THE EFFECT OF EXHAUSTIVE EXERCISE ON LYMPHOCYTE APOPTOSIS

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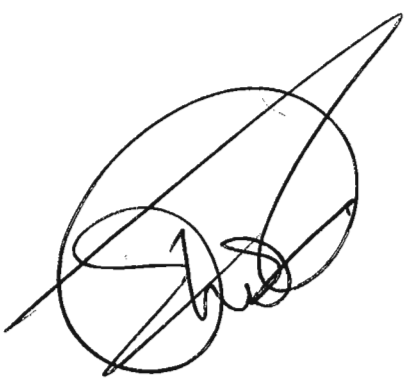
DECLARATION

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

The research was carried out in the Department of Physiology of the Nelson R Mandela Medical School under the supervision of Professor Maurice Mars

ALL LITERARY RESOURCES HAVE BEEN CORRECTLY REFERENCED AND ACKNOWLEDGED.



DURBAN----------DAY OF----------2001

DEDICATION

This work is dedicated to my loving family who supported me throughout my efforts.

My patients and my colleagues

ACKNOWLEDGEMENTS

My sincere thanks and appreciation to the following:

Professor Maurice Mars, Acting Head of Department of Physiology, University of Natal

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Page no.

ETHICS

This study was performed with the approval of the Ethics Committee of The University of Natal.

ABSTRACT

Post exercise lymphocytopenia is a well documented phenomenon. Studies have reported exercise induced DNA damage in leucocytes and have postulated a possible link to apoptosis. Five subjects of differing fitness levels underwent a ramped treadmill test to exhaustion. Venous sampling was undertaken before, immediately post exercise, and 24 and 48 hours after exercise. Single cell gel electrophoresis showed evidence of single strand DNA breaks (as evidenced by an increase in tail moment measurements using the comet assay) in 100% of lymphocytes immediately after exercise, and in the 24 hour and 48 hour post exercise samples. Flowcytometric analysis of lymphocytes revealed a minimal amount of both apoptosis and necrosis at all time intervals. Lymphocyte apoptosis has again been demonstrated after exercise, however the percentage of apoptosis was a maximum of 4.8% at 24 hours. These findings may in part account for the exercise induced lymphocytopenia and reduced immunity demonstrated by numerous previous other studies.

CHAPTER 1

EXERCISE AND APOPTOSIS

1.1 Introduction

Exercise immunology has generated tremendous interest in the sports medical fraternity in recent times and is in fact a sub discipline on its own. The relationship between exercise intensity and the risk of upper respiratory tract infections has been modelled in the form of a J-shaped curve (Niemann, 1994). Moderate exercise is associated with a below average risk for upper respiratory tract infections whereas prolonged high intensity exercise has been associated with an above average risk of URTI's. This is associated with leucocyte subset changes with an acute bout of exercise, where the concentrations of neutrophils are seen to increase during and after exercise whereas lymphocyte numbers increase during exercise and decrease within a 2-4 hour period after exercise thus creating what is termed an open window period for infection. It is postulated that apoptosis is the phenomenon by which immune status is compromised and further that a single bout of high intensity exercise causes apoptosis by lymphocyte DNA damage (Mars *et al,* 1998).

Apoptosis or programmed cell death which first appeared in biochemical literature in 1972, is a critical process for the normal development of multicellular organisms. The process is characterized by a unique and distinct set of structural changes. These changes exhibited by cells entering programmed death by development are also shared by cells dying in a wide variety of circumstances outside of development: T-cell killing, negative selection within the immune system, atrophy induced by

endocrine and other physiological stimuli, normal cell turnover in many tissues, and in tumours and normal tissues following exposure to the appropriate (low) doses of ionising radiation, chemotherapy and even hypoxia (Wyllie *et al.*, 1997).

Analysis of cells using electron microscopy has enabled elucidation of morphological changes that occur during apoptosis. These include chromatin condensation, cytoplasmic shrinkage and plasma membrane shrinkage (Strasser *et al.*, 2000). Blebs form and bud of the cell. The blebs are membrane-invested extensions of cytosol that are usually devoid of organelles and are reversibly extruded and resorbed (Wyllie *et al.*, 1997). Following this, the cells experience an irreversible condensation of cytoplasm, accompanied by an increase in cell density, close aggregation of cytoplasmic organelles and condensation of the nuclear chromatin to form dense granular caps that underlie the nuclear membrane.

Apoptotic cells are rapidly phagocytosed by their viable neighbours or specialist phagocytes. Another characteristic feature of apoptosis is that the dying cells disappear rapidly from the tissue without the generation of any inflammatory response. Visible changes of a cell undergoing apoptosis using light microscopy, occur within a few hours at most.

All of these changes contrast the features of necrosis. Changes associated with a cell undergoing necrotic death are, the swelling of the cell, cytosolic as well as nuclear structural changes as well as a conservation of euchromatin and nuclear pores. The cell ultimately ruptures and the cytosolic contents are released into the extracellular

space, where a significant percentage of these elicit an inflammatory reaction including chemotaxis of neutrophil polymorphs.

Over the past few years the field of apoptosis has found application many different areas and ahs been reported in several thousand of scientific publications. Recent advances have revealed that mitochondria probably play a central regulatory role in the field of apoptosis particularly through the cytochrome c pathway. The release of cytochrome c from mitochondria into the cytosol is an early apoptotic event. Cytosolic cytochrome c will bind to apoptosis protease-activating factor (Apaf-1) and ATP. This complex is capable of activating caspase-9, which is responsible for initiating the proteolytic cascade of events resulting in apoptosis (Green and Reed, 1998). Also, mitochondria and radical species are intimately involved in the programmed cell death that occurs during aging and exercise. Increased oxidative stress from radical oxygen species and reactive nitrogen species changes the cellular redox potentials, depletes glutathione, and decreases reducing equivalents like NADP and NADPH. These intracellular changes are sufficient to induce the formation of mitochondrial permeability transition pores, leading to the subsequent release of cytochrome c and the activation of the caspase cascade.

We hypothesize that during oxidative stress as in exhaustive exercise, tissues may undergo unnecessary and increased apoptosis, leading to pathological dysfunctions from significant cell loss. Similarly, uncontrolled and unnecessary apoptosis may occur during exhaustive exercise resulting in various pathologies.

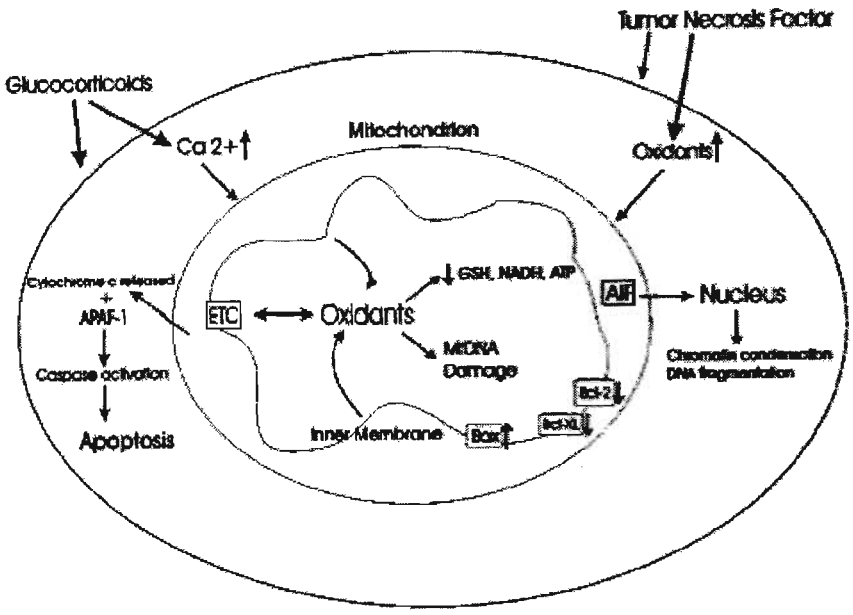

Figure 1. Simplified scheme on possible signals affecting apoptosis after exercise. Exercise causes increases in oxidant production by the electron transport chain (ETC) or oxidant production induced by increases in Ca^{2+} concentration. Oxidants could have a direct impact on the levels of glutathione (GSH), ATP, NADH, and on oxidative mitochondrial DNA damage. Other factors, such as tumour necrosis factor and glucocorticoids, may have similar actions or operate by different mechanisms inducing cell death. All these factors may effect mitochondrial proteins, such as Bcl-2, Bcl-XL , and Bax, and lead to the release of cytochrome c from the mitochondria. Cytochrome c may lead to "the point of no return" and activate caspases, resulting in apoptosis (programmed cell death). Other proteins released from the mitochondria, such as apoptosis inducing factor (AIF) located in the mitochondrial intermembrane space may be caspase-independent and translocate to the nucleus, causing large-scale DNA fragmentation. (Phaneuf and Leeuwenburgh, 2001)

To date only one study by Mars *et al.*, (1998) has demonstrated a possible link to lymphocyte DNA damage (apoptosis) associated with a single bout of high intensity. The object of our study was to determine the effect of a single bout of high intensity exercise on lymphocyte DNA.

CHAPTER 2

THE COMET ASSAY

2.1 Introduction

The single cell gel electrophoresis assay is an attractive and unique technique being employed increasingly in biological systems for the evaluation of DNA damage (Allah *et al.*, 1999). It is a rapid sensitive and relatively inexpensive method of determining DNA damage on a single cell basis.

Techniques, which permit the sensitive detection of DNA damage in studies of environmental toxicology, are constantly sought after. The effects of environmental toxicants are often tissue and cell type specific and therefore require detection of DNA damage in individual cells (Singh *et al.*, 1988).

This assay has previously been used in both *in vitro* and *in vivo* studies to investigate the effects of various agents on DNA damage in a number of mammalian cells (Hartmann *et al.*, 1994). Due to its simplicity, sensitivity and requirement for small sample amounts the SCGE technique has found widespread applications in genotoxicity testing DNA damage and repair studies, and biomonitoring.

The SCGE technique has been successfully used in screening human blood samples for susceptibility to radiation and various chemical mutagens (Vijayalaxmi *et al.*, 1992). It has also been applied to the study of peripheral blood cells from volunteers after physical exercise. The results exhibited a substantial increase in DNA damage

after specific time intervals after physical exercise (Hartmann *et al.*, 1994). Concordet *et al.,* (1993) linked exercise and leucocyte apoptosis in their study which used DNA agarose gel electrophoresis to investigate exercise induced thymocyte involution in rats. In this study glucocorticoid receptor mediated thymocyte apoptosis was shown to occur following 2.5-5 hour treadmill runs to exhaustion.

The detection of the DNA breaks are facilitated by alkaline electrophoresis of cells embedded in agarose and lysis by detergents of high salt concentration (Vijayalaxmi *et al.*, 1992). Breaks in DNA strands migrate in the direction of the anode producing an image resembling that of a comet. Furthermore in addition to measuring DNA strand breakage the alkali comet assay measures alkali labile sites of intermediates in base or nucleotide - excision repair (Gedik *et al.*, 1992; Green *et al.*, 1992). The sensitivity of the comet assay in the evaluation of DNA damage depends on accurate and reproducible measurement of DNA in the comet head and tail regions (Olive *et al.*, 1992).

The aim of the present study was to assess whether a single bout of high intensity exercise causes lymphocyte DNA damage in healthy individuals and to assess whether there is any correlation between DNA damage and apoptosis

2.2 Materials and Method

The chemicals used in the experiments were purchased from the following suppliers: Low melting point agarose and ethidium bromide from Roche biochemicals; Triton X from Sigma Chemicals Co. Ltd., US; Tris and Dimethyl Sulphoxide (DMSO) from

Merck; Ethylenediaminetetra-acetic Acid (EDTA) and Sodium Chloride (NaCl) from Capital Suppliers; Sodium Hydroxide (NaOH) from Saarchem.

2.2.1 Blood preparation and treatments

Whole blood was collected in EDTA Vacutainer tubes by venopuncture from donors. Blood samples were immediately processed for the SCGE assay.

2.2.2 Slide Preparation

The procedure described for the SCGE assay by Singh *et al.*, (1988) was followed with minor modifications: 200 microlitres of 0.75% agarose diluted in Ca^{++} and Mg^{++} free PBS buffer was added to frosted microscope slides, immediately covered with coverslips and kept for 10 minutes in a refrigerator to solidify. Coverslips were subsequently removed and 10µl of whole blood mixed with 90µl of 0.5% low melting point agarose (LMPA) at 37% were added to the first layer. The slides were immediately covered with a coverslip and kept in the refrigerator for another 5 minutes to solidify the LMPA. The coverslips were again removed and a final top layer of 75µl of 0.5% LMPA at 37°C was added and again refrigerated for a further 5 minutes. Coverslips were then carefully removed and the slides were immersed in a trough of cold lysing solution (2.5 M NaCl, 100mM EDTA, 1% Triton X-100, 1% Tris and 10% Dimethyl sulphoxide) which was freshly made up. Slides were kept at 4°C for 1 hour.

2.2.3 Electrophoresis

After the lysis step the slides were removed and placed in an electrophoresis tank. The tank was carefully filled with freshly made alkaline buffer (300mM NaOH and 1mM EDTA, pH 13.0) to a level of approximately 0.30mm above the slides. Slides were allowed to stand in the electrophoresis buffer for 20 minutes to allow for DNA unwinding before electrophoresis. Electrophoresis was conducted for the next 35 minutes at 25v and 300mA using a BioRad compact power supplier. The above steps were conducted in dim light to prevent additional DNA damage.

2.2.4 Staining

After electrophoresis the slides were removed and washed with 0.4% Tris pH 7.5. This was done to remove alkali and detergents that would interfere with the ethidium bromide staining. The slides were allowed to stand in Tris for 5 minutes and this step was repeated thrice. Finally, the slides were stained by placing 40µl of 20µg/ml ethidium bromide solution on each slide and then covering them with a coverslip. A schematic drawing of the SCGE assay is shown in figure 2.

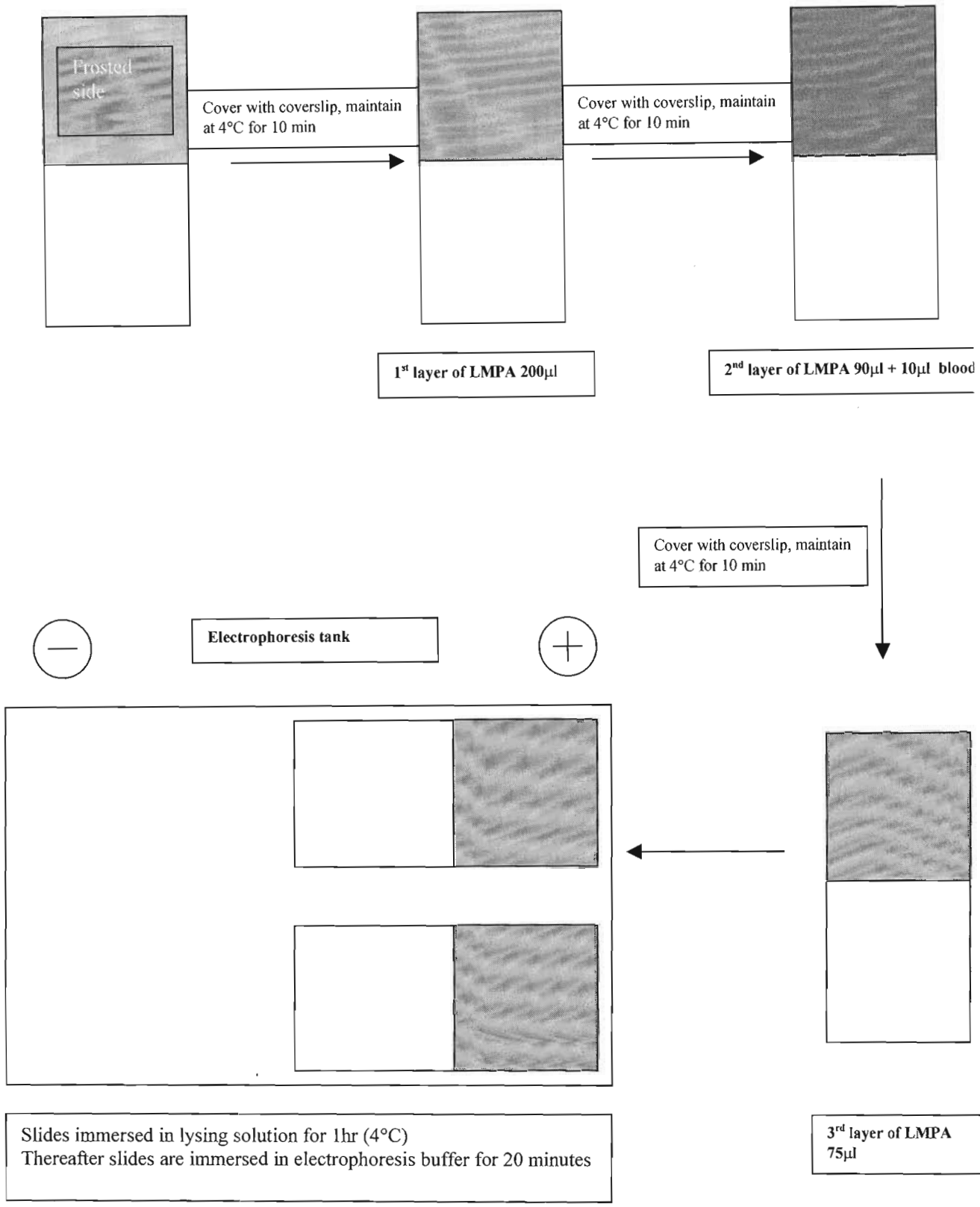

Figure 2 Schematic drawing of the SCGE assay

2.2.5 Image analysis

Observations of cells were made using a Nikon E-400 fluorescent microscope, equipped with an excitation filter of 450-490nm and a barrier filter of 520nm. Images of single cells were taken at 200× magnification using Scion image software. DNA migration lengths were determined on a negative image by measuring the nuclear DNA and the migrating DNA in 50 randomly selected cells (25 from each replicate).

2.2.6 Statistical analysis

Data were represented as the mean tail moment for the cells plus or minus the standard deviation within the various time intervals. Time intervals were compared using nonparametric analysis of variance with *post hoc* testing using Dunn's multiple comparisons test.

2.3 Results

Photographs of the lymphocyte DNA migration after electrophoresis are shown in figure 3.

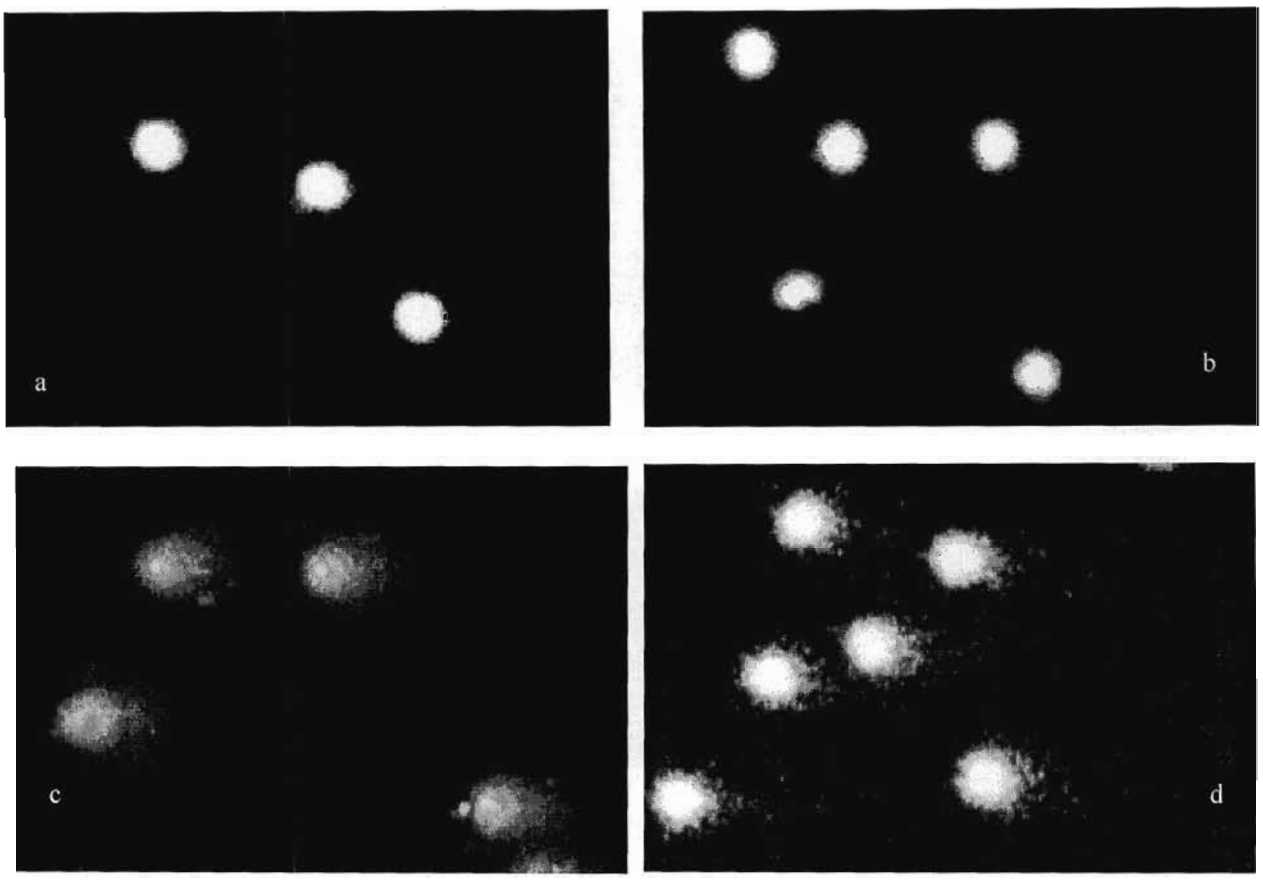

Figure. 3 Lymphocytes exhibiting increased DNA migration with increasing time. 3a Basal cells. 3b. Cells immediately after exercise. 3c. Cells 24 hours after exercise. 3d. Cells 48 hours after exercise.

The mean tail moments, an indicator of the severity of the DNA damage, are shown in table 1 and figure 4.

Table 1. The tail moments are measured in microns and expressed as a mean and one standard deviation. expressed as the mean and one standard deviation. Demonstration of DNA damage induced in human lymphocytes by exercise using the SCGE technique. Data are based on 50 randomly counted cells (25 from each of two replicate slides) per time interval.

	Pre-exercise	Post-exercise	24 hr after exercise	48hr after exercise
Subject 1	35.3 ± 4.5	32.7 ± 2.7	38.5 ± 3.8	26.1± 2.0
Subject 2	36.3 ± 2.7	37.6 ± 6.7	52.6 ± 4.7	90.0 ± 13.0
Subject 3	36.8 ± 3.3	36.9 ± 4.3	52.4 ± 4.4	68.4 ± 10.1
Subject 4	42.4 ± 7.1	32.4 ± 2.8	49.4 ± 6.0	84.9 ± 9.6
Subject 5	35.4 ± 2.3	35.6 ± 3.2	51.0 ± 3.6	40.1 ± 6.8
TOTAL	37.2 ± 5.1	35.0 ± 4.7	48.8 ± 7.0	61.9 ± 26.6

The mean data for each time point for all subjects were compared using a nonparametric analysis of variance (ANOVA) (Kruskal - Wallis test) with *post hoc* testing using Dunn's multiple comparisons test. Non-parametric tests were used because the Bartlett's test showed the standard deviations to be significantly different ($p<0.0001$) and the data sets were shown to be non Gaussian in distribution according to the method of Kolmogorov and Smirnov.

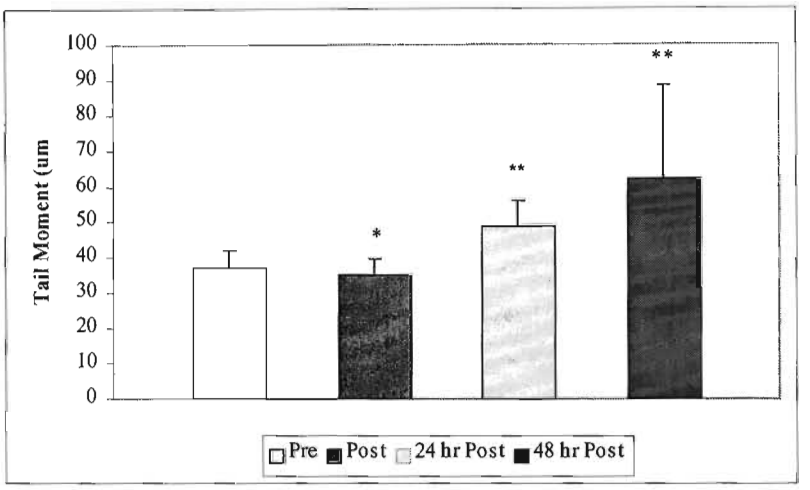

Figure 4. Average of tail moments in all test subjects over time. ANOVA was significant ($p<0.0001$). Post hoc testing showed significant increases in mean tail moments, 24 and 48 hours after testing (** = $p<0.001$) and a significant decrease immediately after exercise (* = $p<0.01$). There was no difference between the 24 hour and 48 hour post exercise samples.

CHAPTER 3

FLOW CYTOMETRY

3.1 Introduction

Cytometry refers to the measurement of physical and/ or chemical characteristics of cells. Flow cytometry is a process in which such measurements are made while the cells or particles pass, preferably in a single file, through the measuring apparatus in a fluid stream (Shapiro, 1988).

A number of physical factors such as acoustic, electronic, optical as well as radiation sensors have, at one time or the other been incorporated into flow cytometers. Optical measurements are the most common at present. It is often useful to employ flow cytometry even in dealing with highly homogenous populations of particles for the purpose of counting the number of particles present in a given volume of sample.

Most of the present interest in, and applications of, flow cytometers, however derive from the utility of the apparatus for the definition and quantification of heterogeneity in cell populations (Shapiro, 1988).

Amongst the most demanding applications of flow cytometry are the identification and subsequent characterization of subpopulations of cells. Both flow sorting and multi-parameter analysis are used for this purpose. Flow sorting utilizes electrical and/or mechanical means to divert cells with pre-selected characteristics from the main stream, and can be used to isolate pure populations of viable cells with more

homogenous characteristics than could be obtained by any other means. Flow sorting is particularly useful in circumstances in which further characterization of the selected cells requires short- or long-term maintenance in culture or analytical procedures which cannot be accomplished by flow cytometry. In other cases, multi-parameter analysis may allow the desired information about a cell population to be obtained without physically isolating the cells (Shapiro, 1988).

Multi-parameter analysis in which values of one (or more) parameter(s) are examined to determine whether to include or exclude values of another parameter from the same cell in a tabulation or distribution, is referred to as gated analysis.

When several parameters are measured, it is frequently possible to identify cell subpopulations without demonstrating the presence of cell-specific chemical markers. The relative light scattering and colour discriminated fluorescence of the microscopic particles is measured and analysis and differentiation of cells is based on size and granularity, and whether the cell is carrying fluorescent molecules either in the form of antibodies or dyes. The result of the cell passing through the laser beam is a scattering of light in all directions and that light scattered in the forward direction is proportional to the square of a sphere and so to the size of the cell or the particle. Light may enter the cell and be reflected and refracted by the nuclear contents of the cell, thus the 90° light scatter may be considered proportional to the granularity of the cell.

In blood leucocyte populations containing lymphocytes, monocytes, and granulocytes, measurements of light scattered by cells at two different angles (less than 2°,

commonly called forward scatter, and about 90°, orthogonal, right-, or wide-angle scatter) from an incident laser beam can discriminate the three cell types sufficiently well to be usable for differential leucocyte counting in clinical haematology.

The cell may be labelled with fluorochrome linked antibodies or stained with fluorescent membrane, cytoplasmic or nuclear dyes. This greatly facilitates differentiation of cell types, the presence of membrane receptors and antigens, membrane potential, pH enzyme activity and DNA content. Most commercial cytometers can make four or more simultaneous measurements on every cell although some may even possess greater capabilities.

The functional units of a typical cytometer are : (a) a light source or laser, and a sensing system which comprises the sample/flow chamber and optical assembly; (b) a hydraulic system which controls the passage of cells through the sensing system; and (c) a computer system which collects data and performs analytical routines on the electrical signals relayed from the sensing system.

The flow chamber is instrumental in delivering the cells in suspension to the specific point that is intersected by the illuminating beam and the plane of focus of the optical assembly. A laminar/sheath flow technique is utilized in most instruments to confine cells to the centre of the flow stream; this also reduces blockage due to clumping. Cells enter the chamber under pressure through a small aperture that is surrounded by sheath fluid. The sample fluid is drawn into a stream by the hydrodynamic focussing effect created by the sheath fluid in the sample chamber. Accurate and precise positioning of the sample fluid within the sheath fluid is critical to efficient operation

of the flow cytometer and adjustment of the relative sheath and sample pressures ensures that cells pass one by one through the detection point (Shapiro, 1988).

In laser based flow cytometers, where fluorescence is measured at right angles to the illuminating beam, chambers may comprise flattened cuvettes to minimize unwanted light reflections. Water cooled laser sources in the range 50mW to 5W output power have been used for fluorescence and light scatter measurements. Air-cooled lasers with a maximum of 100mW output are now more commonly used in commercial instruments.

The advantage of lasers is that they are capable of producing an intense beam of monochromatic light which in some systems may be tuned to several different wavelength. The most commonly used lasers in flow cytometry are: argon lasers, which produces light between the wavelengths 351 and 528nm: krypton lasers, which produce light between 350 and 799nm; helium-neon lasers, which produce lines at 325-441nm (Shapiro, 1988).

3.2 Fluorescence analysis

Fluorescence is excited as cells pass through the laser excitation beam, and this fluorescence is collected by optics at 90° to the incident beam. A barrier blocks laser excitation illumination, while dichroic mirrors and appropriate filters serve to select the required wavelengths of fluorescence for measurement. Photo-multiplier tubes are used to convert the photons of light falling upon the detectors to an electrical impulse, and this signal is processed by an analogue-digital A-D converter, which changes the

analogue to a digital signal. The quantity and intensity of the fluorescence are recorded by the computer system and displayed on a visual display unit as a frequency distribution, which may be single, dual or multi-parameter. Single parameter histograms usually convey information regarding the intensity of the florescence and number of cells of a given fluorescence, so that weakly fluorescent cells are distinguished from those which are strongly fluorescent. Dual parameter histograms of forward angle scatter and 90° light scatter allow identification of the different cell types within the preparation, based on size and granularity (Shapiro, 1988).

3.3 Light scatter and detection

3.3.1 Filters

Light scattered by particles as they pass through a laser or light source must be efficiently detected and fluorescent light of given wavelength requires specific identification. The amount of light scattered is generally high in comparison with the amount of fluorescent light. Photodiodes are therefore used as forward angle light (FAL) sensors that maybe used with neutral density filters, which proportionately reduce the amount of light received by the detector. A beam absorber (diffuser or obscuration bar) is placed across the front of the detector to stop the laser beam itself and any defracted light from entering the detector. The scattered light is focused by a collecting lens onto the photodiode(s), which converts the photons into voltage pulses proportional to the amount of light collected (integrated pulse). These pulses may be amplified by the operator. In some systems with multiple diode upper and lower light may be collected, which may help separate populations of cells or particles.

Fluorescence detectors are usually placed at right angles to the laser beam and sample stream. Stray light is excluded by an obscuration bar in front of an asperic (objective) lens, which collects the light and refracts it into a parallel beam. To detect the components of the beam, filters and mirrors are used to remove unwanted wavelengths of light and direct light to the correct detector(s).

Typically, the first filter used eliminates laser light that still may have passed through. This light may then be diverted to a beam splitter or a dichroic mirror. This mirror reflects light in one band of wavelengths (usually long) while allowing another band to pass through (usually short) it should be noted that there is no direct cut-off here between reflection and transmission. There is a middle band of wavelengths that will do both. For this reason, the colour components are passed through other filters before entering the director. These filters remove the unwanted wavelengths and allow the desired wavelengths to pass to the director. These filters are called band-pass and are designated by whether they transmit long wavelengths (long pass) or shorter wavelengths (short pass). They may be termed by the centre wavelength and band widths. The sensors used are photon multiplier tubes (PMTs). These tubes serve as detectors and also amplifiers of the weak fluorescent signals. These tubes have their own high voltage powers supplies, which provide the boost needed to amplify the signal internally within the PMT. The amount of high voltage and therefore the amplification is adjustable by the operator. A second amplification, also operator controlled, may be made on the PMT signal external to the PMT. PMTs are used only under weak light conditions as they may be damaged by high intensity light such as normal room light.

3.3.1.1 Filter sets.

Filters are used in sets, usually pairs of band-pass filters with a dichroic filter or beam splitter. Filter 1 is an excitation filter which is used to select a specific wavelength from the broad spectrum of light to produce a monochromatic illumination corresponding to the flourophor's absorption band. Filter 2 is an emission filter which blocks the excitation and emission filters. Such light originating from the light source would either be erroneously observed as fluorescence or would make a coloured, rather than black, background. This is termed spectral overlap or filter crosstalk. This very low transmission may be measured spectrophotometrically and should not exceed 0.0001% or 10^{-6} (Shapiro, 1988).

3.3.2 Detection of apoptotic cells by light scatter analysis

The intersection of a cell with the light of a laser beam in a flow cytometer results in light scatter. Analysis of this scattered light provides information on the cell size and structure.

The forward light scatter is associated with cell size while the light measured at right angle to the laser beam (side scatter) correlates with granularity, refractiveness, and the ability of the intracellular structures to reflect the light (Gorczyca *et al.*, 2000).

During the process of death this inherent ability of the cell to scatter light is altered. This is reflected in the morphological changes such as cell swelling or shrinkage,

rupture of the plasma membrane, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies (Gorczyca *et al.*, 2000).

Necrosis is characterized by a rapid initial increase in the cell's ability to scatter light simultaneously in the forward and the right angle direction. This is indicative of cell swelling. By contrast, especially in the latter stages of apoptosis, the intensity of light scatter at both forward and right angles decreases (Gorczyca *et al.*, 2000).

3.4.1 Apoptotic quantification by detection of phosphotidylserine with annexin V-FITC conjugate

Numerous methods have been employed in the analysis of apoptosis, such as universal biochemical, cytometric and antigenic marker procedures. Electron light and ultraviolet microscopy however remain the gold standard in the analysis of apoptosis although these techniques are rather tedious (Gorczyca *et al.*, 2000).

The characteristic morphological, functional and biochemical features of cells undergoing apoptosis or necrosis described in the introduction provided the basis for development of many flow cytometric techniques which enabled the differentiation between apoptosis and necrosis. These methods were all engineered around the changes in size and gross cell structure, in plasma membrane transport function, physical integrity or in chromatin and DNA structure (Gorczyca *et al.*, 2000).

Apoptotic cells possess the ability to trigger their own engulfment by phagocytic cells prior to cell lysis in order to avoid tissue damage and inflammation associated with

necrosis. Timeous generation of recognition signals on the surface of apoptosing cells is therefore a key event in the apoptotic program (Verhoeven *et al.*, 1995).

Phospholipids found within the plasma membrane are asymmetrically distributed between the inner and outer leaflets of the plasma membrane. In live cells phosphatidylserine (PS) is almost exclusively observed on the inner surface of the membrane.

It has recently been shown that the loss of phospholipid asymmetry leading to exposure of PS on the outside of the plasma membrane is an early event in apoptosis.

3.5 Materials and method

3.5.1 Blood preparation and treatments

Whole blood was collected in lithium heparin Vacutainer tubes by venopuncture from subjects. Blood samples were immediately processed for flow cytometric analysis.

3.5.2 Lymphocyte isolation

4ml of whole blood was diluted to 10ml using phosphate buffered saline. Diluted blood was then carefully layered horizontally onto 5ml of histopaque in a sterile 15ml Sterilin tube. Samples were then centrifuged for 30 minutes at 1500 rpm at 25°C. The resulting cell interface/buffy coat layer was separated using a sterile pasteur pipette. The buffy coat layer was then pipetted into a new Sterilin tube, topped up to 10ml

with phosphate buffered saline (PBS) and then centrifuged down at 1500 rpm at 4°C for 5 minutes (1st wash). The supernatant was then decanted and the pellet resuspended in the residual fluid. The tube was then topped up to 10ml with PBS and again centrifuged down at 1500 rpm at 4°C for 5 minutes (2nd wash). After spinning, the supernatant was decanted and the pellet was resuspended in the residual fluid.

3.5.3 Staining of lymphocytes for flow cytometry

Staining solution was made up by adding 20µl of both annexin V-FITC and propidium iodide to 1000µl of incubation buffer. The cells were then treated with 100µl of staining solution and incubated for 15 minutes at 22°C to allow the cells to take up the respective stains.

3.5.4 Flow Cytometric analysis

400µl of incubation buffer was then added to the stained cells. The cells were then analysed on a Becton Dickenson Facs Calibur flow cytometer using a 488 nm excitation and 515 nm bandpass filter for flourescein detection and a filter greater than 560 nm for propidium iodide detection.

Electronic compensation of the instrument was performed to exclude overlapping of the two emission spectra.

3.5.5 Data Analysis

Data were analysed using Apple Macintosh Cellquest software for flow cytometry. 100,000 cells were analysed for levels of apoptosis and necrosis at respective time intervals.

3.6 Results

The results of the annexin V tests showing the percentage of cells found to be apoptotic or necrotic at the different time points are shown in table 2

	Pre-excercise	Post-excercise	24 hr after exercise	48hr after exercise
Subj 1 Apop	3.92	2.62	4.87	0.92
Nec	4.29	3.10	1.76	0.48
Subj 2 Apop	4.63	2.79	4.01	0.74
Nec	3.83	2.92	2.97	0.50
Subj 3 Apop	2.28	2.49	2.53	0.72
Nec	2.02	2.64	1.66	0.40
Subj 4 Apop	2.60	1.64	1.73	0.68
Nec	1.81	1.06	1.55	0.57
Subj 5 Apop	3.60	3.71	4.37	0.13
Nec	3.27	3.79	3.48	0.77
Total Apop	3.4 ± 0.9	2.7 ± 0.7	3.5 ± 1.3	0.6 ± 0.3
Nec	3.0 ± 1.1	2.7 ± 1.0	2.3 ± 0.9	0.5 ± 0.1

Table 2. Percentages of apoptosis and necrosis in test subjects at various time intervals. Values are given as percentage of apoptotic or necrotic cells of 10^5 events run. Repeated measures ANOVA was significant ($p<0.0001$). Post hoc testing using the Tukey Kramer multiple comparisons test showed the tests after 48 hours to be significantly lower than all other time points for both percentage apoptosis and necrosis.

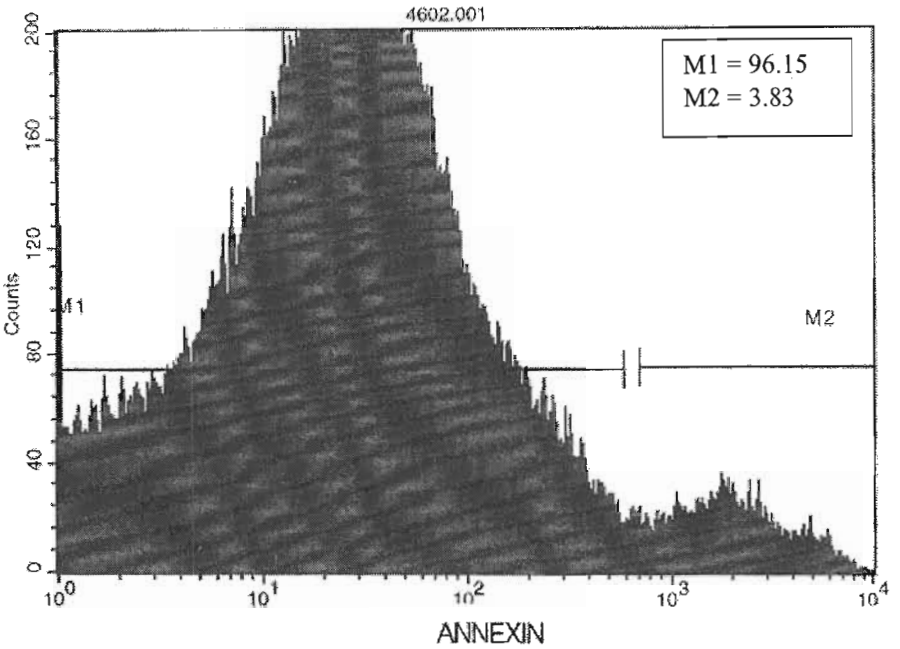

Figure 5a. Percentage of apoptosis in subject before exercise

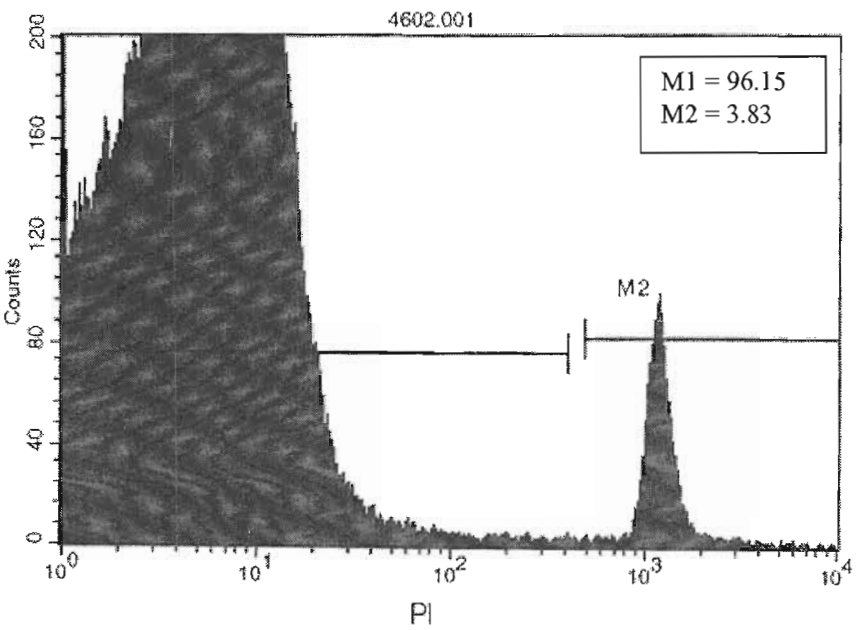

Figure 5b Percentage of necrosis in subject before exercise

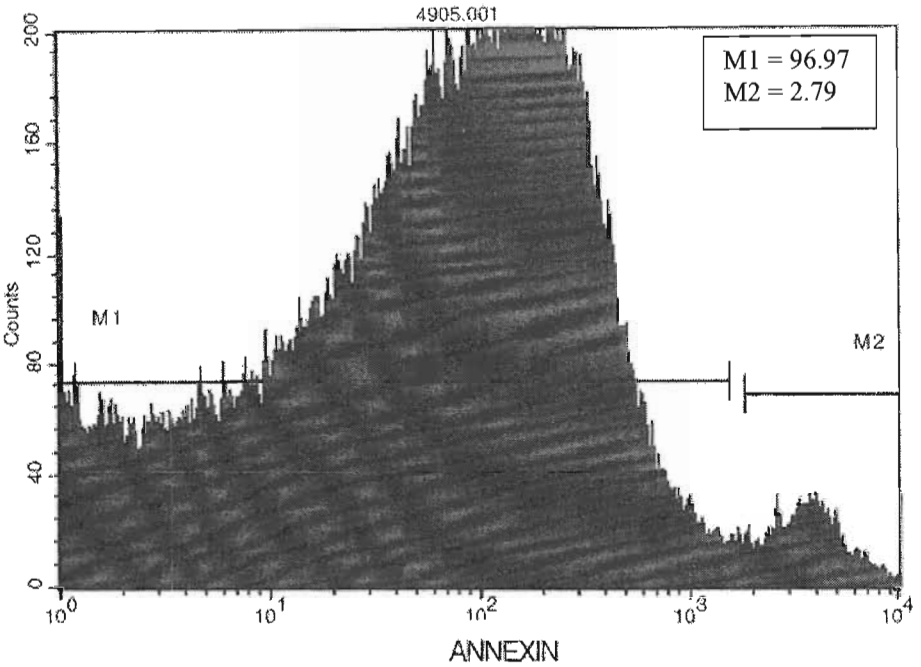

Figure 5c. Percentage apoptosis in a subject immediately after exercise.

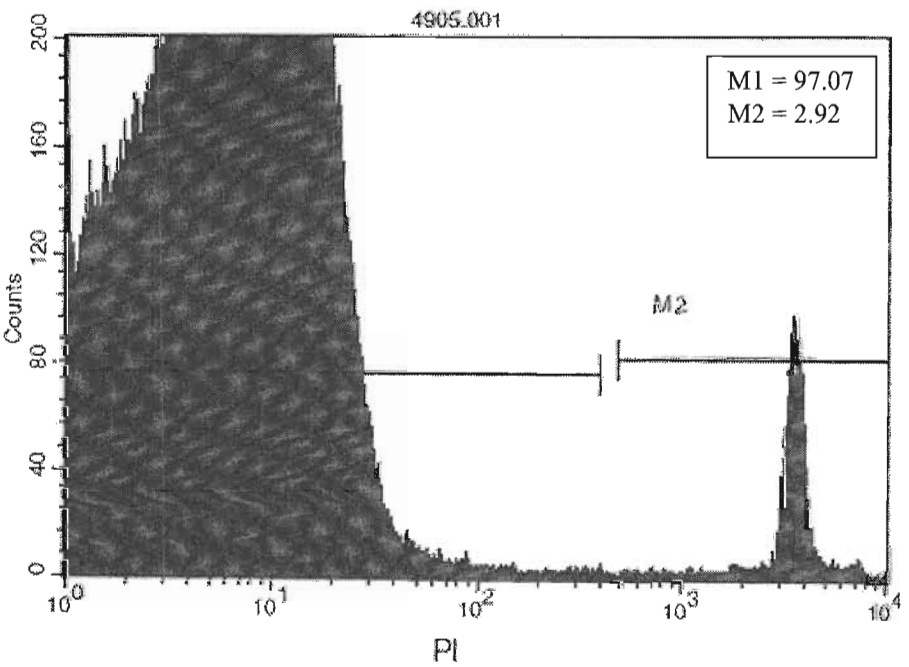

Figure 5d. Percentage of necrotic cells in a subject after exercise.

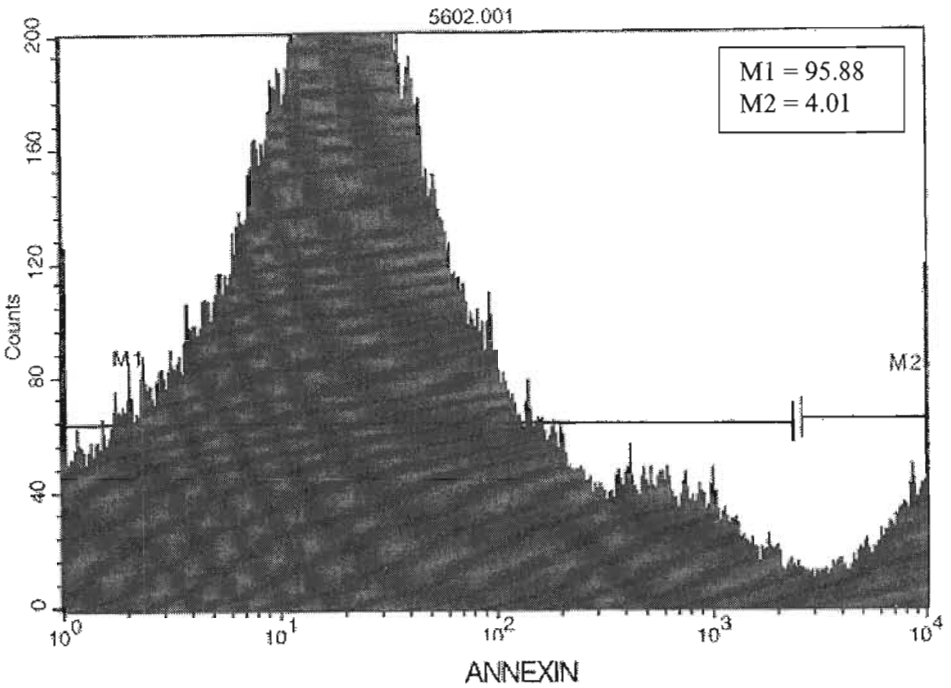

Figure 5e. Percentage of apoptosis in test subject, 24 hours after exercise

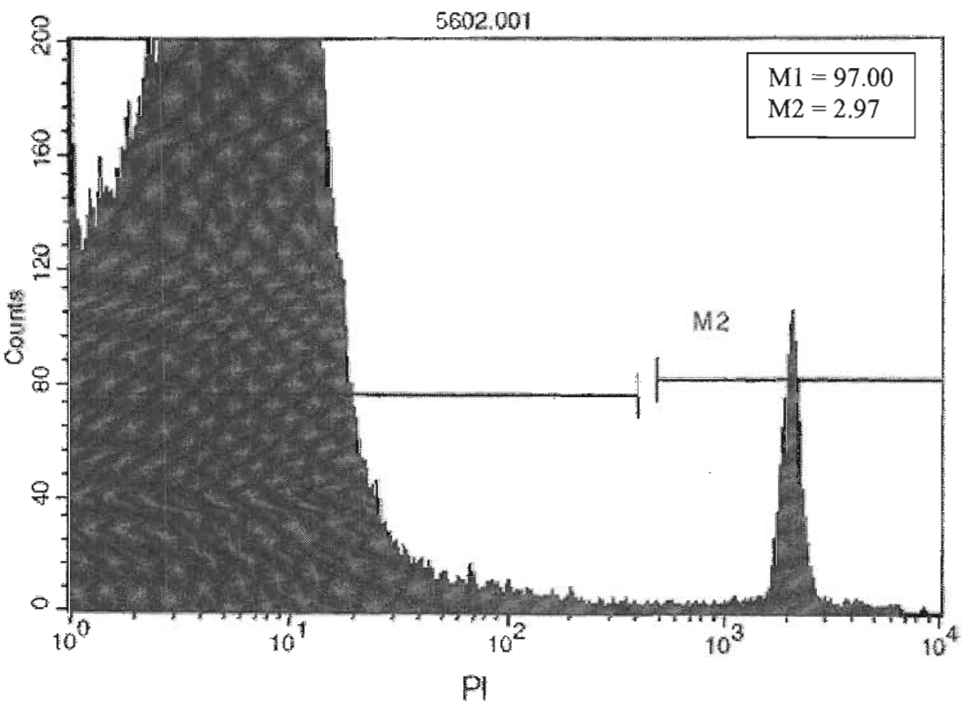

Figure 5f. Percentage necrosis in test subject, 24 hours after exercise.

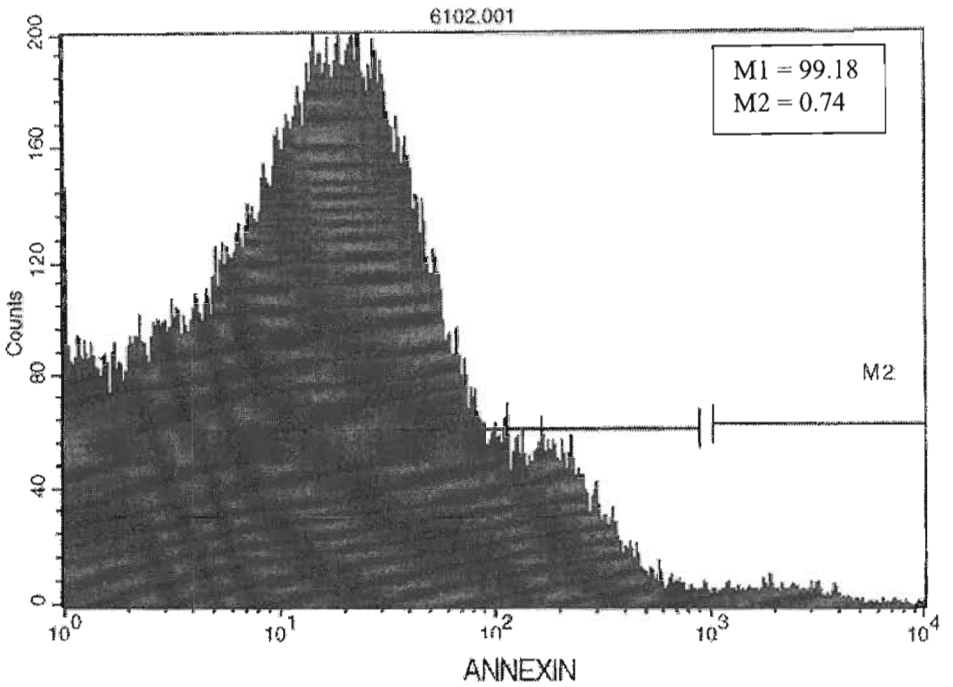

Figure 5g. Percentage apoptosis in subject 48 hours after exercise

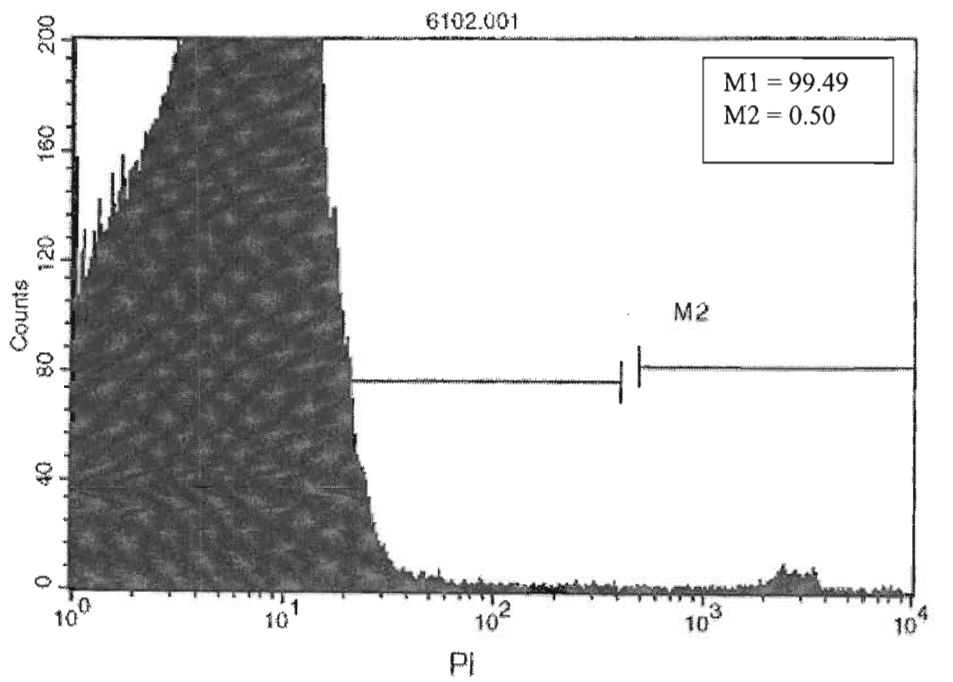

Figure 5h. Percentage necrosis in subject 48 hours after exercise

CHAPTER 4

EXERCISE TESTING

4.1 Maximum oxygen (VO_{2max}) testing

Measurement of the maximum uptake of oxygen by an athlete is probably the best method of predicting the aerobic athletic potential of a specific individual in sports physiology. The measurement of maximum oxygen uptake enables one to assess the capacity to transport and utilize oxygen. This involves the functional capacities of the heart, lungs, muscle and mitochondria.

4.1.1 Definition of VO_{2max} testing

The point in time where oxygen uptake eventually plateaus irrespective of an increase in workload is referred to as VO_{2max}. For subjects who do not develop an oxygen consumption plateau an exercising respiratory exchange ratio of 1.15 is taken to indicate that maximum oxygen consumption has occurred. The ability of an individual to execute work thereafter is a result of the energy transfer reactions of glycolysis. This however leads to an increase in hydrogen ions and the reduction of pyruvate to lactate. Under these conditions exhaustion soon ensues.

The VO_{2max} of marathon runners is approximately 45% greater than that of untrained persons. It has been suggested that the reason for this greater VO_{2max} of marathon runners is partly due to genetics. Those individuals with greater chest sizes in relation

to their body size and stronger respiratory muscles select themselves to become marathon runners (Guyton, 1991). It is more likely that successful marathon runners have a high percentage of oxidative type 1 and type 11a fibres.

4.1.2 The relation between cardiovascular performance and VO_{2max}

During strenuous exercise both the heart rate and the stroke volume are increased to approximately 90% of their maximal levels. The cardiovascular system is much more rate limiting on the VO_{2max} than the respiratory system. This would explain the 40 percent advantage in maximum cardiac output that a marathon runner has over an untrained person (Guyton, 1991).

It was previously thought that the best indicator of cardiorespiratory endurance / physical fitness / athletic potential is the $VO_{2\,max}$ (maximum aerobic capacity). Later research now seems to favour the *maximum work rate,* the running speed at the *lactate turnpoint* together with *running economy* as being more important. The very best predictor of performance at even ultra-long distances is the 10 km running time (Noakes, 1990).

The VO_{2max} is an indirect measure of athletic potential because in effect what is measured is the oxygen consumption at the peak achieved work rate or running velocity. There is strong support for the view that /VO_{2max} is a genetically predetermined value. However it has been shown that the VO_{2max} is trainable in terms of increases that have been recorded from 4% to 93%. An average increase of 15% to 20% is more typical for the average sedentary individual and this far greater than that

seen in active athletes. The reasons for increased /VO_{2max} is controversial and can be summarized into two theories.

Theory 1 is the utilization theory – in which there is said to be limitation of oxidative enzymes in the muscle mitochondria. Mitochondrial oxidative enzymes have been shown to substantially increase with endurance training both in number and size.

Theory 2 is the presentation theory, which is the limitation of oxygen delivery. This proposes that central and peripheral circulatory factors are responsible for the increase in VO_{2max}. More specifically the central factors are an increase in blood volume / cardiac output (via an increase in stroke volume) and an increased perfusion of active muscles as a result of increased capillary density and an increased arterio-venous difference.

Prolonged endurance training of the VO_{2max} allows an athlete to compete at a higher percent of his VO_{2max}. Similarly, with training, the lactate threshold increases thus enabling the athlete to compete at a higher intensity before reaching the lactate threshold.

Factors determining /VO+ are hereditary (25% -50% influence), age related decline, gender, responders and non-responders which is genetically determined and sports specific training.

Running economy which accounts for the differences in VO_{2max} between athletes running at similar speeds is influenced by running experience, body mass, stride

length, age, (adults are more economical than children), gender (males are more economic than females), clothing (aerodrag), hair length, shoe weight and lace, shoe type (air soles are more beneficial) wind speed, (head wind and drafting), running uphill or downhill (running downhill is more economical).

In conclusion it can be stated that successful endurance athletes require muscles that have superior contractility, thus allowing an athlete to achieve a higher work rate because of a higher oxygen consump0tion (VO_{2max}) but the exact VO_{2max} that each athlete achieves is however greatly influenced by his / her running economy. Athletes who exhibit high VO_{2max} values and poor performance may be less economical than those athletes with lower VO_{2max} values and good performances.

4.2 Materials and method

4.2.1 Measurement of maximal oxygen uptake

The most common tests are methods which recruit large muscle groups maintaining intensity and duration for as long as is required to induce maximal aerobic energy transfer.

The method employed in this study involved treadmill running. The treadmill used was a Powerjog treadmill, model EG10 which has a maximal speed of 25 $km.h^{-1}$ and a maximum gradient of 25%. Measurement of the athletes' oxygen consumption was measured using computerised open circuit spirometry (Oxycon Gamma Spirometer).

The computer program used to generate printouts of VO_{2max} etc. was the Oxycon Ergospirometry program.

A continuous treadmill run to exhaustion using graded protocol was used (Table 3)

TABLE 3 The exercise protocol used for exercise testing to determine VO_2max.

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.

Venous blood samples were taken before exercise and immediately after VO_{2max} testing. Subjects returned for further blood sampling 24 and 48 hours after the VO_{2max} test. All blood samples were taken at the same time of day to avoid the effects of diurnal changes in hormone concentrations.

4.3 Results

The results of the VO_{2max} tests are shown in table 4.

TABLE 4 Physical characteristics and results of the VO_{2max} test.

Subject	Age (years)	Height (cm)	Mass (kg)	VO_{2max} (ml. $kg.^{-1}.min^{-1}$)	Maximum Heart rate ($beats.min^{-1}$)
1	35	178.8	67.8	56.3	188
2	42	173.4	59.2	57.2	177
3	43	180.3	70.8	52.2	180
4	33	176	73.8	41.9	199
5	28	178.9	75.6	38.5	193

CHAPTER 5

DISCUSSION AND CONCLUSION

It was found that all five volunteers exhibited an increase in DNA migration after 24 hours of exercise. The degree of DNA migration between individuals varied greatly as we expected. Before the run the subjects showed no sign of DNA damage. Peripheral blood cells exhibited negligible or almost no DNA damage immediately after exercise. There was substantially increased DNA damage 24 and 48 hours after exercise.

It is therefore evident from the results that there was no DNA migration before the exercise whereas after the run most of the cells exhibited increased DNA migration.

The point in time when oxygen uptake plateaus and shows no further increase, or increases only marginally (< 100ml/min) with an increase in work load is referred to as the maximum oxygen uptake, maximum oxygen consumption, maximum aerobic power or simply the $VO_{2max.}$ The additional work that an individual is thereafter able to achieve is a result of energy transfer reactions of glycolysis which results in the accumulation of hydrogen ions and reduction of pyruvate to lactate.

The concentrations of lactate were not quantified in this study. Lactic acid concentrations in other studies however revealed that the values decrease rapidly to control levels after 1 hour of exercising. This therefore excludes the possibility that the cytotoxic effects in the SCGE assay are due to acidic conditions (Hartmann *et al.*, 1994).

Apoptosis induced by the single bout of exercise is possibly a normal regulatory process that serves to remove certain damaged cells without a pronounced inflammatory response, therefore ensuring optimal body function (Phaneuf *et al.*, 2001). DNA fragmentation is invariably a process that characterizes apoptosis in its latter stages. This biochemical process is responsible for the cleavage of DNA by endonucleases to fragments of approximately 180-200 bps. These results have relevance in that DNA damaged (apoptotic) lymphocytes are not functional to allow, for example, viral replication and normal immune function.

Apoptosis due to strenuous exercise would therefore be readily detectable by the SCGE technique which is sensitive in the detection of single stranded DNA breaks. This is clearly evident in the results summarized in table 1.

The most significant observation from the flow cytometric results in all test subjects, is a substantial decrease in both apoptotic and necrotic cells after 48 hours when compared to all other time intervals. As expected once again as in the SCGE assay there is a great degree of variability among the test subjects. This is probably best explained by the difference in individual immune status of each test subject at the respective times. Three of the test subjects showed a marked increase in levels of apoptosis after 24 hours while the other two remained almost the same. This is followed by a sharp decrease in apoptotic cells among all the test subjects. Cells actively undergoing apoptosis possess the ability to trigger their own engulfment by phagocytic cells prior to cell lysis. This is due to the generation of recognition signals on the surface of apoptosing cells (Fadok *et al.*, 1992a; Savill *et al.*, 1993 and Fadok *et al.*, 1992b).

In most of the cells where macrophage recognition has been studied, two such signals have been identified. The first of these signals is the exposure of a ligand for the vitronectin receptor (Hall *et al.*, 1994; Fadok *et al.*, 1992a and Savill *et al.*, 1993). The second of these signals is the relocation of PS to the outer leaflet of the cell membrane (Fadok *et al.*, 1992; Savill *et al.*, 1993 and Fadok *et al.*, 1992). In studies conducted, the ability of macrophages to recognize exposed PS is indicated by the ability of PS vesicles to specifically inhibit the phagocytosis of apoptotic lymphocytes with phosphatydylserine exposed on their surfaces (Fadok *et al.*, 1992). Increased phagocytic activity due to these recognition signals could possibly explain the sharp decrease in apoptotic cells after 48 hours.

The increase in apoptotic levels in test subjects after 24 hours can possibly be attributed to physical stress induced glucocorticoid receptor-mediated apoptosis (Concordet *et al.*, 1993). It is possible that the apoptosis induced by the single bout of exercise is a normal regulatory process that serves to remove certain damaged cells without a pronounced inflammatory response, therefore ensuring optimal body function (Phaneuf *et al.*, 2001).

The results of the necrotic cells by contrast exhibit a significantly lower percentage of cells 24 and 48 hours later as compared to the basal and immediately post exercise values.

Subjects 1,2 and 3 are marathon runners. Subject 4 is a regular indoor soccer player (twice a week) while person 5 leads a relatively sedentary lifestyle. From the results

in table 3 it can be seen that ultradistance runners like subjects 1-3 specializing in races such as the comrades marathon have significantly higher VO_{2max} values than subject 5 who do not train as regularly.

A large number of studies have been conducted with the aim of elucidating the effects of high intensity exercise on the immune system. The result is a well documented paradox between exercise intensity and the incidence of illness, where moderate exercise is generally beneficial to immunity, while prolonged intense exercise may be immunosuppressive (Pyne *et al.*, 1998).

It is well known that various types of stresses, such as heat, anxiety, and physical stress, can influence immune system function (Concordet *et al.*, 1993; Mars *et al.*, 1998). For example, acute exercise stress is associated with a lower lymphocyte functional response. In Nieman's "J-shaped model," it is suggested that exercise can enhance or reduce immunity depending on the frequency, duration, and intensity of the exercise (Nieman 1994).

The response of blood leucocytes to a bout of acute exercise are highly stereotyped. The concentrations of neutrophils seen to increase during and after exercise whereas lymphocyte concentrations increase during exercise and decrease after exercise. The proliferative response of cells to mitogens decreases during and after exercise while the degree of lymphocyte apoptosis increases during and after exercise.

Strenuous exercise stimulates a variety of signals, such as increases in glucocorticoid (GC) secretion, intracellular calcium levels, and reactive oxygen species, which can

potentially induce apoptosis (Carraro and Franceschi 1997). In addition, glutathione depletion, thiol oxidation, DNA damage, and hypoxia have been reported during exercise and may likely contribute to programmed cell death. It has been proposed that apoptotic cell death induced by exercise in very metabolically active tissues, such as the heart and skeletal muscle, may be a normal process to remove partially damaged cells. Certain forms of exercise however, may cause mechanical damage, followed by an inflammatory response, leading to excessive apoptosis.

Mitochondria have primarily been seen as powerhouses for energy production; however, it is now clear that excessive free radical production and the loss of mitochondrial membrane potentials can lead to apoptotic cellular events, including cytochrome c release or decreased Bcl-2 expression. One of the first papers to suggest that apoptosis may play a role in post-exercised animals was by Concordet *et al.* (1993). In this study, rats ran to exhaustion on a treadmill. The T-cells in their thymuses showed signs of programmed cell death (increased DNA fragmentation) immediately after and 24-h post exercise. Moreover, RU-486 (a potent glucocorticoid receptor antagonist) administered 2 hours and 0 hours before the run partially inhibited thymocyte DNA fragmentation. This suggests a relationship between physical stress and glucocorticoid receptor-mediated apoptosis of rat thymocytes.

Damage of myocyte mitochondria due to oxidants and a variety of other factors, such as loss of mitochondrial membrane integrity, could possibly be inducers of apoptosis during exercise. Investigations of apoptosis in skeletal muscle myocytes of dystrophin-deficient (mdx) mice subjected to spontaneous exercise have been conducted. Muscle analysis revealed increases of apoptotic myonuclei after exercise

detected by the TUNEL method using electron microscopy. It is not clear from these studies, however, whether these cells were lost entirely by an apoptotic process. Moreover, expression of ubiquitin (a protein expressed during apoptosis that covalently links to proteins and tags them for degradation) correlated with exercise and with positive myonuclei. Furthermore, this study demonstrated a decrease in mitochondrial Bcl-2, which may be due to exercise-induced apoptosis in the muscle of mdx mice (Sandri *et al.*, 1997).

It was also found that after spontaneous wheel-running, both normal and dystrophin-deficient muscles of mice showed increases in fragmented DNA using the TUNEL method and gel electrophoresis. Ubiquitin increased in muscles of both dystrophic and control exercised mice. In this study, tissues were collected two days after the exercise bout. The apoptotic events observed might very well reflect factors released due to inflammatory processes caused by mechanical damage to metabolically active tissues (Sandri *et al.*, 1995). It is thus conceivable that factors stimulating apoptosis during or immediately following exercise may be different from those involved 24-48 h post-exercise.

Human studies on the effects of apoptosis and exhaustive exercise are scarce. One study investigating human subjects exercising on a treadmill until exhaustion found DNA strand breaks in lymphocytes immediately after exercise, but not 24 and 48-hours post-exercise (Mars *et al.*, 1998). Moreover, flow cytometry revealed lymphocyte apoptosis in 63% of lymphocytes immediately after exercise and in 86.2% of lymphocytes 24 hours after exercise. Although these events could possibly explain the greater incidence of upper respiratory tract infections in highly trained

athletes following exhaustive training periods the results are questionable. Firstly changes of a cell undergoing apoptosis occur only after a few hours at most. It is therefore inconceivable that 63% of lymphocytes were apoptotic immediately after exercise as phosphatidylserine relocation to the outer leaflet of the cell, which is the basis for flow cytometric determination of apoptosis, would invariably take much longer to manifest itself. A further explanation for the divergent results could be explained by the difference in gating of the lymphocyte populations carried out by the different studies and the different methods of analysing apoptosis.

Exercise training is believed to result in a variety of adaptations that may be beneficial in attenuating apoptosis. It has been shown that several critical antioxidants and antioxidant enzymes are up-regulated with exercise training, thus providing additional free radical protection in myocytes (Leeuwenburgh *et al.*, 1997; Powers *et al.*, 1993; Sen *et al.*, 1992). It has therefore been proposed that animals that are exercise-trained should show increased resistance to apoptosis.

Skeletal muscle inactivity due to chronic heart failure could potentially also influence apoptosis. Indeed, recent research showed that apoptosis occurs in skeletal muscle myocytes in about 50% of patients with chronic heart failure possibly due to inactivity (Adams *et al.*, 1999). Importantly, patients with apoptosis-positive skeletal muscle myocytes exhibited a significantly lower VO_{2max}, a higher iNOS expression, and lower Bcl-2 expression as compared with biopsies of healthy patients. Future research is needed to determine the molecular signals enhancing skeletal muscle apoptosis in order to develop a therapeutic rationale for muscle fibre protection and restoration of contractile force.

The possibility that mitochondrial-produced oxidants during exercise have a direct effect on apoptosis has not yet been directly investigated. It seems very plausible that disturbances in mitochondrial homeostasis, i.e., DNA damage, inner mitochondrial membrane damage, and increases in calcium, could eventually result in the release of pro-apoptotic factors, such as cytochrome c from the intermembrane space. Thus, alteration in anti-apoptotic factors, such as Bcl-2 and Bcl-X_L with aging and exercise, could increase the likelihood of an induction of mitochondrial-mediated programmed cell death.

The results of the experiments conducted exhibit a lack of correlation, which may be explained by the different sensitivities of the test for different aspects of cell death.

The SCGE assay has been effective in detecting DNA strand breaks induced by ionising radiation, radiomimetic chemicals and oxygen radicals (Vijayalaxmi *et al.*, 1992; Mackelvey- Martin *et al.*, 1993). The cause of the DNA damage in the SCGE assay is not entirely clear but oxidative stress is a strong possibility. The assay has been proven to be highly sensitive (Olive *et al.*, 1990) and thus the results presented have to be taken as an indication that physical activity under certain circumstances causes DNA damage.

The percentages of apoptotic and necrotic cells in the flow cytometric results are not as high as those anticipated although it is possible to extrapolate certain trends amongst the various test subjects.

There are a number of issues within the sports discipline that warrant experimental and clinical investigation. It is important to achieve reliable and sensitive diagnostic technology for the rapid assessment of immune status and associated risk of susceptibility to illness in the sports field. This area is in desperate need of further investigation because a functional loss of specific immune cells may explain the higher incidence of respiratory tract infections seen in highly-trained athletes.

It is in this regard that we believe that the SCGE assay could be applied being a rapid sensitive and relatively inexpensive method of determining DNA damage on a single cell basis. The results presented here have direct implications for the use of the SCGE assay in studies with human blood cells. This may be beneficial in terms of coaches and athletes being better able to plan training and competitive schedules and in the determination and prevention of the so called "overtrained" athlete who is apparently more susceptible to URTIs as a result of possibly being immuno depressed.

REFERENCES

1. Abd-Allah G.A., R.I. El-Fayoumi, M.J. Smith, R.A. Heckmann and K.L.O'Neil, (1999) A comparative evaluation of aflatoxin B_1 genotoxicity in fish models using the comet assay, *Mutation Research*, 446, 181-188.
2. Carraro U and C. Franceschi. (1997) Apoptosis of skeletal and cardiac muscles and physical exercise. *Aging*. 1-2:19-34.
3. Concordet J. P. and A. Ferry, (1993) Physiological programmed cell death in thymocytes is induced by physical stress (exercise), *American Journal of Physiology*, 265, C626-C629.
4. Fadok V. A., J. S. Savill, C. Haslett, D.L. Bratton, D. E. Doherty, P. A. Campbell and P. M. Henson, (1992a) Different populations of macrophages use either the vitronectin receptor or the phosphatydylserine receptor to recognize and remove apoptotic cells, *Journal of Immunology*, 149, 4029-4035.
5. Fadok V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D.L. Bratton and P. M. Henson, (1992b) Exposure of phosphatydylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages, *Journal of Immunology*, 48, 2207-2216.
6. Gedik C.M., S.W.B. Ewen and A.R, Collins, (1992) Single-cell gel electrophoresis applied to the analysis of UV-C damage and its repair in human cells, *International Journal of Radiation Biology*, 62, 315-320.
7. Gorcyca W., M. R. Melamed and Z. Darzynkiewicz, (2000) Analysis of apoptosis by flow cytometry, Flow Cytometry Protocols (From Methods in Molecular Biology: Vol 91), 217-238.

8. Green M. H. L., J.E. Lowe, S.A. Harcourt, P. Akinluyi, T. Rowe, A.V. Anstey and C.F. Arlett, (1992) UV-C sensitivity of stimulated and unstimulated human lymphocytes from normal and xeroderma pigmentosum donors in the comet assay: A potential diagnostic technique, *Mutation Research*, 273, 137-144.

9. Green D. R., and J. C. Reed, (1998) Mitochondria and apoptosis. *Science* 281:1309 – 1312.

10. Guyton A. C., (1991), Respiration in Exercise, Textbook of Medical Physiology, 8, 945-947.

11. Hall S. E., J. S. Savill, P. M. Henson and C. Haslett, (1994) Apoptotic neutrophils are phagocytosed by fibroblasts with participation of the fibroblast vitronectin receptor and involvement of a mannose/fucose-specific lectin, *Journal of Immunology*, 153, 3218-3227.

12. Hartmann A., U. Plappert, K. Raddatz, M. Grunert-Fuchs and G. Speit, (1994) Does physical activity induce DNA damage, *Mutagenesis*, 9, 269-272.

13. Leeuwenburgh C.; J Hollander.; R Fiebig.; S Leichtweis.; M Griffith.; LL Ji., (1997) Adaptations of glutathione antioxidant system to endurance training are tissue and muscle fiber specific. *American Journal of Physiology* 272, 363-369.

14. Li P.; D Nijhawan.; I. Budihardjo; SM. Srinivasula; M. Ahmad; ES. Alnemri; X. Wang, (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**:479-489.

15. Mackelvey-Martin V.J., M. H. L. Green, P, Schmezer, B.L. Pool-Zobel, M. P. Demeo and A. Collins, (1993) The single cell gel electrophoresis assay (comet assay): A European review, *Mutation Research*, 288, 47-63.

16. Mars M., S. Govender, A. Weston, V. Naicker, A. Chuturgoon, (1998) High intensity exercise: a cause of lymphocyte apoptosis? *Biochemical Biophysical Research Communications*, 249: 366-70.

17. Marzo, I, (1998) The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins. *Journal of Experimental Medicine*, 187: 1261-1271.

18. Nieman, D.C., (1994) Exercise, infection, and immunity. *International Journal of Sports Medicine*, 15:S131-S141.

19. Noakes T, 1990, The Lore of Running, Oxford University Press 25-28.

20. Olive P. L., D. Wlodek, R.E. Durand and J.P. Banath, (1992), Factors influencing DNA migration from individual cells subjected to gel electrophoresis, *Experimental Cell Research*, 198,259-26.

21. Phaneuf S. and C. Leeuwenburgh, (2001), Apoptosis and Exercise, *Medicine and Science in Sports and Exercise*, 33, 393-396.

22. Powers, S. K. D. Criswell, J. Lawler, D. Martin, F.K. Lieu, L.L. Ji, R.A. Herb, (1993), Rigorous exercise training increases superoxide dismutase activity in ventricular myocardium. *American Journal of Physiology* 265:H2094-8.

23. Pyne D. B. and M. Gleeson, (1998), Effects of intensive exercise training on immunity in athletes, *International Journal of Sports Medicine*, 19, S183-S194.

24. Sandri M.; U. Carraro, M. Podhorska-Okolov, C. Rizzi, P. Arslan, D. Monti, C. Franceschi, (1995) Apoptosis, DNA damage and ubiquitin expression in normal and mdx muscle fibers after exercise. *FEBS Lett*, 373, 291-5.

25. Sandri M., M. Podhorska-Okolow, V. Geromel, C. Rizzi, P. Arslan, C. Franceschi, U. Carraro, (1997) Exercise induces myonuclear ubiquitination and apoptosis in

dystrophin-deficient muscle of mice. *Journal of Neuropathology and Experimental Neurology*, 56:45-57.

26. Savill J. S., V. A. Fadok, P. M. Henson and C. Haslett, (1993) Phagocyte recognition of cells undergoing apoptosis, *Immunology Today*, 14, 131-136.
27. Sen C.K., E. Marin, M. Kretzschmar, O. Hanninen, (1992) Skeletal muscle and liver glutathione homeostasis in response to training, exercise, and immobilization. *Journal of Applied Physiology*, **73.** 1265-1272.
28. Shapiro H. M., (1988) Learning about flow cytometry, *Practical Flow Cytometry*, 2, 6-9.
29. Singh N.P., M.T. Macoy, R.R. Tice and E.L. Schneider, (1988) A simple technique for the quantification of low levels of DNA damage in individual cells, *Experimental Cell Research*, 175, 184-191.
30. Susin S.A., N. Zamzami, M. Castedo, E. Daugas, H.G.Wang, S. Geley, F. Fassy, J.C. Reed, G. Kroemer, (1997) The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis. *Journal of Experimental Medicine*, 186: 25-37.
31. Strasser A., L. O. Connor and V.M. Dixit, (2000) Apoptosis signaling, *Annual Reviews in Biochemistry*, 69, 217-245.
32. Verhoeven B., R. A. Schlegel and P Williamson., (1995), Mechanisms of phosphatydylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes, *Journal of Experimental Medicine*, 182, 1597-1601.
33. Vijayalaxmi R., R. Tice and G.H.S. Strauss, (1992), Assessment of radiation-induced DNA damage in human blood lymphocytes using the single-cell gel electrophoresis technique, *Mutation Research*, 271, 243-252.

34. Wyllie A. H., (1997), Apoptosis an overview, *British Medical Bulletin*, 53, 451-465.