during recovery, causing a marked lymphocytopaenia (Nieman and Nehlsen-Cannarella, 1994). Studies to date have examined the influence of exercise on white blood cells, looking at the effect on cell numbers, function and damage (Nieman *et al.*, 1994; Hartmann *et al.*, 1995) however, the cause of damage has not been elucidated

Determination of the proliferative response of human lymphocytes upon stimulation with mitogens *in vitro* is used to evaluate the functional capacity of T lymphocytes. Exhaustive exercise suppresses mitogen stimulated proliferation of separated mononuclear cells by 50%, while short moderate exercise has little or no effect (Weight, 1996). Although moderate exercise appears to stimulate the immune system, there is good evidence that intense exercise can cause immune deficiency (Fitzgerald, 1988). Most of the research has focused on the immune system changes following a single exercise session. Dramatic changes in the number and functional

capacities of lymphocytes have been observed after exhausting exercise. These effects may reflect the transient suppression of cell-mediated immunity sufficient to allow micro-organism, particularly viruses, time to evade early immunological recognition and thus establish infection in the athlete. Exercise to exhaustion results in the temporary reduction in the *in vitro* response to T and B cell mitogen stimulation, a reduction in the CD4:CD8 ratio and changes the NK cell activity. This data was obtained after testing of healthy trained volunteers and sportsmen after a single bout of maximal exercise. Maximal exercise overcomes the problem of any differences in training and fitness, since each subject exercises to his own limit (Fitzgerald, 1988).

The decrease in the mitogenic response is probably due to the relative decrease in T cells compared to NK cells in the post exercise samples. It has been demonstrated that there is a proportionally greater increase in B cells, NK cells and monocytes than in T cells. The T:B ratio decreases, although there does not appear to be a significant change in the T_H:T_S ratio (Weight, 1996). The small percentage of cells that respond to mitogens is due to the differential changes in circulating lymphocyte numbers. Suppression of lymphocyte function has been reported in exhaustively trained athletes (Nieman, 1994). Lymphocytes, neutrophils and macrophages are attracted to the injured muscle cells where they variously phagocytose tissue debris and release soluble products such as cytokines which mediate the inflammatory response (Weight, 1996).

Among the lymphocytes, the number of NK cells usually increases the most during the exercise. The lymphocyte count begins to fall within 5 minutes of ending the exercise and 1 hour later it may be 36% below pre-exercise baseline levels (Nieman *et al.*, 1992). Within minutes after a long race such as a marathon, the blood lymphocyte count tends to be significantly lower than at the pre-race baseline. This correlates with a sharp rise in blood cortisol level during the race. The delayed fall in lymphocytes may in fact relate to a large part to the unopposed action of cortisol as soon as exercise ceases. The action of adrenaline, which brings lymphocytes into the blood, ends soon after the exercise bout, allowing the longer lasting action of cortisol to direct lymphocytes out of the blood into the tissue reservoirs. Usually after 4 to 6 hours of recovery, and nearly always within 24 hours of recovery, the lymphocyte count is back to baseline levels (Eichner and Calabrese, 1994).

In essence, the immune system is enhanced during moderate and severe exercise, and only intense long duration (e.g. marathon) exercise is followed by immunodepression (Pederson *et al.*, 1996). The latter includes suppressed concentration of lymphocytes, suppressed NK and lymphokine

activated killer cytotoxicity cells and secretory IgA in mucosa. During this time of immunodepression, often referred to as the "open window", the host may be susceptible to micro-organisms bypassing the first line of defence. This is of interest to top athletes who perform frequent severe, high intensity exercise without allowing the immune system to recover between each bout. One reason for the "overtraining effect" seen in elite athletes could be that this window of opportunism for pathogens is longer and a degree of immunosuppression may occur if the athlete does not allow the immune system to recover. This condition is accentuated when the athlete initiates a new bout of exercise while still immunodepressed. The decrease in immunity following extreme activity would be compatible with the propensity of "overtrained" individuals to develop URTI (Pederson *et al.*, 1996).

In addition to developing URTI, a considerable body of research has accumulated concerning the effects of exercise and training on indexes of oxygen toxicity and defences against such challenge. Oxygen toxicity may be implicated in the aetiology of a wide variety of pathophysiological conditions e.g. atherosclerosis, retinopathies, muscular dystrophies, some cancers, diabetes, rheumatoid arthritis, ageing and ischemia-reperfusion injury (Sen, 1995). Increased energy demands during physical exercise result in a multifold increase in oxygen supply to the active tissues. The rate of oxygen uptake by the body during exercise may increase by 15 to 20 fold (Sen, 1995). It is in recent years that athletes have begun questioning the detrimental effects of exhaustive exercise. The majority of athletes now know that the body must be protected against the potentially harmful oxygen (O_2) products, which is a paradox since this essential nutrient is also a potential toxin at sea level. There is abundant evidence that O_2 free radicals are involved in the mechanisms of a vast array of diseases described above (Jenkins, 1993).

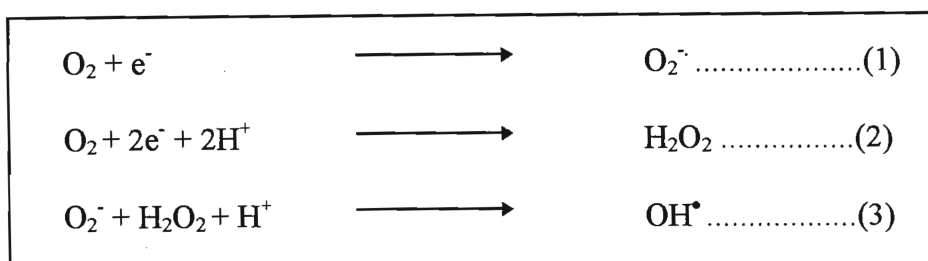
2.4 Free radicals

Free radicals may be broadly defined as molecules or ions containing an unpaired electron and capable of existing freely. Molecular O_2 contains two unpaired electrons with parallel spins and is therefore a radical itself. Ground state molecular O_2, is often therefore referred to as a diradical, triplet O_2, or simply dioxygen. In biological systems, diradical oxygens are reduced to produce energy and water (H_2O). Because molecular O_2 has two unpaired electrons, the reduction process is not simple. According to Pauli's exclusion principle, no more than two electrons may occupy the same atomic orbital, and to do so these electrons must have opposite spins. Univalent reduction of oxygen, refers to the reduction of molecular O_2 by one electron at a time.

Intermediates produced in this process are oxygen derived free radicals that are highly reactive. Some of these reactive intermediates may escape from the process of complete tetravalent reduction and react with biological structures and other components, inciting oxidative damage and producing other reactive oxygen species (ROS) which may include more free radicals and other toxic metabolites (Sen, 1995).

2.4.1 Production of free radicals

Free radicals may be produced by two different pathways. One pathway involves reactions initiated by drugs, alcohol and other foreign toxins. The second involves the enzymatically controlled, one-electron reduction of molecular O_2, an ongoing process that occurs during normal respiration (Kanter, 1994). During respiration O_2 is reduced to form H_2O, however due to certain physical restrictions, oxygen may receive only one electron at a time instead of four. This univalent pathway of oxygen reduction leads to the production of free radicals. Addition of one, two or three electrons leads to the production of the superoxide ($O_2^{\cdot-}$) radicals (reaction 1), hydrogen peroxide (H_2O_2) (reaction 2) and hydroxyl radicals ($OH^{\bullet}$) (reaction 3).



2.4.2 Mechanism of action of free radicals and lipid peroxidation

Membrane lipid peroxidation may alter fluidity and permeability and compromise the integrity of the barrier. Multiple unsaturation points in polyunsaturated fatty acids (PUFA) make them highly susceptible to ROS attack and oxidative damage. The withdrawal of the hydrogen from the PUFA then converts it into an organic radical which may in turn react with an adjacent PUFA and thus alter the membrane's integrity. These ROS which may be generated as a result of increased oxygen consumption in exercising muscle or in neutrophils are carried by the blood to distant targets where oxidative damage may be initiated. These oxygen derivatives may also serve as

precursors for more potent free radicals. The free radicals may be quenched by enzyme systems, however it has been estimated that between 2% and 5% of the total electron flux during normal metabolism "leaks off" to generate free radicals (Boveris and Chance, 1973). These free radicals may attract hydrogens from the PUFA in cells or inclusion membranes. The domino effect that results is known as lipid peroxidation (Jenkins, 1993). The lipid peroxidation sequence is characterised by 3 different steps known as initiation, propagation and termination (Fig 2.1) using the PUFA, linoleic acid as an example. The initiation step results from free radical attack on the methylenic groups between or adjacent to ethylenic groups of PUFA and is followed by a chain of reactions, the propagation step that yields several types of secondary free radicals. These include primary lipid radicals ($L^{\bullet}$), alkoxy radicals ($LO^{\bullet}$) and hydroperoxy radicals ($LOO^{\bullet}$). These free radicals accumulate and thereby disrupt the function of the membrane, causing it to collapse. This reaction is usually terminated when vitamin E, synergistically with vitamin C, converts $LOO^{\bullet}$ to LOOH. In the termination step, a large number of non-radical products, namely alkanes and carbonylic compounds are formed (Vaca *et al.*, 1988). During the various steps of lipid peroxidation, chemical alterations to proteins and DNA may occur. Figure 2.2 shows the possible pathways that may exist when lipid peroxide products and DNA interact. This severe stress can lead to cell transformation or cell death by apoptotic or necrotic mechanisms (Jacob and Burri, 1996).

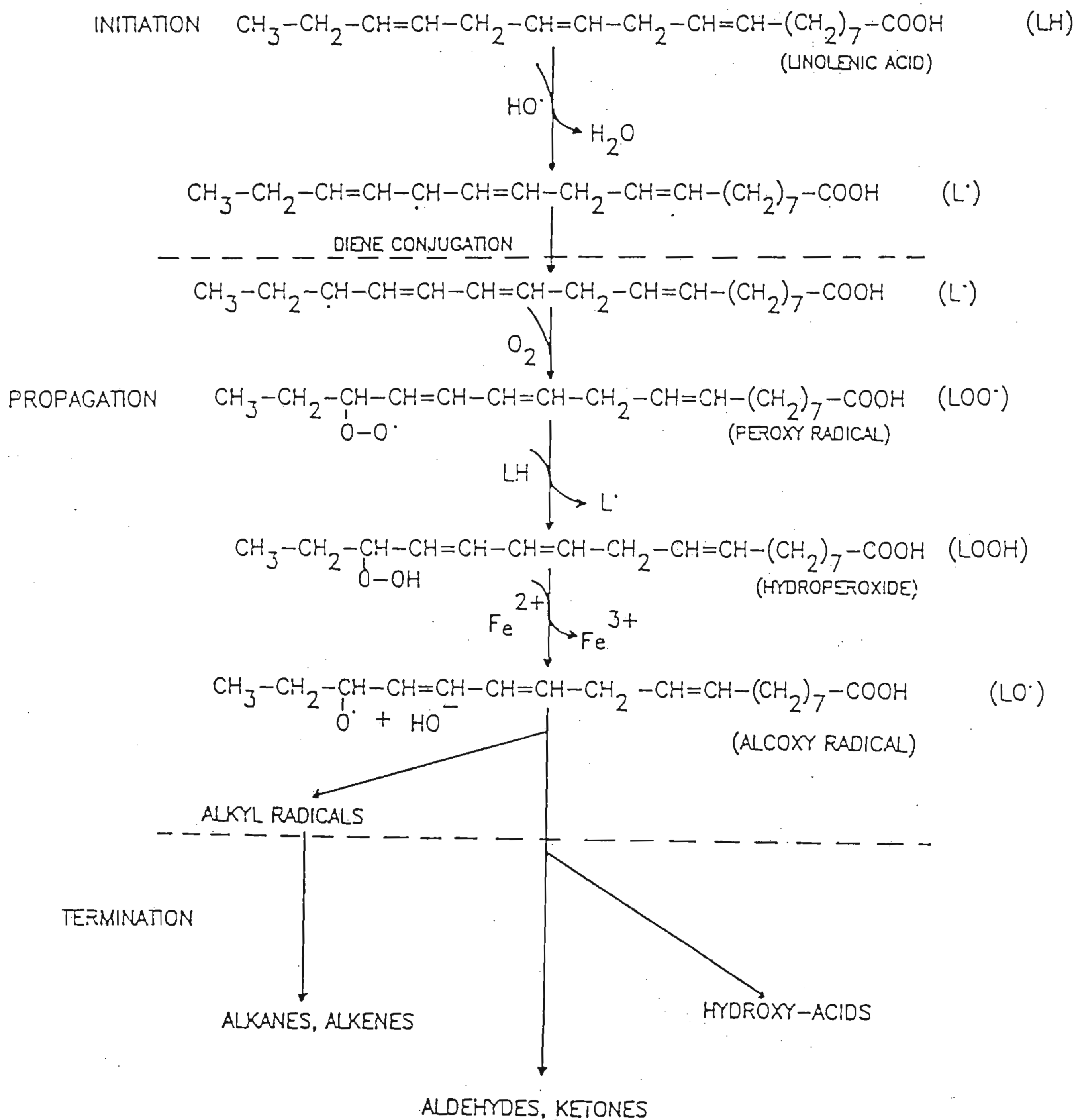


Fig 2.1 Scheme representing the peroxidation of linoleic acid induced by hydroxyl radicals (Vaca *et al.*, 1988).

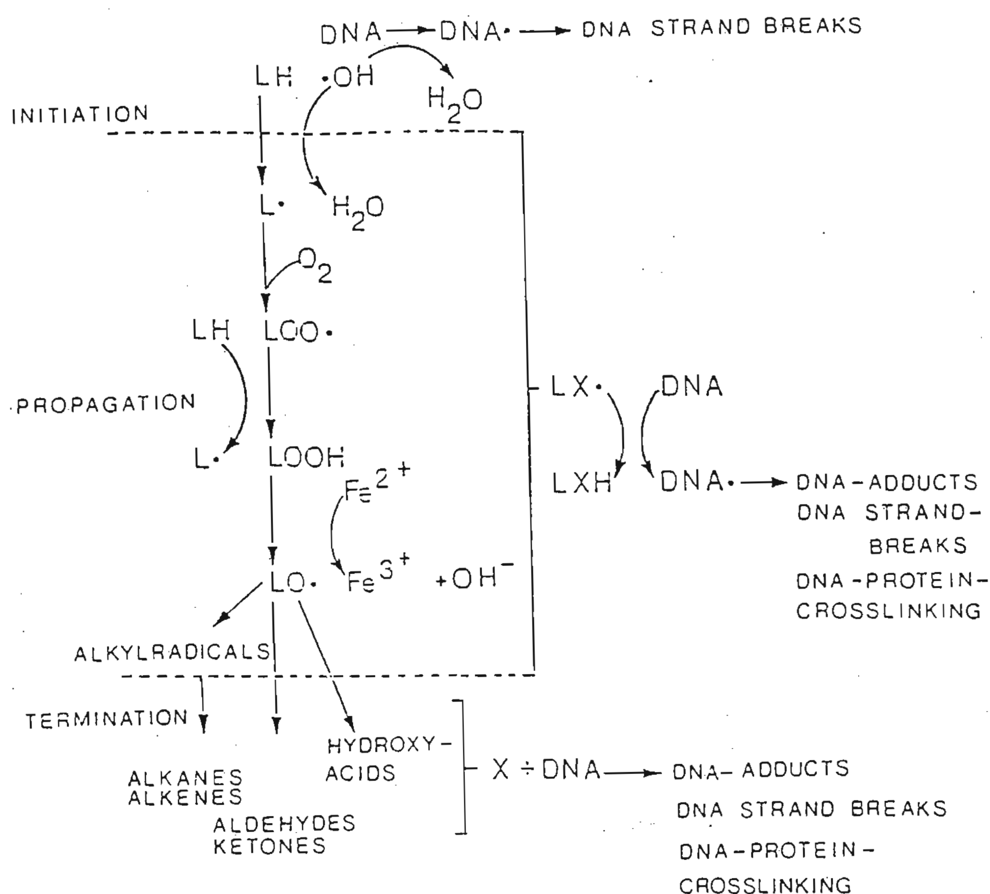


Fig 2.2 Possible pathways for the interaction between lipid peroxidation products and DNA (Vaca *et al.*, 1988).

2.4.3 Production of free radicals during exercise

There are several mechanisms that could potentially lead to the generation of free radicals during exercise. During oxidative phosphorylation in the mitochondria, oxygen is reduced by the mitochondrial electron transport chain to generate adenosine triphosphate (ATP) and H_2O. However during the process some of the molecular O_2 (approximately 2% consumed in the mitochondria) can bind single electrons which leak from the electron carriers in the respiratory chain, resulting in the formation of $O_2^{\cdot-}$ (Boveris and Chance, 1973). The superoxide radicals thus generated are considered to lead to the formation of H_2O_2 and highly reactive $OH^{\bullet}$ radicals in the presence of copper (Cu) and iron (Fe) (Halliwell, 1987). During exercise, oxygen consumption in the exercising muscle can increase as much as 10-40 fold over the resting state (Witt *et al.*, 1992). Furthermore, regular strenuous exercise has been found to increase both the number and size of mitochondria, thus increased flow and metabolism of oxygen in the exercising muscle can enhance the production of $O_2^{\cdot-}$ in the mitochondria. The latter may lead to enhanced generation of H_2O_2

and highly reactive $OH^{\bullet}$ radicals (Singh, 1992). The second possible mechanism is that of ischemia-reperfusion. During exercise, blood flow is shunted away from many organs and tissues and to the working muscles, and part or all of these regions may experience hypoxia (Witt *et al.*, 1992). In addition, during exercise at or above $VO_{2\ max}$. and perhaps at lower intensities it is certain that fibres within the working muscles undergo hypoxia (Wolbarsht and Fridovich, 1989). At the cessation of exercise these regions then undergo reoxygenation, and such reoxygenation can lead to a well known burst of ROS production that occurs during ischemia-reperfusion (Witt *et al.*, 1992).

2.4.4 Exercise and lipid peroxidation

Results describing the effects of exercise on lipid peroxidation by-products are inconsistent. Much of the inconsistency may be explained by the wide variety of methods employed and the different exercise conditions (e.g. intensity, type, duration and training protocols) used in different studies (Sen, 1995). Exercise increases oxygen consumption with the result that the oxygen consumption is elevated 10-15 fold above rest (Sen, 1995). This enhances the potential for free radical generation and increased lipid peroxidation. The increase in oxidant reactions might be initiated in relation to exercise when (a) the electron flow through the cytochrome chain is high, (b) hypoxic tissues provide an abundance of hydrogen ions to react with the increased levels of superoxides produced, and (c) hypoxia leads to freeing of transitional metals (Fe, Cu, Mg) to catalyse radical formation (Jenkins, 1993). Strenuous exercise (80%-100% $VO_{2\ max.}$, < 30 min) is known to cause oxidative stress and various kinds of cell and tissue damage through the generation of these ROS (Hartmann *et al.*, 1995). The lipid peroxidation pathway is the same at rest and during exercise (process described earlier). However the rate at which the reactions occur is believed to increase during exercise. Different methods have been used to detect lipid peroxidation and there is no single biomarker that is best at assessing lipid peroxidation during exercise. The most commonly used biomarkers used to assess lipid peroxidation are thiobarbituric acid-reactive species (TBARS), lipid hydroperoxides and conjugated dienes.

The various methods used have led to inconsistent results. Conjugated dienes appear at the onset of lipid peroxidation and seem to be linked to several steps during lipid peroxidation degradation. Conjugated dienes are polyunsaturated molecules having two double bonds separated by a single one bond. When attacked by oxygen-centred free radicals, one or more of the double bonds shift to become diene conjugated. However, conjugated dienes do not indicate which specific lipid

peroxidation products are being measured (Allesio, 1993). Byarley and Goldfarb (1987) measured conjugated dienes in both plasma and red blood cells before, immediately, 5 minutes and 20 minutes following graded exercise to exhaustion and reported no significant differences when compared to levels before exercise. Duthie *et al.* (1990) also used conjugated dienes as a biomarker of lipid peroxidation and showed that there is no significant increase in the levels of lipid peroxidation after running a half marathon. This indicates that the oxidative stress incurred was insufficient to increase the level of free radical lipid peroxidation.

Another biomarker used is the measurement of lipid hydroperoxides which are formed when hydroperoxides are reduced by the peroxidase activity of haemoglobin. Allesio and Cutler (1990) have reported a 96% increase in the level of lipid hydroperoxides following exhaustive exercise (80% $VO_{2\ max.}$) whereas Kretzschmar and Muller (1993) have found decreased levels of lipid hydroperoxides after exercise in rats and humans. Viinikka *et al.* (1984) have reported no significant increase in the level of lipid hydroperoxidases after moderate intensity (60% $VO_{2\ max.}$) exercise.

The most commonly used method to determine lipid peroxide levels is the TBARS method. This technique is sensitive to malondialdehyde (MDA) which is a product of lipid peroxidation. The problem with this technique is that MDA can arise from other pathways besides lipid peroxidation (Clarkson, 1995). Experiments using this technique have generally shown similar results. A 77% increase above the resting concentration of TBARS in both plasma and serum from highly trained humans taken 30 minutes after exhaustive running exercise has been reported by Kanter *et al.* (1988). Davies *et al.* (1982) reported similar results following running exercises in untrained rats. Laires *et al.* (1993) found an increase in TBARS products in plasma after 3 minutes after a 40 minute run of submaximal intensity.

Hartmann *et al.* (1995) also assessed the effects of high intensity exercise on MDA levels. Five healthy volunteers were subjected to a treadmill run to exhaustion using a ramp protocol. Blood samples taken before exercise, 15 minutes after exercise and 24 hours later, showed that serum MDA was elevated in the 15 minute sample compared to pre-exercise samples but the increase was not statistically significant (p=0,06). In the 24 hour sample, the mean serum level of MDA was similar to that of the control level.

The variable nature of the results may be due to a number of factors, including the mode of exercise, exercise duration, timing of samples and more importantly the method used for

measuring exercise-induced lipid peroxidation. These methods have contributed to contradictory findings.

As mentioned previously, lipid peroxidation can lead to chemical alterations to proteins and DNA This severe stress can lead to transformation or cell death by apoptotic and necrotic mechanisms. Many of the physical and chemical treatments known to evoke oxidative stress are also capable of inducing apoptosis.

2.5 Apoptosis

A type of physiological cell death noted to occur extensively during embryogenesis, was given the name "apoptosis" by Kerr and co-workers in 1972. The term apoptosis is derived from the Greek word meaning "falling off" like leaves from a tree or petals from a flower (Payne *et al.*, 1995). Apoptosis occurs in the maintenance of correct cell numbers in various tissues, in metamorphosis and in various sorts of tissue disorders, including tissue regression, ischemia, nervous diseases, exposure to toxins and tissue atrophy (Uchiyama, 1995). It is also observed in growing tumours and is enhanced during regression. It is a morphologically distinct form of cell death that is involved in many physiological and pathological processes. Because apoptosis is an integral part of the development program, and is frequently the result of a temporal cause of cellular events, it is sometimes referred to as programmed cell death (PCD) (Buttke and Sandstrom, 1994). Another form of cell death also known to occur in nucleated eukaryotic cells is called necrosis. This differs from apoptosis in that it results from complement attack, severe hypoxia, hyperthermia, lytic viral infection, or exposure to a variety of toxins and respiratory poisons (Duvall and Wyllie, 1986). This form of cell death is never programmed; it follows injury in which the cell is mechanically disrupted, or in which homeostasis is compromised. The earliest changes in necrosis occur in the mitochondria, which swell and accumulate calcium (Ca^{2+}). At this stage the cell can no longer make sufficient ATP to control the cell's ion pumps, water enters down a colloidal osmotic gradient, and the cell swells and bursts (Cohen, 1993a).

Two processes must be distinguished in apoptosis, the first called 'priming' involves accumulation into the cell of the machinery which permits apoptosis to take place, the second is 'triggering' of primed cells into apoptosis itself (Steller, 1995). Priming is reversible and may be part of a strategy in the regulation of the cell population, since cells primed for apoptosis are doomed to

die unless rescued by specific growth factors. Apoptosis immediately follows triggering, but the time between priming and triggering may vary (Sandri *et al.*, 1995). Cells undergoing apoptosis were originally characterised at the morphological level and morphology still remains the "gold standard" for identifying apoptotic cells. A cardinal feature of apoptotic cells is the appearance of highly condensed chromatin, segregated into sharply defined bodies within an intact nuclear envelope. Often the DNA is digested by endonucleases, resulting in cell shrinkage and condensation with membrane blebbing, chromatin margination and the DNA fragments into multiple, membrane bound bodies which are eventually engulfed by surrounding cells and removed without inflammation and attendant damage to surrounding tissue (Meikrantz and Schlegel, 1995; Cohen, 1991).

Apoptosis is a process which is triggered by a variety of stimuli which activate endogenous nucleases to cleave chromatin; the result is a series of DNA fragments which first yield fragments of 50-300 kb and then nucleosomic fragments in multiples of 180-200 base pairs (bp) (Idziorek *et al.*, 1995; Zhang *et al.*, 1995). Apoptotic cells show changes after DNA is degraded. The most characteristic biochemical event during apoptosis is fragmentation of nuclear DNA into oligonucleosomal subunits that precedes cell death. The fragmentation of nuclear DNA during apoptosis appears to be due to the activation of a Ca^{2+} dependent nuclear endonuclease that is a normal constituent present in an inactive form in the cell nuclei. The process by which this enzyme becomes activated is not known, though there is an increase in intracellular Ca^{2+} concentration during apoptosis. The integrity of the plasma membrane plays an important role in maintaining the Ca^{2+} homeostasis in the cell (Ramakrishnan *et al.*, 1993).

The pattern of DNA fragments observed results from DNA undergoing double stranded cleavage in the linker regions between nucleosomes. This cutting is not sequence specific but rather relates to the relative accessibility of DNA in the linker, where it is rather loosely associated with histone H1; DNA in the nucleosome is tightly complexed to the H2a/H2b/H3/H4 core. Organisation of this higher order structure around the histone proteins forms the basic architecture of chromatin. Important changes in this structure occur during DNA synthesis and transcription. During apoptosis there are alterations of the higher order structure characterised by internucleosomal double stranded breaks (Ansari *et al.*, 1993). Deoxyribonuclease I (DNAse I) can readily degrade chromatin down to oligonucleotides on the order of 10 bp; it is perhaps a smaller enzyme than the endogenous endonuclease however, it has been proposed as a candidate for the endonuclease activity of apoptosis (Cohen, 1991; Hale *et al.*, 1996).

2.5.1 Cellular changes in apoptosis

The cellular changes in apoptosis are numerous but it is not clear which are directly associated with death and which are of the greatest physiological consequence. Apoptosis is known to occur in a matter of minutes. The reason a cell dies is different from cell to cell type, as is the triggering mechanism (Wyllie, 1987). Often the signal to die comes from the environment, as in exposure to or withdrawal of a hormone or growth factor. The pathway to activation of apoptosis is different in different cells, but the mechanism is the same, that is a common final pathway (Cohen, 1993a). The identification of apoptosis in lymphoid tissue dates as far back as 1972. Since that time lymphocytes have been one of the most studied cell types with respect to apoptosis. Lymphocytes are subject to apoptosis at practically every stage in their development and maintenance, but also failures of normal apoptosis regulation may contribute to lymphocyte related diseases such as lymphomas and Acquired Immune Deficiency Syndrome (AIDS) (Squier *et al.*, 1995). The sequence of apoptosis can be seen in Fig 2.3 using a lymphocyte as an example.

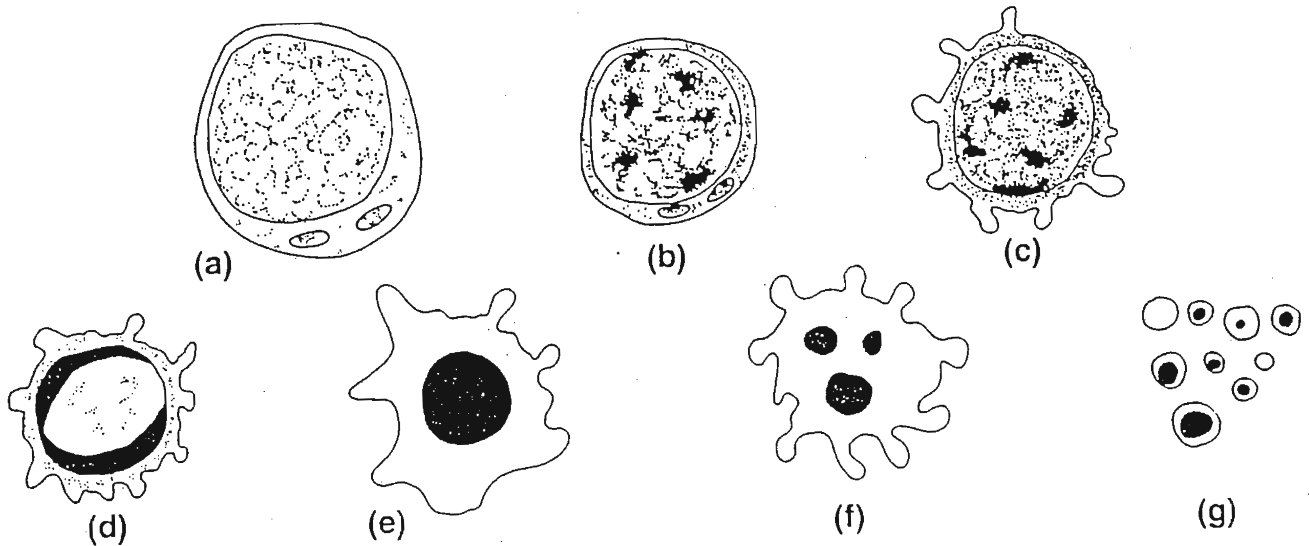


Fig 2.3 The stages of apoptosis in a lymphocyte (Cohen, 1993a).

(a) A cell undergoing apoptosis rounds up, severing junctions with its neighbours (Howie *et al.*, 1994). The normal lymphocyte has sparse cytoplasm and heterogeneous nuclear chromatin with a cell volume of about 90 fico litres (fl). (b) The cell loses approximately 25% of its volume (Cohen, 1993b), and its cytoplasmic organelles become tightly packed. Clumping of the chromatin occurs. At this stage, membrane changes that lead to phagocytosis are present. (c) The cell exhibits zeiosis i.e. the plasma membrane becomes ruffled and blebbed (Cohen, 1993a). (d) The chromatin collapses, becomes condensed and marginated and distinct crescents form along the nuclear envelope (Payne *et al.*, 1995). This is usually observed using cell-permeant DNA dyes and a light microscope. At this stage the cell is still alive and its plasma membrane is functioning as a diffusion barrier. Cell volume is now about 70 fl. (e) The nucleus collapses into a black hole. (f) The collapsed nucleus frequently breaks up into spheres or multiple fragments. This change is often accompanied by DNA fragmentation. Since this cleavage is random, a distribution of DNA fragment sizes is obtained, each one which is an integral multiple of the 180-200 bp subunit (Cohen, 1993a). Some DNA is probably lost from the cell by now, as apoptotic bodies are blebbing off it. (g) The cell fragments into apoptotic bodies and these are phagocytosed almost immediately by phagocytic cells to prevent inflammation.

2.5.2 Oxidative stress as a mediator of apoptosis

Many of the chemical and physical treatments capable of inducing apoptosis are also known to evoke oxidative stress. Both ionising and ultraviolet radiation are capable of inducing apoptosis and both generate reactive oxygen intermediates (ROI) such as H_2O_2 and $OH^{\bullet}$. Some agents which induce apoptosis are not free radicals themselves, but may elicit ROI formation. Doxorubicin, cisplastin and ether linked lipids are antineoplastic agents which induce both apoptosis and oxidative damage in sensitive cells. Alternatively, oxidative stress can be induced by decreasing the ability of a cell to scavenge or detoxify ROI. Drugs such as buthionine sulphoxamide deplete intracellular stores of glutathione (GSH), thereby rendering the cells more susceptible to oxidative stress induced apoptosis (Buttke and Sandstrom, 1994). Molecular O_2, which is the terminal electron acceptor for oxidative phosphorylation plays an essential role in the many metabolic processes associated with an aerobic existence. Oxygen, is however toxic and toxic oxygen free radicals have been implicated in the pathogenesis of many clinical disorders. The normal oxidant by-products produced during the reduction of molecular O_2 to H_2O ($O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$) can damage lipids, proteins, sugars and nucleic acids. Low doses of these ROI can induce apoptosis (Buttke and Sandstrom, 1994). The formation of these ROI as a result of oxidative stress can lead to DNA damage which is characterised by nuclear condensation and fragmentation. Figure 2.4 outlines how oxidative stress can lead to DNA damage.

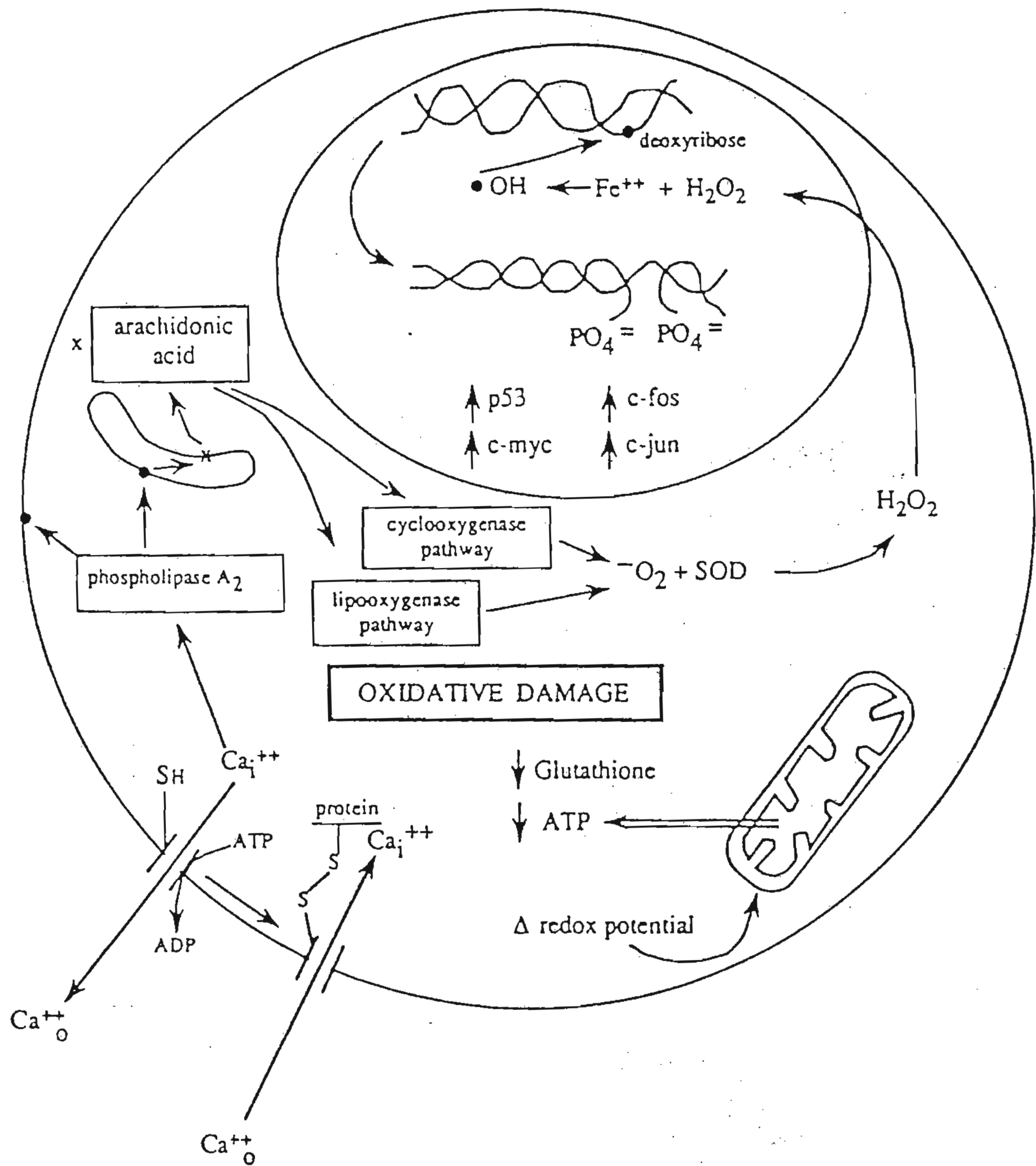


Fig 2.4 Schematic drawing showing how metabolically induced oxidative stress can lead to DNA damage (Payne *et al*., 1995).

The ATP-dependent Ca^{2+} transporter requires its sulphydral group to be in a reduced state (SH) (Payne *et al.*, 1995). A change in redox potential can result in oxidised sulphydral groups (S-S), leading to a decrease in calcium transporter activity, and resulting in increased intracellular levels. The increased Ca^{2+} levels can activate phospholipases, resulting in the release of arachidonic acid from the membranes. Arachidonate enters the lipooxygenase and cyclooxygenase pathways, resulting in the generation of the superoxide anion. The superoxide anion is reduced to H_2O_2 by superoxide dismutase (SOD). Hydrogen peroxide in the presence of ferrous ions (Fe^{2+}) can generate highly reactive $OH^•$ which cause single strand breaks (Payne *et al.*, 1995).

2.5.3 Genetic regulation of apoptosis

The similarity of apoptotic changes in many different cell types and in response to many different sorts of triggers, suggests that apoptosis is an orderly process; there is a cell death program underlying apoptosis (Squier *et al.*, 1995). The basic design of the genetic regulation of apoptosis was identified by studying the nematode *Caenorhabditis elegans* (Wyllie, 1987). In *C.elegans* mutants have been identified in which the 131 cells normally fated to die during development, survive. Genetic analysis has revealed that two genes, *ced-3* and *ced-4*, are essential for programmed death of cells in this species. The *ced-4* seems to be a calcium binding protein and acts upstream from the *ced-3* product, whose sequence suggests that it is a cysteine protease. A mammalian homologue of *ced-3* is the interleukin-1β-converting enzyme, and this gene has been expressed during apoptosis (Squier *et al.*, 1995).

Individual genes have been associated with apoptosis in two ways: either they are expressed in cells undergoing apoptosis, or their modulation affects apoptosis. Among the latter, the *c-myc* proto-oncogene may play a part in regulating the choice between proliferation and apoptosis. Fibroblasts which express the *c-myc* do not undergo growth arrest in low serum concentrations as do wild-type fibroblasts, but readily undergo apoptosis. It may be that the *c-myc* programs a cell to grow and if this is thwarted, the cell may then commit suicide (Evan *et al.*, 1992).

The anti-oncogene *p53* has also been associated with apoptosis. This gene's product arrests cell proliferation and may instead switch the cell to differentiation mode. In many cell lineages, differentiation may equal death, and *p53* will cause apoptosis when expressed in myeloid or epithelial cell lines (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992). The role of *p53* may be to

hold a damaged cell in the G1 phase, while the damage is repaired; cells which try to oppose the G1 block may end up activating the suicide pathway (Lane, 1992).

The putative oncogene *bcl-2* is commonly overexpressed in human follicular B-cell lymphomas. When it is transfected into an IL-3-dependent B-lymphoblastoid cell line, it does not make these cells factor independent. Instead it allows them to survive factor removal, whereas such cells otherwise commit suicide (Vaux *et al.*, 1988). Therefore, *bcl-2* has properties suggestive of an "anti-apoptosis" gene but it does not prevent apoptosis under all circumstances nor does it work in every cell line.

There are some genes whose expression increases in apoptotic cells, although their role in the process, has not yet been determined. The *TRMP-2* gene is expressed in a number of tissues, primarily in the urogenital tract, during apoptosis. It is not seen in tissues undergoing morphogenetic death in the embryo. This gene product may play a role in secretion and lipid transport and could be involved in damage (Cohen, 1993a).

2.5.4 Methods to detect apoptosis

Methods used to detect apoptotic cells range from histological evaluation of membrane blebbing, chromatin condensation, nick translation of ends of fragmented DNA and gel electrophoresis. The comet assay is also a sensitive method of detecting DNA strand breaks in individual cells (Singh *et al.*, 1988; McKelvey-Martin *et al.*, 1993). It is based on the ability of smaller negatively charged DNA fragments to migrate through an agarose gel in response to an electric current. Since apoptosis is characterised by extensive DNA cleavage, the comet assay is useful in detecting not only the initial DNA damage, but also early stages of apoptosis when other methods may lack sensitivity (Olive *et al.*, 1993). The hallmark of apoptosis is endonucleolysis, with nuclear DNA initially degraded at the linker regions to fragments equivalent to single and multiple nucleosomes. The flow cytometric methods of identifying apoptotic cells are based on measurement of cellular DNA to denaturation or altered light scattered properties. At the very early stages of apoptosis, prior to nuclear fragmentation, the presence of DNA strand breaks is detected primarily at the periphery of the nucleus. This suggests that the initiation of DNA degradation during apoptosis starts at the chromatin regions close to the nuclear envelope. There are several advantages that the terminal deoxynucleotidyl tranferase (TdT) assay offer in studying apoptosis:

(a) the reactions are based on the direct labelling of the 3′ hydroxyl termini of DNA breaks, and thus the lesions measured are identifiable at the molecular level;

(b) the DNA breaks occur very early in apoptosis, prior to changes in cell morphology; the method thus detects apoptotic cells which cannot yet be recognisable based on changes in morphology;

(c) since DNA content is measured in addition to the DNA breaks, apoptosis can be related to the cell's position in the cycle or to DNA ploidy if cells of different ploidies are present in the same sample (Gorczyca *et al.*, 1993a).

Apoptosis in most cells is characterised by extensive DNA fragmentation whose frequency and time of appearance after a triggering signal are dependent upon the cell line and DNA damaging agent (Olive *et al.*, 1993).

2.5.5 Apoptosis and exercise

To date there are two published reports that link exercise and DNA damage in WBC in humans. A recent report by Hartmann and co workers (1994) has described evidence of DNA strand breaks in one of three athletes of differing fitness levels. They found that maximum damage was found after 24 hours while the damage was not seen after 72 hours. A further study by Hartmann and co workers (1995) showed a similar increase in DNA strand breakage in the 24 hour sample similar to their previous observation. The biological significance of the findings by Hartmann and co workers (1994, 1995) and the mechanisms underlying the effects seen could not be determined. They demonstrated for the first time that strenuous exercise causes DNA migration and have ascribed this damage as being related to oxidative damage. However, Hartmann and co workers (1994, 1995) have not linked the DNA damage observed, as being related to cells undergoing apoptosis.

2.5.6 Role of antioxidants in preventing apoptosis

The literature regarding the use of antioxidants as modifiers of an apoptotic response is conflicting. In some systems there is little doubt that free radicals and ROS are produced, such as exposure to ionising radiation and in such examples antioxidants have been shown to reduce or

delay apoptosis. Hydrogen peroxide induced apoptosis can be inhibited by trolox, an inhibitor of lipid peroxidation. Agents such as SOD have also been found to attenuate the effects (Clutton, 1997). Hartmann *et al.* (1995) have demonstrated that vitamin E supplementation (1 200 mg daily) for 14 days prior to a run, reduced exercise-induced DNA damage.

2.6 Antioxidants

Evolution of organisms in an oxygen-rich atmosphere has led to the development of endogenous physiological defence systems that co-operate to scavenge and detoxify ROS. In addition a second line of defence is obtained from nutrients or nutrient supplements. An antioxidant may be defined as any substance that, when present in low concentrations as compared to those of an oxidisable substrate, significantly delays or prevents oxidation of the substrate. The possible mechanism by which antioxidants may protect against oxygen toxicity are:

(1) prevention of ROS formation;

(2) interception of ROS attack by scavenging the reactive metabolites and converting them to less reactive molecules and/or enhancing the resistance of sensitive biological targets to ROS attack;

(3) avoiding the transformation of less reactive ROS (e.g. $O_2^{-\cdot}$) to more deleterious forms (e.g. OH^-);

(4) facilitating the repair or damage caused by ROS and triggering the expression of genes that encode antioxidant proteins; and

(5) providing a favourable environment for the effective functioning of other antioxidants (Sen, 1995).

The best known and most investigated antioxidants are vitamin E and vitamin C. In addition, there are many known (viz. Beta carotene and selenium) as well as unidentified antioxidants (Goldfarb, 1993). Vitamin C is an effective water soluble antioxidant, and epidemiological studies suggest that increased ascorbate nutrition is associated with reduced risk of degenerative diseases, especially cancer and cataracts. Studies have also shown that high vitamin E intakes are associated with decreased risk of coronary heart disease, possibly as a result of inhibition of atherogenic forms of oxidised low density lipoprotein (LDL) (Jacob and Burri, 1996). Vitamin C forms the first line of antioxidant defence in plasma under different types of oxidative stress. After

complete oxidation of vitamin C, vitamin E is subsequently oxidised by reaction with oxygen radicals (Dekkers *et al.*, 1996; Frei *et al.*, 1989).

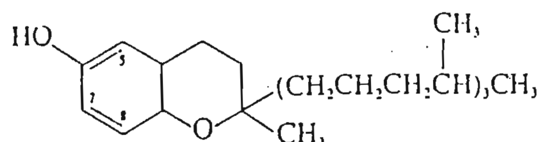
2.6.1 Vitamin E

Physical exercise and especially strenuous exercise accelerates production of free radicals and can result in immune reactions similar to the acute phase response to infection and injury (Pappas, 1993). It has been well documented that vitamin E, which is a major biological antioxidant, is an essential component of the body's defence against the harmful effects of free radicals (Burton and Traber, 1990). Recent studies have shown that vitamin E protects muscles and other tissues (Meydani *et al.*, 1993) and it also prevents exercise-induced DNA damage (Hartmann *et al.*, 1995).

2.6.1.1 Structure and Function

Vitamin E (α-tocopherol), a lipid soluble hydrophobic molecule, was discovered in 1922 by Evans and Bishop (Hoekstra, 1975). Vitamin E is found in the interior of mitochondrial, microsomal and erythrocyte membranes and most other cell membranes (Combs *et al.*, 1975).

Vitamin E is the common name for 8 related compounds termed tocopherols and tocotrienols which consist of a 6-chromanol nucleus with methyl groups at positions 2 and 8 and a branched isoprenoid side chain at position 2 (McCormick, 1986; Tiidus and Houston, 1995). The hydrophobic phytyl chain is closely aligned with acyl chains of lipids in the membrane, while chromanol head is located close to the surface of the membrane. It is the chromanol head which reacts with the radicals (Goldfarb, 1993). There are 4 forms of vitamin E viz. α-, β-, γ- and δ-tocopherol. They differ only in the number and the position of the methyl groups on the chromanol ring (Fig 2.5). α-Tocopherol has the highest biological activity of the various vitamin E derivatives and the greatest free radical scavenging ability (Kanter, 1994).



Tocopherol isomer	*Methyl positions*
α	5,7,8
β	5,8
γ	7,8
δ	8

Fig 2.5 Structure of vitamin E and eight related natural compounds in the vitamin E group (Brewster and Turley, 1987).

The minimum requirement of vitamin E for adults who ingest the minimum of essential fatty acids, is 3-4 mg/day (McCormick, 1986). However, it is now recommended that the daily allowances should be 10 mg for males and 8 mg for females (15 and 12 IU respectively) (Tiidus and Houston, 1995). During exercise, it has been suggested that a significantly higher intake is required and in some studies intakes of 100-800 IU have been evaluated (Pappas, 1993).

Vitamin E is a fat soluble vitamin and is absorbed from the gut into the blood stream via lymph and is transported from the gut by chylomicrons. Most of the absorbed vitamin E reaches the liver where it is secreted in very low density lipoprotein (VLDL) and transported into the plasma. A small portion of vitamin E may be transferred to other lipoproteins and tissues (Pappas, 1993).

Vitamin E has various functions:

1. Quenches singlet oxygens
2. Stabilises superoxide anions
3. Stabilises the hydroxyl radicals

Its primary function though is to protect the membrane by two pathways:

(i) Nicotinamide adenine dinucleotide (NADH)-cytochrome b reductase enzymatic recycling and (ii) vitamin C and dihydrolipoic acid (DHLA) nonenzymatic recycling.

(i) NADH-cytochrome b reductase enzymatic recycling (Fig 2.6).

Enzymatic recycling has been demonstrated in mitochondria, rat liver microsomes and erythrocyte membranes. There are two forms of the NADH-cytochrome b reductase component: soluble types found in the cytosol and membrane bound types that are located on the inner side of the membrane. Electron flow via the NADH-cytochrome reductase bound component is diverted to recycle vitamin E (Constantinescu *et al.*, 1993).

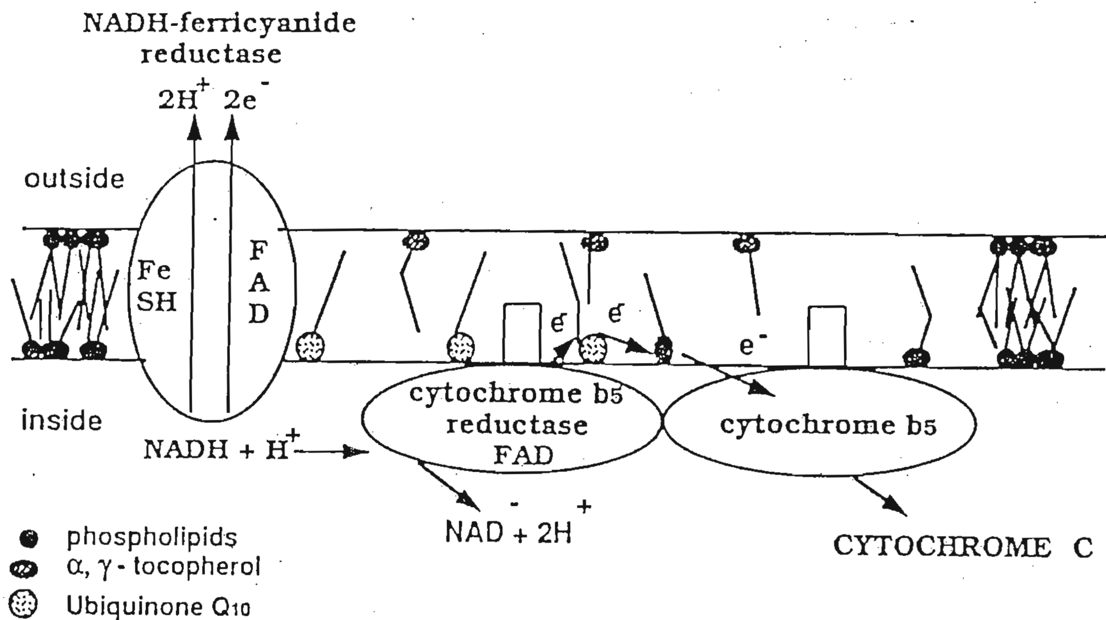


Fig 2.6 NADH-cytochrome b reductase enzymatic recycling of vitamin E (Constantinescu *et al.*, 1993).

(ii) Vitamin C and DHLA nonenzymatic recycling (Fig 2.7)

Nonenzymatic recycling of vitamin E by vitamin C and DHLA has been demonstrated in low density lipoproteins (Kagan *et al.*, 1992) and erythrocyte membranes (Constantinescu *et al.*, 1993). When a proton is donated to a free radical, vitamin E (chromanol) is converted to the vitamin E radical (chromanoxyl). Dihydrolipoic acid is able to synergistically enhance the ascorbate drive reduction of vitamin E radicals and recycling of vitamin E.

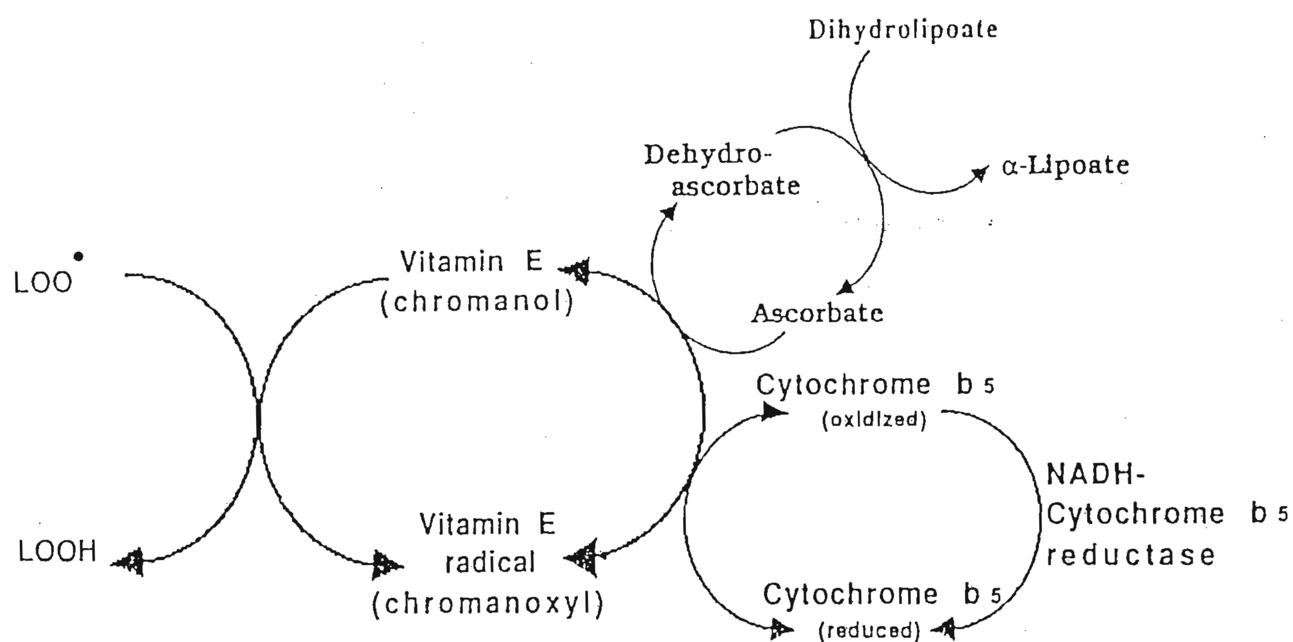


Fig 2.7 Nonenzymatic recycling of vitamin E by vitamin C and DHLA (Constantinescu *et al.*, 1993).

When vitamin E scavenges a radical it becomes a radical itself. It has been proposed that when a peroxyl radical is formed in the tail region, it is forced or projected out of the non-polar region towards the polar region where the chromanol head is located. This enables the chromanol head to be oxidised and enables vitamin C (ascorbic acid) in the aqueous region to interact and regenerate the chromanol region of vitamin E. The ascorbate radical which is now stable and

unreactive is enzymatically reduced back to ascorbic acid by the nicotinamide dinucleotide reaction system (Goldfarb, 1993; Dekkers *et al.*, 1996).

2.6.1.2 Vitamin E and exercise

Most studies to date have focused on the supplementation of vitamin E in animals and humans and its role in preventing exercise-induced oxidative stress. There is a paucity of studies which focus on the effects of exercise and training on plasma vitamin E levels.

Viguie *et al.* (1993) have investigated the effects of 3 consecutive days of prolonged (90 minutes) submaximal exercise (65% $VO_{2\ max}$.) on plasma antioxidant status. Plasma vitamin E levels did not show any significant changes on any one of the 3 days. This has also been observed before and 120 minutes after a half marathon (Duthie *et al.*, 1990) and 30 minutes after completion of a 88 km ultramarathon (Peters *et al.*, 1997). This is however contradictory to other studies that have been carried out. Plasma vitamin E levels have been reported to be significantly elevated following exercise in humans (Maxwell *et al.*, 1993) as well as after a bout of eccentric exercise (Meydani *et al.*, 1993; Cannon *et al.*, 1990). Pincemail *et al.* (1988) attribute the increase in vitamin E levels following intense cycling to exhaustion in humans, to the redistribution of vitamin E in the body as a result of high intensity exercise.

2.6.2 Vitamin C

Vitamin C or ascorbic acid, a water soluble vitamin, can be a potent antioxidant depending on the concentration of ascorbate. Vitamin C is regarded as the first line antioxidant in defence against phagocyte derived reactive oxidants (Peters *et al.*, 1996), as well as the first line of defence in plasma under different types of oxidative stress, and is the only exogenous antioxidant capable of protecting lipids against oxidative damage (Dekkers *et al.*, 1996). Vitamin C has been shown to reduce symptoms of URTI in ultramarathon runners (Peters *et al.*, 1996).

2.6.2.1 Structure and function

When a proton (H^+) is removed from vitamin C, ascorbic acid is called ascorbate. Ascorbic acid is reversibly oxidised to dehydroascorbic acid (Fig 2.8) by reduced glutathione, NADPH or both.

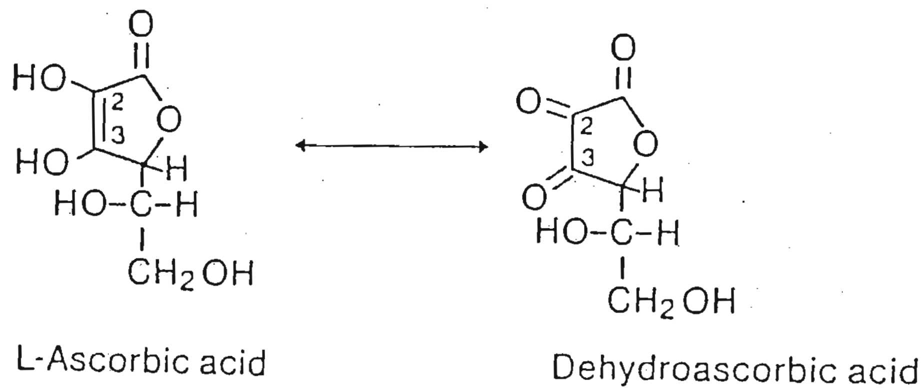


Fig 2.8 Structures of ascorbic acid and dehydroascorbic acid (Brewster and Turley, 1987).

The recommended intake for vitamin C is 30-100 mg/day (Clarkson, 1995). However, for exercising individuals, an additional 1 000 mg/day is recommended to prevent URTI and exercise induced oxidative damage (Peters *et al.*, 1996).

Vitamin C is readily absorbed from the stomach, where it is converted to dehydroascorbic acid. At physiological pH, the uncharged dehydroascorbic acid passes across cell membranes and once in cells, intracellular reduction to the less diffusible ascorbate ion occurs. Vitamin C is concentrated in tissues such as the pituitary gland, adrenal cortex, corpeus luteum and thymus. The retina has 20 to 30 times the plasma concentration (McCormick, 1986).

Vitamin C has numerous biological functions, including collagen, hormone and neurotransmitter synthesis and it also functions directly as an antioxidant by quenching singlet oxygens, reacting directly with $O_2^{\cdot}$, stabilising the $OH^{\bullet}$ and regenerating reduced vitamin E (Kanter, 1994).

2.6.2.2 Vitamin C and exercise

Plasma ascorbic acid has been reported to increase after exercise. An increase in plasma vitamin C of 34% has been noted immediately after a completion of a half marathon with the values decreasing to below pre-exercise values 2-3 days later (Duthie *et al.*, 1990; Gleeson *et al.*, 1987). Increases in vitamin C levels immediately after exercise have also been noted after a bout of eccentric exercise (Meydani *et al.*, 1993; Cannon *et al.*, 1990) and treadmill running to exhaustion (Robertson *et al.*, 1991). Vitamin C supplementation has been shown to decrease the occurrence of URTI, post-exercise muscle soreness and prevention of lipid peroxidation (Peters *et al.*, 1996; Dekkers *et al.*, 1996). Most studies have concentrated on supplementation of vitamin C to trained and sedentary individuals and have shown results which have lead to the conclusion that supplementation of vitamin C does prevent exercise-induced lipid peroxidation.

2.7 Conclusion

A search through the literature has revealed inconsistencies regarding the effects of high intensity exercise on lymphocytes, lymphocyte DNA, lipid peroxidation and antioxidant status. The discrepancies observed in various studies are due to methodological, physiological and morphological differences as discussed above. This study endeavours to fill this gap and identify the reasons for the various changes observed in lymphocytes and lymphocyte DNA after high intensity exercise as well as the effects of high intesity exercise on the antioxidant status of highly trained athletes. In so doing, researchers will be able to establish better exercise prescriptions and training schedules for the elite athlete in preventing the establishment of infections. The thirst for knowledge in the fields of exercise immunology, physiology and biochemistry is ever increasing, and the results obtained will play a significant role to the athlete whose life depends on new findings for optimum performance.

CHAPTER 3

EXERCISE TESTING

3.1 Introduction to maximum oxygen uptake ($VO_{2\ max.}$) testing

Maximum oxygen uptake testing has been suggested as the single best predictor of aerobic athletic potential (Noakes, 1988) and is probably the single most commonly used procedure in exercise physiology. Maximum oxygen uptake testing is not only a measure of aerobic energy turnover, but also offers a precise measure of capacity to transport and utilise oxygen, i.e. the functional capacities of the lungs, cardiovascular system and muscle mitochondria combined (Saltin and Strange, 1991).

3.1.1 Principle of $VO_{2\ max.}$ testing

The oxygen uptake during exercise of increasing intensity shows a linear increase in O_2 consumption. Oxygen consumption will eventually plateau despite increasing exercise intensity (Hill and Lupton, 1923). The region where oxygen uptake plateau's and shows no further increase, or increases only slightly (<100 ml/min.) with an additional workload is called the maximum oxygen uptake, maximum oxygen consumption, maximum aerobic power or simply $VO_{2\ max.}$ (Fig 3.1) (McArdle *et al.*, 1996). Additional physical work is accomplished only by the energy transfer reactions of glycolysis with the resulting accumulation of hydrogen ions and reduction of pyruvate to lactate. Under these conditions, the runner soon becomes exhausted and is unable to continue (McArdle *et al.*, 1996).

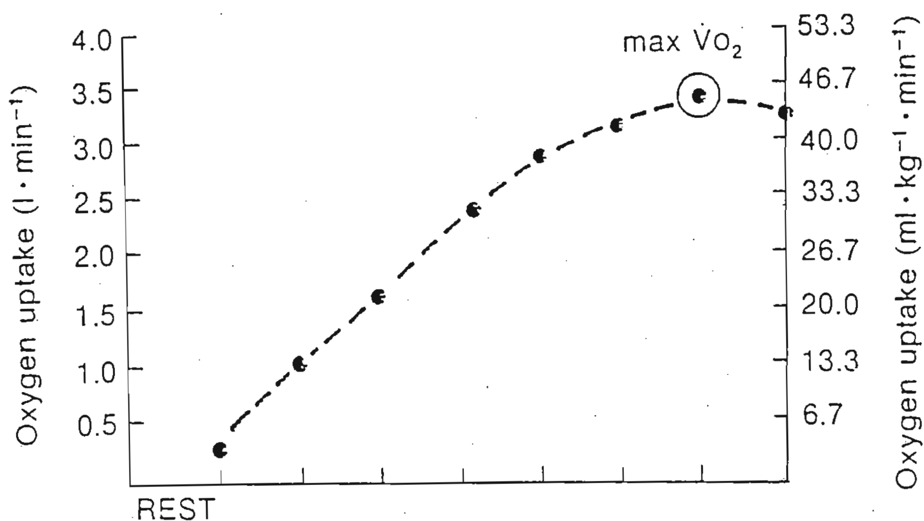


Fig 3.1 Diagram showing the principle behind $VO_{2\,max.}$ testing (McArdle *et al.*, 1996).

The above principle of maximum oxygen uptake testing was used for testing of athletes in the present study. The treadmill exercise protocol used in this study is discussed in section 3.3.

3.1.2 Measurement of Maximal Oxygen Uptake

Maximum oxygen uptake can be measured using a variety of exercises that activate large muscle groups as long as the intensity and duration of the effort are sufficient to engage maximal aerobic energy transfer. The usual exercise modes include treadmill running or walking, bench stepping and stationary cycling. The mode used for this particular study was treadmill running since all the subjects involved in this study were runners. The treadmill used was a Powerjog treadmill, model EG10 which has a maximum speed of 25 $km.h^{-1}$ and a maximum gradient of 25% (Fig 3.2). The athletes oxygen consumption and CO_2 production were measured via computerised open circuit spirometry using an Oxycon Gamma Spirometer (Oxycon Gamma, Mijnhardt, Bunnik, Holland). The computer program used to generate printouts of $VO_{2\,max.}$ etc. was the oxycon ergo-spirometry program.

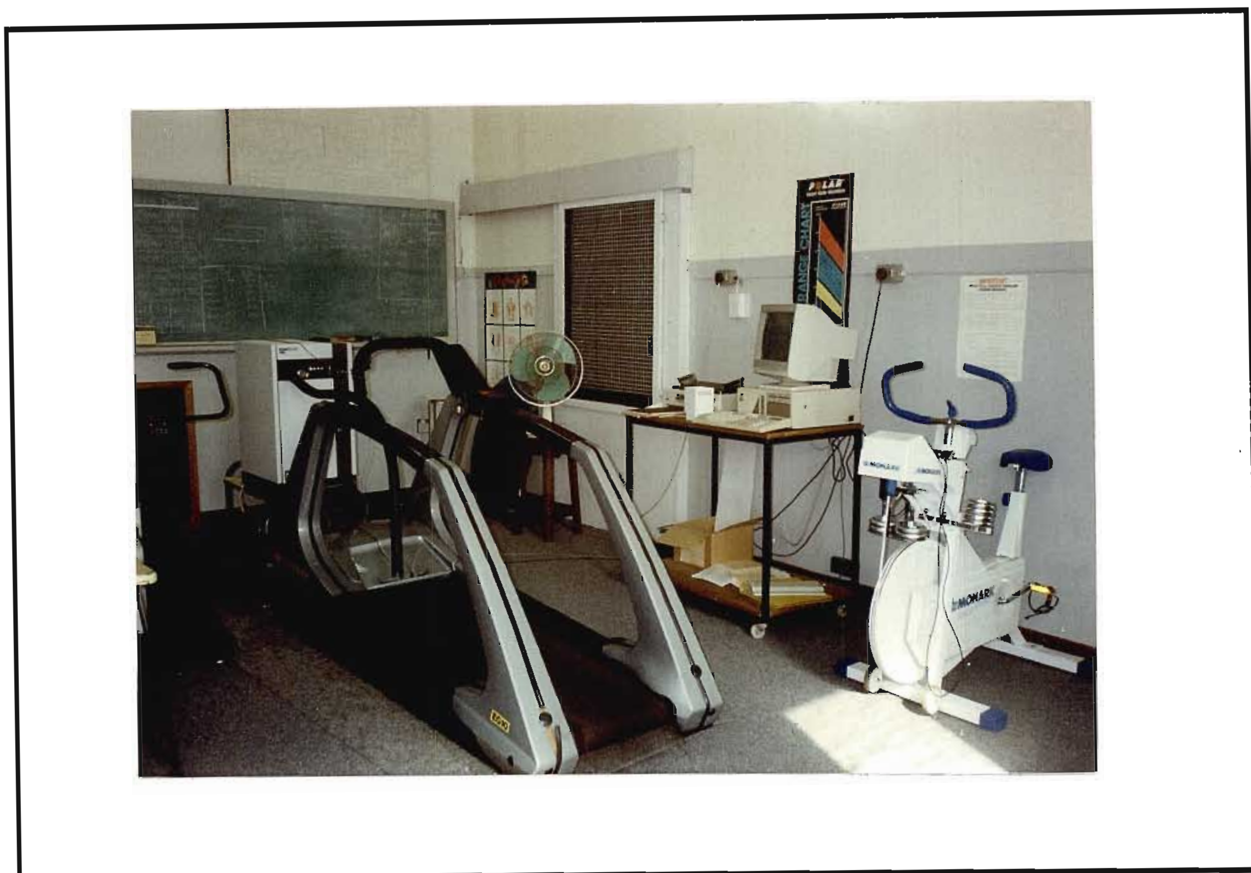


Fig 3.2 Photograph showing the Powerjog treadmill (Model EG 10, Birmingham, England) and the Computerised Metabolic Analysis System (Oxycon Gamma, Mijnhardt, Bunnik, Holland) used in determining the athletes $VO_{2\ max}$.

3.2 Selection and Exclusion criteria for subjects for inclusion in the trial

Institutional permission was obtained from the Faculty of Medicine's Ethics Committee and the research protocol was approved by the Post Graduate Research Committee of the Faculty of Medicine, University of Natal.

Eleven male athletes between the ages of 18 and 55 were recruited for this study. Each subject completed an informed consent form for inclusion in the trial (Appendix 3.1). In addition, the subjects completed a medical history questionnaire (Appendix 3.2) to ascertain possible exclusion from the trial. Exclusion criteria were that they had no previous viral and bacterial infections in the past three months.

3.3 Exercise test protocol

A continuous treadmill run to exhaustion using a graded protocol was used (Table 3.1).

Table 3.1 The exercise protocol used for exercise testing to determine $VO_{2\ max}$.

Speed (km.h^{-1})	8	9	10	11	12	12	12	12	12
Time (min)	0-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13
Treadmill grade (%)	3	4	5	6	7	8	9	10	11

Before the test was started, the athletes were fitted with a heart rate monitor (Polar Sports Tester, Polar Electroy, Kempele, Finland) to record their maximal heart rate during the test. All athletes started at a speed of 8 km.h^{-1} at a 3% gradient for 5 minutes and this represented the warm-up stage. Thereafter the speed and gradient was increased by 1 km.h^{-1} and 1% respectively after every minute until a maximum speed of 12 km.h^{-1} was reached. Thereafter, the speed was kept constant and the gradient was increased by 1% every minute until the athlete became exhausted and terminated the test.

3.4 Blood sampling

Venepuncture was done by qualified personnel. Fifteen millilitres of blood were taken at each time interval. Blood samples were taken before exercise and this served as the control, immediately after exercise, 24 hours and 48 hours after the exercise test. Blood was collected in 3 tubes: (1) 5 ml for vitamin E and lipid peroxide analysis was collected in a test tube containing EDTA (potassium salt 1 mg. ml^{-1}) as an anticoagulant, (2) 5 ml for vitamin C analysis was collected in a test tube containing lithium heparin as an anticoagulant and (3) 5 ml for detection of DNA damage was collected in a sterile plain test tube. Blood collected in the EDTA and lithium heparin test tubes were centrifuged at 2 500 revolutions per minute (rpm) for 7 minutes at room temperature. After centrifugation, the plasma (1,3 ml) was removed from both tubes and placed into 1,5 ml eppendorf tubes. The plasma samples were immediately stored at -72^{0}C in an ultrafreeze until analysed. Whole blood collected in the plain test tube was used to determine DNA damage using the single cell gel electrophoresis (SCGE) assay.

3.5 Results and Discussion

Table 3.2 Physical characteristics and results of the $VO_{2\ max.}$ test.

Subject	Age	Height (cm)	Mass (kg)	$VO_{2\ max.}$ (ml. kg^{-1}. min^{-1})	Maximum heart rate (MHR)	Age Predicted MHR[a]	V'e (l/min)
1	40	186	96	47,4	175	180	126,2
2	25	183	73	69,2	182	195	166,9
3	21	183	77	64,4	195	199	114
4	20	164	60	59,9	198	200	98,2
5	19	183	76	67,6	201	201	115,3
6	23	180	83	51,9	196	197	127
7	51	186	91	47,7	169	169	95,3
8	26	179	76	71,0	185	194	150,7
9	25	168	63	81,9	189	195	146,6
10	49	166	67	46,5	165	171	77,5
11	27	175	69	72,7	193	193	132,1

[a] MHR (beats. min^{-1}) = 220-age (yrs.)
V'e (l/min) = pulmonary or minute ventilation

Maximum oxygen uptake and the individual data are listed in Table 3.2 (age, height, weight, maximum values for heart rate, age predicted maximum heart rate and V'e). There were three athletes with maximum oxygen uptakes above 70 ml.kg^{-1}.min^{-1}. The subjects viz. 9, 11 and 8 who had $VO_{2\ max.}$ values of 81,9 ml.kg^{-1}.min^{-1}, 72,7 ml.kg^{-1}.min^{-1} and 71,0 ml.kg^{-1}.min^{-1} respectively were all Provincial triathletes. Subject 9's $VO_{2\ max.}$ was similar to that obtained by the world record holder in the 3 000 m from Kenya in 1967 who had a $VO_{2\ max.}$ of 82,0 ml.kg^{-1}.min^{-1} (Saltin and Astrand, 1967).

The 3 oldest athletes viz. subjects 1, 7 and 10 had the lowest $VO_{2\ max.}$ values of 47,4; 47,7 and 46,5 ml.kg^{-1}.min^{-1} respectively. Subject 1 was a life saver while subjects 7 and 10 were ultradistance runners specialising in races such as the Comrades Marathon in South Africa. From this result it can be seen that subject 1, who was a swimmer, has a $VO_{2\ max.}$ almost as high as subjects 7 and 10. It should be noted that all subjects in this study were primarily runners during

training and/or during competition. The reduction in $VO_{2\ max.}$ with age is well documented (Saltin and Astrand, 1967; Astrand and Saltin, 1961). It has been noted that after age 25, $VO_{2\ max.}$ declines steadily at a rate of about 1% per year, so that by age 55, it is about 27% below values usually attained by 20 year olds (McArdle *et al.*, 1996). This could explain the low values of the three subjects as compared to the highest values obtained in subjects 8, 9 and 11 who were almost half their age.

The mean V'e for the subjects listed in Table 3.2. was 122,27 l/min with a mean oxygen uptake of 61,9 $ml.kg^{-1}.min^{-1}$. The highest individual value for V'e was 166,9 l/min obtained by subject 2 who was a South African hockey player and long distance runner. The MHR varied within the group between 165 and 201 with a mean of 186,2 beats/min (mean age = 29,6 years). The relatively high mean V_E maximum values recorded were as a result of intense exercise.

During quiet breathing at rest, the normal breathing rate is approximately 12 breaths per minute and the average tidal volume (volume of air moved during either the inspiratory or expiratory phase of each breath) is approximately 0,5 l per breath. Consequently, the volume of air breathed each minute, or minute ventilation (V'e) is 6 l/min.

V'e = 12 breaths per minute x 0,5 l = 6 l/min.

Significant increases in minute ventilation result from an increase in either the rate or depth of breathing or both. During strenuous exercise, the breathing rate of healthy adults usually increases between 35 and 45 breaths per minute and rates as high as 60 to 70 breaths per minute have been measured in elite athletes during maximal exercise (Casaburi, 1994). In male endurance athletes, minute ventilation may increase to 160 l/min in response to maximal exercise (McArdle *et al.*, 1996). From the results obtained it can be seen that the V'e increased dramatically with subject 2 showing an increase of 28 times the normal while the average change was approximately 20 times the normal.

During exercise the oxygen demand for the respiratory work is great. Pulmonary ventilation might be a limiting factor in the sense that further increases in ventilation will not increase the availability of O_2 without lowering the partial pressure of oxygen (Po_2) and the higher the ventilation the smaller is the total proportion of total O_2 that is available for usual work (Astrand and Saltin, 1961). The functional capacity of an individual usually decreases with age, deterioration often varies widely at a particular age according to lifestyle characteristics. Although all physiological

measures do decline with age, not all decline at the same rate. Functions such as heart rate, which shows very little if any ageing effect at rest, usually show an appreciable decrement during maximal exercise. The heart rate is increased in exercise by a decrease in parasympathetic and an increase in sympathetic neural activity triggered largely by stimulating input from the central command in the brain (McArdle *et al.*, 1996).

The most noted change in cardiovascular function with age is a decline in the maximum exercise heart rate. This apparent age effect is progressive with advancing years and it reflects reduced medullary outflow of sympathetic activity (β-adrenergic stimulation). A rough approximation of MHR with age is expressed by the following relationship (McArdle *et al.*, 1996).

MHR (beats. min^{-1}) = 220- age (years)	.....(McArdle *et al.*, 1996)

As can be seen from the results all athletes achieved their approximate maximum heart rates for their age. Subjects 1, 7 and 10 who were 40 yrs., 51 yrs. and 49 yrs. respectively, had the lowest maximum heart rates. Maximum cardiac output is reduced with age resulting in their lower MHR. Contributing to this reduced capacity for blood flow is a reduction in the heart's stroke volume, which may account for as much as 50% age-related decline in $VO_{2\ max.}$ and this clearly evident in their observed $VO_{2\ max.}$ (McArdle *et al.*, 1996).

From the results obtained it can be seen that individual differences and specificity are important to understanding the capacities of an individual. Variations in the type of sports and fitness levels of the subjects may be crucial. There are many interdependent factors which influence the physical performance. In general these factors are energy output (i.e. from anaerobic and aerobic processes), neuromuscular function (i.e. strength and technique) and psychological factors. These factors play different roles in the various sports events.

CHAPTER 4

VITAMIN C and E

4.1 Introduction

Cells, organelles and tissues are rich in unsaturated fatty acids and are susceptible to free radical damage. Lipid peroxidation, which involves the conversion of unsaturated fatty acids to lipid peroxides ultimately leads to changes in membrane structure and alterations in enzyme activity. Vitamin C and vitamin E are components of the defence system which the body has developed to protect against lipid peroxidation. Vitamin E is lipid soluble and is located within cell membranes whereas vitamin C (water soluble) is found in plasma. Together these vitamins help to reduce the activity of radical induced reactions and assist in membrane stabilisation. Vitamin C concentrations in plasma were measured spectrophotometrically while High Pressure Liquid Chromatography (HPLC) was used to determine vitamin E concentrations in plasma.

4.2 Vitamin C

4.2.1 Principle of the vitamin C assay

Plasma vitamin C can be measured both spectrophotometrically and by HPLC. The spectrophotometric method, however has proved to be simple, inexpensive method when compared to that of HPLC. Studies by Lee *et al.* (1982) have shown a good relation when both methods were used simultaneously.

Total plasma vitamin C concentration was determined using the 2,4-dinitrophenylhydrazine (2,4-DNPH) method (Brewster and Turley, 1987) in which ascorbic acid is oxidised to dehydroascorbic acid in the presence of copper (Cu^{2+}). The dehydroascorbic acid is treated with 2,4-DNPH to form dinitrophenylhydrazone. The hydrazone dissolves in strong sulphuric acid to produce an orange-red complex which is measured spectrophotometrically at 500 nm (Fig 4.1).

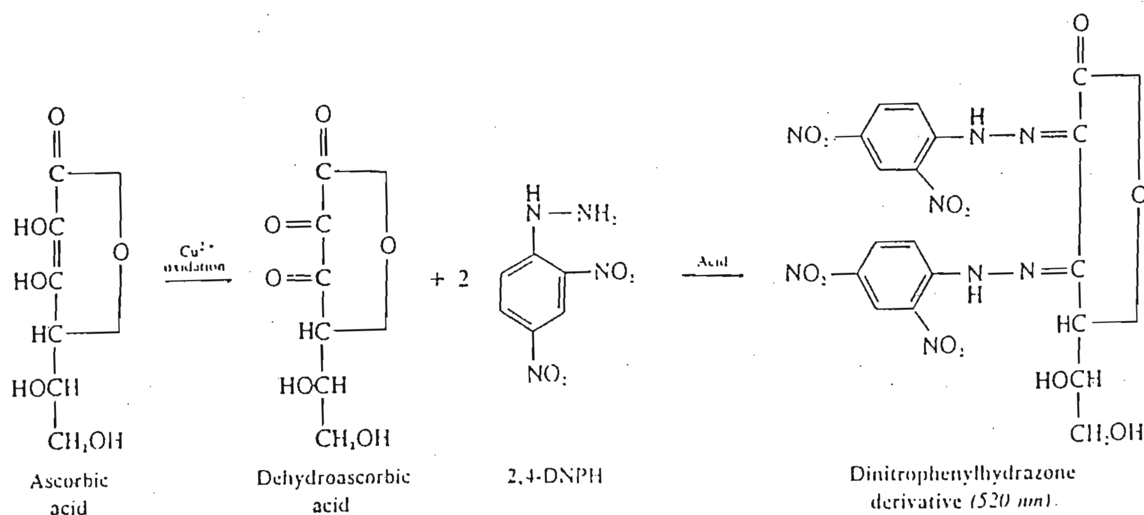


Fig 4.1 Principle of the 2,4-DNPH assay (Brewster and Turley, 1987).

4.2.2 Ultraviolet and visible spectroscopy

Ultraviolet (UV) and visible spectroscopy is an analytical method for measuring the amount of light absorbed by a substance in solution (Wilson and Goulding, 1989). These solutions absorb light of one wavelength and transmit light of another wavelength. As absorbency is related to the concentration of substance in solution, it can be used quantitatively to determine the concentration of the substance.

Light or electromagnetic radiation consists of photons that are associated with wave function and the distance between peaks in the wave is defined as the wavelength: $\lambda = c/v$ where λ is the wavelength of radiation, c is the speed of light, and v is the number of waves passing a certain point per unit time. i.e. frequency in Hertz (Hz).

Photons of different wavelengths have different energies that are given by: $E = hv = hc/\lambda$ where h is the Plank's constant. Light can be divided into regions according to the wavelength:

(1) UV ~200 - 400 nm

(2) Visible region ~400 - 700 nm

Molecules possess both kinetic energy and the energy associated with their bonding electrons. There are 3 types of bonding energies namely: electronic, vibrational and rotational. When molecules absorb electromagnetic radiation, there is a corresponding change in one or more of these bonding energies. The molecules consist of different energy levels and when the molecule is not absorbing photons, the electronic, vibrational and rotational energies are all at their lowest

values which is in the ground state. Thus the molecule is raised to a higher energy level when bombarded by photons. However, the energy gained from the photon absorption is lost by collision with other molecules. The energy is transformed into kinetic energy and the molecule returns to the ground state with the liberation of heat.

The quantitative aspects of light absorption is determined by the Beer-Lambert Law (equation 4.1) which is as follows:

$$- \text{Log } I/Io = - \text{Log } T = A = abc \quad \text{(equation 4.1)}$$

where A = absorbance

a = constant, called the extinction coefficient

b = path length

c = concentration of the substance under investigation and

I and Io are the intensity of the transmitted and incident light respectively.

The extinction coefficient can be determined by measuring the absorbency at different concentrations of the substance. Thus a plot of absorbance versus concentration yields a linear curve whose slope is the coefficient when the cell length is 1 cm.

The instrument consists of light sources, a tungsten lamp for the visible region, and a deuterium lamp for the UV region and monochromators which are optical systems which produce parallel beams of monochromatic radiation. The radiation is usually based upon refraction by a prism of diffraction grating. Prisms are of two types, namely glass for the visible region and quartz for the UV region. Light emerging from the monochromator consists of a bandwidth of wavelengths. Photocells convert quanta of radiation to electrical energy which is then amplified, detected and recorded. Photomultiplier tubes are more sensitive than photocells because the emitted electrons are accelerated by a high potential and generate secondary electrons by collision with the gas phase producing a larger current. The narrowest possible slit width should be used in order to obtain reliable data. Cuvettes are purchased as matching pairs, commonly known as the reference and test optically transparent cells. Special care should be taken when handling such cuvettes as scratched windows absorb light and reflect radiation, resulting in incorrect data. Cuvettes have an optical path length of 1 cm and require at least 3 ml of sample for accurate readings whereas optical microcells, only require up to 0,5 ml of sample.

4.2.3 Materials and Method

4.2.3.1 Vitamin C

Trichloroacetic acid (TCA), 2,4 DNPH, thiourea, cupric sulphate ($CuSO_4.5H_2O$), sulphuric acid (H_2SO_4) and the vitamin C stock standard were purchased from Saarchem, Durban.

For total plasma vitamin C concentration determination, a protein free filtrate was prepared within 30 minutes of drawing blood into heparinized tubes as described previously in section 3.4.

Plasma (1 ml) from all subjects was pipetted into 5 ml test tubes and this was followed by the addition of 10% TCA (1 ml) and chloroform (0,5 ml). The tubes were shaken vigorously for 10-15 seconds and centrifuged at 2 500 rpm for 7 minutes in a bench top centrifuge (4^0C). The clear supernatant was removed and placed into clean, sterile test tubes. The reagents for the analysis were prepared as follows (Appendix 4.1):

(1) The blank consisted of 10% TCA (0,5 ml), double distilled (dd) H_2O (0,5 ml) and colour reagent (0,4 ml). The colour reagent was made up by adding 2,4 DNPH (5,0 ml), $CuSO_4.5H_2O$ (0,1 ml) and thiourea (0,1 ml).

(2) The test sample consisted of the clear supernatant (1 ml) collected after centrifugation at 4^0C and the colour reagent (0,4 ml).

(3) The standard was prepared by mixing the vitamin C working standard (0,5 ml), 10% TCA (0,5 ml) and colour reagent (0,4 ml).

All the test tubes were stoppered and placed in a 56^0C water bath for 1 hour. After 1 hour the tubes were cooled to room temperature and 2 ml of cold 85% H_2SO_4 was added slowly with mixing. The tubes were then allowed to stand at room temperature for 30 minutes. They were vortexed twice and the absorbance was read at 500 nm using a Milton Roy Spectronic 601 UV spectrophotometer.

Concentration of Vitamin C in plasma was calculated as follows:

$$\frac{\text{Abs}_{\text{vitamin C in plasma}}}{\text{Abs}_{\text{vitamin C standard}}} \quad \text{x} \quad 113{,}6 \quad = \quad \mu\text{mol/l (vitamin C in plasma)}$$

The reproducibility of this test has been studied by Ziegler *et al.* (1987) who showed a reproducibility of 2,4% with respect to dehydroascorbic acid and a reproducibility of 1% with respect to ascorbic acid.

4.3 Vitamin E

4.3.1 Introduction

Vitamin E (α-tocopherol) can be measured in serum, red blood cells, platelets and lymphocytes. Serum is most frequently used for vitamin E analysis. Several techniques have been developed for measuring vitamin E concentrations and these include erythrocyte haemolysis, fluorometric techniques and HPLC. Of the methods cited, HPLC of plasma offers an increasingly attractive approach to vitamin E quantitation in the laboratory. This approach is not only sensitive and simple, but also affords separation of the analytes from interferents and permits specific quantitation of α-tocopherol, the most biologically active isomer (Brewster and Turley, 1987).

4.3.2 High Pressure Liquid Chromatography

The aim of chromatography is to separate components of a sample within a reasonable period of time into separate bands or peaks as they migrate through the column. In HPLC, the stationary phase consists of absorbent particles which are contained in a densely packed stainless steel column and the mobile phase is pumped through the column under pressure. The principle of separation is based on polarity (Holme and Peck, 1983).

There are two main types of absorbent packings. The first of these is silica in which the stationary phase is more polar than the mobile phase and is therefore described as the normal phase HPLC (NP-HPLC). Polar groups such as alkylnitrile $[Si\text{-}(CH_2)_nCN]$ and alkylamine $[Si\text{-}(CH_2)_nNH_2]$

groups can be bonded to the packing to modify polarity. The other type of packing is where silica is coated by covalent bonding, for reversed phased HPLC (RP-HPLC). In this form of chromatography, the mobile phase is more polar than the stationary phase. The elution order is generally related to the increasing hydrophobic nature of the solute. The choice of the mobile phase depends on the type of the stationary phase and the nature of the sample mixture to be separated. However, the mobile phase should have the following requirements: (1) be pure; (2) not react with the packing; (3) be compatible with the detector and (4) dissolve the sample.

The technique requires apparatus consisting of a solvent delivery system, an injection device, a column, a continuously monitoring detector and a recorder. All components provide the advantage of automation, continuous monitoring of a chromatogram and the ability of being directly interfaced with computer hardware to provide data processing (Fallon *et al.*, 1987).

There are many factors that are of interest to the chromatographer with the resolution and control of separation being the most important (Krstutovic and Brown, 1982). Mathematical equations are used to facilitate the choice of conditions for optimisation. The equation of resolution (R_S) is obtained by integrating individual expressions such as the column efficiency (N), selectivity (α) and the capacity factor (k) into a general expression for chromatographic performance, and is given as follows (equation 4.2):

$$R_S = {}^{1/4} (\sqrt{N}) (\alpha - 1/\alpha) (k_2/k_2 + 1) \text{....................(equation 4.2)}$$

where the column efficiency, as measured by the theoretical plate number (N), is a function of column parameters such as the solvent flow rate, the particle size of the column packing and the viscosity of the solvent. The factor N can be determined as follows (equation 4.3):

$$N = 16\,(v/w)^2 \text{..(equation 4.3)}$$

where v is the retention time for a retained component and w is the peak width at the baseline. The selectivity factor (α) is a function of the thermodynamics of the exchange between the stationary and mobile phases and is as follows (equation 4.4)

$$\alpha = V_2 - V_0/V_1 - V_0 \qquad \text{(equation 4.4)}$$

where V_1 and V_2 are the retention measures of two components 1 and 2 respectively, and V_0 is a measure of retention of the unretained component.

The capacity factor k is a function of the ratio of the volumes of the mobile and stationary phases in the column and is defined as (equation 4.5):

$$k = V_1 - V_0/V_0 \qquad \text{(equation 4.5)}$$

where V_1 is a measure of retention of component 1 and V_0 is the void volume.

A decrease in k, will cause a decrease in resolution and where k for two peaks is zero, then both peaks co-elute and no separation is observed. Increased k values will lead to an increase in resolution and analysis time. Increasing the selection will improve resolution without significant changes in analysis time and if $\alpha < 1$, then two peaks will be resolved. However, improving the resolution is merely a trial and error experience and the most effective option available, is to adjust the nature and/or the solvent strength of the mobile phase.

4.3.3 Materials and Method

Ethanol (HPLC grade) and hexane (HPLC grade) were purchased from Merck and methanol (HPLC grade) was purchased from Anatech. The dl-α-vitamin E and dl-α-vitamin E acetate stock standards were purchased from Sigma Chemical Company (USA) (Appendix 4.2).

Plasma samples were prepared as described in section 3.4.

For plasma vitamin E concentration determination, all the steps in the procedure were performed in indirect, subdued light, at room temperature. Ethanol (500 µl) and vitamin E internal standard (1 µg/ml; 250 µl) were added to plasma (250 µl) in plastic tubes which were then tightly sealed. The mixture was vortexed for 1 minute. Thereafter, hexane (500 µl) was added to the tube and the mixture was vortexed for a further minute. The mixture was then centrifuged at 2 500 rpm for

5 minutes. After centrifugation the upper hexane layer was removed and placed in a clean test tube. Hexane (500 μl) was added again to the mixture, vortexed and centrifuged as described previously. The upper hexane layer was removed and was added to the hexane layer from the previous extraction. The combined hexane layers were evaporated in a water bath (50°C) and the vitamin E was reconstituted with the addition of ethanol (50 μl). The freshly prepared extract (25 μl) was then ready for injection into the chromatographic system.

Before injection, chromatograms for vitamin E and vitamin E acetate internal standard were prepared to determine the elution times of both standards. Three concentrations of vitamin E and the vitamin E internal standard viz. 20 μg/ml, 10 μg/ml, 5 μg/ml were prepared as follows:-

20 μg/ml	vitamin E standard (1 000 μl) + vitamin E acetate standard (1 000 μl)
10 μg/ml	vitamin E standard (500 μl) + vitamin E acetate standard (1 000 μl)
5 μg/ml	vitamin E standard (250 μl) + vitamin E acetate standard (1 000 μl)

In each case, vitamin E and the vitamin E acetate standard mixture (50 μl) were injected into the chromatographic column. Vitamin E standard eluted at a retention time of 8,6 minutes whereas the vitamin E acetate internal standard eluted at a retention time of 11 minutes (Fig 4.2).

The HPLC system used was a Hewlett-Packard HP 1090 liquid chromatography system with in-built pump and Diodo Array Detector (DAD), automatic sampler/automatic injector and computer Ventra XM2 with Ultra VGA 1280 screen. The column used was a Licrospher 100 RP C18 (250 mm x 4 mm) (Merck) column. The mobile phase consisted of methanol (98%)/ water (2%) and the system was set at a flow rate of 1,0 ml/min at a pressure of 1 650 kPa. The samples were arranged appropriately on an automatic sampler, set to inject 25 μl of sample. The fluorescence detector was set at 292 nm and the HPLC system was set to analyse the samples automatically. Foljtikova and Binkova (1991) have shown that the determination of vitamin E levels using the method of HPLC has a reproducibility of 3,7%.

Statistical analysis

The results were tested for significance of difference between the means (±SD) of pre- and (i) immediate post-exercise values, (ii) 24 hour post-exercise values and (iii) 48 hour post-exercise values using analysis of variance (ANOVA). The level of significance employed was ($p \leq 0,05$).

4.4 Results

4.4.1 Vitamin C and E concentrations

The vitamin C and vitamin E concentrations obtained during the 48 hour period of the study are presented in Table 4.1.2 and Table 4.2.2 respectively.

The mean concentration before exercise, immediately after exercise, after 24 hours and after 48 hours for the 11 subjects were 70,11 ± 30,18 μmol/l; 46,93 ± 20,44 μmol/l; 60,98 ± 52,80 μmol/l and 61,97 ± 24,24 μmol/l respectively. Due to no readings being obtained because of insufficient sample in 2 subjects after 24 hours and in one subject after 48 hours, the mean plasma vitamin C concentrations are given with respect to 9 subjects only.

The mean concentration of total ascorbate (ascorbate plus dehydroascorbate) in plasma before exercise was 70,41 ± 33,70 μmol/l (n = 9). Immediately after exercise the concentration of vitamin C decreased to 46,41 ± 22,64 μmol/l (n = 9). Over the next 2 days there was an increase in vitamin C concentrations to levels detected before exercise. After 24 hours the concentration was 60,98 ± 52,80 μmol/l (n = 9), with the level approximately the same after 48 hours (59,97 ± 24,81 μmol/l, n = 9). The decrease in vitamin C levels immediately after exercise and the increasing trend over the remaining 2 days was not statistically significant when compared to the pre-exercise values ($p = 0{,}16$, $n = 9$).

The concentration of vitamin E in plasma increased from 2,11 ± 0,88 μg/ml before exercise to 2,30 ± 0,93 μg/ml (n = 11) immediately after exercise (n = 11). After 24 hours the concentration decreased to 2,11 ± 0,83 μg/ml (n = 11), similar to levels observed before exercise. After 48 hours there was an increase in vitamin E levels (2,20 ± 0,68 μg/ml, n = 11) above that of the baseline level, however the overall changes observed over the 48 hour period ($p = 0{,}82$, $n = 9$) was not significant when compared to the pre-exercise values..

4.4.2 Vitamin C and E concentrations for individual subjects

Minimum and maximum vitamin C concentrations were as follows:
From the results presented in Table 4.1.1, it can be seen that Subject 7 (51 yrs.), who was the oldest subject in this study, had the lowest vitamin C concentration before exercise

(37,75 μmol/l), however he also displayed an extremely high vitamin C concentration after 48 hours (112,20 μmol/l). Of the 11 subjects studied, subject 6 (23 yrs.) was characterised by a consistently high vitamin C concentration before exercise (141,97 μmol/l), immediately after exercise (84,94 μmol/l) as well as 24 hours after exercise (182,11 μmol/l). The lowest vitamin C level observed immediately after exercise (24,12 μmol/l) and after 48 hours (30,41 μmol/l) was seen in subject 2 (25 yrs.). After 24 hours, the lowest vitamin C concentration was seen in subject 4 (20 yrs.). Of the 11 subjects, 90,9% (n = 10) showed a decrease in vitamin C concentrations immediately after exercise while 9,1% (n = 1) showed an increase. After 24 hours, 44,4% (n = 4) showed an increase in vitamin C concentrations while 55,6% (n = 7) of the subjects showed a decrease in vitamin C concentrations compared to before exercise. After 48 hours, 50% (n = 5) showed an increase in vitamin C concentrations while a similar number showed a decrease in vitamin C concentrations (50%, n = 5).

Minimum and maximum vitamin E concentrations were as follows:
From the results presented in Table 4.2.1, it was observed that subject 3 (21 yrs.) exhibited the lowest vitamin E concentrations before exercise (1,10 μg/ml) as well as 24 hours after exercise (1,21 μg/ml). The highest vitamin E level before exercise (4,11 μg/ml) was noted in subject 6. Assessment of the 11 subjects immediately after exercise revealed that subject 2 (25 yrs.) showed the lowest vitamin E level (1,47 μg/ml). The highest vitamin E concentration immediately after exercise was seen in subject 7 (4,58 μg/ml) who also exhibited the highest value after 48 hours (3,82 μg/ml). After 24 hours the highest vitamin E concentration was seen in subject 8 (26 yrs.; 3,47 μg/ml) while the lowest vitamin E concentration observed after 48 hours was seen in subject 10 (1,53 μg/ml) who was 49 years old, the second oldest in the study (Table 4.2a). Of the 11 subjects, 72,7% (n = 8) showed an increase in vitamin E concentrations while 27,3% (n = 3) showed a decrease in vitamin E concentrations immediately after exercise. After 24 hours, 63,6% (n = 7) showed an increase in vitamin E concentrations while 36,4% (n = 4) of the subjects showed a decrease in vitamin E concentrations, compared to before exercise. After 48 hours, 54,5% (n = 6) showed an increase in vitamin E concentrations while 45,5% (n = 5) showed a decrease when compared to levels before exercise.

Table 4.1.1 Vitamin C concentrations (μmol/l) for 11 subjects, before exercise, immediately after exercise, 24 hours and 48 hours after exercise.

Subject	Before exercise (control)	Immediately after exercise	24 Hours after exercise	48 Hours after exercise
1	92,63	48,24	65,36	75,15
2	62,57	24,12	32,18	30,41
3	72,35	43,34	-	-
4	45,79	25,52	7,34	37,75
5	54,18	40,20	85,99	63,97
6	141,91	84,94	182,11	38,45
7	37,75	35,56	66,41	112,20
8	97,17	37,75	31,81	52,78
9	49,63	38,10	12,23	60,82
10	52,08	83,54	65,36	68,16
11	62,57	54,88	-	80,04
x	**70,11 ± 30,23**	**46,93 ± 20,24**	**60,98 ± 53,38**	**61,97 ± 29,63**

Key: (-) denotes no reading due to insufficient sample.

(x) denotes the mean (± SD) plasma vitamin C concentrations from all subjects at the various time intervals

Table 4.1.2 Mean (± SD) vitamin C concentrations obtained during the 48 hour period.

	Before exercise (control)	Immediately after exercise	24 hours after exercise	48 hours after exercise
Number	9	9	9	9
Vitamin C (μmol/l)*	70,41 ± 33,70	46,41 ± 22,64	60,98 ± 52,80	59,97 ± 24,81

* Data represents mean ± standard deviation (SD) over the 48 hour period.

The significance of difference observed over the 48 hour period when compared to the pre-exercise values was p = 0,16.

Table 4.2.1 Vitamin E concentrations (μg/ml) for 11 subjects, before exercise, immediately after exercise, 24 hours and 48 hours after exercise.

Subject	Before exercise (control)	Immediately after exercise	24 hours after exercise	48 hours after exercise
1	2,81	2,87	2,97	2,40
2	1,67	1,47	1,53	1,60
3	1,10	1,50	1,21	1,71
4	1,39	1,84	1,35	1,79
5	1,25	1,71	1,36	1,62
6	4,11	2,21	1,87	2,49
7	2,62	4,58	2,85	3,82
8	2,29	3,14	3,47	2,07
9	1,90	1,86	1,75	2,56
10	1,54	1,74	1,69	1,53
11	2,50	2,35	3,15	2,62
x	**2,11 ± 1,04**	**2,30 ± 1,12**	**2,11 ± 1,00**	**2,20 ± 0,91**

Table 4.2b Vitamin E concentrations obtained during the 48 hour period.

	Before exercise (control)	Immediately after exercise	24 hours after exercise	48 hours after exercise
Number	11	11	11	11
Vitamin E (μg/ml)*	2,11 ± 0,88	2,30 ± 0,93	2,11 ± 0,83	2,20 ± 0,68

* Data represents mean ± SD over the 48 hour period.

The significance of difference observed over the 48 hour period when compared to the pre-exercise values was $p = 0{,}82$.

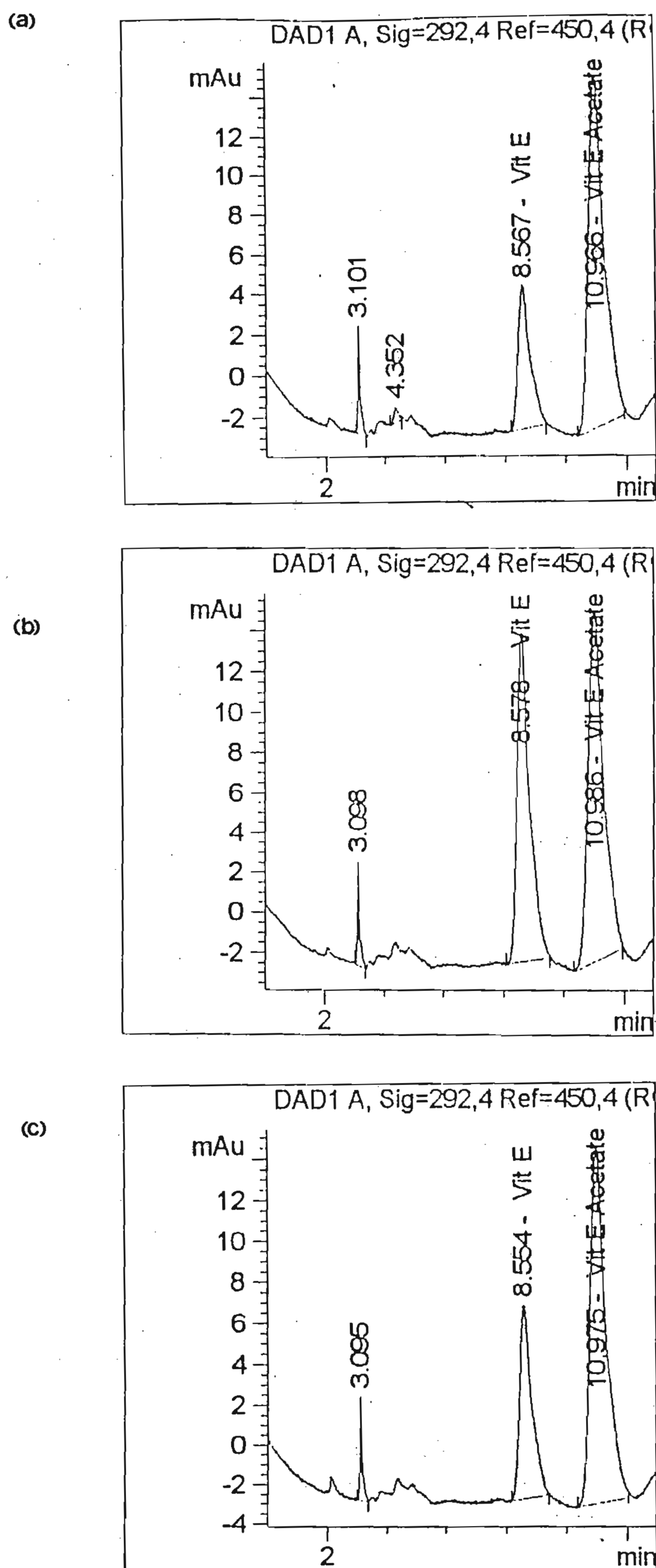


Fig 4.2 Chromatograms of vitamin E and vitamin E acetate standards at various concentrations.
(a) 20 µg/ml vitamin E standard (1 000 µl) + vitamin E acetate standard (1 000 µl)
(b) 10 µg/ml vitamin E standard (500 µl) + vitamin E acetate standard (1 000 µl)
(c) 5 µg/ml vitamin E standard (250 µl) + vitamin E acetate standard (1 000 µl)
Chromatograms for vitamin E (elution at 8,6 minutes) and vitamin E acetate (elution at 11 minutes) standards. Wavelength (292 nm), solvent (methanol 98% and water 2%), Flow rate 1,0 ml/min, column (C18 RP-HPLC column), pressure (1 650 kPa).

4.5 Discussion

Polyunsaturated fatty acids are highly susceptible to oxidative damage and are converted to lipid peroxides by the process of lipid peroxidation (Sen, 1995). The body's defences against oxidative stress are the antioxidants synthesised in the body and the antioxidant vitamins taken up in the diet (Witt *et al.*, 1992). The antioxidants synthesised in the body include antioxidant proteins (e.g. catalase and transition metal-binding proteins) and various small molecules, some of which are the end products of the metabolic pathways (e.g. bilirubin and urate) (Maxwell, 1995). Their levels in the body cannot be manipulated by simple means. On the other hand, the levels of antioxidant vitamins such as vitamin C and vitamin E can be increased by dietary means and supplementation. The antioxidants studied, vitamin C and E function synergistically in trapping free radicals, thereby preventing this process from taking place. Furthermore, vitamin C together with DHLA is responsible for the nonenzymatic recycling of vitamin E and thus vitamin C is more effective than any of the endogenous antioxidants, providing a protective rather than a pro-oxidant role (Frei *et al.*, 1989).

The reference range for vitamin C concentrations in persons on a normal American diet is from 34 to 110 µmol/l. Vitamin C concentrations of 23 to 79 µmol/l are due to the dietary vitamin C intake (for adults) of ≥ 40 mg/day. Persons whose concentrations are less than 11 µmol/l, are clearly deficient (Brewster and Turley, 1987). The normal reference range for serum total vitamin E in adults is 11,6 to 46,4 µmol/l. Serum vitamin E concentrations depend on the serum lipid content and therefore reference values for α-vitamin E are not well defined. Consequently further experimentation is required for accurate determination for reference ranges for serum vitamin E (Brewster and Turley, 1987).

The results of the present study showed a decrease in vitamin C concentrations immediately after exercise [46,41 ± 22,64 µmol/l (24,12-84,94 µmol/l)] as compared to before exercise [70,41 ± 33,70 µmol/l (37,75-141,91 µmol/l)]. There was no significant difference in vitamin C concentrations over the 48 hour period when compared to the pre-exercise values ($p = 0,16$). The non-significant decrease in vitamin C concentration immediately after exercise may suggest that vitamin C may be consumed at a high rate to possibly buffer and maintain membrane vitamin E concentrations at a constant concentration and also to buffer oxidants. The concomitant non-significant increase in lipid peroxide concentration seen immediately after exercise ($p=0,21$)

(section 5.4.1) is possible evidence of oxidant induced damage in exercising muscle. In plasma, vitamin C is the only exogenous antioxidant that can completely protect the lipids from detectable peroxidative damage induced by aqueous peroxyl radicals (Frei *et al.*, 1989). Vitamin C can only function as an antioxidant when transitional metal ions are absent (Halliwell, 1990). In the presence of transition metal ions (e.g. Fe^{3+} and Cu^{2+}), high concentrations of ascorbic acid may actually act as a pro-oxidant (Buettner, 1986). Vitamin C appears to trap virtually all peroxyl radicals in the aqueous phase before they can diffuse into the plasma lipids. Once ascorbate has been consumed completely, the remaining water soluble antioxidants, urate, bilirubin etc. can trap only part of the aqueous peroxyl radicals.

Compared to the concentrations of vitamin C levels in human plasma, ascorbate levels in human tissues are generally far higher. They are particularly high in the cornea, lens and aqueous humor of the eye and in adrenal and pituitary glands (Goldfarb, 1992). The brain, liver, spleen, kidneys and the pancreas also contain high concentrations of vitamin C (Hornig, 1975). These tissues, beside requiring vitamin C for enzymatic reactions, need particularly good antioxidant protection because of relatively high fluxes of oxidants due to high metabolic rate coupled to high oxygen consumption during exercise (Frei *et al.*, 1989). Vitamin C is also essential for many metabolic roles (e.g. in collagen synthesis and hormone production) and also helps to oxidise air pollutants in the respiratory tract (Halliwell, 1994). It is also involved in the absorption of iron and prevents the oxidation of folic acid which is a coenzyme required for the transmethylation synthesis of nucleoproteins and blood cell production (Pearson, 1994). The non-significant decrease in vitamin C concentration immediately after exercise may be as a result of the need for vitamin C to carry out all these functions.

After falling, the vitamin C concentrations increased after 24 hours [60,98 ± 52,80 μmol/l (7,34-182,11 μmol/l)] and remained at a constant level after 48 hours [59,97 ± 24,81 μmol/l (30,41-112,2 μmol/l)]. This increasing trend may be due to the efflux of ascorbate from the adrenal gland mediated by post-exercise increases in plasma cortisol. Plasma cortisol levels are known to increase after heavy exercise (McArdle *et al.*, 1996) and may remain elevated for hours thereafter (Duthie *et al.*, 1990). Prolonged, high plasma concentrations of cortisol ultimately lead to excessive protein breakdown, tissue wasting and negative nitrogen imbalance. Cortisol secretion also accelerates lipid mobilisation for energy. With rapid and large increases in plasma cortisol, the liver splits the mobilised lipids into its simple ketoacid components. Above normal ketoacid concentrations in the extracellular fluid can result in the elevation of hydrogen ions (H^+),

which may then act as electron donors and lead to the potentially dangerous condition called ketosis (a form of acidosis) (McArdle *et al.*, 1996). During respiration, oxygen may receive only one electron and the addition of H^+ leads to the formation of $O_2^{\cdot-}$, H_2O_2 and $OH^{\bullet}$. The resultant increase in vitamin C concentrations is to quench these radicals before the initiation of lipid peroxidation (Kanter, 1994).

The increase in vitamin C concentration after 24 hours and its similar level after 48 hours may also be due to dietary intake. It should be noted that none of the subjects were on, or received any form of supplementation during the study and neither were they on a controlled diet. Sources of vitamin C may include citrus fruits and juices, salad greens, tomatoes and green peppers (Maxwell, 1995; McArdle *et al.*, 1996) and these may have been sources of vitamin C in their daily diets. The decrease in vitamin C concentrations immediately after exercise suggest that it was required to perform its functions maximally at this point and the increase thereafter was probably as a result of dietary intake and it being needed briefly in the post exercise period.

The results differ from those observed by Duthie *et al.* (1990), Robertson *et al.* (1991) and Meydani *et al.* (1993). All observed an increase in plasma ascorbic acid after exercise, however the increases were not significant. Duthie *et al.* (1990) observed the increase in plasma vitamin C immediately after completion of a half-marathon while Robertson *et al.* (1991) observed the increase immediately after $VO_{2\ max.}$ testing in 26 subjects, using a graded treadmill run to exhaustion similar to that which had been used in this study. The only difference to these reported studies is the method used to assess total plasma vitamin C levels. The HPLC method was used to ascertain vitamin C levels in the above studies. In the present study, the 2,4 DNPH method was used to measure the reduced (ascorbic acid) and oxidised (dehydroascorbic acid) form of vitamin C, however, the DHLA concentration was not measured. In the present study, thiourea was added to reduce interference from non-ascorbate chromagens such as fructose and glucoronic acid (Brewster and Turley, 1987). Lee *et al.*(1982) used the HPLC and 2,4 DNPH methods simultaneously and the results correlated well (r = 0,97).

Vitamin E is a lipophilic radical inhibitor. Correspondingly, its action is limited mainly to the region of the lipophilic membrane (Simon-Schnass, 1993). Vitamin E directly scavenges most species of free radicals including $O_2^{\cdot-}$, H_2O_2 and lipid peroxides by using the hydroxyl group on the chromanol head to donate a proton or accept an electron. The resulting vitamin E radical will ultimately react with itself or with another peroxyl radical to form non-reactive degeneration by-products (Burton and Traber, 1990).

The results of the present study show that vitamin E concentrations increased non-significantly immediately after exercise [2,30 ± 0,93 μg/ml (1,47-4,58 μg/ml)] as compared to before exercise [2,11 ± 0,88 μg/ml (1,10-4,11μg/ml)]. A possible explanation is that vitamin E concentrations may have been maintained at the expense of vitamin C which had decreased immediately after exercise. Frei *et al.* (1989) have stated that only after vitamin C has been consumed, will the remaining antioxidants carry out their function. The peroxyl radicals that escape these remaining antioxidants in the aqueous phase will diffuse into the plasma lipids, where they initiate lipid peroxidation (Singh, 1992). The initiation and propagation of peroxidation in the lipids are then strongly inhibited by vitamin E (Goldfarb, 1993). It can thus be inferred that a minimal amount of peroxyl radicals were not taken up by vitamin C and thus there was no significant increase of vitamin E from the tissue reserves (Gohil *et al.*, 1987)

The mean concentration of vitamin E decreased non-signficantly to control levels after 24 hours [2,11 ± 0,83 μg/ml (1,21-3,47 μg/ml)] and increased slightly after 48 hours [2,20 ± 0,68 μg/ml (1,53-3,82 μg/ml)], however not significantly. Pincemail *et al.* (1988) have suggested that vitamin E may be mobilised from tissues to help prevent lipid peroxidation in skeletal muscle. Free radicals have been implicated in skeletal muscle damage and inflammation after strenuous exercise (Dekkers *et al.* 1996). This damage to exercising muscle results in loss of muscle function, release of muscular enzymes and muscle pain. Skeletal muscle is composed of 20% protein (McArdle *et al.*, 1996). Proteins in turn are composed of amino acids and it is the function of vitamin E to repair these oxidised amino acids (Simon-Schnass, 1993) and thus restore the structure of skeletal muscle. The non-significant increase in vitamin E concentrations seen after 48 hours may be due to the mobilisation of vitamin E from cells membranes (e.g. erythrocyte membranes) to skeletal muscle to repair damaged muscle after a bout of high intensity exercise (Pincemail *et al.*, 1988). The increase may also be due to the ability of vitamin E to lower the exercise-induced leakage of enzymes from lysosomes and mitochondria (β-glucouronidase and mitochondrial glutamic-oxaloacetic transaminase) (Sumida *et al.*, 1989), possibly because these membranes, especially the mitochondrial membranes, are more likely to be oxidatively damaged during exercise.

The results are similar to those obtained by Viguie *et al.* (1993) who showed no significant changes in plasma vitamin E levels after 3 consecutive days of prolonged exercise (90 minutes) at submaximal intensity (65% $VO_{2\,max.}$). This had also been observed before and 120 minutes after a

half marathon (Duthie *et al.*, 1990). The non-significant rise in plasma vitamin E levels seen in this study have also been reported following a bout of eccentric exercise (Meydani *et al.*, 1993) and after intense cycling to exhaustion (Pincemail *et al.*, 1988). The increase in vitamin E levels immediately after exercise may be attributed to the redistribution of vitamin E in the body (Meydani *et al.*, 1993). All the studies mentioned used the HPLC method to assess vitamin E levels. The results also suggest that exercise of different intensities does not affect the levels of vitamin E in plasma.

In summary, the results of the present study, demonstrated that vitamin E concentrations were constant over the 48 hour period following high intensity exercise, at the possible expense of vitamin C which showed a non significant reduction.

CHAPTER 5

LIPID PEROXIDES

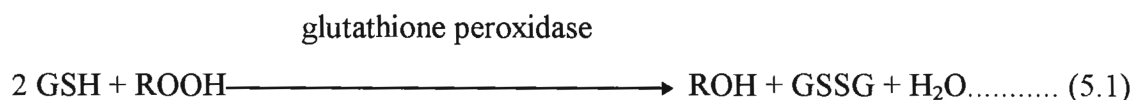
5.1 Introduction

Lipid peroxidation occurs as a result of the generation of free radicals. During exercise the oxygen consumption of an individual is increased 10 to 15 fold above rest and as a result free radicals are produced to a greater extent. These free radicals initiate lipid peroxidation in the membranes of muscle tissues, organelles and various cells. Furthermore, during exercise, damage to active tissues occurs and oxidative stress reactions are known to increase in damaged tissues. To suppress the effects of free radicals, an elaborate defence system consisting of vitamins and enzymes has evolved in all species.

Biological oxidation occurs by mechanisms similar to chemical peroxidation. Peroxidation in various biological systems has been measured by the thiobarbituric acid reaction, which measures only one indirect decomposition product, MDA (Draper *et al.*, 1993). A more satisfactory quantitation of peroxidation can be achieved by measuring the initial peroxidation product. While there exists a large number of methods for measurement of peroxides, these methods are often non-specific for hydroperoxides and are relatively insensitive (Heath and Tappel, 1976). Procedures for the determination of hydroperoxides have included catalytic dye bleaching, formation of phenolpthalein or triiodide, coupled oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) and horseradish peroxide-coupled oxidations. The method based on coupled oxidation of NADPH is the most widely applicable to peroxide species (Frew *et al.*, 1983) and was used to determine the level of lipid peroxides in the trained athletes after a single bout of high intensity exercise. The advantage of using this method is that it can be used with a variety of solvent systems and can detect hydroperoxides easily and specifically at a concentration as low as 3 nmol/ml (Heath and Tappel, 1976).

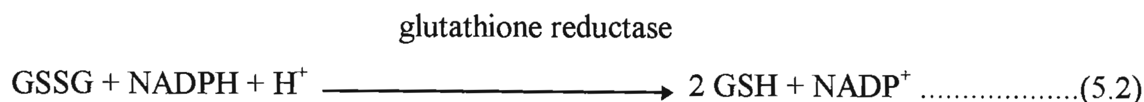
5.2 Principle of the assay

The method based on the coupled oxidation of NADPH involves the coupling of glutathione peroxide and glutathione reductase (Frew *et al.*, 1983). In the glutathione redox cycle, glutathione peroxidase catalyses the reaction of hydroperoxides with glutathione (GSH) as the reductant, where R may be H or an organic residue (reaction 5.1)



$$2\ GSH + ROOH \xrightarrow{\text{glutathione peroxidase}} ROH + GSSG + H_2O \quad (5.1)$$

This reaction is followed by measuring the coupled oxidation of NADPH in the presence of glutathione reductase (reaction 5.2):



$$GSSG + NADPH + H^+ \xrightarrow{\text{glutathione reductase}} 2\ GSH + NADP^+ \quad (5.2)$$

5.3 Materials and Method

Glutathione peroxidase (GSHPx) was purchased from the Sigma Chemical Company (USA). Glutathione reductase (type III from yeast) and NADPH were purchased from Boehringer Mannheim. Sodium azide (NaN_3), sodium dihydrogen orthophosphate (NaH_2PO_4), ammonium suplhate (NH_4SO_4), ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide (H_2O_2) (6%), arsenic oxide (As_2O_3), potassium permanganate ($KMnO_4$) and potassium iodide (KI) were purchased from Saarchem, Durban.

Plasma samples were prepared as described previously in section 3.4. The H_2O_2 (1-5 μM) standard curve, blank and plasma lipid peroxide samples were prepared as follows (Appendix 5.1):

(1) The blank, which was required to zero the spectrophotometer, consisted of 0,1M phosphate buffer [NaH_2PO_4 (0,01M) and dithiothreitol (1 mmol/l)], pH 7,75 (250 μl), 0,01 M EDTA (25 μl), 0,052 M NaN_3 (50 μl), 1 x 10^{-6} M GSHPx (50 μl), 0,038 M GSH (50 μl), 1,4 x 10^{-3} M NADPH (25 μl), 5 units/ml glutathione reductase (50 μl) and dd H_2O (0,125 ml).

(2) The freshly prepared standards (1-5 μl) consisted of the same reagents as the blank, except the standard (0,125 ml) replaced dd H_2O.

(3) The plasma lipid peroxide samples consisted of the same reagents as the blank except that the plasma sample (0,125 ml) replaced dd H_2O.

The blank was thoroughly mixed, placed in a cell holder and the wavelength set at 340 nm. Each standard (1-5 μM) and the plasma lipid peroxide samples were decanted into cuvettes and the extent of NADPH oxidation was monitored using a Milton Roy Spectronic 3000 Array spectrophotometer. All the measurements were performed at 25°C and the extent of NADPH oxidation was completed within 2-5 minutes. A standard curve was drawn using the absorbance values obtained at its corresponding concentrations (Fig 5.1). The absorbance values for each plasma lipid sample was read and the concentration of the plasma lipid samples were determined from the standard curve and multiplied by 8 (i.e./ml).

Statistical analysis

The results were tested for significance of difference between the means (±SD) of pre- and (i) immediate post-exercise values, (ii) 24 hour post-exercise values and (iii) 48 hour post-exercise values using analysis of variance (ANOVA). The level of significance employed was ($p \leq 0,05$).

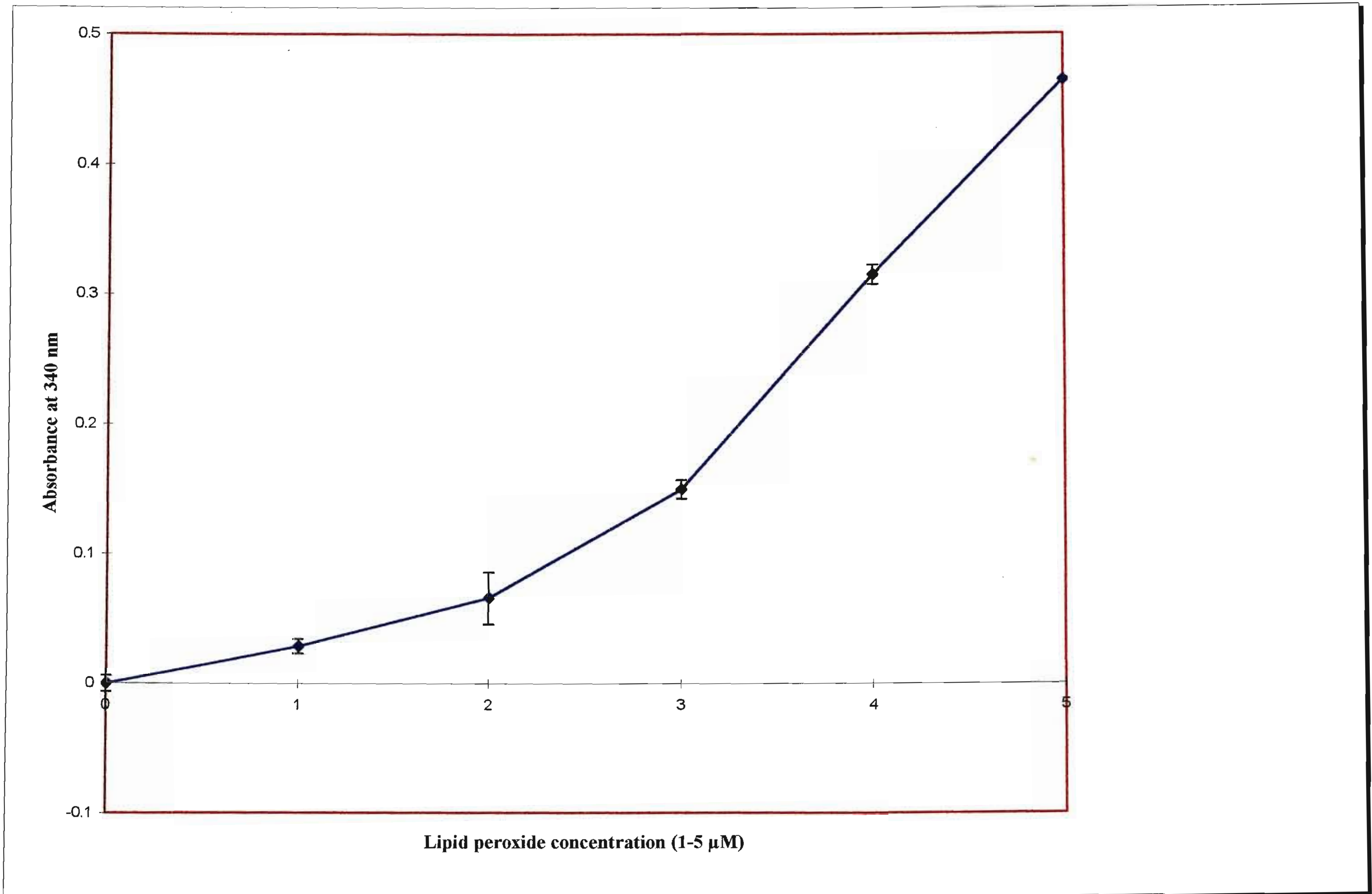


Fig 5.1 **Lipid peroxide standard curve with absorbance at 340 nm as a function of lipid peroxide concentration (µmol/0,125 ml).**

5.4 Results

5.4.1 Lipid peroxide concentrations

The mean concentration before exercise, immediately after exercise, after 24 hours and after 48 hours for the 11 subjects were 27,62 ± 13,08 μmol/l; 32,50 ± 9,15 μmol/l; 26,39 ± 11,59 μmol/l and 35,62 ± 29,53 μmol/l respectively. Due to the absence of readings as a result of insufficient sample in 1 subject after 24 hours and in 1 subject after 48 hours, the mean plasma lipid peroxide concentrations are given with respect to 10 subjects only.

The lipid peroxide concentrations obtained over the 48 hour period are presented in Table 5.1b. The plasma lipid peroxide concentrations before exercise was 28,99 ± 12,94 μmol/ml (n = 10). This level increased non-significantly immediately after exercise to 32,97 ± 9,50 μmol/ml (n = 10), however a decrease was observed after 24 hours (26,39 ± 11,59 μmol/ml, n = 10). The level reached after 24 hours was similar to the level seen before exercise. After 48 hours there was a non-significant increase in the lipid peroxide concentrations (35,62 ± 29,53 μmol/ml, n = 10) to values above those seen immediately after exercise. The changes in the lipid peroxide concentrations over the 48 hour period, when compared to the pre-exercise value was not significant (p = 0,21).

5.4.2 Lipid peroxide concentrations for individual subjects

Minimum and maximum lipid peroxide concentrations were as follows:
The lipid peroxide concentrations are presented in Table 5.1a. The lowest lipid peroxide concentrations obtained throughout this study were observed in subject 7 (51 yrs.). Before exercise the lipid peroxide concentration attained in subject 7 was 2,10 μmol/ml. This level increased immediately after exercise to 20,92 μmol/ml. A subsequent decrease was noted after 24 hours (8,36 μmol/ml) and this decreasing trend persisted after 48 hours (2,79 μmol/ml) to a level approaching control levels (2,10 μmol/ml). The maximum concentration observed before exercise was seen in subject 9 (47,46 μmol/ml) and immediately after exercise in subject 8 (44,60 μmol/ml). After 24 hours, the highest lipid peroxide concentration was observed in subject 6 (40,42 μmol/ml) while subject 1 displayed the highest concentration after 48 hours (108,72 μmol/ml). Immediately after exercise, 63,6% (n = 7) of the subjects showed an increase

in lipid peroxide concentrations while 40% (n = 4) showed a decrease. After 24 hours, 36,4% (n = 4) of the subjects showed an increase in lipid peroxide concentrations while 60% (n = 6) showed a decrease in lipid peroxide concentrations. After 48 hours, an increase in lipid peroxide concentrations was observed in 60% (n = 6) of the subjects while 40% (n = 4) showed a decrease in lipid peroxide concentrations when compared to levels seen before exercise.

Table 5.1.1 Lipid peroxide concentrations (μmol/ml) before exercise, immediately after exercise, 24 hour and 48 hours after exercise.

Subject	Before exercise (control)	Immediately after exercise	24 Hours after exercise	48 Hours after exercise
1	33,48	44,6	40,42	108,72
2	29,69	25,12	21,18	20,21
3	13,94	27,87	-	-
4	39,57	22,30	18,46	21,61
5	28,84	26,48	27,18	23,00
6	29,97	39,02	40,42	30,80
7	2,10	20,92	8,36	2,79
8	24,39	44,60	34,84	30,18
9	47,46	44,60	27,87	59,23
10	15,33	30,66	10,39	20,92
11	39,02	31,36	34,74	38,75
x	**27,62 ± 13,13**	**32,50 ± 9,15**	**26,39 ± 13,57**	**35,62 ± 30,00**

Key : (-) denotes no reading obtained due to insufficient sample

(x) denotes the mean plasma lipid peroxide concentrations of all subjects at the various time intervals.

Table 5.1.2 Mean (±SD) lipid peroxide concentrations taken during the 48 hour period.

	before exercise (control)	Immediately after exercise	24 hours after exercise	48 hours after exercise
Number	10	10	10	10
Lipid Peroxide (μmol/ml)*	28,99 ± 12,94	32,97 ± 9,50	26,39 ± 11,59	36,52 ± 29,53

* Data represents mean ± SD over the 48 hour period.

The level of significance when compared to the pre-exercise values was $p = 0,21$.

5.5 Discussion

There are several means by which free radicals are generated during exercise. These are: (1) the increased intake of oxygen which itself is a diradical; (2) the production of intermediates, such as O_2^-, H_2O_2 and $OH^\bullet$, that are increased due to partial reduction of oxygen; (3) the increase in epinephrine and other catecholamines that can produce oxygen radicals when they are metabolically inactivated; and (4) the production of lactic acid that can convert a weakly damaging free radical (superoxide) into a strongly damaging one (hydroxyl) (Clarkson, 1995). These free radicals react with unsaturated fatty acids and initiate a chain of events known as lipid peroxidation. Lipid peroxidation can cause damage to cells by producing an increase in membrane fluidity, inability to maintain ionic gradients, cellular swelling and tissue inflammation (Allesio, 1993). The toxic effects of lipid peroxidation may be opposed by the antioxidants vitamin C and E.

The non-significant rise in lipid peroxide concentrations before exercise from 28,99 ± 12,94 µmol/l to 32,97 ± 9,50 µmol/l immediately after exercise can be attributed to the rise in peroxyl radicals known to occur in plasma immediately after high intensity exercise (Pincemail *et al.*, 1988). However, this result suggests that the oxidative stress incurred was insufficient to increase the level of lipid peroxide oxidation. The peroxyl radicals produced during oxidative damage in muscle, when released extracellularly can destroy adjacent host cells and tissues (e.g. muscle) in the vicinity of inflammatory reactions (Peters, 1997). These radicals are removed from plasma by antioxidants, vitamin C and E, however a few may elude the defence formed by these antioxidants and cause lipid peroxidation in adjacent membranes especially in skeletal muscle. Another source which may initiate lipid peroxidation immediately after exercise is free iron. Free iron is harmful because it is able to diffuse into membranes and can interact with vitamin C to initiate lipid peroxidation. Running and other sports with high mechanical impact can cause destruction of red blood cells which may then serve as a potential source of free iron (Sjodin *et al.*, 1990).

In addition to vitamin C and vitamin E, there are several inducable antioxidant enzymes such as SOD, catalase (CT) and glutathione peroxidase (GP) (Criswell *et al.*, 1993). Superoxide dismutase scavenges peroxide radicals and produces H_2O. Glutathione peroxidase catalyses the reduction of H_2O_2 to H_2O and a large range of lipid hydroperoxides to hydroxy acids. Catalase

also reduces H_2O_2 to H_2O (Kanter, 1994). The action of these enzymes, as well as vitamin C and E have been known to reduce lipid peroxide concentrations and oxidative stress (Goldfarb, 1993; Robertson *et al.*, 1991). The increase in vitamin C and vitamin E concentrations in the 24 hour post-exercise samples (section 4.5) could account for the reduction in lipid peroxide concentration observed after 24 hours.

In this study, during the post-exercise recovery period (48 hr) there was a non-significant increase in the lipid peroxide concentration. This rise in lipid peroxide concentration may indicate the occurrence of ischemia in certain organs and tissues during exercise followed by reperfusion during recovery. During exercise there is a redistribution of blood away from many organs and tissues (e.g. kidney, splanchnic region) to the working muscles. Some of these tissues may become transiently hypoxic and thus, on reperfusion may be susceptible to peroxidation (Maxwell *et al.*, 1993; Lovlin *et al.*, 1987). Along with the beneficial effects of reperfusion, some component of the re-introduction of oxygen (re-oxygenation) is responsible for further damage, which among others might be the release of oxygen-derived free radicals. Reperfusion on relaxation allows the production of superoxide radicals (Maxwell *et al.*, 1993; Kellog and Fridovich, 1975; Wolbarsht and Fridovich, 1989).

These results compare favourably with those of Duthie *et al.* (1990) and Allesio (1993). In both studies there were increases in lipid peroxide concentrations immediately after a half marathon and graded exercise run to exhaustion, however not significantly. In both these studies, the method of measuring conjugated dienes was used to assess lipid peroxide levels. Duthie *et al.* (1990) have attributed the slight increase, although not significant, to the fact that the oxidative stress incurred was insufficient to cause significant free radical mediated peroxidation of fatty acid components of the cell membranes. The slight increase in lipid peroxide concentrations immediately after exercise suggests that the protective effect of the antioxidant defence systems was sufficient to counteract the effects of the free radicals and prevent high levels of lipid peroxidation.

These results differ from those of Allesio and Cutler (1990), Kanter *et al.* (1988), Laires *et al.* (1993). Kanter *et al.* (1990) reported a 77% increase in lipid peroxide concentrations above resting levels in both serum and plasma from highly trained athletes immediately after exhaustive running. Allesio and Cutler (1990) reported a 96% increase in lipid peroxide concentrations following exhaustive exercise while Laires *et al.* (1993) reported a 67% increase immediately after a 40 minute run. It should be noted that in these three studies, lipid peroxide

levels were measured using the TBARS method. This technique is sensitive to MDA which is a product of lipid peroxidation. The TBARS test for MDA has been controversial. The reaction requires acid for catalysis and for maximum complex formation and should be carried out at a pH of 2-3. An excess of strong acid can inhibit colour development (Bird and Draper, 1984). The disadvantages of using acid are: (1) heating with strong acid alters the structure of TBARS, leading to degradation products and (2) different values have been obtained when different acids are used. As lipid peroxidation is a rapid and complex process, accurate estimation is problematic. Some lipid peroxides have very short half lives and with the diversity of peroxide compounds, direct measurement is difficult and can be misleading.

The results of the present study demonstrated that lipid peroxide concentrations showed a non-significant increase immediately after exercise. The continuous production of lipid peroxides may have been opposed by the free radical trapping ability of vitamin C acting synergistically with vitamin E. In addition, the method used to determine lipid peroxide concentrations seems to be an underlying factor in similarities and differences seen in previously reported studies. Other factors that need to be considered are the intensity of the exercise, blood sampling times and the diet, which was not controlled in this study.

CHAPTER 6

APOPTOSIS

6.1 Introduction

There are a variety of strategies possible in developing methodologies that differentiate live from apoptotic cells. The more widely used methods, which are based on characteristic biochemical and morphological changes in apoptotic cells (Payne *et al.*, 1995), include light and transmission electron microscopy (Falasca *et al.*, 1996; Oka *et al.*, 1996; Samaha *et al.*, 1995), analysis of DNA fragmentation using gel electrophoresis (Douglas *et al.*, 1995; Zhang *et al.*, 1995; Samaha *et al.*, 1995), or DNA quantification assays (Sandri *et al.*, 1995), *in situ* labelling techniques and analysis of nuclear chromatin by fluorescent DNA binding dyes and flow cytometry (Douglas *et al.*, 1995; Momoi *et al.*, 1996; Liegler *et al.*, 1995). In this particular study, four different methods were used to detect apoptosis in lymphocytes after a single bout of high intensity exercise. The methods employed were the single cell gel electrophoresis (SCGE) assay, an *in situ* cell detection assay using a labelling technique, results being quantified by flow cytometry, light and electron microscopy.

6.2 DNA Damage

6.2.1 Introduction

Techniques which permit the sensitive detection of DNA damage have been useful in the studies of environmental toxicology (Hartmann and Speit, 1994), carcinogenesis (Lewensohn *et al.*, 1982) and ageing (Niedermuller *et al.*, 1985). Since the effects of environmental toxicants, cancer and ageing are often tissue and cell-type specific, techniques were developed to detect DNA damage in individual cells. Rydberg and Johanson (1978) were the first to directly quantitate DNA damage in individual cells by lysing cells embedded in agarose on slides, under mild alkali conditions to allow the partial unwinding of DNA. After neutralisation, the cells were stained with acridine orange or ethidium bromide and the extent of DNA damage was quantified by measuring the ratio of green (indicating double stranded DNA) to red (indicating single stranded DNA) fluorescence using a photometer. This technique was not widely

used as numerous critical steps were involved in the processing. To improve the sensitivity for detecting DNA damage, Singh *et al.* (1988) developed a microgel electrophoresis technique called the SCGE assay. In this technique cells are embedded in agarose gel on microscope slides, lysed by detergents and high salt, and then electrophoresed for a short period under neutral conditions. Cells with increased DNA damage display increased migration of DNA from the nucleus toward the anode. However the neutral conditions only permit the detection of double stranded breaks and since many agents induce from 5 to 2 000 fold more single stranded breaks than double stranded breaks, alkaline conditions have now replaced the neutral conditions (Singh *et al.*, 1991). Alkaline conditions result in the degradation of cellular RNA, which sometimes interferes with the quantitation of the ethidium bromide stained samples. Alkaline conditions also optimise DNA denaturation and the migration of single stranded DNA, thus permitting an evaluation of single stranded DNA breaks and alkali labile sites.

The importance of this assay lies in its ability to evaluate DNA damage and repair in proliferating or nonproliferating cells, to detect intercellular differences in DNA damage and its requirement for very small samples. The DNA in the nucleus is supercoiled and tightly packed. Single stranded breaks which are detected by the SCGE assay releases supercoiling and induces a relaxed structure. Fluorescent strands seen outside the cell are indicative of extensive DNA damage and represent the relaxed structure of the DNA. This technique has been used widely to assess DNA damage induced in human WBC by the carcinogenic metals, arsenic and cadmium (Hartmann *et al.*, 1994), for detecting radiation induced DNA damage in humans (Tice and Strauss, 1995) and to assess DNA damage after physical activity (Hartmann *et al.*, 1994). This technique was used to examine the effect of a single bout of high intensity exercise on lymphocytes and lymphocyte DNA in trained athletes.

6.2.2 Materials and Method

All chemicals used in the SCGE assay were purchased from Saarchem unless otherwise stated. The procedure of the SCGE assay was carried out using aseptic techniques. Contact with hazardous chemicals was minimised by using sterile gloves and a mask (Appendix 6.1).

Low melting point agarose (LMPA) (0,5%, 0,5g) was melted in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) (100 ml) by heating in a microwave (60°C) until a clear solution was achieved. The molten agarose was then cooled to 35°C. A first layer of LMPA (0,5%) was poured onto one end of 2 microscope glass slides (300 μl each) (Lasec, 76 x 26 mm) covering an area of approximately 19 x 26 mm^2 (Fig 6.1). The slides were covered with a coverslip (60 x 20 mm) and maintained at 4°C for 10 minutes to allow the agarose to solidify. The solid agarose formed a matrix, the density of which was determined by the concentration of the agarose. The first layer on the slide was used to promote even and firm attachment for the second and third layers. A second layer of molten LMPA (96μl) was mixed with whole blood (4μl) and the mixture was rapidly pipetted onto the first solidified layer of LMPA on the microscope glass slides covering an area of 19 x 26 mm^2. The slides were covered with a coverslip and maintained at 4°C for 10 minutes. The coverslips were then gently removed after solidification of the second layer. A third layer of LMPA (75μl, 0,5%) was then placed on the previous two solidified layers on the microscope slides, covered with a coverslip and maintained at 4°C for 10 minutes. The coverslips were then gently removed and the slides were then gently immersed in a freshly prepared lysing solution (250 ml) consisting of 2,5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), 1% Triton X100, 10% DMSO for 1 hour at 4°C, to lyse the cells and permit DNA unwinding.

After 1 hour the slides were removed, drained and placed side by side in a horizontal electrophoresis tank with the preset gel ends of the microscope slides closest to the anode. The tank was filled with fresh electrophoresis buffer made up of 1 mM Na_2EDTA and 300 mM NaOH (pH 13), to a level approximately 0,25 cm above the slides. The primary role of the buffer is to impose some constant number of charges on the cell, provide good conditions of good solubility and to pass some current. The pH and the ionic strength of the buffer will affect the migration of the cells during electrophoresis. If the electrophoresis buffer is omitted, electrical conductance is minimal and the cells will migrate slowly, if at all (Sambrook *et al.*, 1989). The slides were left in the buffer for 20 minutes to allow for unwinding of DNA before electrophoresis.

Electrophoresis was then conducted at room temperature for 20 minutes at 25V using a Sahndon Southern and Vocam electrophoresis power supply. Twenty five volts was used to obtain maximum resolution of the migrating cells. After electrophoresis, the slides were removed from the tank and were washed gently to remove alkali and detergents which would interfere with ethidium bromide staining, by placing them on a tray and flooding them slowly with 3 changes of 0,4 M Tris (3 ml), pH 7,5 for 5 minutes.

After neutralisation the slides were stained with 50 µl of ethidium bromide (20 µg/ml) and covered with a coverslip. Ethidium bromide is a dye that intercalates between stacked base pairs of the DNA molecule, extending the length of linear and nicked DNA and making them more rigid. All the steps were conducted in the dark to prevent any additional DNA damage. The slides were then viewed with a Nikon UV fluorescent microscope with an excitation filter of 515-560 nm and a barrier filter of 590 nm. Images of 50 randomly selected lymphocytes (25 cells from each of the two replicate slides), covering an area of 19 mm x 26 mm^2 were identified on the basis of cell size and location of the nucleus. These images were analysed and photographed using a Nikon camera.

Statistical analysis

The results were tested for significance of difference between the means (±SD) of pre- and (i) immediate post-exercise values, (ii) 24 hour post-exercise values and (iii) 48 hour post-exercise values using analysis of variance (ANOVA). The level of significance employed was ($p \leq 0,05$).

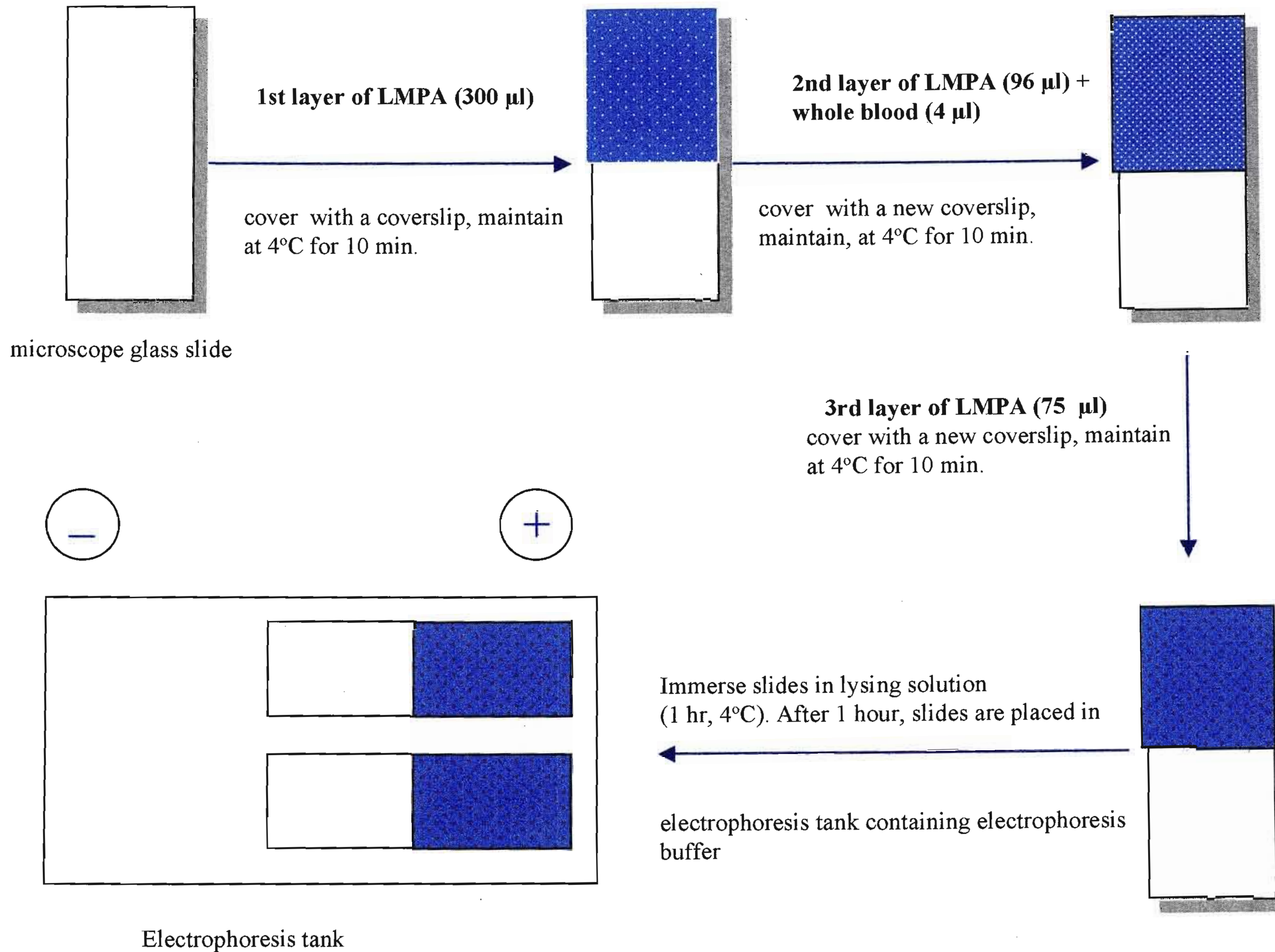


Fig 6.1 Schematic drawing of the SCGE assay

6.2.3 Results and Discussion

Analysis of the SCGE assay revealed four different patterns of DNA distribution. These were DNA damage as evidenced by fluorescent strands exterior to the cells, and three different forms of DNA aggregation with DNA situated centrally, peripherally or at one pole of the cell (Table 6.1). The SCGE assay revealed exteriorised DNA in all samples immediately after exercise while DNA damage was seen in only one subsequent sample. Table 6.1 summarises the effects of exercise on DNA distribution within the nucleus after cells are processed in the SCGE assay. Results indicate that exercise causes a marked alteration in the number of lymphocytes that showed structural DNA damage as well as the location of the DNA within the nucleus over a 48 hour period.

Before exercise, lymphocyte DNA damage was minimal as evidenced by the location and staining pattern of the DNA within the cell. Most of the lymphocytes appeared normal with the DNA visualised as a central core (Fig 6.4), while a small number of cells showed DNA located at the periphery of the cell. The medium and large sized lymphocytes which were studied have a nucleus which covers approximately 90% of the cell area. Their chromatin is condensed and the nucleus is intensely stained with ethidium bromide, a characteristic that facilitates the identification of the lymphocyte (Ham and Cormack, 1979). This result is similar to those observed by Hartmann *et al.* (1994, 1995) who observed no DNA damage before a bout of strenuous exercise, however the peripheral location of DNA was not reported by Hartmann *et al.* (1994,1995).

Immediately after exercise, all subjects showed DNA damage in some lymphocytes. Cells with extensive DNA damage displayed migration of the DNA from the nucleus towards the anode (Fig 6.5). In addition to DNA damage, there was also a change in the location of the DNA within the cell. The DNA was located centrally, at the periphery of the cell (Fig 6.6) and at one pole of the cell (Fig 6.7) closest to the anode. Table 6.2 summarises the mean values, standard deviations and the range of the various localities of DNA at the various time intervals. There was a significant reduction ($p = 0{,}0026$) in the number of cells which showed DNA as a central core at all times after exercise. This was accompanied by a significant increase in the peripheral and polar location of DNA ($p = 0{,}0359$ and $p = 0{,}0022$ respectively) over the 48 hour period.

The migration of DNA from single cells under electrophoresis seen immediately after exercise is based on the organisation of DNA within the nucleus. DNA is a polymer of deoxyribonucleotide units. A nucleotide consists of a nitrogenous base (i.e. adenine, thymine, cytosine or guanine), a

sugar, and one or more phosphate groups which carry a negative charge. The sugar in dexoyribonucleotide is deoxyribose indicating that the sugar lacks an oxygen atom that is present in ribose, the parent compound (Fig 6.2). Two helical polynucleotide chains are coiled around a common axis and these chains run in opposite directions with the nitrogenous bases on the inside of the helix whereas, the phosphate and deoxyribose units are on the outside (Stryer, 1988). The DNA thus occurs as loops attached to the framework or nuclear matrix and is negatively supercoiled by virtue of its organisation within the nucleosomes (Cook and Brazell, 1976). DNA may be linear, circular or supercoiled. The axis of the double helix can be twisted to form a superhelix. A circular DNA without any supercoiling is known as a relaxed molecule. Supercoiling is biologically important for two reasons. Firstly, supercoiled DNA has a more compact shape than the relaxed structure and secondly, it affects the capacity of the double helix to unwind and thereby affects its interactions with other molecules. Negative supercoiling is when the DNA superhelices in the left handed direction as compared to positive supercoiled DNA which is wound in the right-handed direction (Fig 6.3) (Stryer, 1988).

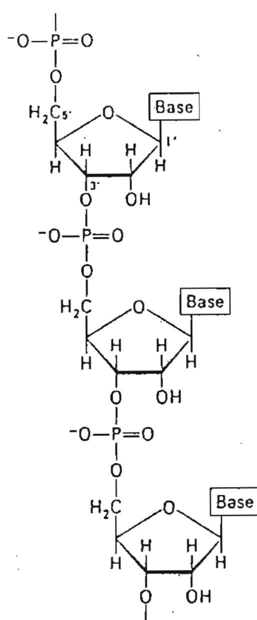


Fig 6.2 Structure of part of a DNA chain (Stryer, 1988)

Fig 6.3 Schematic diagram of DNA molecules showing (a) negative supercoiling and, (b) positive supercoiling (Stryer, 1988)

When cells are permeabilised with detergent and the nuclear proteins are extracted with high salts, the DNA remains within a residual nucleus like structure, the nucleoid. However, if the nucleoid DNA contains breaks, the structure changes dramatically and a halo extends around the original form of the nucleus (Vogelstein *et al.*, 1980). The presence of breaks in DNA relaxes supercoiling and the loops, instead of being constrained within the bounds of the nuclear matrix, are free to extend outside the nucleoid. Electrophoresis accentuates the migration of the uncoiled DNA. The DNA has an overall negative charge as a result of the negative charge imparted by the phosphate groups. During electrophoresis the loop which is negatively charged is able to extend towards the anode (+) as seen in cells immediately after exercise.

DNA located at the periphery and at one pole of the cell was also seen after 24 hours and 48 hours. This pattern of DNA distribution is a characteristic feature of apoptosis as observed under the electron microscope (Payne *et al.*, 1995). The condensation of DNA at the periphery and at one pole is a result of fragmentation of nuclear DNA. The fragmentation of nuclear DNA during apoptosis appears to be due to activation of Ca^{2+} and Mg^{2+} dependent endonucleases that are present in the inactive form. The process by which these enzymes are activated is unknown, though there is an increase in intracellular Ca^{2+} concentration during apoptosis. Cohen and Duke (1984) have shown that thymocyte nuclei contained considerable quantities of this (or a similar) Ca^{2+}/Mg^{2+} dependent endonuclease and that the pattern of cleavage seen with isolated nuclei incubated with these ions was identical to that seen when intact cells were incubated with dexamethasome or corticosterone. The actual role of calcium is controversial. There are reports on both sides of the question of the need for external Ca^{2+} in thymocyte apoptosis (Kaiser and Edelman, 1977; Bansal *et al.*, 1990). Cohen and Duke (1984) have found that the calcium ionophore A23187 in Ca^{2+} containing medium will induce some DNA fragmentation in thymocytes but the same is not seen in any tissue culture line (Allbritton *et al.*, 1988). The theory of Ca^{2+} involvement is further complicated by the observation that A23187 induced fragmentation is prevented by the protein synthesis inhibitor cyclohexamide, which would not be the case if a simple activation of an endonuclease by Ca^{2+} was all that was involved (Wyllie *et al.*, 1984). The growing amount of evidence however, suggests that Ca^{2+} mobilisation from internal stores is probably required for apoptosis (Cohen, 1991).

There are several endonucleases of different cellular origins that have been reported as potential candidates for fragmenting DNA. DNAse I has been proposed as the candidate for the endonuclease activity since it is Ca^{2+} and Mg^{2+} dependent and has a pH optimum of 7,5

(Hale *et al.*, 1996). DNAse I is localised in the endoplasmic reticulum and the perinuclear region of the Golgi complex of the cell (Polzar *et al.*, 1993). DNAse I gains access to the nuclear DNA as a result of raised intracellular Ca^{2+} levels which occur at the onset of apoptosis This causes the endoplasmic reticulum to disperse and dissociate releasing DNAse I (Hale *et al.*, 1996). Another endonuclease is Nuc 18 which was isolated from the nuclei of apoptotic thymocytes and is dependent on the glucocorticoid receptor (Caron-Leslie *et al.*, 1991). This endonuclease is also dependent on Ca^{2+} and Mg^{2+} for activity. Its nuclear localisation and cation dependence suggest that it plays a role in apoptosis (Hale *et al.*, 1996).

Destruction of the DNA may be a simple way of speeding the disposal of an effete cell, removing activated lymphocytes from circulation, or it may have evolved to ensure that no damaged or senescent DNA would find its way to another cell or virus. There is little evidence that destruction of DNA leads directly to cell death from cessation of transcription (Squier *et al.*, 1995). The pattern of DNA distribution is also similar to those observed by Arends *et al.* (1990) and Dini *et al.* (1996). Arends *et al.* (1990) studied the exposure of apoptotic and morphologically normal, live cells to 0,2 M HCl. When viewed under fluorescence microscopy after staining with acridine orange (a metachromatic fluorochrome that binds DNA stoichiometrically), the morphologically normal cells had a bright centre, characteristics of long loops of attached DNA while those cells undergoing apoptosis presented smaller profiles with smooth, sharply defined margins. These cells also exhibited more intense nuclear staining and one or more central cavities, consistent with partial loss of DNA after chromatin digestion (Arends *et al.*, 1990). Dini *et al.* (1996) studied the effects of a variety of apoptogenic agents on U937 myelomonocytary cells. They observed chromatin condensation, in the periphery involving most of the nuclear edge.

Results also show that there is an increase in the number of cells which showed DNA at one pole of the cell immediately after exercise as compared to before exercise. This number increased further after 24 hours and then decreased after 48 hours to the level seen immediately after exercise (Table 6.2). The sudden rise and drop in number could be attributed to apoptotic cells being eliminated from the bloodstream after recognition by macrophages.

The results of the present study differed from those observed by Hartmann *et al.* (1994, 1995), who observed DNA damage in WBC, 6 hours after the end of strenuous exercise which reached its maximum after 24 hours. After 72 hours, DNA migration decreased to about control levels.

The contrasting results observed in the studies by Hartmann *et al.* (1994, 1995) could be due to the differences in methodology of the SCGE assay, constituents of the blood sample analysed, exercise protocol employed, the differences in fitness levels of the subjects as well as the blood sampling times. Hartmann *et al.* (1994,1995) followed the procedure of Singh *et al.* (1988) with minor modifications whereas in this study, the exact procedure of Singh *et al.* (1988) was followed. Modifications were in respect to the concentrations of the different layers of LMPA used which was 0,75% and 0,5% respectively while in this study, 0,5% LMPA was used to prepare all 3 layers. In this particular study a particular subset of WBC (lymphocytes) were analysed as compared to the entire WBC population. Their tests involved a multiple step test where the volunteers ran as long as possible with increasing speed while the gradient was kept constant. In the second test the volunteers ran for 45 minutes with a fixed individual speed. In the first study, the 3 volunteers participated in diverse sports activities, no sports activities and occasional sports activities respectively while in the second study, the 8 volunteers participated in occasional sports activities. Blood samples were taken before the start (control) of the exercise test, 6 min after the end of the test as well as 6, 24, 48,72 and 96 hours later. In the present study, all the subjects were trained athletes who participated in sporting activity on a daily basis. The exercise protocol employed was a graded treadmill run to exhaustion which involved the manipulation of the speed and gradient and blood samples were taken before exercise, immediately after exercise, 24 hours and 48 hours after exercise.

The results of the present study shows that strenuous exercise causes DNA damage in lymphocytes in trained athletes. The damaging effect on DNA may be linked to the involvement of oxidative stress on the lymphocytes. Reactive oxygen species are generated during exhaustive exercise and can cause various kinds of tissue damage (Meydani *et al.*, 1993; Packer, 1984). Two of the potential sources of superoxide radical production are semiquinones in the mitochondria and xanthine oxidase in the endothelial cells (Duarte *et al.*, 1993). Inflammatory cells are also involved in the generation of H_2O_2, a potent initiator of lipid peroxidation. The production of these radicals are further exaggerated during exercise which results in a 10-40 fold increase in oxygen consumption over the resting state. This increases the possibility for the involvement of ROS as a mediator of apoptosis.

The study has also shown that the experimental procedure of the SCGE assay and the results achieved are consistent with an interpretation in terms of detection of DNA strand breaks and

alkali labile sites. The SCGE assay has shown to be a very simple technique for detecting DNA strand breakages in individual cells.

Table 6.1 Number of lymphocytes and location of DNA within the lymphocyte over various time intervals.

Subject	0 min control sample			Immediately after exercise			24 hours after exercise			48 hours after exercise		
	1	2	3	1	2	3	1	2	3	1	2	3
1	46	4	-	(6) 14	19	11	24	9	17	29	13	8
2	50	-	-	(5) 12	25	8	20	7	23	33	11	6
3	48	2	-	(3) 28	16	3	*	*	*	*	*	*
4	31	19	-	(3) 30	17	-	28	10	12	31	12	7
5	47	3	-	(4) 38	5	3	34	6	10	35	15	-
6	50	-	-	(6) 28	16	-	29	6	15	36	12	2
7	49	1	-	(8) 23	9	10	33	7	10	(2) 30	5	13
8	38	12	-	(5) 27	18	-	23	19	8	31	14	5
9	50	-	-	(7) 24	9	10	23	14	13	27	14	19
10	36	14	-	(5) 20	15	10	22	18	10	28	14	8
11	38	12	-	(3) 23	16	8	*	*	*	28	13	9

Key : 1 - no. of cells showing DNA located centrally

2 - no. of cells showing DNA located at the periphery

3 - no. of cells showing DNA located at one pole of the cell

no's in brackets represents cells with DNA damage

* denotes no value obtained due to insufficient sample

- denotes characteristic not seen in sample

Table 6.2 Statistical analysis of the number of lymphocytes reported as mean (± SD) and their location of DNA over 48 hours

Location of DNA within nucleus	0 minutes control sample	Immediately after exercise	24 hours after exercise	48 hours after exercise
Centre[a]	43,9 ± 6,4 Range: 32-50	24,3 ± 7,3 12-38	22,4 ± 12,0 0-34	28 ± 9,7 0-36
Periphery[b]	6,1 ± 6,8 0-19	15,9 ± 5,2 5-25	8,7 ± 6,3 0-19	11,2 ± 4,6 0-15
At one pole of the cell[c]	0 ± 0 -	5,7 ± 4,5 0-11	10,7 ± 6,7 0-23	6,1 ± 4,1 0-13
Damage		5 ± 1,7 3-8		

[a] p = 0,0026 (significant change from baseline to 48 hours after exercise)

[b] p = 0,0359 (significant change from immediately after exercise and 48 hours as compared to baseline)

[c] p = 0,0022 (significant change from baseline to 48 hours after exercise)

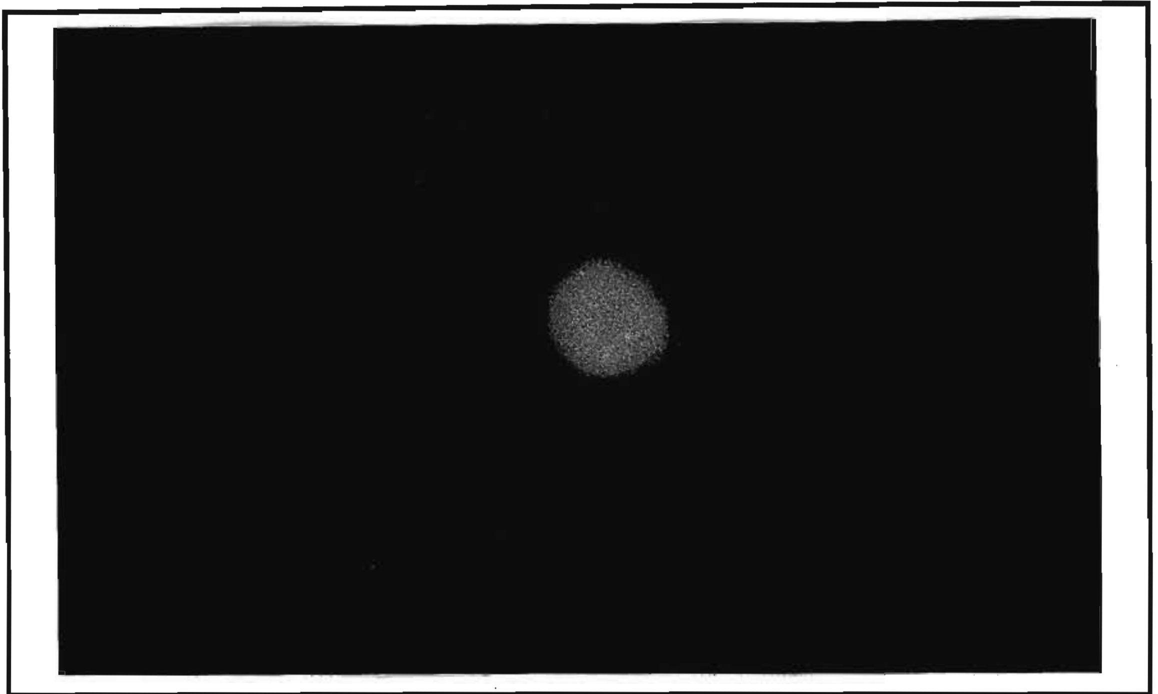


Fig 6.4 A human lymphocyte processed in the SCGE assay before exercise showing DNA as a central core (x 2 000).

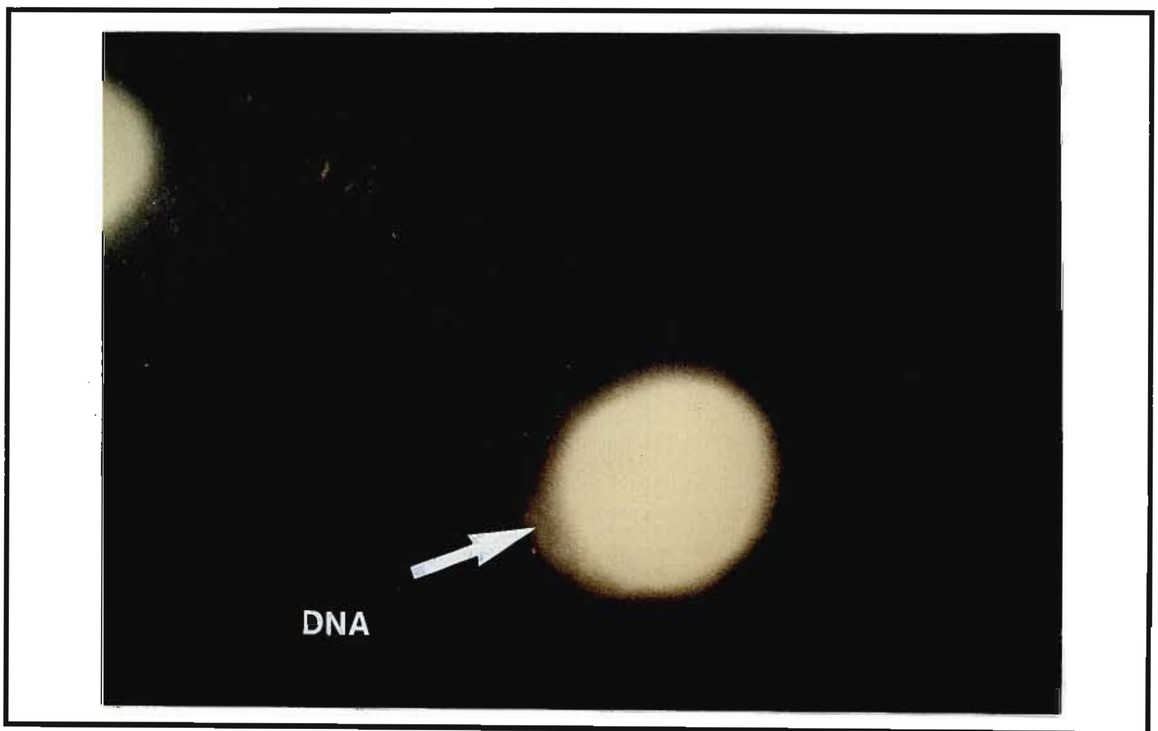


Fig 6.5 A human lymphocyte processed in the SCGE assay immediately after exercise showing DNA migration out of the cell (x 2 000).

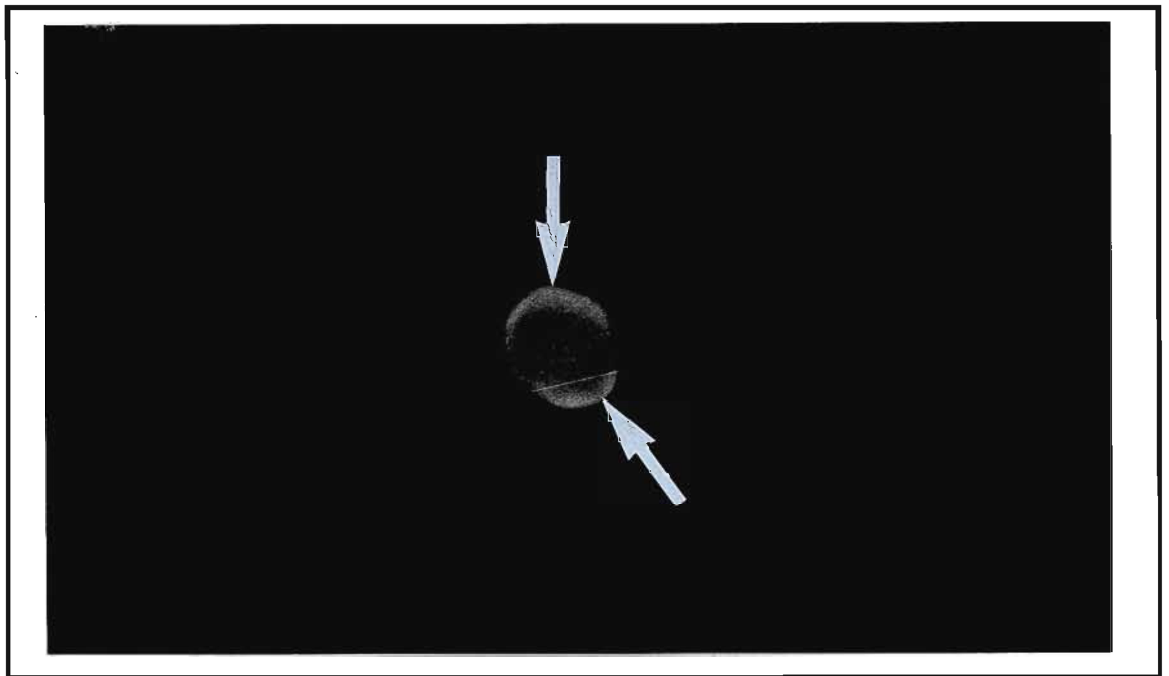


Fig 6.6 A human lymphocyte processed in the SCGE assay immediately after exercise showing location of DNA at the periphery. This pattern was also observed after 24 hrs and 48 hrs (x 2 000).

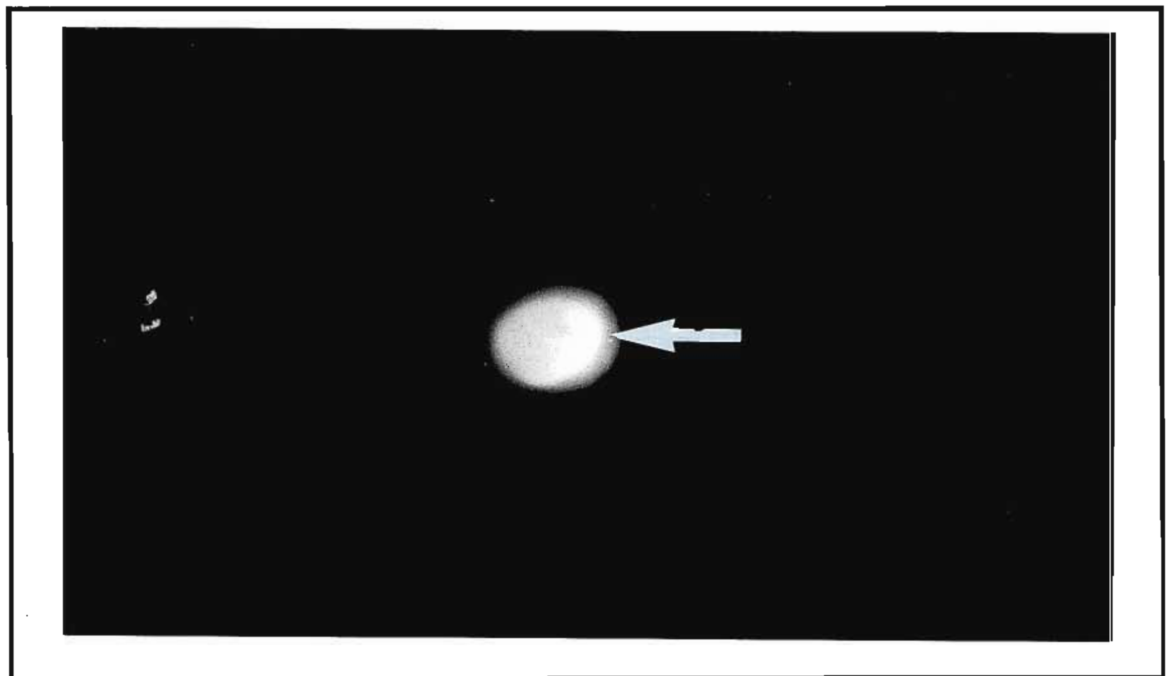


Fig 6.7 A human lymphocyte processed in the SCGE assay immediately after exercise showing location of DNA at one pole of the cell. This pattern was also observed after 24 hrs and 48 hrs (x 2 000).

6.3 *In Situ* End Labelling

6.3.1 Introduction

Methods that enable the detection of DNA strand breaks in cells are being used increasingly in the study of apoptosis. Endonucleolysis is considered the key biochemical event of apoptosis, resulting in cleavage of nuclear DNA into oligonucleosome-sized fragments, therefore this process is commonly used for the detection of apoptosis by the typical "DNA ladder" on agarose gels during electrophoresis (Arends and Wyllie, 1991; Sen, 1992; Arends *et al.*, 1990). This method however cannot provide information regarding apoptosis in individual cells nor relate cellular apoptosis to histological localisation or cell differentiation. This can be done by enzyme *in situ* labelling of apoptosis induced DNA strand breaks.

Gorczyca *et al.* (1993a) compared two different assays that allow the detection of DNA strand breaks, the Nick Translation Assay and the Terminal Deoxynucleotidyl Transferase Assay (TdT) and found the latter to be superior. The TdT assay involves using a DNA polymerase which catalyses the addition of deoxynucleotides to the 3′-hydroxyl termini of DNA molecules. The basis of the TdT assay therefore, is the labelling of the free 3′ hydroxyl termini produced by DNA strand breaks (Bromidge *et al.*, 1995).

DNA polymerase as well as TdT have been used for the incorporation of labelled nucleotides to DNA strand breaks *in situ* (Gorczyca *et al.*, 1993b; Schmied *et al.*, 1993). The tailing reaction using TdT or TUNEL (dUTP nick end labelling) technique has several advantages in comparison to other labelling techniques:

(1) label intensity of apoptotic cells is higher with TUNEL than with other labelling techniques, resulting in increased sensitivity (Gorczyca *et al.*, 1993a; Gold and Schmied, 1994).

(2) kinetics of nucleotide incorporation is very rapid with TUNEL (Gorczyca *et al.*, 1993a; Gold and Schmied, 1994).

(3) TUNEL preferentially labels apoptosis in comparison to necrosis, thereby discriminating apoptosis from necrosis and from primary DNA strand breaks induced by antitumour drugs or radiation (Gorczyca *et al.*, 1993a).

(4) the reactions are based on the direct labelling of the 3′ hydroxyl termini of DNA breaks, and thus the lesions measured are identifiable at the molecular level.

(5) the DNA breaks occur very early in apoptosis, prior to changes in cell morphology; the method thus detects apoptotic cells which cannot yet be recognised on changes in morphology (Gorczyca *et al.*, 1993b).

(6) since DNA content is measured in addition to the DNA breaks, apoptosis can be related to the cell's position in the cycle or to DNA ploidy if cells of different ploidies are present in the same sample (Gorczyca *et al.*, 1993b). Apoptosis in most cells is characterised by extensive DNA fragmentation whose frequency and time of appearance after a triggering signal are dependent upon the cell line and DNA damaging agent (Olive *et al.*, 1993).

6.3.2 Principle of *in situ* cell death detection kit, Fluorescein

Cleavage of genomic DNA during apoptosis may yield double stranded, low molecular weight DNA fragments as well as single stranded breaks ("nicks") in high molecular weight DNA. These DNA strand breaks can be identified by labelling 3′-OH termini with modified nucleotides in an enzymatic reaction (Fig 6.8). In the *in situ* cell death detection kit, TdT is used to label DNA strand breaks by catalysing the polymerisation of nucleotides to free 3′-OH ends in a template independent manner. Fluorescent labels in incorporated polymers are detected and quantitated by flow cytometry.

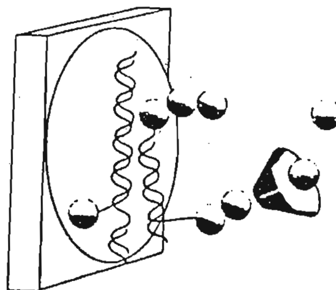


Fig 6.8 DNA of fixed cells labelled by addition of fluorescein dUTP at strand breaks by terminal transferase.

6.3.2.1 Flow Cytometry

Flow cytometry (FCM) is an optical system for measuring light scatter and fluorescence signals of single particles in a fluid stream (Gabriel *et al.*, 1992). It is based on the concept of counting cells which was first introduced in 1934. Since then there has been widespread instrumentation development with respect to counting of cells. The flow cytometer has achieved importance in the fields of diagnostic procedures.

Results are based on the simultaneous measurement of the many physical and biochemical parameters of single cells and in one step, data from many thousands of cells may be collected. Flow cytometry has been used widely in the field of exercise immunology and sports medicine. It has been used in dealing with the fundamentals of the acute immune response to exercise (Shinkai *et al.*, 1992); interactions between acute exercise and training and infections, cancer and autoimmune diseases (Gabriel *et al.*, 1991).

6.3.2.2 Principle of flow cytometry in detecting apoptotic cells

A cell transversing through the focus of the laser beam in a flow cytometer scatters the laser light. Analysis of the scattered light provides information about the cell size and structure. The intensity of light scattered in a forward direction correlates with cell size. The intensity of light scattered, measured at right angle to the laser beam (side scatter), correlates with granularity, refractiveness and the presence of intracellular structures that can reflect light. The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of the plasma membrane, and in the case of apoptosis, chromatin condensation, nuclear fragmentation, and shedding of apoptotic bodies. During apoptosis there is a decrease in forward light scatter, which is a result of cell shrinkage. There is also sometimes a transient increase in right angle scatter and this can be seen in some cell systems. This may reflect an increased light by condensed chromatin and fragmented nuclei. In later stages of apoptosis, the intensity of light scattered at both the forward and right angle direction, is decreased (Darzynkiewicz *et al.*, 1997).

6.3.3 Materials and Methods

6.3.3.1 Isolation of Lymphocytes

Three athletes underwent a treadmill run to exhaustion as described previously in section 3.3. Blood (10 ml) samples were collected in EDTA (potassium salt 1 mg/ml) test tubes before exercise, immediately after exercise, 24 hours and 48 hours after the exercise test. Lymphocytes were separated from whole blood using Histopaque 1077 (Sigma Chemical Co.). Fresh blood (10 ml) was added to 40 ml of PBS. This mixture was added slowly with a plastic pipette to 3 tubes, each containing 5 ml of Histopaque 1077. The tubes were then centrifuged at 2 000 rpm for 30 minutes at 20°C. After centrifugation the clear supernatant was aspirated and discarded. The opaque layer containing the mononuclear cells (lymphocytes) was then carefully aspirated and transferred to a sterile test tube. The isolated lymphocytes were then washed by resuspending the lymphocytes in 8 ml of PBS. The test tube was centrifuged as described above. After centrifugation the supernatant was aspirated and discarded. The lymphocytes were then resuspended in PBS (600 μl) and prepared for the TUNEL assay.

6.3.3.2 Preparation of lymphocytes and TUNEL reaction mixture

The *in situ* cell death detection kit Fluorescein, was purchased from Boehringer Mannheim. The kit contained 2 reagents: (1) Reagent 1 was the enzyme solution which contained terminal deoxynucleotide transferase from calf thymus in storage buffer (10 x concentration) and (2) Reagent 2 (Label solution) was a nucleotide mixture in reaction buffer (1 x concentration).

The preparation of the lymphocytes for analysis using the TUNEL assay was carried out in 3 steps. This involved cell fixation, cell permeabilisation and labelling of the isolated lymphocytes.

a) Cell fixation

The isolated lymphocytes in PBS (600 µl) were placed in 3 test tubes (200 µl each). Test tube 1 served as the positive control, test tube 2, the negative control and test tube 3 was the test sample. Before cell fixation the lymphocytes were resuspended and washed in 1% Bovine Serum Albumin (BSA)/PBS solution (500 µl) (Appendix 6.2). The 3 test tubes were then centrifuged at 2 500 rpm for 5 minutes at 4°C. After centrifugation the supernatants were removed and discarded. Cell fixation was then carried out by adding paraformaldehyde solution (4% in PBS, pH 7,4) (500 µl) (Appendix 6.2) to the cell suspensions. The tubes were agitated gently to allow for resuspension and were left at room temperature for 30 minutes.

b) Cell permeabilisation

After 30 minutes, the test tubes were centrifuged as described above. The supernatants were removed and discarded. The lymphocytes were then resuspended and washed 3 times in 1% BSA/PBS solution (500 µl). Resuspension and centrifugation were carried out after each wash as described above. The cell suspensions were then resuspended in freshly prepared permeabilisation solution (1% Triton X100 in 1% sodium citrate) (100 µl) (Appendix 6.2). The test tubes were then immersed in ice (4°C) for 2 minutes to allow for permeabilisation.

c) Labelling and analysis

After 2 minutes the reaction was stopped by the addition of 1% PBS/BSA solution (500 µl). The tubes were centrifuged as before and the supernatants discarded. Before labelling of the DNA with the TUNEL reaction mixture was carried out, DNAse 1 (10 µl) was added to test tube 1 (positive control) and left for 10 minutes at room temperature. This reaction was stopped after 10 minutes by the addition of 1% PBS/BSA solution (500 µl). The test tube was centrifuged as described above and the supernatant discarded. The TUNEL reaction mixture was then prepared by adding reagent 1 (enzyme solution) (20 µl) to reagent 2 (label solution) (80 µl). The mixture was vortexed for 30 seconds to equilibrate the components. Labelling of the lymphocytes was then carried out as follows:

The TUNEL reaction mixture (50 µl) was added to test tubes 1 and 3 (test sample). Reagent 2 (50 µl) was added to test tube 2 (negative control). The 3 test tubes were then incubated in a water bath at 37 °C for 1 hour. After 1 hour the samples were washed twice in 1% PBS/BSA

solution (500 μl), with resuspension and centrifugation as described previously. The supernatants were removed and discarded. The lymphocytes were then resupended in 1% PBS/BSA solution (500 μl), vortexed and then analysed by FCM.

6.3.4 Results and Discussion

The 3 subjects who had participated in this part of the study were subjects 1, 8 and 9. Before exercise subjects 1 and 8 demonstrated a small percentage of apoptotic cells as compared to subject 9 (Table 6.3). The presence of apoptotic cells before exercise is a normal physiological event. In most organs or cell systems in the adult body, a steady state is achieved so that there is no net increase or decrease in size and number of cells (Cohen, 1993b). Most of the cells in the haemopoetic system have short life expectancies, from less than a day for blood neutrophils to a few weeks or months for monocytes and lymphocytes (Squier *et al.*, 1995). After development of lymphocytes in the primary lymphoid organs, lymphocytes are exported to the secondary lymphoid organs to carry out their function of defence. Upon exposure to an antigen, B and T cells become activated, enter the cell cycle, proliferate and differentiate. Once the foreign threat has been overcome, and they have served their effector functions such as producing antibodies (B cells) or secreting cytokines or killing target cells (helper and cytotoxic T cells), the lymphocytes must be removed. The death of activated cells serves to limit an immune response by killing cells which are no longer needed (Ekert and Vaux, 1997). Cell death may be essential for deletion of cells with inappropriate specificities and to counterbalance mitosis in lymphoid organs. It can also allow for the deletion of cells which may have developed the potential to recognise and generate a response to self-antigens, but which may have eluded the selection process in the thymus (Fossum and Ford, 1985; Ekert and Vaux, 1997). The apoptotic cells seen before exercise would therefore be cells being destroyed to maintain homeostasis.

Subject 9 demonstrated a high percentage of apoptotic cells before exercise (64,9%). A possible reason for the high percentage of apoptotic cells is that subject 9 had participated in a training session in the evening, preceding the day of the exercise test. This result suggests that although apoptosis is known to occur in a matter of minutes (Wyllie, 1987), its recognition and complete elimination from the bloodstream may take a few hours, or that the signal inducing apoptosis in lymphocytes continues for some time after the cessation of exercise.

All 3 subjects showed an increase in apoptosis in lymphocytes immediately after exercise in the range of 30,5%-48,3%. Subject 9's high percentage of apoptotic cells may be an additive process to the already high percentage of apoptotic lymphocytes seen before exercise or may be attributed to his high $VO_{2\ max.}$ (81,9 $ml.kg^{-1}.min^{-1}$) (Table 3.2). Oxygen intake is known to increase 10-15 fold during exercise. As a result of this increase, there is a greater chance for the generation of superoxide radicals. These radicals react with cellular macromolecules, either damaging them directly or setting in motion a chain reaction wherein the free radical is passed from one macromolecule to another, resulting in extensive damage to cellular structures such as membranes (Buttke and Sandstrom, 1994). This has led to the hypothesis that oxidative stress can be a mediator of apoptosis. This hypothesis has been supported at several levels: (1) the addition of ROI or a depletion of antioxidants can result in apoptosis (Zhong *et al.*, 1993); (2) apoptosis can be associated with the induction of ROI (Larrick and Wright, 1990); and (3) apoptosis can be blocked by the addition of compounds of antioxidant abilities (Iwata *et al.*, 1992).

After 24 hours there was an increase in the percentage of apoptotic cells in subjects 1 (87,9%) and 8 (82,9%) while there was a slight decrease in subject 9 (87,9%). These results suggest that the oxidative stress incurred as a result of high intensity exercise, generates a high level of superoxide radicals. These results are similar to those observed in the SCGE assay (Table 6.1) and under the electron microscope (section 6.4) which shows an increase in apoptosis after 24 hours as compared to before exercise and immediately after exercise. In the SCGE assay, more than 50% of the number of cells showed DNA located at the periphery and one pole of the cell, immediately after and 24 hours after exercise. These characteristics are typical features of apoptosis (Payne *et al.*, 1995). Electron microscopy revealed an increase in apoptotic cells at these times as evidenced by nuclear displacement to one side, indentation of the nucleus, decrease in cell size and swelling of the endoplasmic reticulum (ER), mitochondria and Golgi (section 6.4.3).

After 48 hours there was a dramatic decrease in the number of apoptotic cells with numbers decreasing to almost control levels in subjects 8 (11,5%) and 9 (5,1%). Subject 1 (95,2%) showed a high percentage of apoptotic cells. The high percentage in subject 1 is attributed to his participation in a training session the previous evening. These results suggest that recovery is complete after 48 hours only if no exercise is performed during the 48 hour period.

These results can been seen more clearly when one looks at the histogram of subject 8 obtained using FCM (Fig. 6.9 a-d). Before exercise there were few apoptotic cells (0,9%) (Fig 6.9 a).

Immediately after exercise there is a clear shift in the graph towards the right (Fig 6.9 b), indicating an increase in side scatter and cellular changes that reflect smaller sized cells and an organisation of the nucleus within the cytoplasm, which is a typical appearance of apoptotic cells (Darzynkiewicz *et al.*, 1997). After 24 hours there is a further shift towards the right (Fig 6.9 c), indicating an increase in the number of cells showing apoptosis. After 48 hours the graph shifts back to the left (Fig 6.9 d) indicating fewer apoptotic cells. The high peak observed after 48 hours is attributed to the greater number of cells counted as compared to the previous times.

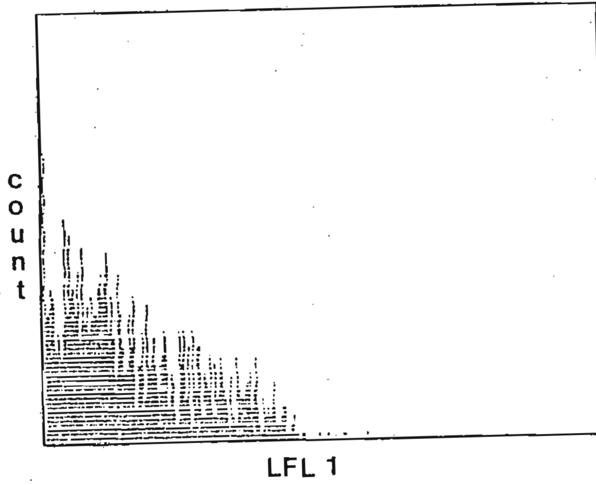
From the results observed it can be seen that high intensity exercise causes an increase in apoptosis. This effect lasts for approximately 24 hours and returns to normal after approximately 48 hours. Fortuitously, the additive effects of previous and subsequent exercise of lower intensity were also shown in subjects 1 and 9.

At present, DNA fragmentation is considered to be the most characteristic feature of apoptosis. The advantage of using the TUNEL assay is that it is a quantitative assay facilitating the early detection of DNA strand breaks during apoptosis, prior to the loss of any significant DNA content or nuclear fragmentation. This study has also revealed that FCM can provide rapid, quantitative and objective assays of cell viability which may be employed for the enumeration of apoptotic cells. However, regardless of the particular method that has been used to identify the mode of cell death, flow cytometric analysis should always be confirmed by the inspection of cells under the light or electron microscope. Morphological changes during apoptosis are unique and they should be the deciding factor when ambiguity arises regarding the mechanisms of cell death.

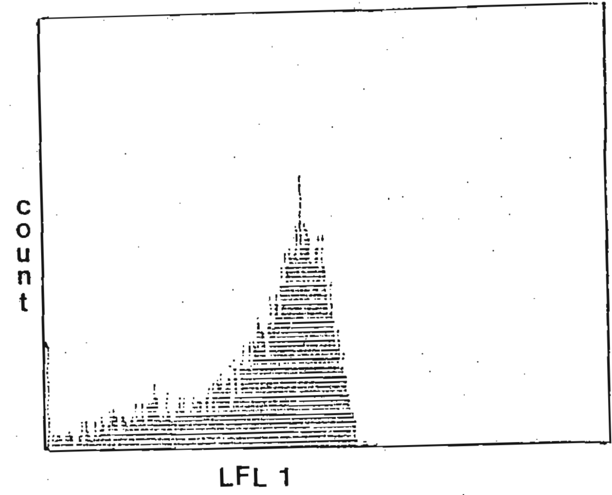
Table 6.3 The percentage of apoptosis in lymphocytes in subjects 1,8 and 9 using the TUNEL assay.

Subject	**0 Minutes control sample**	**Immediately after exercise**	**24 Hours after exercise**	**48 Hours after exercise**
1	8,7	57	87,9	95,2
8	0,9	35,9	82,9	11,5
9	64,9	95,4	87,9	5,1

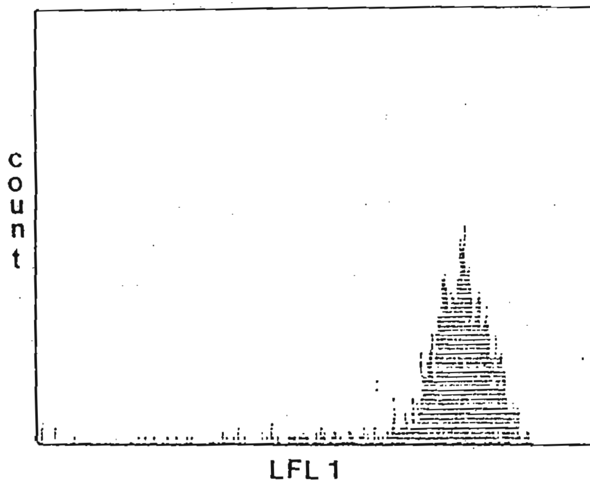
(a)



(b)



(c)



(d)

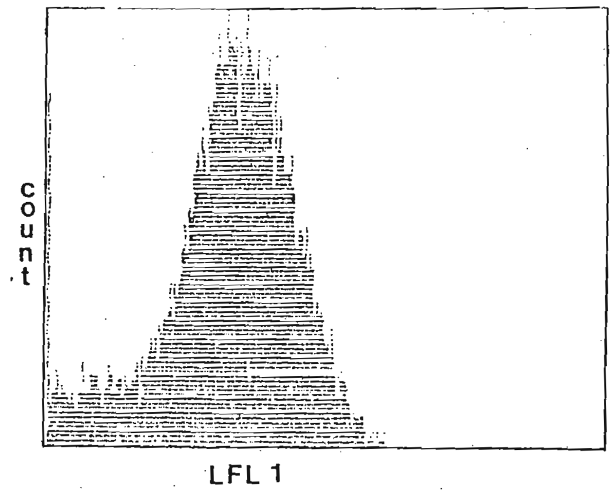


Fig 6.9 a-d Histogram of lymphocytes processed by the TUNEL assay as analysed using the flow cytometer to show the number of apoptotic cells at various time intervals. a) before exercise (n = 13 358), b) immediately after exercise (n = 17 338), c) after 24 hours (n = 5 119), d) after 48 hours (n = 18 958). n = number of cells counted.

6.4 Microscopy

6.4.1 Introduction

The electron microscope has provided the missing link between the details of tissue and cell organisation as seen by the light microscope and the details of molecular architecture as revealed by new biochemical and physiological techniques (Carr and Toner, 1982). Cells undergoing apoptosis were originally characterised at the morphological level and morphology still remains the "gold standard" for identifying apoptotic cells. The morphological criteria for identifying apoptotic cells are condensation of nuclear chromatin, compaction of cytoplasmic organelles, blebbing of the cell surface and a decrease in cell volume (Payne *et al*., 1995; Wyllie *et al*., 1984). In this study, the morphology and frequency of apoptotic cells was determined by light microscopy using toluidine blue staining of sections and confirmation of apoptosis was determined using the electron microscope.

6.4.2 Materials and Methods

6.4.2.1 Isolation and Preparation of Lymphocytes

The isolation and preparation of lymphocytes is described in section 6.3.3.1.

6.4.2.2 Transmission Electron Microscopy

Hanks Balanced Salts Solution (HBSS) was purchased from Polychem. Araldite and 200 mesh copper grids were purchased from Wirsams and lead citrate, uranyl acetate and toluidine blue from Capital Enterprises (SA) (Appendix 6.3).

The processing schedule for transmission electron microscopy (TEM) listing the fixation, dehydration and embedding procedure are summarised in Table 6.4.

Table 6.4 Processing schedule procedure of lymphocytes for TEM.

Step	Solution	Time
1	**Fixation** Cells are fixed in 1% glutaraldehyde in HBSS (1 ml)	30 minutes
2	**Buffer rinse** Rinse in HBSS (1 ml)	2 x 5 minutes
3	**Post fixation** Osmium at 4°C (1 ml)	1 hour
4	**Buffer rinse** Rinse in HBSS (1 ml)	2 x 5 minutes
5	**Dehydration**- 70% alcohol (1 ml)	15 minutes
	Dehydration- 90% alcohol (1 ml)	15 minutes
	Dehydration-100% alcohol (1 ml)	15 minutes
	Dehydration-100% alcohol (1 ml)	15 minutes
6	**Ethanol :araldite** 50:50 (1 ml)	30 minutes
7	**Araldite** (1 ml)	1,5 hours in oven (60°C)
8	**Araldite** (1 ml)	48 hours (60°C)

6.4.2.2.1 Light microscopy

Resin embedded sections were cut on a Reichert Ultracut microtome. Thick sections (1μm) for light microscopy were cut and the sections were mounted on a drop of water on glass slides and heat fixed at 60°C. The sections were stained with 4% toluidine blue in a 50:50 distilled water:acetone solution containing borax (10 g). The sections were examined under a Nikon FX-3 photomicroscope.

6.4.2.2.2 Ultramicrotomy

The resin embedded sections were then trimmed to produce a "mesa" with a trapezoidal shape. Sixty to eighty nanometer thin sections were then cut for electron microscopy. The sections were picked up on uncoated 200 mesh copper grids and doubled stained with 1% uranyl acetate in 50% ethanol (1 ml) and Reynold's (1963) lead citrate (1 ml) for 2 minutes in each solution.

Sections were viewed and photographed on a JEOL JEM transmission electron microscope (60kV) in the Electron Microscope Unit at the University of Natal.

6.4.3 Results and Discussion

A quantitative analysis of the number of cells undergoing various alterations in subjects 1 and 8 are shown in Table 6.5.

Before exercise, most of the lymphocytes appeared normal (80%) (Fig 6.10) while other lymphocytes were smaller with indented nuclei (19%) (Fig 6.11). Lymphocytes consist of 3 classes: small lymphocytes, medium sized lymphocytes and large lymphocytes. The chromatin of small lymphocytes is mostly condensed and their nuclei have sometimes showed indentation (Ham and Cormack, 1979). The nucleus of the small lymphocyte makes up approximately 90% of the cell volume with the result that very little cytoplasm is visible (Junqueira *et al.*, 1995). Under the electron microscope, the lymphocyte contains a great deal of condensed chromatin and little extended chromatin. The small lymphocyte reveals almost no organelles except free ribosomes. It is therefore not equipped to perform any specialised function as it has not developed the organelle equipment required for performing specialised functions. It does however, possess the organelle equipment essential for growth. Lymphocytes observed during this study were of the medium and large sized class. These lymphocytes appeared to show more differentiation with the cytoplasm containing several mitochondria. These lymphocytes contained condensed chromatin (Ham and Cormack, 1979) and a nucleus which occupied approximately 90% of the area of the cell. This is one of the distinguishing characteristics that facilitates the identification of the lymphocyte.

Immediately after exercise the number of normal lymphocytes and those with indented nuclei decreased (26,3% and 30,4% respectively). The remaining cells in this group showed cellular alterations which included a decrease in cell size (17,4%) and displacement of the nucleus to one

side (8,9%) (Fig 6.12). Some cells showed elongation, indentation (Fig 6.13) and microsegregation (Fig 6.14) of the nucleus. The ER was most affected as evidenced by swelling of the cisternae (9,3%) (Fig 6.14). Swelling of the cristae of the mitochondria (4,3) was also observed (Fig 6.15). Lymphocytes also showed an increase in cellular protrusions (3,4%) (Fig 6.16) and vacuolation which contained amorphous/granular material (Fig 6.14).

Cell shrinkage and water loss as observed by Falasca *et al.* (1996) leads to the peculiarities in the antigenic makeup of the apoptotic cell membrane. Morphological changes including cell shrinkage and chromatin condensation, characteristic of apoptotic cells, has been shown in peripheral blood lymphocytes from cats infected with feline immunodeficient virus (Momoi *et al.*, 1996). Cell shrinkage as indicated by smaller cells and dense cytoplasm was observed after exercise. The phenomenon of cell shrinkage is not well understood. It might be due to loss of isotonic KCl, since there is no compensatory swelling such as follows the loss of water (Squier *et al.*, 1995). One possibility is that there is a contraction of the cytoskeleton elements, leading to a rise in hydrostatic pressure of cell fluid. Alterations in the cytoskeleton could also explain the membrane protrusions (Squier *et al.*, 1995).

Nuclear alteration is one of the most constant and important accompaniments during the process of apoptosis. DNA, histones and other proteins present in the nucleus constitute the chromatin. Chromatin condensation is a result of DNA fragmentation (Uchiyama, 1995). The condensation of chromatin into dense masses and the nucleus displaced to one side has been seen in TK 6 human B lymphocyte cells examined 20 hours after 7,5 Gy irradiation (Olive *et al.*, 1993) and in peripheral blood lymphocytes after induction of apoptosis using heat shock at 43°C (Falasca *et al.*, 1996). Condensation of chromatin along the nuclear envelope has also been observed in P815 mastocytoma cells after low temperature induced apoptosis (Liepens and Bustamante, 1994) as well as after micrococcal nuclease digestion of normal thymocyte nuclei (Arends *et al.*, 1990). Chromatin condensation has also been observed in U937 cells treated with a variety of apoptogenic agents and have concluded that this condensation may be as a result of two different pathways, by budding and by cleavage. Budding results in chromatin condensation to form large clumps and during cleavage, the chromatin condenses into tiny, regularly shaped crescents involving most of the nuclear edge (Dini *et al.*, 1996). Budding was not observed in this study probably as a result of the rapidity of this process as well as the apoptogenic agent being different. The condensation of chromatin shows that chromatin is cleaved to oligonucleosome chains in the course of apoptosis. The morphology of apoptosis is associated with the same

endonucleolytic chromatin cleavage seen after treatment of thymocytes with ionophore A23187 (Wyllie *et al.*, 1984). Chromatin cleavage appears only in cells recognised as apoptotic by morphology as seen during this study (Fig 6.12). This provides strong evidence that the condensed chromatin which provides the morphological hallmark of the apoptotic nucleus consists of fragmented chromatin molecules, presumably resulting from endogenous endonuclease activation (Wyllie *et al.*, 1984; Arends *et al.*, 1990).

Microsegregation of the nucleus is one of the characteristic features observed in the apoptotic process. The nucleus is known to split into several fragments which may be contained in separate cytoplasmic small round bodies (Uchiyama, 1995). These round bodies are referred to as apoptotic bodies and are intact. These are phagocytosed by the nearest cell capable of phagocytosing it, usually as soon as the process of apoptosis has begun and usually before the cell dies (Cohen, 1991). These apoptotic bodies were not observed probably due to their quick clearance by macrophages or may have been eliminated during the method of lymphocyte isolation.

Arends *et al.* (1990) and Cohen (1993a) have reported a dilatation of the ER during the process of apoptosis. Swelling usually indicates membrane damage. The reason for the swelling of the ER is biochemical in nature. Biochemical alterations may affect the integrity of the membrane leading to influx of fluid and retardation of protein synthesis. During apoptosis the cell is known to quickly decreases its synthesis of RNA and proteins. (Cohen, 1993a).

Mitochondrial swelling, may be attributed to the production of ROS as reported by Zamazani *et al.* (1995). It has been shown that ROS may participate as effector molecules in apoptosis since lymphocytes undergoing glucocorticoid or superantigen induced death, exhibit a reduction in mitochondrial transmembrane potential preceding nuclear degradation. Also, members of the Bcl-2 family of apoptosis-regulatory proteins are located preferentially in the outer mitochondrial membrane, and at least in one system, localisation to this compartment is indispensable for apoptosis modulation. Reduction in the transmembrane potential, concomitant with uncoupling of respiratory transport and ATP synthesis causes increased generation of ROS which in turn are important mediators of apoptosis (Zamazani *et al.* 1995; Clutton, 1997). With this there is inhibition of mitochondrial DNA replication, blockage of the respiratory chain, and induction of mitochondrial ROS generation which may result in apoptosis of lymphocytes. This can account for the swelling of the mitochondria seen in cells after exercise also taking into

account that an increased oxygen consumption may result in a greater turnover of mitochondria due to the greater demand for energy.

The cellular protrusions of the plasma membrane may be as a result of shifts in phospholipid distribution between the inner and outer leaflets and altered lipid packing. These changes lead to the recognition of the lymphocyte by phagocytes (Squier *et al.*, 1995).

Twenty four hours after exercise, the lymphocytes contained nuclei which showed indentation (36,4%) (Fig 6.17) and micosegregation (Fig 6.18). The nuclei were also smaller and displaced to one side (50,9%) similar to those seen immediately after exercise (Fig 6.19). There was also an increase in cellular processes (4,2%) and microvilli (Fig 6.20). Swelling of the Golgi (1,6%) (Fig 6.21) and cristae of the mitochondria (0,7%) and cisternae of the ER (3,0%) (Fig 6.22), although uncommon were also observed. There was also an increase in the number of mitochondria.

An increase in number of mitochondria can be attributed to aerobic system changes that usually accompany training. All the subjects in this study were endurance trained athletes with aerobic training being the major component in their training schedules. As a result of aerobic training, mitochondria have been known to be larger and more numerous. Associated with the increased structural machinery for cellular respiration is a greatly increased capacity to generate ATP aerobically during oxidative phosphorylation. The increase in mitochondria is an important factor in increasing a person's ability to sustain a high percentage of aerobic capacity during prolonged exercise (McArdle, 1996).

Forty eight hours after exercise, only a small percentage of the cells showed the nucleus displaced to one side (1,9%) while the rest of the cells appeared normal (75%) (Fig 6.23). Results indicate that there are still a few apoptotic cells after 48 hours as evidenced by nuclear displacement (1,9%), however the numbers had decreased. This could represent damaged cells observed, immediately after exercise and 24 hours later, undergoing complete apoptosis. The decrease in numbers of apoptotic cells may be as a result of clearing of these cells by macrophages.

Table 6.5 Number of lymphocytes (%) showing cellular alterations during the various time intervals.

Cellular Alterations	No. of cells showing alterations (%)			
	0 min	**immediately after exercise**	**24 hours**	**48 hours**
Normal lymphocytes	80,0	26,3	3,2	75,0
Normal cells with indented nuclei	19,0	30,4	-	18,6
Decrease in cell size	1,0	17,4	-	2,4
Displacement of nucleus to one side	-	8,9	50,9	1,9
Indentation of nucleus	-	-	36,4	2,1
Cellular protrusions	-	3,4	4,2	-
Swelling of ER	-	9,3	3,0	-
Swelling of mitochondria	-	4,3	0,7	-
Swelling of Golgi	-	-	1,6	-

Key: - denotes characteristics not seen in the sample

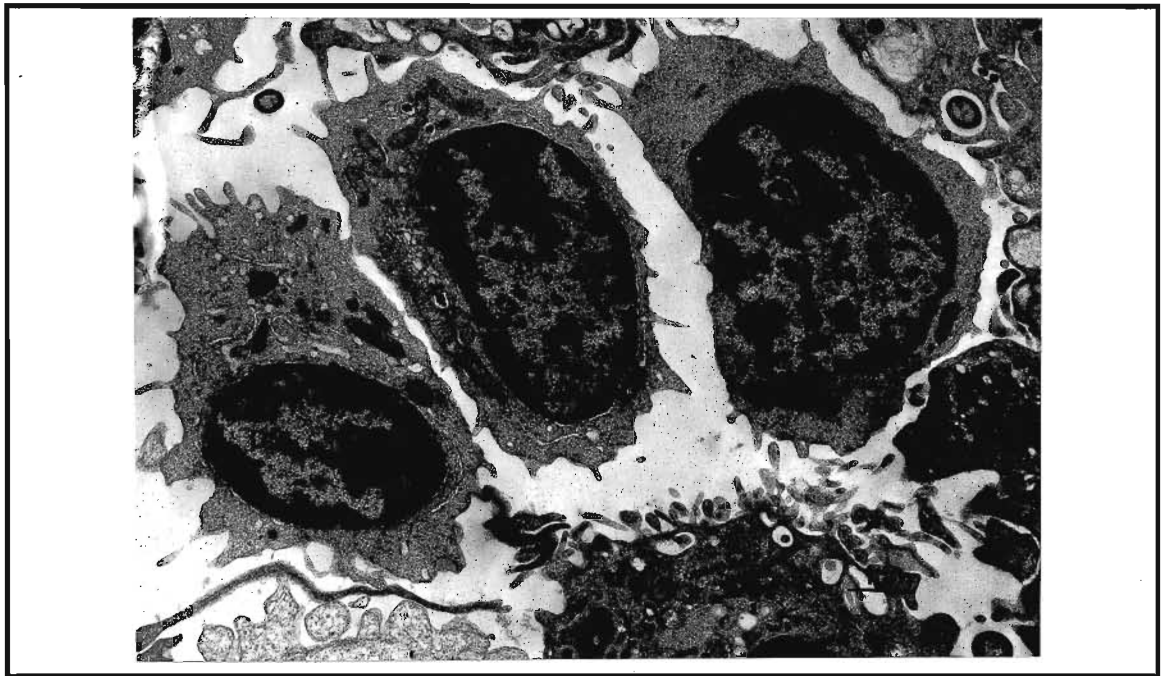


Fig 6.10 Electron micrograph of normal lymphocytes before exercise showing centrally located nucleus (N) and one cell showing displacement of nucleus (DN) to one side (x 5 000).

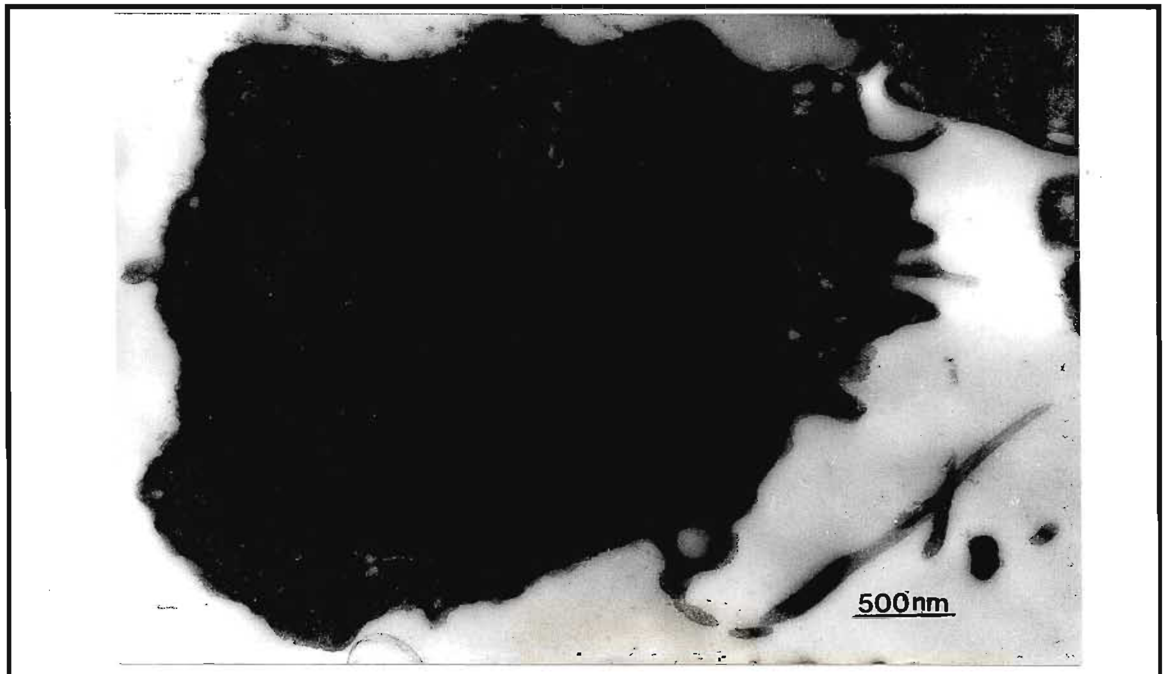


Fig 6.11 Electron micrograph of a small lymphocyte before exercise showing an indented nucleus (↑) (x 10 000).

Fig 6.12 Electron micrograph of lymphocytes after exercise showing a decrease in cell size and displacement of the nucleus to one side of the cell (x 6 000).

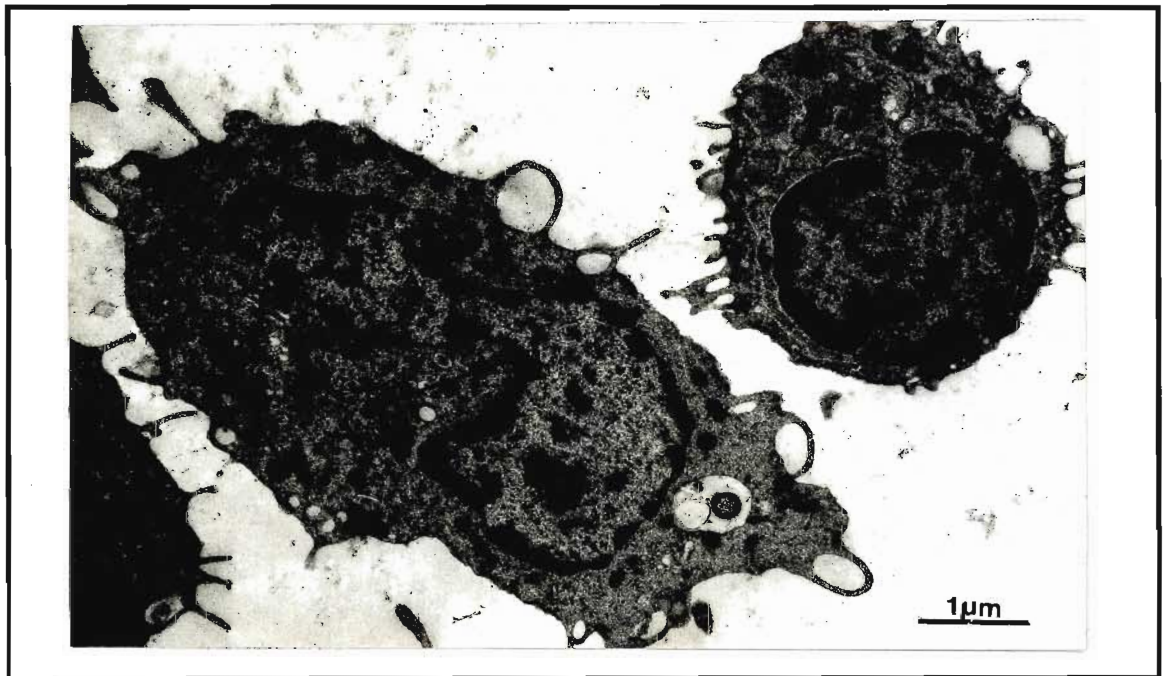


Fig 6.13 Electron micrograph of lymphocytes after exercise showing elongation of the cell and nucleus (E) and indentation of the nucleus (↑) with one cell showing displacement of the nucleus (DN) to one side (x 5 000).

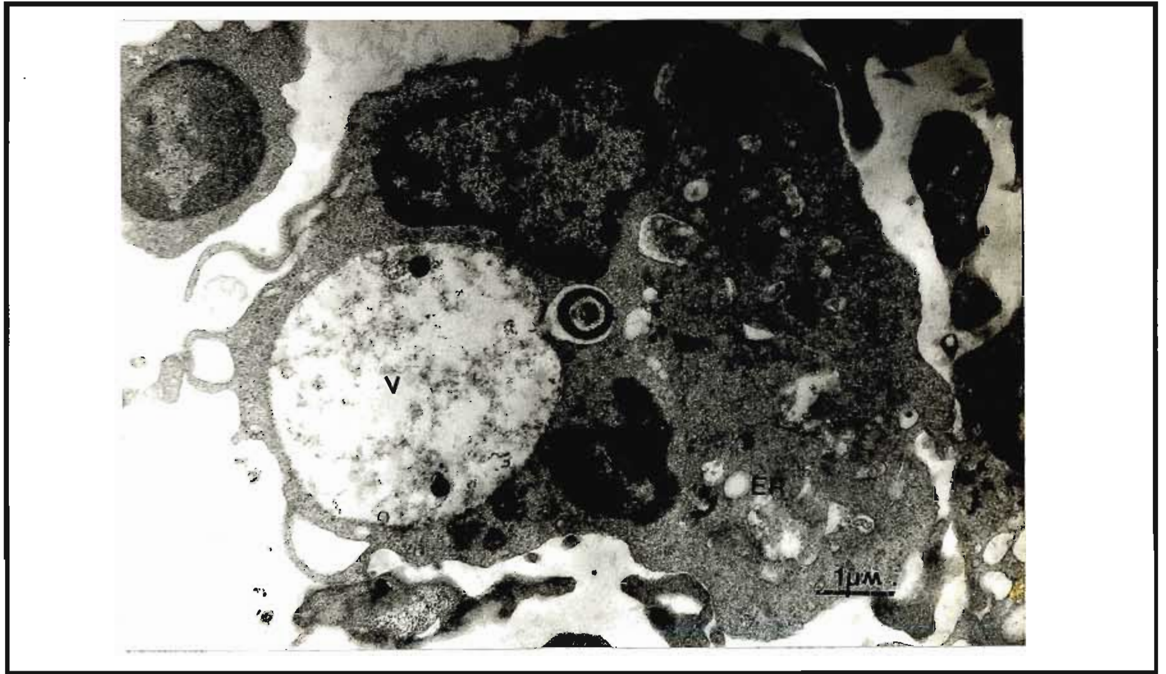


Fig 6.14 Electron micrograph of a lymphocyte after exercise showing microsegragation of the nucleus (N) and swollen cisternae of the endoplasmic reticulum (ER). The cell also exhibited vacuolation (V) which contained amorphous/granular material (x 6 000).

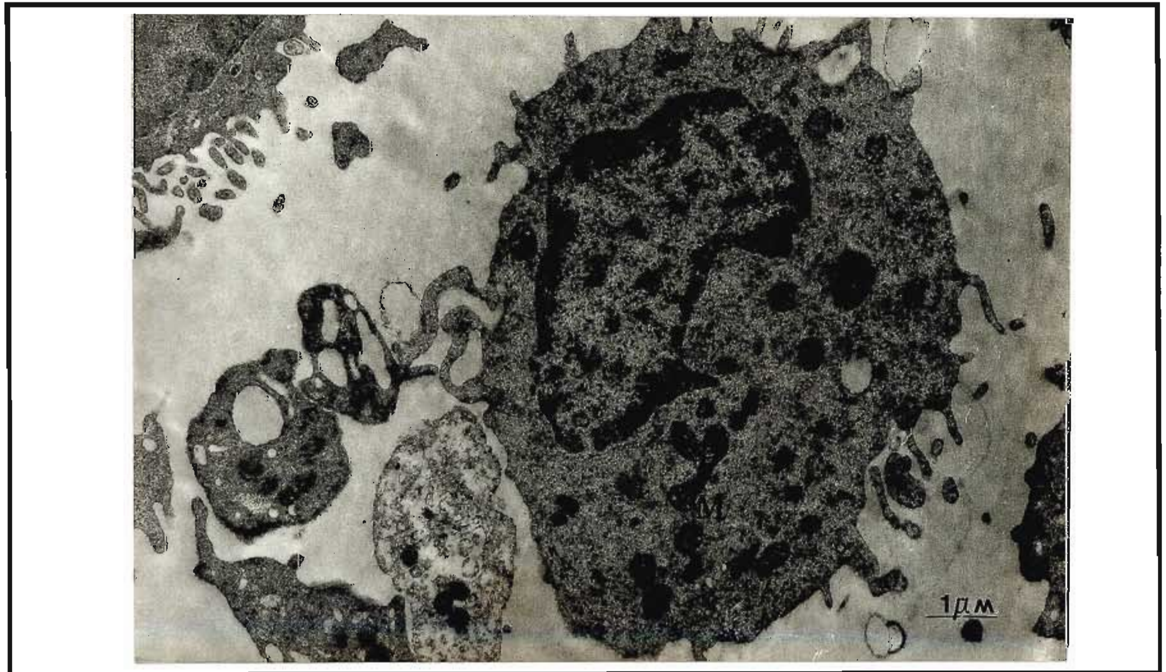


Fig 6.15 Electron micrograph of a lymphocyte after exercise showing swollen cristae of the mitochondria (M) (x 8 000).

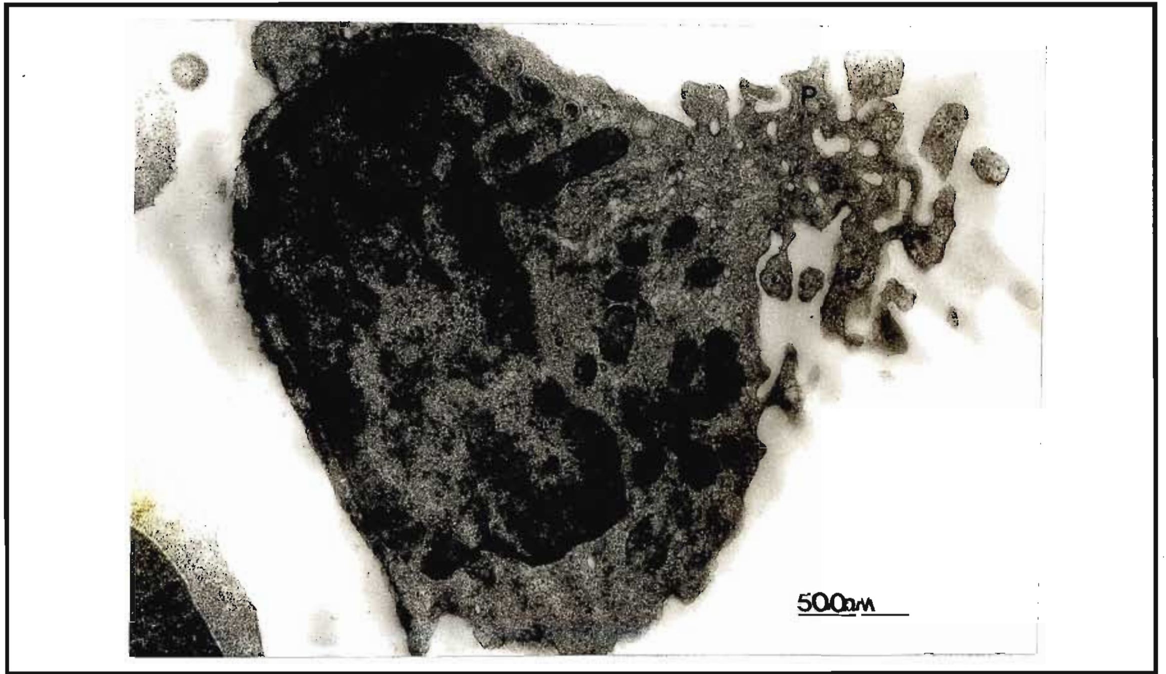


Fig 6.16 Electron micrograph of a lymphocyte after exercise showing an increase in cellular protrusions (P) (x 10 000).

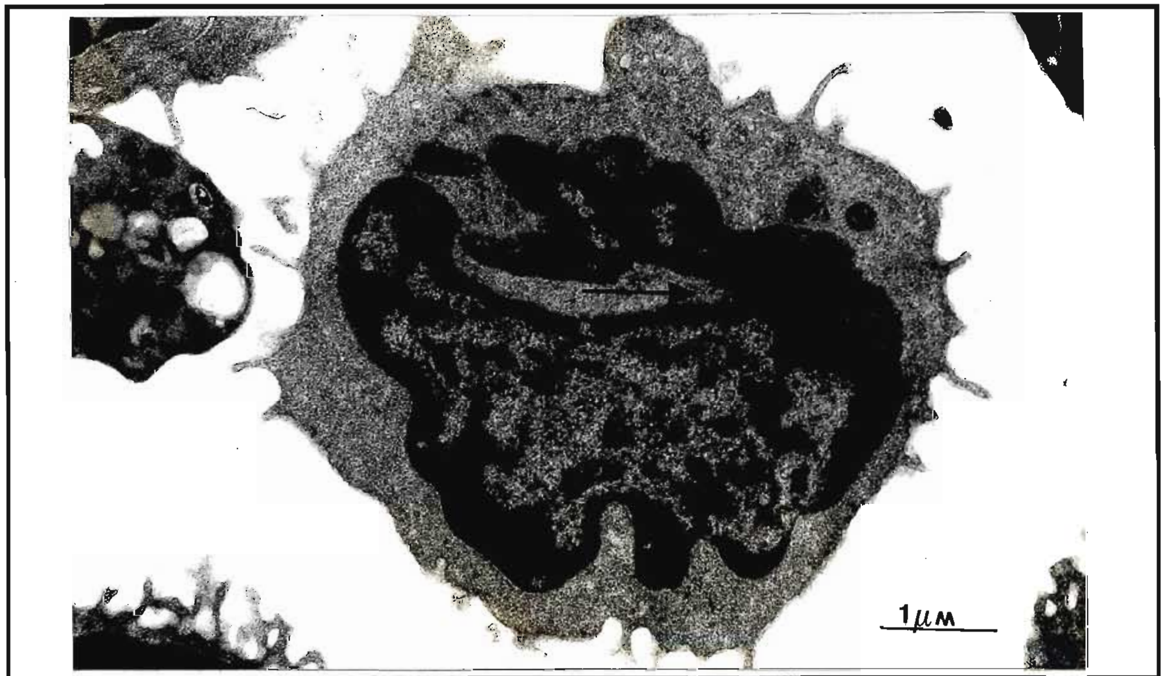


Fig 6.17 Electron micrograph of a lymphocyte, 24 hours after exercise showing indentation of the nucleus (↑) (x 8 000).

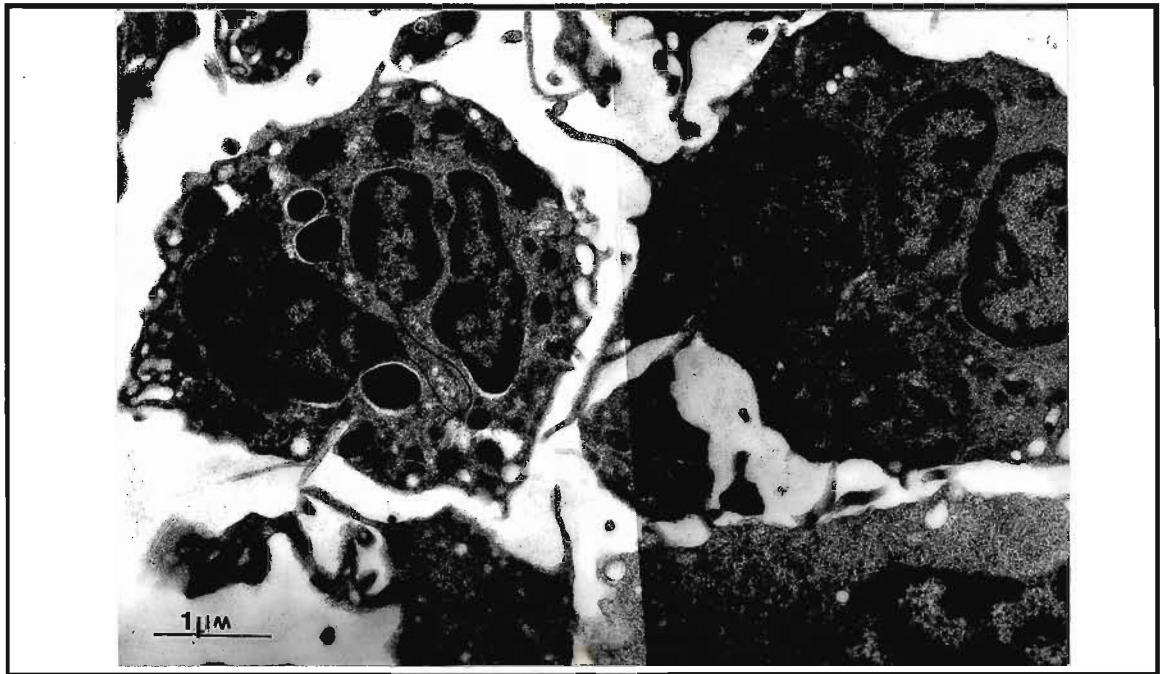


Fig 6.18 Electron micrograph of lymphocytes, 24 hours after exercise showing microsegregation of the nucleus (N) (x 6 000).

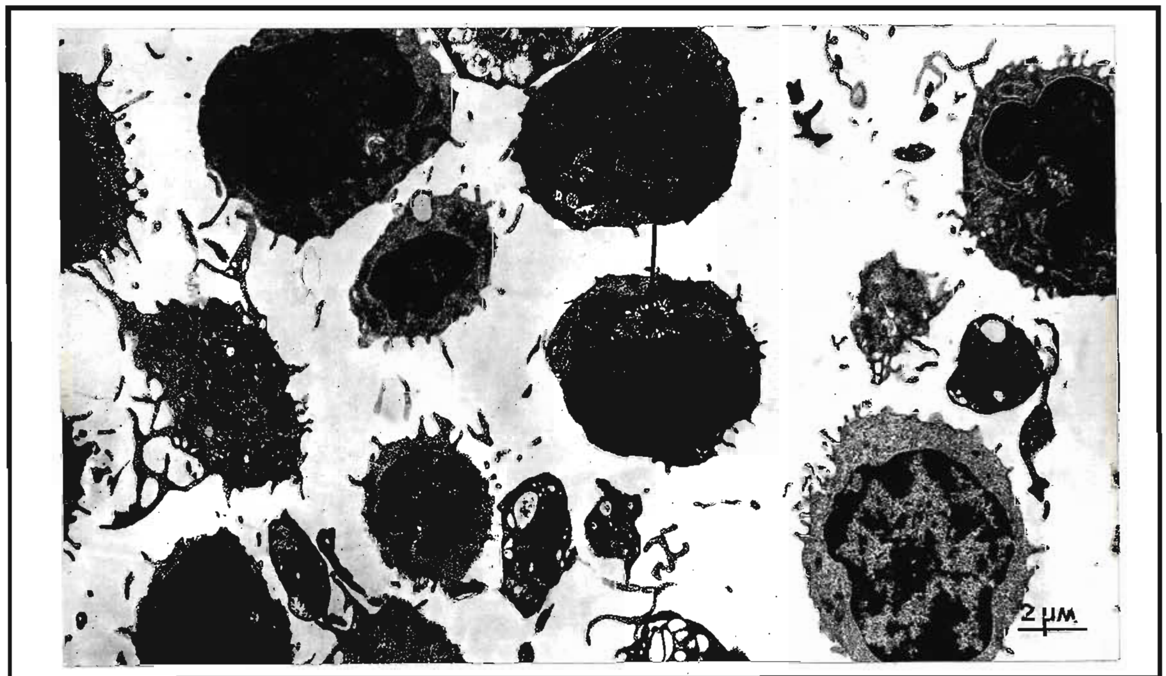


Fig 6.19 Electron micrograph of lymphocytes, 24 hours after exercise showing smaller nuclei (sN) and displacement to one side (↑) (x 3 000).

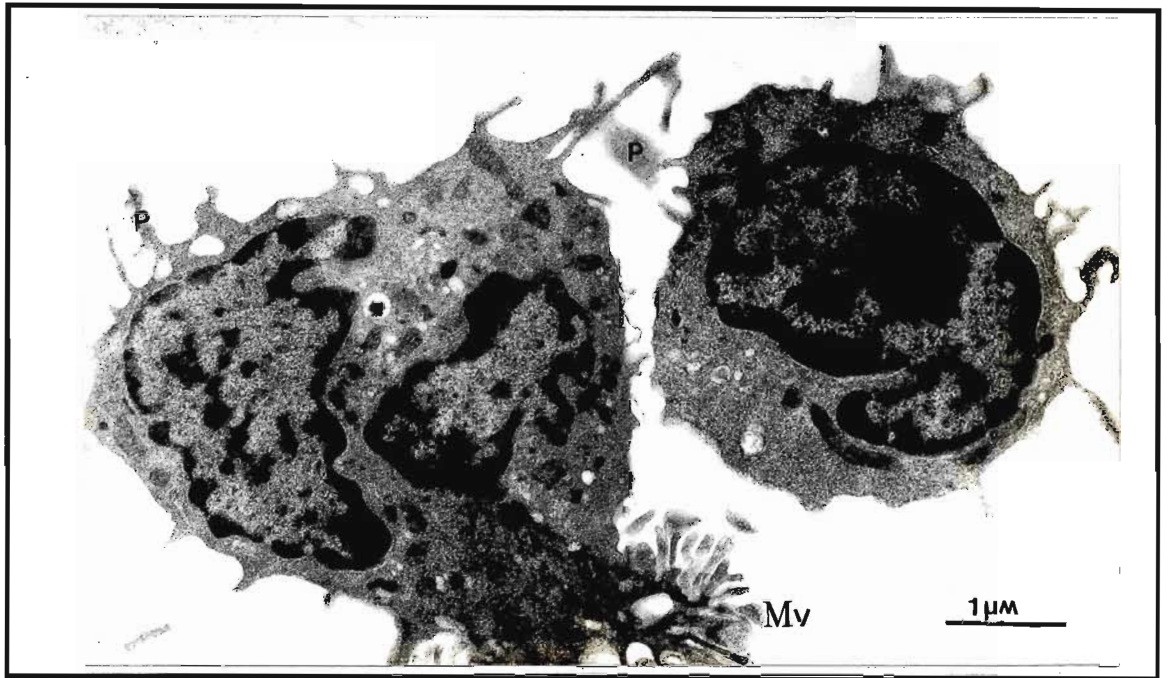


Fig 6.20 Electron micrograph of lymphocytes, 24 hours after exercise showing an increase in cellular processes (P) and microvilli (MV) (x 6 000).

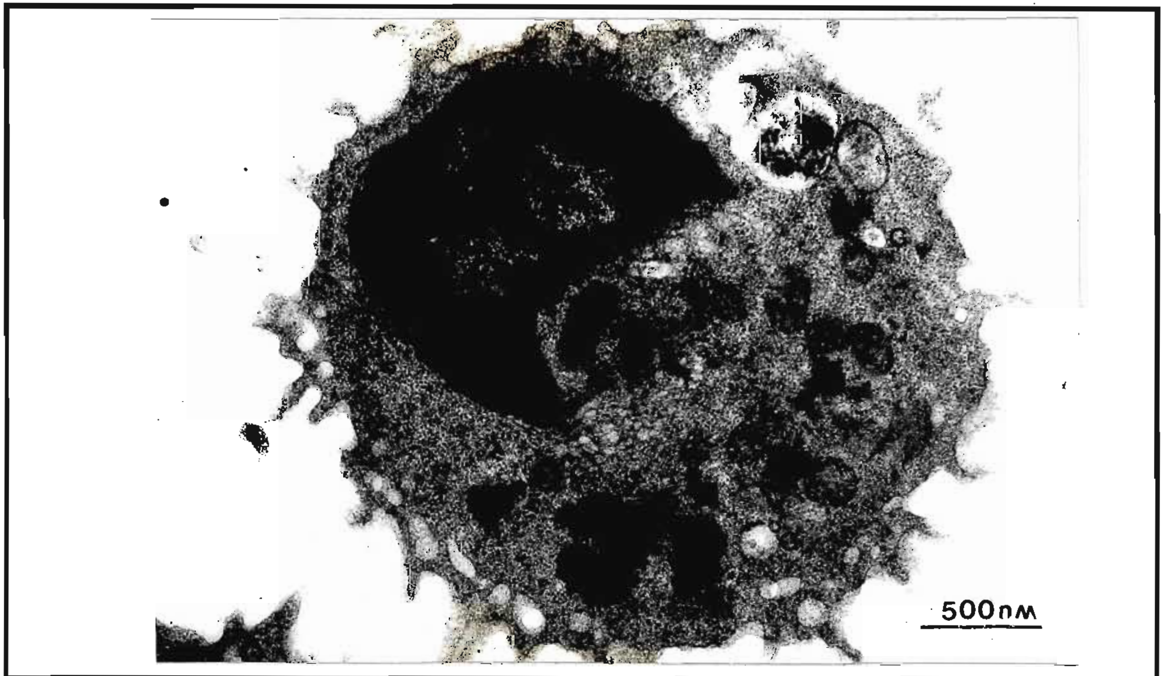


Fig 6.21 Electron micrograph of lymphocyte, 24 hours after exercise showing swollen Golgi (G) (x 12 000).

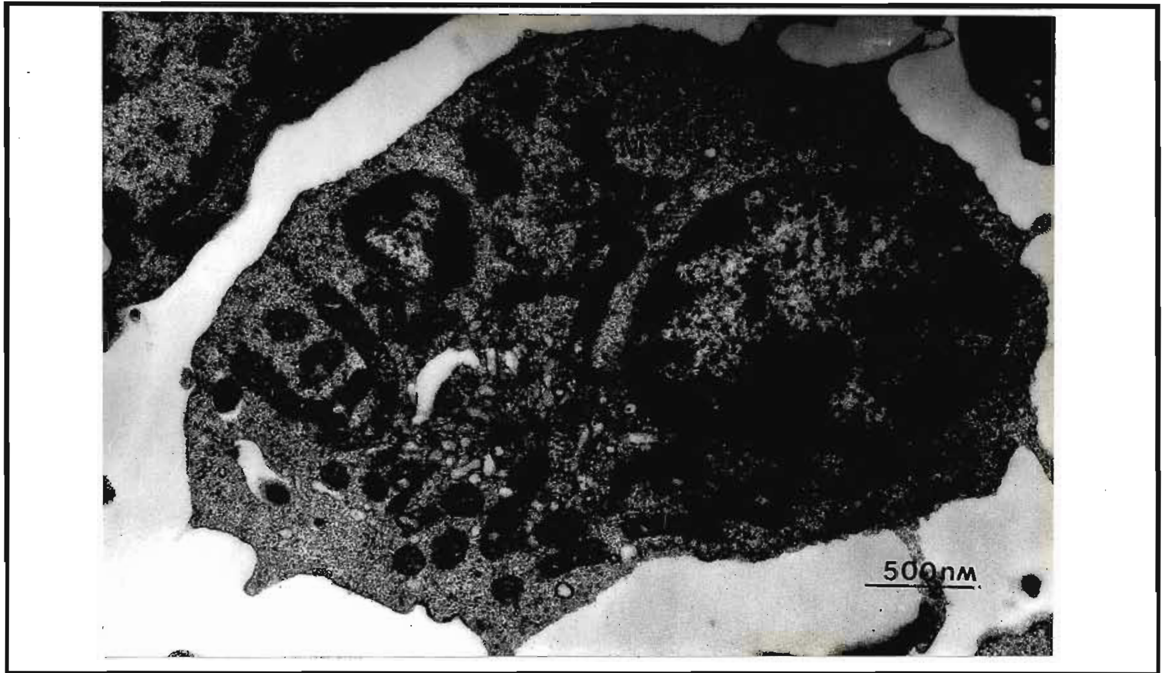


Fig 6.22 Electron micrograph of a lymphocyte, 24 hours after exercise showing swollen cristae of the mitochondria (M) and swollen cisternae of the endoplasmic reticulum (ER) (x 10 000).

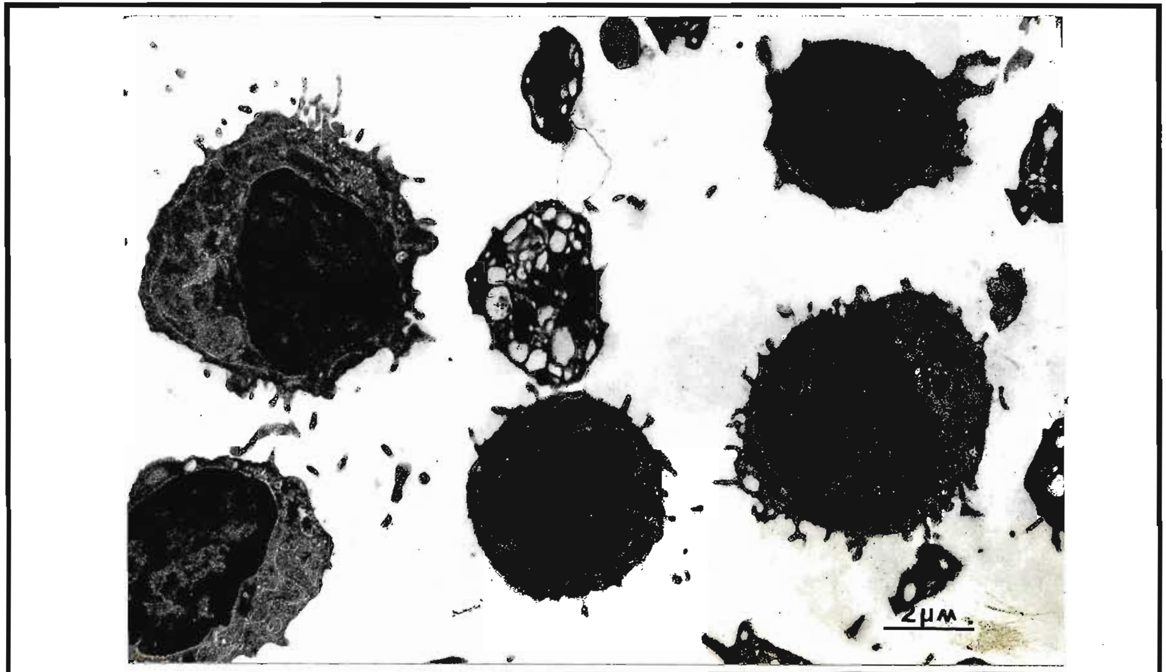


Fig 6.23 Electron micrograph of lymphocytes, 48 hours after exercise showing normal lymphocytes with a centrally located nucleus. Only a small percentage of cells showed displacement of nucleus to one side (DN) (x 4 000).

CHAPTER 7

GENERAL DISCUSSION, CONCLUSION AND FUTURE STUDIES

Exactly how and why the immune system is challenged by exercise has been the subject of concerted research effort over the last decade. What has emerged is a highly inconsistent puzzle because of the extreme complexity of the immune system and the barely quantifiable factors that influence it. Further disparity is caused by the large variation in age, gender, fitness levels of the subjects, the extent or lack of training, the duration and type of exercise protocol and the timing and frequency of the sampling. Host defence parameters measured in these studies range from simple determination of leucocyte counts to sophisticated evaluations of lymphocyte subsets and functions. Large variations in the reliability of both *in vivo* and *in vitro* assay techniques further compromise the reliability of studies in this field.

Pederson and Ullum (1994) have proposed an "open window" hypothesis for the possible increase in upper respiratory tract infections (URTI) seen in athletes. During moderate and severe exercise, the immune system functioning is enhanced but this is followed by a period of post-exercise immunodepression. Total lymphocyte count increases during exercise but the count falls to below normal following severe exercise. What causes the count to decrease is the burgeoning question. It is during this immunodepression, that this window of opportunism is longer and the degree of immunodepression more pronounced. It has been hypothesised that severe immunodepression may occur if the athlete does not allow the immune system to recover but initiates a new bout of exercise while still immunodepressed. The resultant decrease in immune function following extreme activity would be compatible with the propensity of "overtrained" individuals to develop URTI.

The results of the present study lends support to the hypothesis that severe exercise causes immunodepression with the resulting decrease in lymphocyte count as evidenced by lymphocyte DNA damage using varying techniques. The results of the SCGE assay revealed a degree of DNA damage in all subjects immediately after a bout of high intensity exercise with an increase in apoptotic cells after 24 hours. The apoptotic cells were characterised by migration of DNA outside the cell, condensation of DNA at the periphery of the cell and at one pole of the cell. The fluorescent strands of DNA outside the lymphocyte were attributed to single stranded breaks in supercoiled DNA resulting in the relaxation of the DNA molecule. The number of cells which

displayed the characteristics of apoptosis, however decreased after 48 hours. The SCGE assay also proved to be an efficient and simple technique for detecting DNA damage in individual cells.

These results correlated well with that of the *in situ* end labelling technique and with transmission electron microscopy. All subjects showed an increase in apoptotic cells immediately after exercise similar to that observed in the SCGE assay. The number of apoptotic cells increased after 24 hours and decreased after 48 hours. The results also suggest that although apoptosis is known to occur in minutes, its recognition and complete elimination from the bloodstream may take a few hours, or that the signal inducing apoptosis continues for some time after the cessation of exercise. The *in situ* end labelling technique was based on labelling of DNA strand breaks with fluorescent label with quantification of apoptotic cells by FCM. Using the *in situ* end labelling technique it was also possible to evaluate with high efficiency the possible effects of previous and subsequent exercise of lower intensity on the number of apoptotic cells. This part of the study also revealed the rapid, quantitative and objective evaluation in using FCM for the enumeration of apoptotic cells.

Electron microscopy also proved to be an invaluable tool in correlating morphological changes with those observed using the SCGE assay and FCM. The characteristic features of apoptosis viz., condensation of DNA at one pole of the cell, cell shrinkage, microsegregation of the nucleus, swelling of the ER, mitochondria and Golgi complex were observed immediately after exercise and after 24 hours. These characteristic features however were not present after 48 hours. The changes within the nucleus and within the cellular organelles provided a good correlation between cause and effect of high intensity exercise on lymphocytes and lymphocyte DNA damage.

Destruction of the DNA which is the most prominent feature of apoptosis has evolved to ensure that no damaged or senescent DNA would find its way to another cell or virus. It is used as a defence against viral infection as viruses need the use of the cell's machinery to replicate. Defensive apoptosis in response to viral infection can be cell suicide, when a cell detects the virus and activates the intrinsic apoptotic program. These apoptotic cells need to be eliminated from the bloodstream before they come into contact with any viruses. This elimination is accomplished by macrophages which recognise and engulf apoptotic cells. Apoptosis is necessary for maintenance of homeostasis, however, the results indicate that exercise of high intensity accentuates the number of apoptotic cells with an associated decrease in normal circulating lymphocytes.

One of the mediators of apoptosis is known to be oxidative stress. Oxidative stress results in the production of free radicals and ROS, which in sufficient amounts can cause cell damage and cell death. These radicals are quenched by the action of antioxidants whose purpose is to reduce cell damage. Results of the present study show that there was a non-significant decrease in vitamin C concentration immediately after exercise with no significant difference in concentrations over the 48 hour period. Vitamin E concentration, although increasing minimally after exercise were also not significantly altered over the 48 hour period. This suggests that vitamin E concentrations may have been maintained at the expense of vitamin C. It was also found that lipid peroxide concentrations increased immediately after exercise however, not significantly suggesting that the continuous formation of lipid peroxides was opposed by the free trapping ability of vitamin C and E as well as the other antioxidants and inducable antioxidant enzymes.

The results of the present study shows that high intensity exercise causes an increase in apoptosis in lymphocytes as evidenced by the results of the SCGE assay, and supported by the results obtained using FCM and the morphological changes observed using electron microscopy. We can conclude that athletes who exercise at high intensity need time (48 hours) to allow the lymphocyte component of the immune system to recover before they begin another exercise bout. Exercise, stress and illness can be viewed as three points to a triangle. Each has independent effects on the immune system, while being mutually interactive. For every individual there is probably an optimal level of regular physical activity conducive to resistance to illness.

This study concentrated on the effects of a single bout of high intensity exercise on lymphocytes. No distinctions were made with regards to race and diet and these need to be considered in future studies. Far less is known phenomenologically or mechanistically about either chronic intermittent exercise stress or chronic exercise stress with superimposed acute episodes. Whether the results obtained in this study will apply to continuous exercise of high intensity or exercise of lower intensity and longer duration is an unanswered question. Future studies would have to concentrate on which subset of lymphocytes are undergoing apoptosis during exercise or whether this phenomenon is applicable to all cell types of the immune system. Also other forms of stress need to be considered (e.g. heat and cold) when evaluating apoptosis during exercise.

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APPENDIX

APPENDIX 3.1

INFORMATION TO SUBJECTS

Informed consent for inclusion in a clinical/experimental trial

Name of Student: S.N.Govender

Address: Physiology Department, Natal Medical School

Phone No: 2604440/2604364

I will require to run on a treadmill for 15 minutes or until exhaustion at a specific speed and with the treadmill at a specific gradient. Fifteen millimetres of blood will be taken before the run, immediately after the run, 24 hours and 48 hours after the run. Lymphocyte DNA will be analysed for DNA damage using a specific technique called the single cell gel electrophoresis (SCGE) assay. The levels of lipid peroxide activity as well as the levels of the antioxidants, vitamin C and vitamin E will be checked. The reason for taking blood at these times is to determine the effect of exercise on lymphocyte DNA immediately after exercise and during the process of recovery. Little discomfort will be experienced as you will be required to exercise on a treadmill ergometer. You will be given instructions on running on a treadmill and breathing through the collection system and allowed to practice. We will ask you to keep running for 15 minutes or until you feel that you are unable to maintain the pace set by the treadmill. At that point, you may grasp the safety rail and the treadmill will be stopped.

Although you will be undergoing exercise to the point of temporary exhaustion, there is very little risk involved if you are a normal, healthy individual. If you stumble or fall, the treadmill will be stopped immediately, and the consequences would be similar to if you had fallen on a track. The blood sampling to be performed will be done by qualified personnel. A hollow needle will be inserted into a peripheral vein in your arm and 15 ml of blood will be drawn. There will be little discomfort associated with the procedure; there may, however, be slight bruising at the point of puncture.

I have read and understand the above explanation of the purpose for this test and agree to participate. I also understand that I am free to withdraw my consent at any time.

NAME: DATE:

APPENDIX 3.2

MEDICAL HISTORY QUESTIONNAIRE

Name:

Date:

Age: Sex:

Address: (Home):

(Work):

Telephone (work): (Home):

PAST HISTORY

Allergies:

Respiratory defects:

Cardiovascular System:

Hypertension:

Diabetes:

Fits:

Menstrual Irregularities

Recent Illness: Date:

Medications:

Other:

Height: Weight:

Blood Pressure:

APPENDIX 4.1

10% Trichloroacetic acid

10 g of TCA was dissolved in 100 ml distilled H_2O.

2% 2,4-DNPH

2 g of the crystalline compound was dissolved in 100 ml of 4,5 M H_2SO_4 (75 ml H_2O and 25 ml concentrated H_2SO_4). The solution was filtered and stored in a brown bottle at 4°C.

10% Thiourea

10 g of thiourea was dissolved in 100 ml 50% ethanol and refrigerated at 4°C.

1,5% $CuSO_4.5H_2O$

1,5 g $CuSO_4.5H_2O$ was dissolved in100 ml distilled water.

Combined colour reagent

The reagent was prepared just before use by adding 5,0 ml DNPH, 0,1 ml $CuSO_4.5H_2O$ and 0,1 ml thiourea.

85% H_2SO_4

20 ml distilled H_2O was added to 180 ml concentrated H_2SO_4. The solution was mixed and stored in a glass stoppered bottle in the refrigerator at 4°C.

Vitamin C

Stock: 1 % w/v stock solution in distilled H_2O

The fresh stock standard was prepare before use. 1,0 g ascorbic acid was dissolved in 100 ml distilled H_2O.

Working solution: 2 mg/100 ml working solution in distilled H_2O.

The working solution was prepared before use by diluting the stock standard 1:500 in distilled H_2O.

APPENDIX 4.2

dl-α-vitamin E - 5 g/l stock solution in distilled H_2O

- 50 mg/l working solution in ethanol

dl-α-vitamin E - 5 g/l stock solution in ethanol

- 50 mg/l working solution in ethanol

APPENDIX 5.1

Phosphate buffer

NaH_2PO_4 (Mwt = 119,98 g/mol) (0,01 M) was prepared by adding NaH_2PO_4 (0,1199 g) to a 100 ml beaker. In the same beaker, dithiothreitol (Mwt = 154,2 g/mol) (1 mM) was prepared by dissolving 0,0155 g. The contents were dissolved in H_2O (80 ml) and the pH was adjusted to 7,75 with the addition of 0,1 M NaOH.

EDTA

EDTA (Mwt = 372,24 g/mol) (0,01 M) was made by dissolving 0,3722 g in 100 ml of H_2O.

0,052 mol/l NaN_3

NaN_3 (Mwt = 65,01 g/mol) (0,052 M) was made by dissolving 0,3380 g in 100 ml of H_2O.

1×10^{-6} mol/l GSHPx

Glutathione peroxidase (1×10^{-6} mol/l) was made as follows:

Molecular weight of GSHPx, as supplied by Sigma, for one subunit is 21 000. A fixed amount of 2,0 mg was given.

The concentration of the stock solution is

$$\frac{2,0 \times 10^{-3}}{21\ 000} \times 1\ 000\ \mu l \text{ (in one eppendorf)} = 9,5238 \times 10^{-5}\ M$$

The concentration of the working solution is prepared as follows:

$$\frac{1,0 \times 10^{-6}\ M}{9,5238 \times 10^{-5}\ M} \times 1\ 000\ \mu l \text{ (in one eppendorf)} = 10,5\ \mu l$$

Thus 10,5 μl of the stock solution was diluted with phosphate buffer (989,5 μl).

0,038 mol/l Reduced glutathione

Reduced glutathione (GSH) (Mw = 307,3 g/mol) (0,038 M) was made by dissolving 0,1167 g in 10 ml of H_2O.

$1,4 \times 10^{-3}$ M NADPH

NADPH (Mw = 833,4 g/mol) ($1,4 \times 10^{-3}$ M) was made by dissolving 0,0116 g in 10 ml of H_2O.

5 U/ml Glutathione reductase

Glutathione reductase (5 U/ml) was prepared as follows:

The stock solution was 5 mg/ml and (600 U/ml);

Thus $\frac{5\ U/ml}{600\ U/ml} \times 1\ 000\ \mu l = 8,3\ \mu l$

Thus 8,3 μl of the stock solution was diluted with ammonium sulphate (991,7 μl).

Arsenic Oxide

Approximately 1 g of As_3O_4 (Mwt = 197,84) was dried for 2 hours at 110°C and then desiccated

0,0210 mol/l $KMnO_4$

One litre of dd H_2O containing 3,222 g Mn_2O_4 (Mwt = 158,04 g/mol) was boiled for 30 minutes, cooled and decanted into a brown bottle.

0,0025 mol/l KI

KI (Mwt = 166,0 g/mol) (0,0025 M) was prepared by dissolving 0,0207 g in 50 ml of dd H_2O.

H_2O_2 (6%) - 1,62 mol/l in distilled H_2O

H_2O_2 (6%) molar concentration was determined in 3 steps namely;

(1) Molar concentration of As_2O_3 was determined initially

(2) Molar concentration of Mn_2O_4 was determined

(3) Molar concentration of H_2O_2 was determined using Mn_2O_4 molar concentration

(1) Arsenic oxide was weighed in triplicate and its molar concentration determined as follows:

Average mass of As_2O_3 = 0,2502 g

Mwt of As_2O_3 = 197,81 g/mol

Molar concentration of As_2O_3 = $\frac{0,2502\ g}{197,81\ g/mol} = 1,26485 \times 10^{-3}$ M

(2) The molar concentration of Mn_2O_4 was determined as follows:

Arsenic oxide (0,2502 g) was transferred to a 400 ml beaker prior to the addition of NaOH (20% w/v) and the contents stirred occasionally for 8-10 minutes. After stirring, H_2O (100 ml), concentrated HCl (10 ml) and the indicator KI_3 (few drops) were added. The mixture was titrated against Mn_2O_4 until a faint pink colour persisted for 30 seconds.

Average volume of Mn_2O_4 from titration = 48,13 ml

As_2O_3 (1,26485 x 10^{-3} M) and Mn_2O_4 titration (48,13 ml)

$$\text{mol } As_2O_3 \times \frac{2 \text{ mol } HAsO_3^{2-}}{\text{mol } As_2O_3} \times \frac{2 \text{ mol } MnO^{4-}}{5 \text{ mol } HAsO_3^{2-}} \times \frac{1\,000}{\text{volume}}$$

$$1{,}26485 \times 10\text{-}3 \times \frac{2}{1} \times \frac{2}{5} \times \frac{1\,000}{48{,}13} = 0{,}0210 \text{ M}$$

Molar concentration for Mn_2O_4 = 0,0210 M

(3) The molar concentration of stock H_2O_2 (6%) was determined as follows:

Hydrogen peroxide stock (6%) (25 ml) was added to a 500 ml volumetric flask (A) with the balance consisting of dd H_2O (475 ml). After shaking, 25 ml was removed from flask (A) and added to a second volumetric flask (B) that contained H_2O (200 ml), 1:5 H_2SO_4 dilution (20 ml) and a few drops of KI_3. The mixture was titrated against Mn_2O_4 until a faint pink colour persisted for 30 sec.

Average volume of Mn_2O_4 from titration = 19,33 ml

Using Mn_2O_4 (0,0210 M) and Mn_2O_4 titration (19,33 ml)

$$0{,}0210 \; Mn_2O_4 \times \frac{19{,}33 \text{ ml}}{1\,000 \text{ ml}} \times \frac{5 \text{ mol}}{2 \text{ mol}} \times \frac{1\,000 \text{ ml}}{25{,}0 \text{ ml}}$$

Molar concentration of H_2O_2 (6%) = 0,040593 M

H_2O_2 standard (1-5 µM) curve was determined as follows:

H_2O_2 (6%) = 0,040593 = 4,0593 x 10^4 µM therefore,

$$\frac{1 \; \mu M}{4{,}0593 \times 10^4 \; \mu M} \times 100\,000 \; \mu l \text{ (volumetric flask)} = 2{,}46 \; \mu l$$

$$\frac{2\ \mu M}{4{,}0593 \times 10^4\ \mu M} \times 50\,000\ \mu l = 2{,}46\ \mu l$$

$$\frac{3\ \mu M}{4{,}0593 \times 10^4\ \mu M} \times 50\,000\ \mu l = 3{,}69\ \mu l$$

$$\frac{4\ \mu M}{4{,}0593 \times 10^4\ \mu M} \times 50\,000\ \mu l = 4{,}92\ \mu l$$

$$\frac{5\ \mu M}{4{,}0593 \times 10^4\ \mu M} \times 50\,000\ \mu L = 6{,}15\ \mu l$$

Thus, 2,56; 2,46; 3,69; 4,92 and 6,15 μl respectively of stock H_2O_2 (6%) were diluted with dd H_2O (50 or 100 ml volumetric flask), making concentrations of 1-5 μM.

APPENDIX 6.1

0,5% LMPA

0,5 g of LMPA was dissolved in 100 ml PBS.

2,5 M NaCl

14,6 g of NaCl was dissolved in 100 ml dd H_2O.

100 mM EDTA

0,37 g of EDTA was dissolved in 100 ml dd H_2O.

1% Triton-X 100

1 ml of Triton X 100 was added to 100 ml dd H_2O.

1 % Dimethylsulphoxide

10 ml dimethysulphoxide was added to 100 ml dd H_2O.

Electrophoresis buffer:

1 mM Na_2EDTA was made up by adding 0,09 g Na_2EDTA in 250 ml dd H_2O. 300 mM NaOH was prepared by adding 3 g NaOH in 250 ml dd H_2O. The two solutions were added together and mixed thoroughly by stirring.

0,4 M Tris

1,2 g of Tris was added to 25 ml of dd H_2O and the pH was adjusted to pH 7,5.

APPENDIX 6.2

1% BSA/PBS

0,2 M Phosphate buffer solution:

Stock A: 3,12 g of sodium dihydrogen orthophosphate (Mwt =) in 100 ml distilled H_2O.

Stock B: 2,83 g disodium hydrogen orthophosphate (Mwt =) in 100 ml distilled H_2O.

To make up solution with pH 7,4:

10 ml stock A + 40 ml stock B + 50 ml dd H_2O.

1g of BSA was added to 100 ml of PBS (pH 7,4) and mixed properly.

4% Paraformaldehyde solution

4 g of paraformaldehyde was added to 96 ml PBS (pH 7,4) and mixed thoroughly.

1% Triton X 100 in 1% sodium citrate

1% sodium citrate was made by dissolving 1g sodium citrate in 100 ml distilled water. Therafter 1 ml of Triton X 100 was added to the 1% sodium citrate solution.

APPENDIX 6.3

1% Glutaraldehyde in phosphate buffer (pH 7,4):

Mix 0,4 ml of 25% glutaraldehyde in 9,6 ml of 0,2 M phosphate buffer solution (pH 7,4) (Appendix 6.2).

Toluidine blue

1 g of toluidine blue (305,83 g/mol) and 10 g Borax was dissolved in a 50 ml H_2O and 50 ml acetone solution. The stain was filtered before use.

Uranyl acetate

A saturated solution of 50:50 ethanol/water with uranyl acetate (424,15 g/mol) was prepared in a 5 ml test tube. The mixture was allowed to stand for 4 hours, then centrifuged for 25 minutes to pellet any particles in the suspension immediately before use.

Lead Citrate

2,66 g lead nitrate

3,52 g sodium citrate

60 ml distilled water

16 ml 1N NaOH

The lead nitrate was dissolved in 60 ml distilled water. Sodium citrate was added to the solution until a precipitate formed. The mixture was left to stand at room temperature for 30 minutes. after 30 minutes the 16 ml 1N NaOH was added to the solution and the solution turned clear. The stain was filtered and stored at 4°C until use.