EXERCISE AND DNA DAMAGE AND REPAIR IN MIDDLE AGED MEN

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M. A. AIKMAN

Preface

"Because of His grace I am what I am."

-1 Cor 15:9

I wish to thank the following people for their assistance and support:

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The author

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Declaration

This dissertation is submitted in article format and includes a review article (Chapter 2) on "Exercise and oxidative damage in healthy persons" as well as a research article (Chapter 3) entitled "The effect of different aerobic exercise intensities on oxidative DNA damage and repair in men between 40 and 55 years of age". The co-authors of these articles, Dr S.J. Moss, Prof. P.J. Pretorius and Prof. F.H. van der Westhuizen hereby give permission to the candidate, Mr M.A. Aikman, to include the two articles as part of this Master's dissertation. The contribution (advisory and supportive) of these co-authors was kept with in reasonable limits, thereby enabling the candidate to submit his dissertation for examination purposes. This dissertation, therefore, serves as partial fulfilment of the requirements for the M.Sc. degree in Human Movement Studies within the School for Biokinetics, Recreation and Sport Science in the Faculty of Health Sciences at the North-West University (Potchefstroom Campus).

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Conference Presentation

The following presentation, based on this dissertation, has been delivered:

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Abstract

EXERCISE AND DNA DAMAGE AND REPAIR IN MIDDLE AGED MEN

Regular physical activity (PA) leads to an increased quality of life by means of certain physiological adaptations. Regular PA is beneficial to the human body and its functionality, including the physiological, biochemical and even psychological modalities. During PA an increased burden is placed on all physiological mechanisms due to the increased energy demand, resulting in an adaptation of the physiological systems. Currently the biochemical mechanisms by which these adaptations occur are not well understood or defined.

During the flow of electrons through the electron transport chain in the mitochondria free radicals and reactive oxygen species (ROS) are produced. PA results in increased ROS production. The relationship of different exercise intensities and ROS production with resulting DNA damage is unclear. These free radicals and ROS disturb the pro-oxidant anti-oxidant balance resulting in oxidative stress. When this balance is disturbed oxidative stress could lead to potential oxidative damage. Oxidative damage occurs in lipid, protein and nucleic acid macromolecules. ROS can attack DNA bases or deoxyribose residues to produce damaged bases and/or single and double strand breaks. When the DNA is repaired and the damages are replicated it could cause mutations or apoptosis, affecting the cell function and physiology.

The purpose of this study was to investigate the influence of different aerobic intensities on oxidative DNA damage and repair in middle aged men by means of the Comet assay. Five PA males and five physically inactive males were assigned to an experimental and control group respectively. The subjects did not differ significantly at baseline. The VO_2max of each subject was determined at baseline. Subjects were then randomly assigned to 60, 70, 80 and 90% of individual baseline VO_2max intensities for an acute exercise intervention of 30 minutes on a bicycle ergometer. Blood sampling was done at baseline, post-exercise and 24 hours post-exercise for oxygen radical absorbance capacity (ORAC) and hydroperoxide analysis (dROM). Peripheral blood was obtained for DNA damage testing by means of Comet analysis at baseline, post-exercise, 5, 15, 30 minutes, and also 6, 12, 24, 48 and 72 hours after exercise. The results obtained indicated that subjects who regularly participate in PA had an increased baseline

reading of ORAC and dROM values. ORAC levels after each acute exercise session increased, with the highest increase in the control group, with a decrease in the direction of baseline readings 24 hours post exercise. A biphasic damage-repair cycle over the 72 hour period was observed with the Comet analysis. The most damaged cells occur directly after acute exercise. The highest incidence of DNA damage over a 72 hour period was observed at 70% VO_2max, with the least amount of damage after 90% VO_2max.

In conclusion the study indicates stress proteins or other kinds of physiological reaction to minimize the damaging effect of oxidative stress, is in place to restore the cell's homeostasis. Thus PA results in the development of oxidative DNA damage. To minimize DNA damage the optimal intensity for acute physical exercise is between 70-80% VO_2max. At higher intensities the release of stress proteins are initiated to buffer the damaging effect of oxidative stress and to restore homeostasis.

Keywords – Physical activity, Exercise, Free radicals, Oxidative damage, Comet assay, ORAC analysis, dROM analysis

Opsomming

OEFENING EN DNA SKADE EN HERSTEL IN MIDDELJARIGE MANS

Gereelde fisieke aktiwiteit (FA) het 'n beter kwaliteit van lewe tot gevolg. Hierdie verbeterde kwaliteit kom teweë as gevolg van sekere fisiologiese aanpassing en daarom is gereelde FA voordelig vir die liggaam en hoe dit funksioneer. Gedurende FA word 'n verhoogde spanning op die fisiologiese sisteme van die liggaam geplaas. Hierdie verhoogde spanning lei na sekere spesifieke fisiologiese aanpassings in die liggaam. Tot op hede word die biochemiese meganisme van hierdie aanpassings nog nie ten volle verstaan of begryp nie.

Tydens oksidatiewe fosforilasie vloei elektrone deur die mitochondria en vrye radikale en suurstof derivate word as byprodukte geproduseer. FA lei na 'n verhoogde produksie van hierdie vrye radikale. Dit is tans nog onduidelik wat die verhouding is tussen verskillende oefeningsintensiteite en die vrye radikaal vorming wat bydra tot DNA skade. Hierdie vrye radikale versteur die pro-oksidant anti-oksidant balans, wat aanleiding gee tot oksidatiewe stres. Indien die bogenoemde balans so versteur word dat die oksidatiewe stress dramaties styg, kan die spanning lei na die ontwikkeling van oksidatiewe skade. Oksidatiewe skade kan aangetref word in die lipiede, proteïene en nukleïensuur makro-molekules. Vrye radikale val die DNA basisse aan en dit lei na die beskadiging van basisse en/of enkel of dubbel DNA string breke. Wanneer die DNA gerepliseer word met hierdie breke in, kan dit lei na die vorming van geen mutasies of selfs sel apoptose.

Die doel van die studie was om te bepaal wat die effek van aerobiese oefeninge teen verskillende intensiteite was op die mate van DNA skade en herstel by middeljarige mans, m.b.v. die Komeet analise. Vyf fisiek aktiewe mans en vyf fisiek onaktiewe mans is in 'n eksperimentele en kontrole groep verdeel. Hierdie groepe se basislyn metings het nie betekenisvol van mekaar verskil nie. Voor aanvang van die eksperiment was elke proefpersoon aan 'n maksimale aerobiese oefening (VO_2maks) onderwerp. Proefpersone was dan ewekansig onderwerp aan 4 oefeninge teen intensiteite van onderskeidelik 60, 70, 80 en 90% van hul basis VO_2maks. Een akute oefensessie was 30 minute lank en is op 'n fiets ergometer uitgevoer. Bloed is voor aanvang getrek (basislyn), gevolg deur nog trekkings direk na afloop van die toets en 24 uur

post-oefening. Hierdie bloed is gebruik vir die analise van die anti-oksidant kapasiteit (ORAC analises) en vrye radikaal kapasiteit (dROM analises). Perifere bloed was geneem vir Komeet analise, m.b.v. vingerprikke voor aanvang, direk na oefening, en 5, 15 en 30 minute later. Opvolg prikke is ook 6, 12, 24, 48 en 72 uur later geneem. Resultate verkry met die ORAC analises dui daarop dat persone wat gereeld FA is, 'n hoër rustende anti-oksidant kapasiteit toon. Hulle toon ooreenkomstig ook hoër dROM waardes wat dui op hoër vrye radikaal konsentrasies. ORAC waardes van die kontrole groep styg drasties direk na afloop van die oefening, maar keer soos die eksperimentele groep na 24 uur terug na basislyn metings. Die Komeet analises toon dat FA wel 'n mate van DNA skade ontwikkel. Oor 'n 72 uur periode word 'n bi-fasiese patroon van skade-herstel opgemerk. Skade aan die selle is na afloop van al 4 oefensessies die hoogste direk na voltooiing daarvan. Die bi-fasiese patroon het al meer afgeplat hoe meer tyd verloop het na die oefensessie. Dit blyk dat 'n oefensessie teen 70% VO_2maks die meeste skade ontwikkel. Dit is belangrik om te let dat die 90% VO_2maks oefening by beide groepe byna geen DNA skade ontwikkel nie.

Die resultate wat verkry is uit hierdie studie toon dat optimale fisieke aktiwiteit teen 'n intensiteit van tussen 70 en 80% VO_2maks gedoen moet word om DNA skade te minimaliseer. Teen hoër intensiteite word stress proteïene vrygestel om die beskadigings effek van oksidatiewe stress te verminder en die sel se homeostase te normaliseer.

Sleutelwoorde: Fisieke aktiwiteit, Oefening, Vrye radikale, Oksidatiewe skade, Komeet analise, ORAC analise, dROM analise

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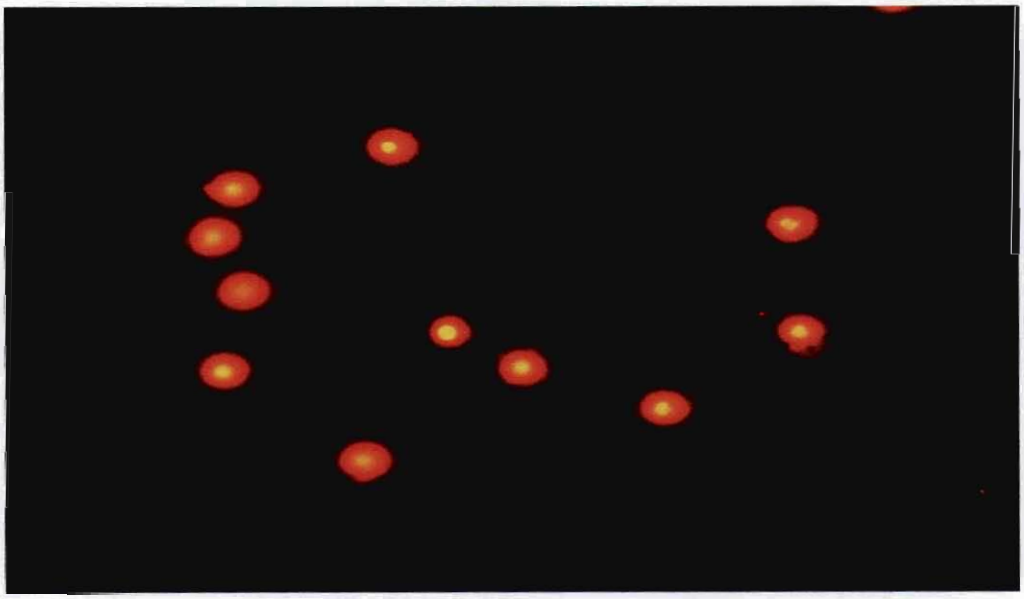
List of Abbreviations

A	**AA**	Ascorbic acid
	ACSM	American College of Sports Medicine
B	**BMI**	Body Mass Index
C	**CARR U**	Cartelli units
	CASP	Computerized image analyses systems program
	CK	Creatine kinase
	CPK	Creatine phosphokinase
	CVD	Coronary vascular disease
D	**dROM**	Free radical capacity determination
G	**GSH**	Glutathione peroxidase
	GSSG	Oxidized glutathione
H	**$HO^{\cdot}$**	Hidroxyl radical
	H_2O_2	Hydrogen peroxide
	HP	Hydroperoxide
L	**LH**	Lipid hydroperoxide
M	**MDA**	Malondialdehyde

	MPO	Myeloperoxide
	m/min	meter per minute
N	**NO**	Nitric oxide
	NO_2	Nitric dioxide
O	**ORAC**	Anti-oxidant capacity determination
	$O_2^{\cdot -}$	Superoxide anion radical
	O_3	Ozone
P	**PA**	Physical activity
R	**RNS**	Reactive nitrogen species
	ROS	Reactive oxygen species
	RONS	Reactive oxygen and nitrogen species
S	**SCGE**	Single-cell gel electrophoresis
	SOD	Superoxide dismutase
T	**TBARS**	Thiobarbituric acid reactive substances
V	**VO_2max**	Maximal oxygen consumption
8	**8-OHdG**	8-hydroxydeoxyguanosine

Chapter 1

Introduction



1.1. INTRODUCTION

The necessity of oxygen during the production of energy is clearly illustrated through mitochondrial oxidative phosphorylation (Vander *et al.*, 1998; Leeuwenburgh & Heinecke, 2001). In the mitochondrial electron transport chain, oxygen is reduced to water through various steps. During this process oxidants and reactive oxygen species (ROS) such as superoxides, hydrogen peroxide and hydroxy radicals are produced in the mitochondria (Jacob & Burri, 1996; Radák *et al.*, 1999; Leeuwenburgh & Heinecke, 2001). This occurs through enzymatic and non-enzymatic reactions (Radák *et al.*, 1999). These free radicals are responsible for the damage of lipids, proteins and nucleic acids (Radák *et al.*, 1999). The rate at which ROS are produced at certain physiological conditions is parallel to the amount of oxygen that is consumed during physical activity (Liu *et al.*, 2000).

During physical activity the demand for oxygen increases, resulting in a higher consumption of oxygen (Allesio, 1993). Jenkins (1988) states that the aerobic metabolism could rise as much as 10 times the value of the resting aerobic metabolism during physical activity. Sen (1995) found the same according to the rate of oxygen transport. The rate of oxygen transport could rise to 20 times the resting value. As mentioned, a higher oxygen intake results in a more frequent production of ROS and oxidants. Thus, the higher the intensity of the physical activity, the higher the production of ROS, and this results in more damage to lipids, proteins and nucleic acids. Studies conducted by Lovlin *et al.* (1997) and Møller *et al.* (1996) found that physical activity at higher intensities leads to the additional production of ROS.

High-intensity physical activity leads to an increase in ROS. Sometimes this amount exceeds the defensive capacity of the aerobic metabolism (Ji, 1995). This amount could increase to such an extent that it exceeds the amount of antioxidants available. Antioxidants defend the body against the effects of ROS. If the ROS concentration rises above that of the defensive antioxidants, lipid oxidation occurs in the muscles (Davies *et al.*, 1982; Salmine & Vihko, 1983). As a result the body experiences oxidative stress. Oxidative stress is defined as an imbalance between the oxidant and antioxidant systems. This imbalance could occur while a person is participating in a physical activity (Leeuwenburgh & Heinecke, 2001). Oxidative stress is measured by the amount of lipid and protein oxidation, DNA damage and the occurrence of endogenous antioxidants in the body (Liu *et al.*, 2000). In the study by Lovlin *et al.* (1997), a correlation between higher oxygen consumption and oxidative DNA damage was found.

It is currently accepted that regular physical activity leads to an increase in quality of life (Holloszy, 1993). Research demonstrated that active persons have a lower incidence of cardiovascular disease, as well as a reduced chance of developing certain types of cancers (Radák *et al.*, 1999). It has been shown that physical activity lowers the chance to develop osteoporosis and diabetes (Leeuwenburgh & Heinecke, 2001) although the underlying biomechanical mechanism is not yet fully understood (Radák *et al.* 1999). There is some indication that ageing, degenerating illnesses and some types of cancer could develop as a consequence of oxidative DNA damage and lipid and protein oxidation (Ames & Saul, 1986; Ames & Shigenaga, 1992; Ames *et al.*, 1993; Halliwell, 1994; Umegaki *et al.*, 2000). The human body can adapt to certain types of stress by changes in the physiology to reduce the production of ROS by increasing the amount of antioxidation defence systems (Radák *et al.*, 2001).

Many studies have indicated a correlation between physical activity and oxidative damage. Most of these studies were done either on rats (Radák *et al.*, 1999; Liu *et al.*, 2000; Radák *et al.*, 2000; Umegaki *et al.*, 2000) or young, trained male subjects (Duthie *et al.*, 1990; Niess *et al.*, 1998; Poulsen *et al.*, 1999; Møller *et al.*, 2001). In these studies the subjects were stressed at high-exercise intensities and/or for long periods of time. It is believed that oxidative DNA damage only occurs with these types of activities. A study by Duthie *et al.* (1990) showed that no modification in the antioxidant capacity occurs when subjects are subjected to low intensity and/or short-distance exercises. Thus, to produce additional ROS to cause damage to the lipid, protein or nucleic acids, a too short, sub-maximal exercise session would be of no significance.

As previously mentioned, physical activity is beneficial to a person's quality of life. It is believed that to experience optimal benefits from physical activity, a person must participate in organised physical activity three to five times a week for 30 to 60 minutes at each session. These activities must be performed at an intensity of 70% of the age-predicted maximal heart rate (ACSM, 2000). This intensity is calculated by Karvonen's formula (Karvonen *et al.*, 1957):

- (220 – Age – Resting heart rate) × 0,7 + Resting heart rate.

According to the ACSM (2000), optimal exercise intensity is measured by maximal oxygen uptake (VO_2-max). This VO_2-max is an accurate indication of a person's functional capacity.

Physiological adaptations that result in health benefits will only occur when physical activity is performed at least three times a week (ACSM, 2000). Exercise sessions must be altered with a

day's rest in between. Repair and adaptation occur during the rest days. It is, therefore, important to know when a person has fully recovered before subjecting the person to another exercise session. It was mentioned above that the biochemical mechanisms behind the benefits of physical activity are not yet fully understood. Thus, it is important to investigate the intensity at which DNA damage occurs as a biomarker of oxidative damage as well as the period it takes to recover from the damaging effects of exercise. Due to the occurrence of chronic disease in older persons, physical activity may be the most beneficial to the older population.

1.2. PROBLEM STATEMENT

The research questions that are posed in this study are to determine the specific exercise intensity that leads to oxidative DNA damage as well as the time required for DNA repair in males between the ages of 40 and 55 years. The antioxidant capacities and the rate of DNA repair of these subjects after exercise will also be investigated.

1.3. STUDY OBJECTIVES

This study has the following objectives:

- To determine the antioxidant and free radical capacity of conditioned and unconditioned males between the ages of 40 and 55 years.
- To determine the exercise intensity where DNA damage occurs in conditioned and unconditioned males between 40 and 55 years of age.
- To determine the rate of DNA repair after exercise in conditioned and unconditioned males between 40 and 55 years of age.

1.4. HYPOTHESIS

The following hypotheses are postulated:

- Conditioned males between the ages of 40 and 55 years will have a higher antioxidant capacity than unconditioned males of the same age group.
- DNA damage will occur at higher exercise intensities in conditioned males between the ages of 40 and 55 years than in unconditioned males between 40 and 55 years.
- DNA repair will be more extensive in conditioned males in the age group of 40 to 55 years than in the unconditioned counterparts.

1.5. PROPOSED CHAPTERS

Chapter 1: Introduction

Chapter 2: Literature review

Article 1: *Exercise and oxidative damage in healthy persons.*

Chapter 3: Research study

Article 2: *DNA damage and repair at different aerobic exercise intensities in middle aged men.*

Chapter 4: Summary, Conclusion and Recommendations

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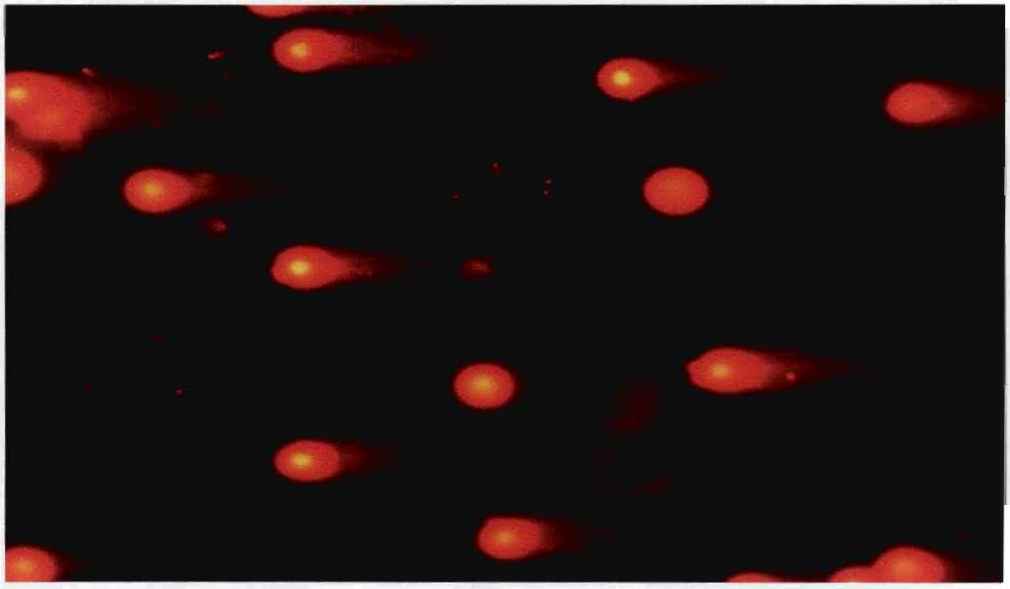
Chapter 2

Literature Review



Exercise and oxidative damage in healthy persons

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TITLE PAGE

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SHORT TITLE: Exercise and oxidative damage

ABSTRACT

Regular physical activity (PA) leads to an increase in the quality of life and reduces the incidence of chronic disease. The physiological adaptations and benefits of PA could be as a result of specific cellular alterations and adaptations, although the biochemical mechanism(s) through which adaptations occur are not well understood. The human body's antioxidant capacity could be compromised, resulting in the production of excess reactive oxygen species (ROS). These free radicals may react with cellular components to initiate damage to cellular macromolecules (nucleic acids, proteins and lipids). To understand the effect of PA on DNA integrity, a review of healthy human subjects was performed. The results indicated that young male subjects were the population mostly investigated. The studies focused on aerobic activity as an exercise intervention, with once-off exercise bouts more prevalent than training. The methods used to determine oxidative stress were as varied as the types of activities to which the individuals were subjected. The parameters measured included: 8-hydroxydeoxyguanosine (8-OHdG), plasma creatine kinase (CK), lipid peroxidation, protein carbonyls, and the single-cell electrophoresis assay (Comet assay). The main conclusion that can be drawn from this investigation is that exercise results in oxidative stress, however, the amount of stress is difficult to quantify due to the diversity in parameters measuring oxidative damage. This makes it difficult to identify definite patterns or to make firm conclusions. Agreement should be reached on the parameters that measure oxidative damage and its effects, to enable scientists to determine the true effect of various modes of exercises on human biochemistry.

Key words: Exercise, Free radicals, Oxidative stress, Oxidative damage, DNA-damage

INTRODUCTION

Regular physical activity (PA) leads to an increase in the quality of life by improving physiological, biochemical and psychological functioning of the human body (American College of Sports Medicine (ACSM), 2000). Certain benefits are associated with regular PA and exercise – such as improved cardiovascular and respiratory functioning and fitness, reduction of risk factors for coronary heart disease, decreased mortality and morbidity, an enhanced feeling of well-being, a decrease in anxiety and depression and enhanced performances at work and recreational or sport activities. The risks associated with the development of osteoporosis and diabetes (Blair, Kohl, Barlow, Paffenbarger, Gibbons & Macera, 1995; Leeuwenburg & Heinecke, 2001) is also lowered through regular PA. In addition decreased risks for the development of obesity, hypertension and certain infections were observed (Halliwell & Gutteridge, 1999). The immune function and mediators, as well as the oxidative capacity, were improved through the increased enzymatic antioxidant defence against oxygen free radicals with training together with the improvement of other oxidants (Jacob & Burri, 1996). Although the mechanisms involved in the advantages of regular PA are not clear, various physiological reactions to regular PA have been observed.

Physiological adaptations to regular PA

During regular PA an increased burden is placed on all physiological mechanisms due to the increased energy demand on the muscles. Aerobic metabolism, for example, could be raised during PA to as much as 10 times the value of the resting aerobic metabolism (Jenkins, 1998). Sen (1995) observed a marked increase in the rate of oxygen transport, as much as 20 times the resting value. Vigorous PA and other activities involving large muscle groups usually lead to a large energy expenditure compared to activities with a low or moderate intensity, as well as activities where smaller muscle groups are involved (ACSM, 2000). To accrue the benefits of PA, regular training three to five days a week should be performed for at least 30 minutes at an intensity of 70-80% of the age adapted maximum heart rate (Karvonen, Kentala & Mustala, 1957; ACSM, 2000).

A vast amount of literature is available on the adaptations that occur in the physiological system during acute and chronic exercise (Allesio, 1993; Jacob & Burri, 1996; Jenkins, 1998; Leeuwenberg & Heinecke, 2001; Heled, Shapiro & Shani, 2004; Hicks & Bennett, 2004). The most significant conclusion from the literature is that the body's response to exercise is similar to the fight and flight reactions and/or training as a result of continued stress on the human body. It

is evident from the literature that these responses culminate in the adaptation of virtually all the physiological systems and are collectively beneficial for the body. This is illustrated by the examples of regular training, which decreases major oxidation reactions, such as lower lipid and protein oxidation, resulting in a more effective metabolism (Jenkins, 1998; Liu, Yeo, Övervik-Douki, Hagen, Doniger, Chyu, Brooks & Ames, 2000). In addition, the efficiency of the immune system is enhanced and a favourable pro-oxidant-anti-oxidant balance is established (Jacob & Burri, 1996; Leeuwenberg & Heinecke, 2001). In trained persons, the response of the body is to adapt to stress (exercise) through physiological changes, with the purpose of reaching a steady state in a shorter period than in untrained persons. Regular PA has the benefit of increased muscle mass (Jacob & Burri, 1996) and muscle enzyme levels, while muscle tension is decreased, which result in improved muscle and physiological functioning (ACSM, 2000).

All the above-mentioned adjustments and benefits are the result of specific cellular and sub-cellular adaptations (Radák, Naito, Kaneko, Tahara, Nakamoto, Takahashi, Cardozo-Pelaez & Goto, 2002). Although regular exercise has a significant beneficial effect on the human body, much needs to be learnt about the biochemical mechanisms through which these adaptations occur. This compels an in-depth study of the molecular and cellular reactions induced by PA to help understand and explain the mechanism(s) behind the beneficial effects of PA. Although the majority of the experimental work on PA and its relationship to physiological processes in humans were performed with young healthy subjects (Hartmann, Pfuhler, Dennog, Germadnik, Pilger & Speit, 1998; Mars, Govender, Weston, Naicker & Chuturgoon, 1998; Allesio, Hagerman, Fulkerson, Ambrose, Rice & Wiley, 1999; Radák, Apor, Pucsok, Berkes, Ogonovszky, Pavlik, Nakamoto & Goto, 2003), only a few studies involved older subjects (Kim, Oberman, Fletcher & Lee, 2001; Radák *et al.*, 2002).

Aging as a consequence of the action of free radicals, a hypothesis that was formulated two decades ago, still attracts a lot of attention (Tortora & Anagnostakos, 1987). The basis of this theory is that the highly reactive oxygen species (ROS) and other free radical species are responsible for the eventual malfunctioning of the vitally important macromolecules, namely proteins, lipids and nucleic acids (Tortora & Anagnostakos, 1987). This impairment could lead to sub-optimal functional cellular mechanisms and to gradual deterioration of bodily functions (Bernadier & Everts, 2001; Bokov, Chaudhuri & Richardson, 2004). Concomitant to this is an increase in the incidence of diseases such as certain types of cancer, rheumatic inflammation, type II *Diabetes mellitus* and muscular dystrophy (Radák *et al.*, 2002).

Aging is a process of progressive failure of the body's homeostatic adaptive responses (Bokov *et al.*, 2004) and is associated with sarcopenia (loss of muscle mass) and dysfunction in motor coordination (Radák *et al.*, 2002). The ACSM (1998) divides men between 30 and 65 years in two groups, namely middle adulthood (30–44 years) and later adulthood (45–65 years). It may be assumed that individuals in these categories have specific characteristics, e. g. metabolic and immunologic features and functioning of muscles, and yet very data is available on these subjects since most of the reports in the literature on the effect of exercise and oxidative damage concern younger subjects (Møller, Loft, Lundby & Olsen, 2001; Sato, Nanri, Ohtam, Kasai & Ikeda, 2003; Radák *et al.*, 2003; Aoi, Naito, Takanami, Kawai, Sakuma, Ichikawa, Yoshida & Yoshikawa, 2004).

Since research is lacking regarding this group of men, it is important that more research should be done on the role of PA in oxidative damage and repair in adults (Cadenas & Davies, 2000). Therefore the purpose of this review is to determine the influence of various PA on the formation of DNA damage and repair.

Free radicals: Friend or Foe?

Halliwell and Gutteridge (1999) define free radicals as any chemical species capable of independent existence that contains one or more unpaired electrons. In the human body free radicals, oxidants and ROS are mainly produced in the mitochondria by the electron transport chain (Jacob & Burri, 1996) and other pathways and events that could produce ROS include peroxisomal metabolism, enzymatic synthesis of nitric oxide (NO), phagocytic leukocytes, heat, exhaustive exercise or pathologic conditions such as activated neutrophils (Halliwell & Gutteridge, 1999).

The damage to cellular components as a result of free radicals and ROS are called oxidative stress and is defined as a disturbance in the pro-oxidant-antioxidant balance in favour of the former (Joulia, Steinberg, Faucher, Thibault, Christophe, Nathalie & Yves, 2003). According to Halliwell and Gutteridge (1999) the two main reasons for the development of oxidative stress are, firstly, diminished antioxidants and, secondly, an increased production of ROS and reactive nitrogen species (RNS).

Oxidative stress primarily targets cellular content depending on the cell type, the type and severity of the imposed stress and the eventual effect of free radicals on the lipid, protein and nucleic acid macromolecules. Damage to lipids occurs via peroxidation which is usually

measured by the quantification of MDA, a relatively stable intermediate of lipid peroxidation (Lamprecht, Greilberger & Oettl, 2004). The peroxidation of lipids is initiated by a free radical through abstracting a hydrogen atom from unsaturated fatty acids, leading to the formation of lipid radicals. These radicals combine with molecular oxygen to propagate the chain of events in lipid peroxidation (Kowaltski & Vercesi, 1999).

Oxidative damage to proteins is accompanied by an increase in the number of carbonyl residues (Lui *et al.*, 2000). These protein carbonyls are quantified chemically or with an immunoassay (Lambrecht *et al.*, 2004). According to Meccoci, Fanó, Fulle, MacGarvey, Shinobu, Polidori, Cherubini, Vecchiet, Senin & Beal, (1999) mitochondrial proteins in human skeletal muscle are particularly susceptible to free radical oxidative damage and it is through the peroxidation of proteins that oxidants could cause damage to mitochondrial membranes and the cytoplasmic structures (Lui *et al.*, 2000).

ROS can attack DNA bases or deoxyribose residues to produce damaged bases and/or single and double strand breaks (Marnett, 2000). Studies aiming at determining this kind of damage mostly make use of the marker 8-OHdG (Lambrecht *et al.*, 2004). In addition, oxygen radicals oxidise lipids or proteins to generate intermediates that can react with DNA to form adducts (Marnett, 2000). If this damage is replicated, it could cause mutations or apoptosis and eventually pathological conditions. Bohr (2002) showed that persistent DNA damage may cause an arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability.

Therefore, oxidative stress which causes macromolecular damage could result in irreversible damage to cells through the loss of homeostatic function and may lead to cell injury and even transformation or cell death through apoptotic or necrotic mechanisms (Halliwell & Gutteridge, 1999). Two kinds of tissues which are particularly prone to oxidative damage are muscle and central nervous system tissue because both these tissues contain post-mitotic cells and are therefore prone to accumulate oxidative damage over time (Meccoci, 1999).

Protection against oxidative stress

Under mild oxidative stress, cells may adapt primarily as a result of the up-regulation of the synthesis of the antioxidant defence system in an attempt to restore the oxidant-antioxidant balance to protect the body against increasing oxidative stress (Halliwell & Gutteridge, 1999). It is necessary for the body to counteract the effects of oxidative stress and damage. To minimise these effects, the rate of ROS formation is slowed by the activity of repair systems or by the

removal of ROS by the degradation of the whole molecule, or a combination of these processes (Radák, Kaneko, Tahara, Nakamoto, Ohno, Sasvari, Nyakas & Goto, 1999b). Human cells possess a complex network of mechanisms to withstand the generation of ROS and to protect the cells against oxidation of macromolecules by scavenging ROS. This is done by antioxidants, for example superoxide dismutase (SOD), glutathione peroxidase (GSH), ascorbic acid (AA) and vitamin E. The dietary intake of antioxidants is thought to play a major role in this (Møller & Loft, 2002). Şandraş, Yilmaz, Öztok, Cakir and Karakaya (2001) found that patients receiving vitamin E supplementation tend to develop less oxidative damage than their placebo counterparts. It was found that long-term endurance PA training enhanced the production of antioxidant enzymes and the reduction of oxidant production (Leeuwenburgh & Hollander, 1997).

THE RELATIONSHIP BETWEEN PHYSICAL ACTIVITY AND OXIDATIVE STRESS

Exercise and PA are characterised by a high rate of adenosine diphosphate (ADP) formation due to the increased adenosine triphosphate (ATP) breakdown and higher levels of ADP are associated with the activation of the oxidative phosphorylation energy system (Leeuwenburgh & Heinecke, 2001). PA could also elevate the oxygen transport to up to 20 times the resting value (Sen, 1995). These factors result in an increased energy metabolism during activity relative to a sedentary state.

A higher metabolic rate leads to increased haemoglobin turnover and haemoglobin autoxidation, resulting in increased carbonyl content of the globin moiety (Cazzola, Russo-Volpe, Cervato & Cestaro, 2003). During extremely high intensity, long-term aerobic exercise lipid peroxidation occurs, and could be detected in serum by measuring the formation of malondialdehyde (MDA) (Esterbauer, Gebicki, Puhl & Jurgens, 1992). Because of the increased breathing rate, air-borne pollutants, such as nitric dioxide (NO_2) and ozone (O_3), are inspired more readily. These free radicals could lead to an increased rate of oxidative damage through the respiratory tract (Lambrecht *et al.*, 2004). Research found that after a long-duration aerobic exercise, alterations in proteins could be observed with urine analysis (Mastaloudis, Leonard & Traber, 2001). Exercise results in proteinuria, as a result of increased levels of ROS and RNS (Lambrecht *et al.*, 2004). Since analytical methods also substantially differ among reported studies comparing results across studies are very problematic.

The flip side of the ROS is the antioxidant capacity that scavenge the free radicals to maintain a steady state in the body and in doing so antioxidants play an important role in the protection of cells against oxidative damage (Sato *et al.*, 2003). Oxidative damage is induced when the natural antioxidant capacity (antioxidants and antioxidant enzymes) of the body is overwhelmed (Umegaki, K., Daohua, P., Sugisawa, A., Kimura, M., Higuchi, M., 2000). PA increases the risk of an imbalance between the rate of oxidant formation and the functioning rate of the antioxidants (Radák *et al.*, 1999b). Excess ROS generated may overwhelm natural cellular antioxidant defences (Sacheck, Milbury, Cannon, Roubenoff & Blumberg, 2003) altering the body's ability to repair and protect damaged tissue (Radák *et al.*, 2003). The magnitude of DNA damage associated with PA depends on the rate of oxygen consumption, production of superoxide radicals and the balance of the antioxidant and pro-oxidant cellular mechanisms (Allesio *et al.*, 1999). Other factors that could influence the induction of oxidative stress include the initial level of fitness, the type and intensity of the exercise and any additional dietary antioxidant supplements (Allesio *et al.*, 1999; Tsai, Hsu, Hsu, Cheng, Lui, Hsu & Kong, 2001). Therefore, free radicals induced through PA react with various cellular components to initiate cellular damage if the antioxidant defences are inadequate (Hartmann & Niess, 1998). This review summarises different studies that investigated the effect of exercise and training on oxidative stress in order to shed light on the relationship between the dose-response of PA and DNA damage.

Exercise and oxidative damage in human subjects: a compilation of the literature

In order to understand the magnitude and nature of the effect of PA on cellular and subcellular integrity, with the focus on the genetic material, due to variation in type, intensity, duration and frequency of PA, a compilation of the available literature was performed (Table I). Publications available to us that investigated the influence of PA on DNA damage and repair from June 1998 until December 2006 were included in this survey.

The summary of 17 studies (Table I) gives an overview of the research that has been performed. In these studies the effect of oxidative stress and/or DNA damage as a result of different exercise or training modalities were investigated. The summarised literature indicates that studies are generally performed on very small sample sizes. An average of 8 subjects were investigated per study, with most of the studies researching changes in DNA damage of young, healthy men with a mean age of about 24 years.

Table I: Exercise and Oxidative Damage in Human subjects – A compilation of the literature

AUTHORS	SUBJECTS	AGE	EXERCISE MODE	EXERCISE PRESCRIPTION	MARKERS	EFFECT	OUTCOME/ CONCLUSION
Vollaard *et al.*, 2006	8 males	30±6	Two 4 week training periods. Each period 2 week build-up, followed 2 weeks either ↑ or same intensity training, preceding with a	15 minute cycle time trail	- Oxidatively modified heme - Methemoglobin - Glutathione redox status	- ↑ Oxidatively modified heme = after time trail - ↑ $GSSG^{12}$ = after time trail - ↓ Methemoglobin = after time trail - ↓ Glutathione = after time trail - ↓ GSH/GSSG = after time trail - ↑ performance = after taper	Short taper period improve performance without significant changes in exercise – induced oxidative stress levels
Pittaluga *et al.*, 2006	6 professional athletes and 12 non-agonists	23.5±2.1	VO_{2max}		- Total anti-oxidant capacity - Vitamin C - GSH - GSSG	- ↑ GSSG, micronuclei hemolysis in training group	The trained group indicated a more chronic oxidative insult, with the non-agonist group showing a balanced oxidative profile. This balance is more susceptible to exercise-induced variations
Watson, MacDonald-Wicks, Garg, 2005	20 exercised trained and 20 sex and age matched sedentary subjects				- Dietary antioxidants - Physical activity - Supplement antioxidants - Oxidative stress	- Athletes & controls = similar Plasma F2-isoprostanes antioxidant enzyme activities and uric acid levels - Athletes = ↓ Total antioxidant capacity	Findings suggest that athletes who consume diet rich in antioxidants have elevated plasma alpha-tocopherol and beta-carotene
Mastaloudis *et al.*, 2004	11 males and 11 females		Long, slow aerobic exercise	McDonald Forest Ultra marathon Race (50km)	DNA damage with comet assay method	- DNA damage = ↑ in mid race	Running induce DNA damage, although

						- Normal 2 hours post race	different effects observed in male and female runners
Joulia *et al.*, 2003	8 triathlon athletes		Breath holding exercises	During 3 months, for 3times a week. 1 hour of 20 seconds breath holding, with 40 seconds normal breathing (60 repetitions)	- TBARS - GSH	- ↓ resting TBARS - GSH ↔	Succession of apnoea and recovery on reoxygenation induced oxidative stress with sustained apnoea. After exercise training decreased effect of oxidative stress.
Radák *et al.*, 2003	6 male students	22 – 24 years	Long, slow aerobic exercise	Completion of Budapest marathon	-DNA glycolcylases (hOGG1 and Endo-III)	- ↑ hOGG1 after race - ↑ Endo-III after race	Severe aerobic exercise alters the activity of DNA repair enzymes.
Sato *et al.*, 2003	15 males (17 active and 9 sedentary)	19 – 29 years	VO_{2max} Session at 50% of VO_{2max}		- 8-OHdG - TBARS	- ↓ 8-OHdG in active subjects - TBARS ↔	Exercise (chronic/ acute) elevated the DNA repair system, preventing oxidative DNA damage.
Mastaloudis *et al.*, 2001	8 males and 3 females	45 ± 3 years	Long, slow aerobic exercise	McDonald Forest Ultra marathon Race (50km)	- AA -Deuteratedtocopherols - Plasma prostanes	- During race = ↑ AA; ↑ Plasmaprostane - 1 hour after race = ↑ AA - 24 hours after race =↓ AA ↓ Deuterated tocopherols ↔Plasma prostanes Sedentary: - ↔ deuterated tocopherols - ↔ Plasma prostanes	Endurance exercise increase vitamin E turnover rate, indicating exercise leads to oxidative stress. Increased AA, α-tocopherol and uric acid may reflect enhanced anti-oxidant defence
Møller *et al.*, 2001	7 males and 5 females	26.1± 4.9 years	Maximal exhaustive bicycle exercise test	Test were repeated 2 times once at sea level and one at altitude	- 8-OHdG - Comet assay	- ↑ 8-OHdG one day after altitude test - ↑ in comet assay during	Acute hypoxia increases DNA damage after exercise, it seems if

						altitude test	antioxidant defences are insufficient to avoid DNA damage.
Tsai *et al.*, 2001	14 male runners	20 – 24 years (mean age 21 years)	Long, slow aerobic exercise	Completion of the 2000 Taiwan 42 km marathon race	- 8-OHdG excretion - Plasma CK[13] - DNA base oxidative damage (FPG sites)	Before race = ↓ FPG Post race = ↑ FPG ↑ 8-OHdG - 24h post race = ↑ FPG - 1 week post race = ↑ 8-OHdG - 24h - 2 weeks post race = ↑Plasma CK	Long duration massive exercise leads to oxidize DNA damage that last long after exercise.
Radák *et al.*, 2000	5 male ultra marathon runners	26 – 45 years	Long duration, massive aerobic exercise	Participate in the 7th Vienna-Budapest marathon over 4 days	- 8-OHdG content - Serum CK	- 8-OHdG = ↑ after day 1 ↓ following 3 days - Serum CK = ↑ day 1 – 3 ↓ day 4	Post 4-day competition oxidative DNA damage ↑. Possible adaptation through regulation of antioxidant system and ↑ in oxidative stress resistance.
Allesio *et al.*, 1999	9 males and 3 females	Mean age 25.2 ± 3.2 years	A maximal exhaustive bicycle exercise test, followed by a maximal isometric exercise	Complete a VO_{2max} and 1 week later perform a maximal isometric grip exercise	- MDA - LH[14] - Protein oxidation - Antioxidant activity - Lactate levels	Aerobic = ↔ MDA ↔ LH ↑ Protein oxidation (67%) ↑ Antioxidant (25%) ↑ Lactate levels (479%) Isometric = ↔ MDA ↑ LH ↑ Protein oxidation (12%) ↑ Antioxidant activity (9%) ↑ Lactate levels (221%)	Although aerobic and isometric exercise types of stress differ, it still leads to oxidative damage.
Radák *et al.*, 1999a	12 females (6 active and 6 control)	20 – 23 years	Eccentric muscle contraction exercise	Maximal isometric contraction test. Active group = 200 eccentric	- NO[15] content - 8-OHdG	- ↑ NO content - ↑ 8-OHdG	Muscle soreness due to eccentric contractions ↓ max. force generation.

				muscle contractions. 24 hours later, retest of maximal isometric contraction			Could be associated with ↑ NO and 8-OHdG content = ↑ oxidative damage to DNA.
Hartmann *et al.*, 1998	5 male and 3 female triathlon athletes	27 – 33 years	Complete a short distance triathlon	Swim = 1,5 km Cycling = 40 km Run = 10 km	- Oxidative DNA base damage - 8-OHdG - Leukocyte migration	DNA migration = 20 min. – 120h post triathlon - ↑biphasic - ↔8-OHdG	DNA effects after exercise are secondary effects that do not originate from oxidized DNA bases
Mars *et al.*, 1998	11 healthy males	29.6 years	One bout of exhaustive treadmill running		Single cell gel electrophoresis Lymphocyte DNA change	-Pre-exercise = ? DNA damage -After exercise = DNA damage -24 – 48h post = DNA damage, no single strand DNA breaks	After intensive exercise, lymophocyte apoptosis occurred = ↓ immunity
Niess et al., 1998	12 males	27.3 ± 4.1 years	Aerobic exercise	Complete a 21.1 km race	- Total leukocyte content	- ↑ 1 h post race - normal 24 h post race	Heavy endurance exercise induced DNA damage in leukocytes by ROS, one day after race.
Umegaki *et al.*, 1998	16 males (8 trained and 8 untrained)	20.1 ± 0.6 years (Train) 20.8 ± 0.5 years (Untrain)	Aerobic exercise	Complete a VO_{2max} and 1 week later exercise for 30 minutes, against 85% of VO_{2max}, on a treadmill	Chromosomal damage in lymphocytes	No spontaneous damage to lymphocytes directly post or 30 min. post exercise	Different changes in chromosomal damage between trained or untrained groups due to enhanced DNA repair system and/or antioxidant capacity.

1. ORAC = Oxygen radical absorbance capacity; 2. TBARS = Thiobarbituric acid-reactive substances; 3. MPO = Myeloperoxide; 4. ROS = Reactive oxygen species; 5. VO_{2max} = Test for Maximal oxygen consumption; 6. 8-OHdG = 8-hydroxydeoxyguanosine; 7. RONS = Reactive oxygen and nitrogen species; 8. MDA = Malondialdehyde; 9. GSH = Redused glutathione; 10. AA = Ascorbic acid; 11. CPK = Creatine phosphokinase; 12. GSSG = Oxidized Glutathione 13. CK = Creatine kinase; 14. LH = Lipid hydroperoxides; 15. NO = Nitric oxide; ↑ = Increase; ↓ = Decrease; ↔ = Unchanged and *m*/min = meter per minute; h = hour; min. = minutes.

The main focus in most of the studies was a single exercise bout with running the preferred modality of intervention (Mars *et al.*, 1998; Niess, Baumann, Roecker, Horstmann, Mayer & Dickhuth, 1998; Pittaluga, Parisi, Sabatini, Ceci, Caporossi, Catani, Savini & Avigliano, 2006). These exercises varied from a single run at 50% of the VO_2max on a treadmill (Sato *et al.*, 2003) to the completion of a four-day supra-marathon race (Radák, Pucsuk, Boros, Josfai & Taylor, 2000). In only a third of the studies swimming, cycling or a triathlon was used as exercise modality. The effect of anaerobic exercise and the resulting oxidative stress was investigated to a much lesser extent. Only one study reported the effect of a resistance program on oxidative DNA damage (Radák *et al.*, 1999b). The only conclusion that can be made from the studies in which long-duration, massive aerobic exercises were investigated is that these activities lead to oxidative DNA damage. All these studies indicate that the repair time of the damaged DNA varies considerably (Radák *et al.*, 2000; Tsai *et al.*, 2001; Mastaloudis, Yu, O'Donnel, Frei, Dashwood & Traber, 2004). Similar inconclusive results were found after one bout of exhaustive exercise (Mars *et al.*, 1998; Møller *et al.*, 2001). This may be the cause of the observed diversity in the results obtained by the various studies, because the weight-bearing activities (like running) may lead to additional cellular damage and inflammation (Radák *et al.*, 1999b). This kind of damage is minimised during non-weight bearing activities (like cycling or swimming), where there is hardly any impact on the joints and the effect of carrying body weight is reduced.

The parameters measured to determine whether exercise leads to oxidative stress were as varied as the activities used to inflict cellular damage. The most common marker used to indicate oxidative stress, was 8-OHdG (Poulsen, Weimann & Loft, 1999). Other parameters that were applied to a lesser extent to measured oxidative stress were plasma creatine kinase (CK) (Tsai *et al.*, 2001), lipid peroxidation (Vollaard, Cooper & Shearman, 2006) and protein carbonyls. The single-cell gel electrophoresis or comet assay, which is widely used in studies with human subjects, was applied in several of the studies and demonstrated that various types of exercise lead to some form of oxidative DNA damage (Hartmann *et al.*, 1998; Mars *et al.*, 1998; Møller *et al.*, 2001; Mastaloudis *et al.*, 2004).

The variation in 8-OHdG levels taken from the literature indicates that much more research is needed before clarity about exercise and oxidative stress can be reached. There seems to be a tendency for 8-OHdG content to increase after an exercise bout and stay elevated for as long as one week (Møller *et al.*, 2001; Tsai *et al.*, 2001), although the study of Radák *et al.* (2000). No change was observed in the 8-OHdG levels in a study that compared chronic trained subjects with sedentary subjects after a maximal exercise bout (Lui *et al.*, 2000). Although used less

frequently than 8-OHdG, other biomarkers such CK, lipid peroxidation and protein carbonyls showed less variation as indicators of oxidative stress. All the studies that used CK as an indicator showed an increase after an exercise session, with a maximum concentration reached 24–48 hours after exercise (Niess *et al.*, 1998; Radák *et al.*, 2000). No change in lipid peroxidation was found after aerobic exercise, but an increase was observed after the completion of an anaerobic exercise bout (Allesio *et al.*, 1999). This may be explained by the plasma volume shift that have a diluting effect on metabolites when participating in PA. The carbonylation of proteins seemed to be an effective parameter to determine whether oxidative stress did occur (Lui *et al.*, 2000; Radák *et al.*, 2002) and it also appears that proteins are more readily carbonylated during aerobic exercise than during anaerobic exercise (Allesio *et al.*, 1999). Consensus also still has to be reached on which proteins are effected, because different proteins apparently carbonylate at different rates (Radák *et al.*, 2002).

The only common feature observed in the literature is that various types of exercise resulted in different levels of oxidative DNA damage and that different studies have observed different DNA repair times. However, available research indicate that the major benefit of exercise to the human body is an adaptive response to minimise oxidative stress and damage (Hartmann & Niess, 1998; Joulia *et al.*, 2003; Sacheck *et al.*, 2003; Aoi *et al.*, 2004). The antioxidant capacity, which decreases the effect of free radicals on DNA, increases as a result of exercise or training (Umegaki *et al.*, 1998). Literature confirms that PA induces oxidative damage to DNA, lipids and proteins, but more research is needed to reach absolute clarity about this and to determine the underlying mechanisms involved.

CONCLUSION

Published studies on the effect of PA on oxidative damage with the focus on DNA damage and repair, indicate a diversity of determinants that are currently being studied, which makes it difficult to identify definite patterns to make firm conclusions (Lamprecht *et al.*, 2004). Agreement must be reached on a set of parameters to measure oxidative stress and its effects, so as to enable scientists to determine the effect of various modes of exercise on the human body. This will enable scientists to compare different studies, so that advances in the explanation of these effects could be guaranteed. A significant shortcoming in the existing studies is that mainly young, active and healthy subjects were involved. Since middle aged people are specifically prone to chronic diseases, like coronary vascular disease (CVD), diabetes mellitus and hypertension and because PA decreases the chances for developing chronic diseases, it is safe to recommend that there is a pressing need to better understand the effect of exercise training in

older subjects (Fatouros, Jamurtas, Villiotou, Pouliopoulou, Fotinakis, Taxildaris & Deliconstantinos, 2004). Currently a shift in research focus from healthy subjects to patients with chronic disease can be observed in publications, especially in research focussing on Type II *Diabetes Mellitus*, which is a global concern. Although exercise does result in an increase in the production of additional free radicals, subjects that are regularly physically active, present with biochemical profiles that are adapted to neutralise the additional free radicals compared to sedentary subjects (Dandona, Thusu, Cook, Snyder, Makowski, Armstrong & Nicotera, 1996). Therefore, the importance to ensure that exercise prescription does not result in damage of, for example, the DNA, thereby doing more harm than good.

One conclusion that can be drawn from the available literature is that excessive oxidative stress may develop if the body is stressed through exhaustive aerobic or anaerobic exercise. The large variety of exercise protocols applied in the studies makes it difficult to construct specific patterns regarding the amount and type of exercise that will cause excessive oxidative stress. A possible explanation for this is that different goals were pursued by these various studies. This review emphasizes the importance of standardised protocols for measuring the effects of exercise and the resulting oxidative stress so that meaningful conclusions can be made. The recommendation made by Lamprecht *et al.,* (2004) that consensus on the markers for determining DNA damage and repair should be reached, is certainly a step in the right direction. Although exercise training seems to decrease the effect of oxidative damage, it is still unclear which PA and physiological adaptations in the body are responsible for this effect.

There is therefore a need for more in-depth studies which investigate the effect of exercise on the formation and repair of DNA damage, especially in older subjects, to clarify the optimal exercise intensity for health and for the prevention of chronic disease.

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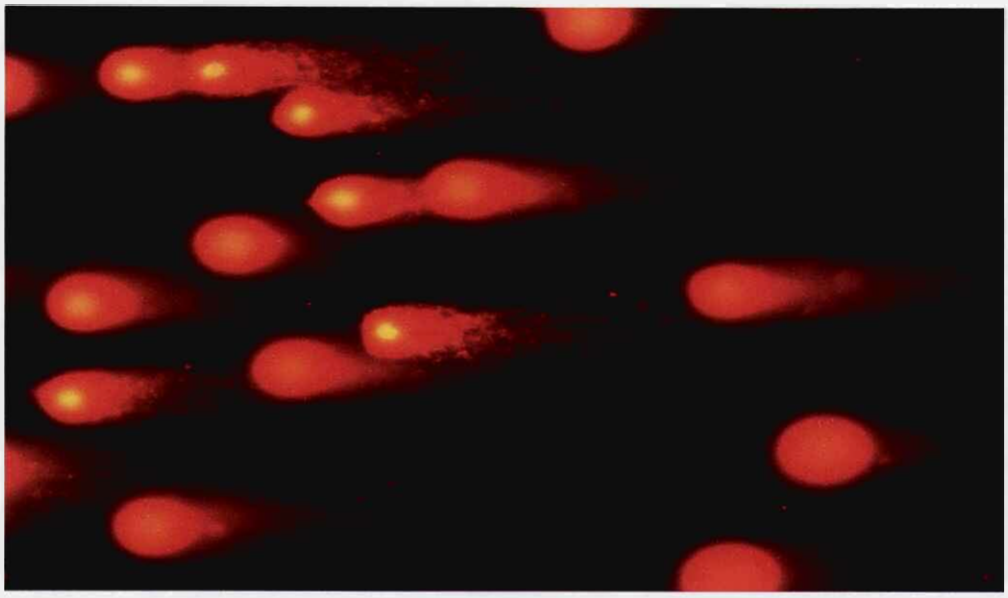
Chapter 3

Research study



DNA damage and repair at different aerobic exercise intensities in middle aged men

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TITLE PAGE

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DNA damage and repair at different aerobic exercise intensities in middle aged men

ABSTRACT

Physical activity (PA) increases the risk of producing an imbalance between oxidant and antioxidant rate of formation and functioning. The purpose of this study was to investigate the influence of different exercise intensities on oxidative DNA damage and repair in middle-aged men by means of Comet assay. Physically inactive and active males were assigned to an experimental and control group, respectively. Baseline VO_2max of each subject was determined, after which they were assigned to different exercise intensities on a bicycle ergometer, in a random order, according to baseline VO_2max. Baseline, post-exercise and 24 hours post-exercise measurements for oxygen radical absorbance capacity (ORAC) and total oxidant status analysis (dROM) were performed as well as DNA damage assessment by means of Comet analysis. ORAC levels increased after acute exercise and decreased to baseline 24 hours post-exercise. A biphasic DNA damage-repair cycle was observed with the Comet analysis. The highest amount of damaged cells was measured directly after acute exercise and the highest incidence of DNA damage over a 72 hour period was observed following exercise at 70% VO_2max, with the least amount of damage following exercise at 90% VO_2max. It was concluded that less DNA damage occurred with exercise at 90% VO_2max. than with exercise at 70% VO_2max. in middle-aged men.

Keywords: Exercise, Oxidative damage, Comet assay, ORAC, dROM

Word count: 209

INTRODUCTION

Lifelong regular physical activity (PA) reduces the incidence of cardiovascular disease and certain types of cancer, thereby prolonging the life span of a human being and slowing down the aging process [4, 15, 34, 36]. Immune function and mediators as well as the oxidative capacity improve through PA [17]. Jacob and Burri [17] have found that PA increases the enzymatic antioxidant defence against oxygen free radicals and other oxidants. All of this could be the result of specific cellular alterations and adaptations [32]. In order to achieve these benefits and adaptations, the ACSM [1] recommends that a person should accumulate 30 minutes or more of regular exercise at an intensity of 70-85% of a person's age predicted maximum heart rate on most days of the week. The biochemical mechanisms of these adaptations are currently not well understood or defined, but there are indications that events such as changes in nitric oxide (NO) release in the circulatory system may be a mediator [6].

Reactive oxygen species (ROS) are produced mainly in the mitochondria by the electron transport chain. Macromolecular damage may occur as a result of the increased levels of these radicals and is generally referred to as oxidative stress [40]. Two main reasons for the development of oxidative stress are, firstly, diminished levels of antioxidants and, secondly, an increased production of ROS and reactive nitrogen species (RNS). Oxidative stress primarily targets cellular content depending on the cell type and the nature and the severity of the stress imposed and could result in irreversible damage to cells [20]. Severe oxidative stress to lipids, proteins and nucleic acids can lead to adaptation, cell transformation, cell injury or cell death through apoptotic or necrotic mechanisms [12, 17].

PA increases the risk of producing an imbalance between the rate of oxidant forming and their removal by antioxidants [34]. Therefore, excess generated ROS may overwhelm the body's natural cellular antioxidant defence, thereby diminishing the ability of the body to protect itself

[30, 35, 36]. During PA, the demand for oxygen increases, resulting in a higher consumption of oxygen and consequently a higher rate of ROS production, resulting in oxidative damage to lipids, proteins and nucleic acids [2, 21]. This oxidative damage as a result of excess ROS may lead to chronic diseases and cancer.

Several studies have confirmed the correlation between PA and oxidative DNA damage. Most of these studies were performed on either rats [20, 32, 34, 40] or young, trained human male subjects [8, 24, 26, 28]. In some of these studies, the subjects involved were stressed at high-exercise intensities and/or for long periods of time, but in the majority of these studies the effect of a single exercise session on the amount and type of oxidative stress was determined [3, 22, 23, 30]. Although oxidative damage was determined in these studies, no consensus could be found on the intensity or duration of exercise which develops oxidative stress. For physiological adaptation, it is important to know whether a person has recovered fully from a previous exercise session. Studies widely used the 8-hydroxydeoxyguanosine (8-OHdG), plasma creatine kinase (CK), lipid peroxidation as well as protein carbonyls parameters as an indicator of oxidative stress and damage [32, 33, 36, 39]. In these studies baseline measurements of 8-OHdG tended to be increased in exercised subjects. Serum CK also increased one to three days after acute aerobic exercise.

Limited research has focused on the effect of exercise training on oxidative stress on DNA damage and repair. Only a few studies were conducted to determine these effects of exercise on older persons. Therefore, the purpose of this study was to investigate the effect of different aerobic exercise intensities on oxidative DNA damage and repair in middle aged men in order to determine the optimal intensity at which physical training should be performed to result in adequate physiological adaptation and with minimal DNA damage.

MATERIALS AND METHODS

Study design

A cross-sectional design between an experimental and control group was used to determine the influence of an acute physical exercise intervention of randomly-assigned aerobic intensities, over a 72 hour period, on oxidative DNA damage and repair in middle aged men.

Subjects

The study consisted of an experimental and control group of men between 40 and 55 years of age with no history or present symptoms of coronary heart disease or pulmonary disease. The subjects were free from hypertension, diabetes mellitus and orthopaedic disabilities. All the subjects were non-smokers, taking no medication and with a body mass index (BMI) of 24 – 27 kg/m^2. The control group consisted of three males who had been physically inactive for the previous 12 months, while the experimental group consisted of five physically active males participating in organised activities three or more times per week during the previous 12 months. All subjects were requested to refrain from alcohol consumption and terminate any consumption of supplements 10 days prior to each exercise intervention session. PA was not permitted 72 hours before onset of the exercise intervention session. Ethical approval to perform this study was obtained from the Ethical Committee of the North-West University (Potchefstroom Campus).

Physical activity intervention

Before the onset of the acute intervention, the maximal aerobic capacity (VO_2max) of each subject was determined by means of a direct VO_2max protocol using a gas analysis system (MetaMax I Air Analyzer) and a bicycle ergometer (Monark 824E, Sweden). Based on these VO_2max results, target heart rates were determined for each individual at the different intensities at which the acute exercise intervention was to be administered. Each subject of the experimental

and control group was randomly assigned to intensities of 60, 70, 80 and 90% of their individual baseline VO_2max heart rate for an acute exercise intervention of 30 minutes on a bicycle ergometer (Monark 824E, Sweden). The initial and final three minutes of each session were used as warm-up and cool down periods. The subjects were blinded to the intensities. Subjects used a Polar heart rate monitor (Polar S210, Finland) to monitor the predetermined heart rates at the various intensities with the resistance of the bicycle ergometer manipulated to keep the subject's heart rate at the required beats per minute.

Blood sampling

Blood was drawn from the *vena cephelia* by means of a butterfly infusion at baseline, immediately post exercise and 24 hours post exercise. Each subject acted as his own control. Two Vaccutainer® tubes were filled for plasma and serum preparation, which were used for the total oxidant status analysis with the dROM test kit and oxygen radical absorbance capacity (ORAC) analysis. Blood was also obtained by a finger prick for the Comet assay (see below). Samples were taken immediately before the start, at the end of exercise, and 5, 15 and 30 minutes post exercise, with follow-up samplings at 6, 12, 24, 48 and 72 hours. These samples were collected in heparin capillary tubes and were stored on ice until processing within two hours post collection.

ORAC and total oxidant status analyses

The antioxidant capacity of the plasma was determined using the ORAC method. Deproteinated (perchloric acid treated) samples were immediately prepared and frozen at -80 °C until use. AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) was used as a peroxyl radical generator and Trolox as standard. Anti-oxidant-induced protection of peroxyl radical-induced decay of fluorescein was measured fluorimetrically (excitation 485 nm, emission 530 nm) over 2 hours and expressed as μM trolox equivalents [7].

Assessment of reactive oxygen species was determined with the measurement of total oxidant status in serum using the dROM test kit as instructed by the suppliers (DIACRON International s. a. s., Grosseto, Italy) [7]. The assay measured hydroperoxyl and alkoxyl radicals in serum kinetically at 546 nm for 3 minutes at 25°C in a BioTek (FL600) plate reader. The linear slopes obtained were compared to a serum standard and was expressed as Carratelli units (CARR U) [7].

Comet assay/Single-cell gel electrophoresis (SCGE)

The amount of DNA damage and repair was determined by means of SCGE, (as described by Singh *et al.* [38]), and adjusted for local laboratory conditions [41]. The images were captured with the computerized image analyses systems programme (CASP) imaging software (http://www.casp.sourceforge.net) and the percentage tail DNA of at least fifty comets was measured per slide. These comets were classified according to the relative amount of DNA in the tail [18].

SPSS 14.0 (Chicago) software was used for the statistical analyses. Baseline characteristics of the variables were determined with descriptive statistics. Statistical significant differences between the changes in dROM and ORAC for the different intensities and post intervention times, a one-way analysis of variance (ANOVA) was performed. The percentage change from baseline to end was corrected for baseline values. For the DNA damage at the various intensities, a one-way ANOVA was performed. Significance was set at $p< 0.05$.

RESULTS

The purpose of this study was to determine the effect of different aerobic exercise intensities on oxidative DNA damage and repair in middle aged men by means of comet analysis. The baseline characteristics of the participants (Table 1) indicated no significant differences between the

experimental and control groups except that the experimental group was, on average, about five years younger than the control group. An unexpected observation was the slightly higher aerobic capacity of the control group relative to the trained group.

Table 1: Characteristics of the subjects at baseline

CHARACTERISTIC	CONTROL GROUP (N=3)	EXPERIMENTAL GROUP (N=5)
Age	50.0 ± 3.5	44.8 ± 4.4
BMI (kg/m^2)	27.1 ± 1.7	27.0 ± 3.3
VO_2max (ml O_2/kg/min)	32.4 ± 10.9	30.6 ± 2.0

ORAC and total oxidant status

From the pre- and post-exercise ORAC values for the control and experimental groups given in Table 2, a relatively large degree of inter-individual variation was evident and consequently no

Table 2: Effect of exercise intensity on the anti-oxidant capacity (ORAC)

Intensity	Groups	Pre-exercise (μM)	Post-exercise (μM)	24-hour post-exercise (μM)
60%	Control	807 ± 129	1054 ± 81	1100 ± 88
	Experimental	1059 ± 154	1397 ± 767	1108 ± 272
70%	Control	1328 ± 109	1231 ± 136	1104 ± 229
	Experimental	1697 ± 1052	1702 ± 1243	1559 ± 1073
80%	Control	1074 ± 407	1243 ± 457	1022 ± 235
	Experimental	1560 ± 1033	1503 ± 764	1458 ± 724
90%	Control	1179 ± 263	1253 ± 117	1256 ± 118
	Experimental	946 ± 574	1197 ± 671	1362 ± 777

significant changes in the anti-oxidant capacity were observed at any exercise intensity. The ORAC values of the experimental group were slightly higher than that of the control group and slightly increased directly after the exercise at all the exercise intensities.

The dROM values obtained pre- and post-exercise at different intensities (Table 3) revealed lower levels of oxidative stress in the control group than in the experimental group at baseline. The oxidative stress levels decreased in both the experimental and control group at 60 and 70% of VO_2max, but increased in both groups at 80 and 90% of VO_2max intensity.

Table 3: The mean levels of oxidative stress at various exercise intensities for the control (N = 3) and experimental (N = 5) groups

Intensity (VO_2max)	Groups	Pre-exercise (Carr U±sd)	Post-exercise (Carr U±sd)	24h post exercise (Carr U±sd)	Carr U	Stress level[#]
60%	Control	274 ± 54	251 ± 64	253 ± 54	< 300	Below
	Ex	364 ± 142	318 ± 60	445 ± 175		border
70%	Control	305 ± 40	273 ± 46	254 ± 29	300-320	Border
	Ex	313 ± 66	319 ± 37	364 ± 120	321-340	Low
80%	Control	248 ± 28	236 ± 34	213 ± 39	341-400	Middle
	Ex	353 ± 123	372 ± 175	396 ± 119	401-500	High
90%	Control	243 ± 58	318 ± 86	284 ± 60	> 500	Very high
	Ex	385 ± 232	479 ± 200	405 ± 180		

sd= Standard deviation; Ex = Experimental group; [#] = Stress levels according to Ioro [29]

Measuring oxidative stress (as represented by total oxidant status, dROM assay) 24 hour post-exercise showed a decrease in the control group at all the exercise intensities, while an increase

was observed in the experimental group for all tested intensities. Exercise at 90% VO_2max intensity reached levels of high oxidative stress as classified by Ioro [16].

The percentage change in oxidative stress (Carr U), as adjusted for baseline values (Figure 1), revealed that no significant differences between the trained and untrained subjects. This may be due to both the trained and control group displaying similar physiology as indicated by the similar VO_2 max test results.

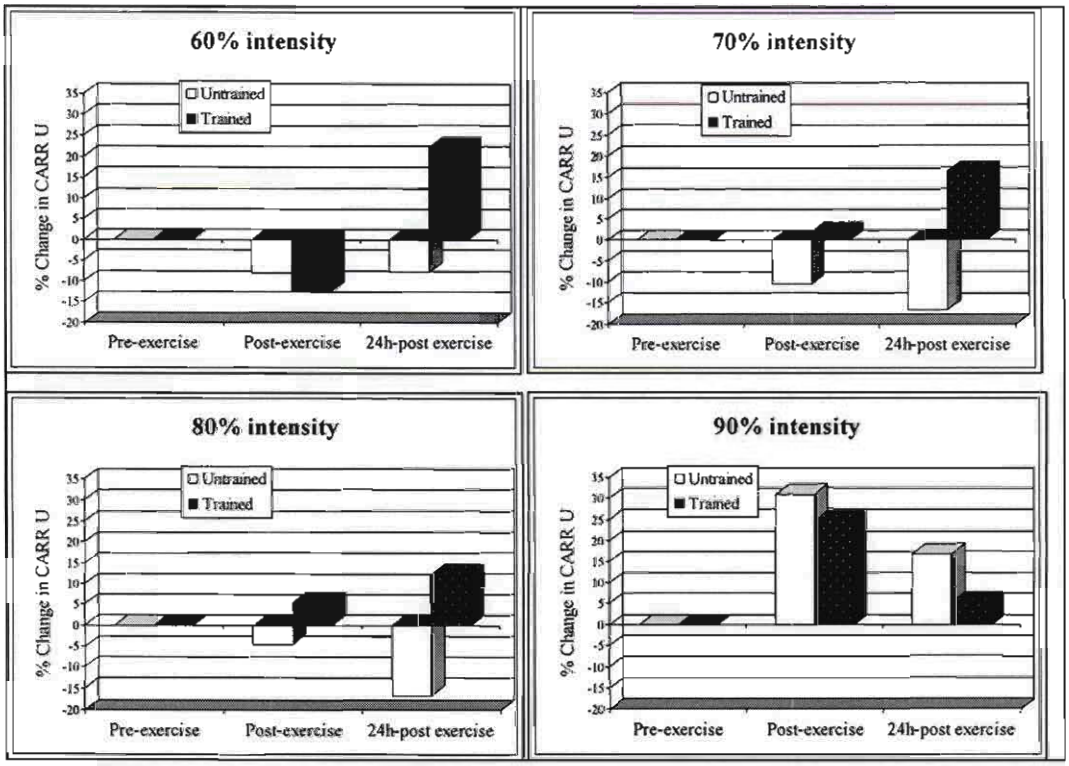

Figure 1: The percentage change in oxidative stress from pre- to post & 24h post acute exercise

Comet assay/Single-cell gel electrophoresis (SCGE)

Combining the results of all the participants obtained during the first 24 hours for each of the exercise intensities (Figure 2) shows that the inter-individual variations were more pronounced at the lower intensities and that the subjects tended to react similarly at the more strenuous

intensities. A peak in the comet tail length occurred at about 5 minutes post exercise, where a decrease was observed to reach a maximum after 15-30 minutes post exercise. This decrease was

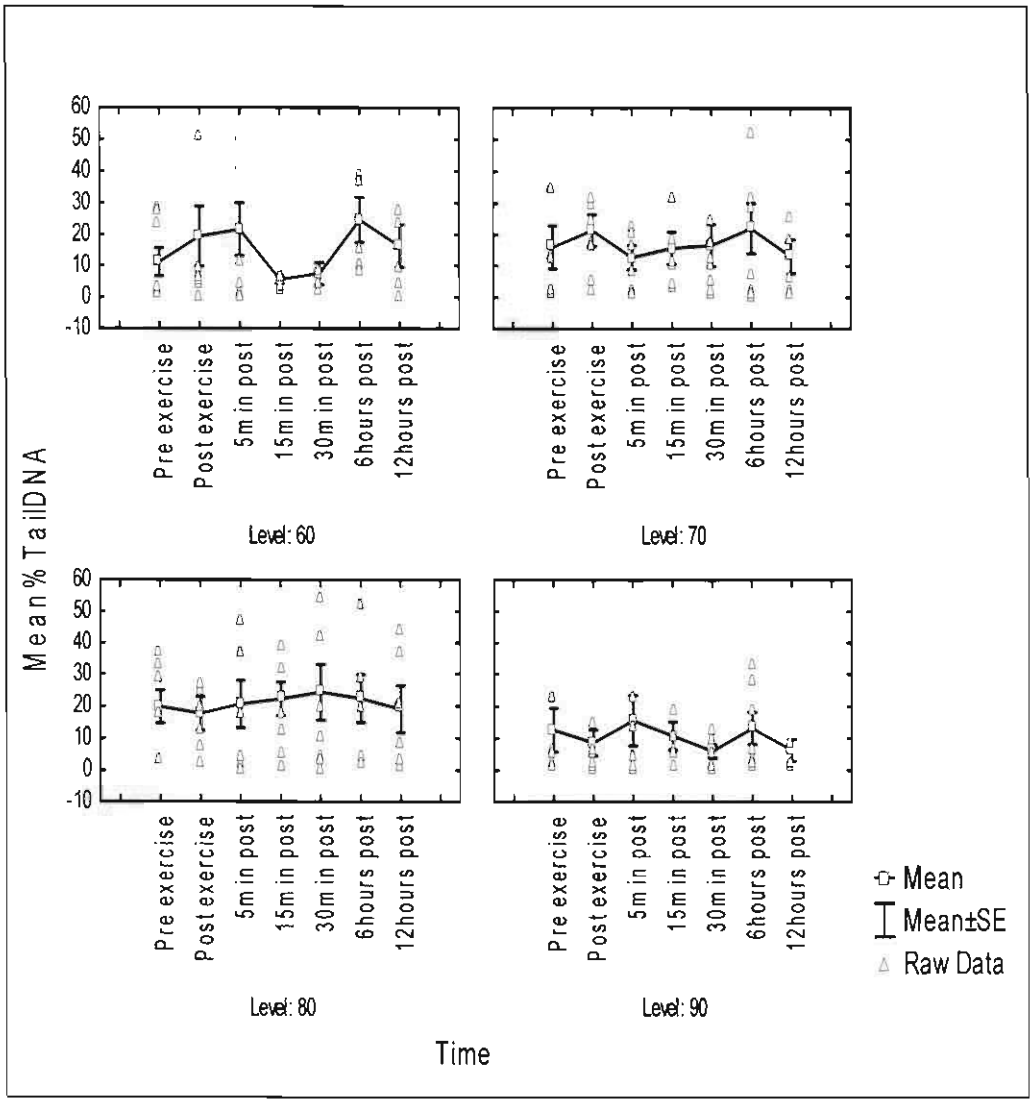

Figure 2: Mean percentage Tail DNA for trained and untrained subjects at different intensities

very pronounced at the 60% intensity. A second peak in the tail length was evident at 6 hours post exercise. It was also evident that the biphasic nature in the percentage tail DNA observed over post exercise time period tended to level out as the exercise intensity increased.

In an effort to compensate for the large inter-individual variations observed, the percentage tail DNA measured at the various exercise intensities was expressed relative to the pre-exercise value. The results of this manipulation (Figure 3) to a large extent confirmed the observations made above. The marked changes in the percentage tail DNA measured at 60% exercise intensity dramatically decreased to very small values at the higher intensities. Negative values indicate that DNA damage was fully repaired, which was observed only after exercising at 80% and 90%.

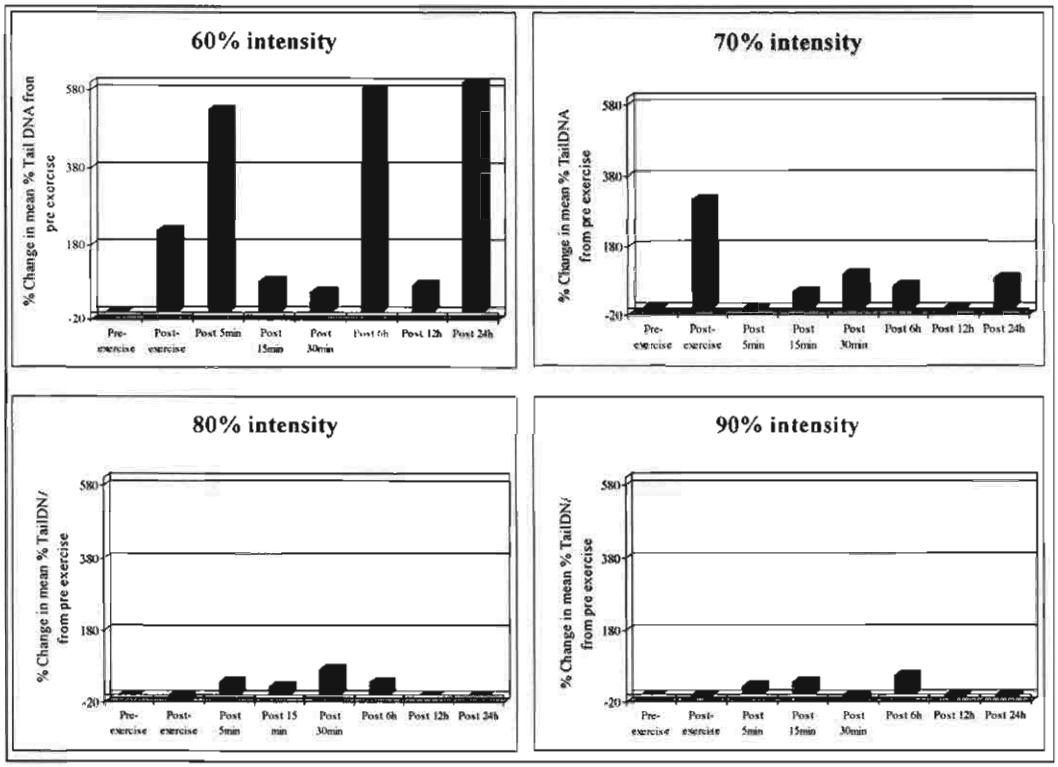

Figure 3: Percentage change in tail DNA relative to pre-exercise values for the different intensities

To facilitate the interpretation of the results obtained with the comet assay, the comets were grouped into different classes on the basis of the percentage tail DNA. Class 0 represents those comets with the least amount of tail DNA and class four those having the most tail DNA. The relative changes in the total number of comets in the different classes indicate the level of DNA damage as well as the degree of DNA repair that occurred. This is reflected in the number of comets in classes 0 and 1 and those of classes 2 and 3. Class 4 was not taken into account

because of the insignificant number present at all intensities and time points. The number of comets in the different classes measured at the various exercise intensities are presented in Figure 4. This result indicates that with the increase in exercise intensity from 60 to 70% of VO_2 max. the number of comets in classes 2 and 3 increased with those in classes 0 and 1 decreasing simultaneously. With a further increase in exercise intensity (80-90% of VO_2max.) a shift in the number of comets to classes 0 and 1 at the cost of those in classes 2 and 3, resulted in more comets in classes 0 and 1 than present at 60% exercise intensity.

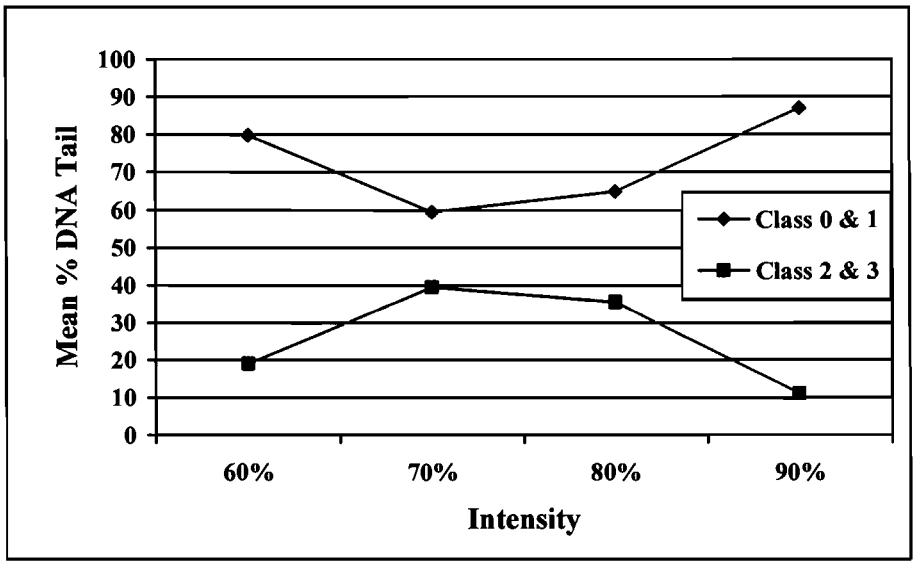

Figure 4: Trends in % DNA damage for all classes over the various exercise intensities for the trained and untrained subjects combined

DISCUSSION AND CONCLUSION

The purpose of this study was to determine the influence of different exercise intensities on oxidative DNA damage in middle-aged men, as identified by comet analyses. Several studies have indicated that acute and chronic exercise result in DNA damage [14, 19, 23, 40]. They confirm the occurrence of oxidative DNA damage during exercise. During these studies, the

exercises were performed at a selected intensity and with various modes of exercise such as walking and running.

Anti-oxidant (ORAC) and oxidant status (dROM) determination

The results of the anti-oxidant and free radical capacities for both the control and experimental groups showed large inter-individual differences. Variation in the response of the anti-oxidant and free radicals may partly be explained by variables such as genetics, diet, and life style which were neither determined nor controlled during the study.

No significant variations were observed in either group for the ORAC and dROM values. The experimental group's ORAC and dROM readings were higher than in the untrained group. This finding supports the findings of studies where indicators of anti-oxidant and free radical capacities were also higher for trained subjects, compared to untrained subjects [27, 40]. No significant changes in anti-oxidant measurements were observed after subjects were submitted to a single bout of exercise. [9].

These findings also indicate that, although DNA damage was observed in the subjects, with various exercise intensity, the damage could not be clearly correlated to the anti-oxidant or oxidant status as measured by the specific methods used. The higher levels of free radicals in the trained group may be a protective function of free radicals for athletes to support the immune system where free radicals play a major role.

Comet assay/Single-cell gel electrophoresis (SCGE)

The comet analyses showed a bi-physic damage-repair cycle over a 24-hour period at all exercise intensities. This cycle was observed in all subjects, indicating an initial increase in oxidative DNA damage. Repair processes appeared to be immediately up-regulated as part of the body's

defence mechanisms in order to counteract any possible cellular and sub-cellular damage that might occur. This occurrence of a bi-physic damage-repair cycle over a 72-hour period mirrored the findings of Hartmann *et al.* [13]. The maximum amount of DNA damage observed occurred at approximately 6 hours post exercise, independent of the specific exercise intensity, also mirrored observations made by Hartmann *et al.* [14].

The large increase in the amount of oxidative DNA damage at 60% intensity could be a result of poor and uneconomic muscle activity which requires more ATP to generate the acquired force for the sub-maximal exercise efforts [25]. During the flow of electrons through the electron transport chain in the mitochondria, ROS are produced by enzymatic and non-enzymatic reactions [5, 19, 34]. This electron flow will be more pronounced during uneconomic muscle activity, leading to more free radical production which might explain the higher incidence of oxidative damage at lower PA intensities.

After the exercise session at 90% VO_2max., very little oxidative DNA damage was observed in either of the groups, contrary to the expected higher amount of oxidative damage as a result of the large amount of oxygen consumed during this session. Since no significant increase in the antioxidant capacity after the exercise bout was observed, one must look for another mechanism to explain the lower levels of DNA damage observed at higher intensities. This is contrary to the observations made by Gianni *et al.* and Schneider *et al.* [10, 37] which identified an exercise-related decrease in oxidative stress. A possible explanation for the results obtained in this study is found in the notion of "exercise as an anti-oxidant" in which it is proposed that an enhanced adaptation of cells (the body) is brought about by increasing the appropriate signal transduction pathways [11]. This approach finds support in the review published by Vollaard *et al.* [42]. The suggestion by Gianni *et al.* of an indirect role in increasing the antioxidant capacity of a cell by up-regulating complex IV activity of the respiratory chain during exercise [10] also lends support

to this notion. Such a reaction could put the cellular repair mechanisms on alert to counteract the effects of the acute exercise bout at the higher intensities. It is not clear where exercise induced apoptosis of lymphocytes [43] fits in to explain these results since we have seen no comets resembling apoptotic cells.

We concluded from this study that contrary to expectation observed DNA damage was greater at an exercise intensity of between 70-80% VO_2max in physically fit middle aged males but appeared to decrease at 90% VO_2 max. This phenomenon may be explained by the hypothesis made by Radak *et al.* [31], that adaptive mechanisms are initiated by transcription factors, resulting in increased activities of the antioxidant enzymes, and more effective repair and housekeeping by the DNA repair enzymes and proteasome complex. In other words less damage was observed at 90% VO2 max, because effective repair had already started. Future research should be directed to the physiological ability to sustain DNA repair at repeated high intensity exercises.

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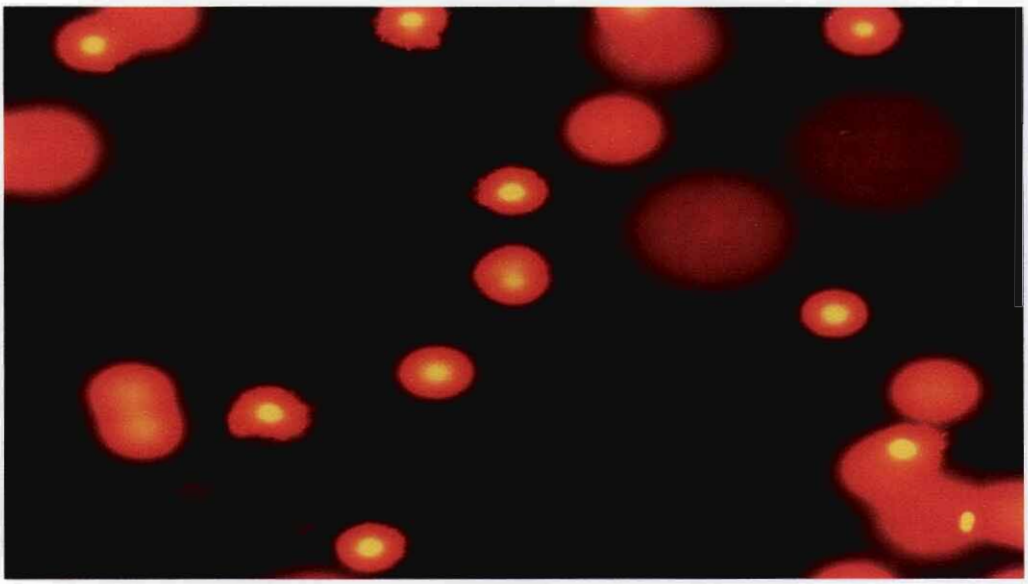
Chapter 4

Summary, conclusions and recommendations



4.1. SUMMARY

4.2. CONCLUSION

4.3. STUDY LIMITATIONS

4.4. RECOMMENDATIONS

4.1. SUMMARY

The purpose of this study was to investigate the influence of different aerobic exercise intensities on oxidative DNA damage and repair in middle aged men. The results of this study will give an indication of the optimal intensity at which physical training should be performed and the possible mechanisms involved in the DNA damage and repair regulation of the middle aged men. Chapter 1 consists of a short introduction, the problem statement and the purpose of the study. The hypothesis for the study was also stated.

Chapter 2 consists of a literature review in article format. The article gives a description of the effect of physical activity on the body, the formation of free radicals and the implications of these free radicals on the body and DNA. The article also reviews a few articles about what is currently being done in the field of physical activity and DNA damage as a result of free radicals. Proof that physical activity on a regular basis will improve a person's quality of live, although the biochemical adaptations are not yet fully understood. In reviewing the articles it is clear that definite barriers for research should be drawn up in this field. No definite method or testing modality is currently standardised. More research should be done on the identification of specific parameters. Though this article indicates that physical activity will lead to oxidative damage, no clear answer was obtained on the exercise intensity at which damage will occur.

In Chapter 3 the focus of the research article will be on how different aerobic exercise intensities will lead to different oxidative DNA damage. In this article an experimental group and a control group, of healthy men between 40 and 55 years of age, were subjected to an acute exercise intervention with four varying intensities (60, 70, 80 and 90% VO_2max). The pattern of DNA damage-repair is analysed over a 72 hour period by means of the Comet analysis. The subjects' anti-oxidant (ORAC) and free radical (dROM) capacities is measured. The values of the ORAC and dROM analysis indicated that conditioned men have a higher anti-oxidant capacity, although this could be because of the higher amount of free radicals that were more readily available. The values of these analyses tend to increase after exercise and return to normal 24 hours post exercise. The most dramatic increase was observed in the unconditioned male subjects. Patterns observed through the Comet analysis indicated a bi-phasic pattern of DNA damage-repair over a 72 hour period. Both subject groups indicated some form of oxidative damage, where the unconditioned group once again showed higher levels of damage. The most cells were affected by free radicals immediately after the acute exercise session and a drastic repair action following immediately. Exercise at 70% VO_2max resulted in the most DNA damage, while 90% VO_2max

indicates the least amount of oxidative damage of all the exercise intensities in both groups. The deviations in the damage-repair bi-phasic pattern tend to be smaller over time.

4.2. CONCLUSION

The conclusions are based on the hypotheses stated earlier.

The first hypothesis postulated that conditioned males between the ages of 40 and 55 years will have a bigger antioxidant capacity than unconditioned males of the same age group.

During the course of this study it was indicated that conditioned males between 40 and 55 years of age do have a larger anti-oxidant capacity. Therefore, the first hypothesis can be accepted. It is also important to remember that the experimental group's free radical capacity was also higher. Also of importance to note is that the increase in both anti-oxidant and free radicals was more definite in unconditioned males than in conditioned subjects. The amount of free radicals was reduced to baseline readings 24 hours post exercise.

The second hypothesis stated that DNA damage will occur at higher exercise intensities in conditioned males between the ages of 40 and 55 years than in unconditioned males between 40 and 55 years.

This hypothesis should be discarded. Tendencies observed in the results of the Comet assay indicate that maximal DNA damage occurs after an exercise session of 70% VO_2max. After 80% VO_2max exercise session a slightly less amount of cells are damaged by free radicals than at 70% VO_2max. The most interesting observation made from this study was the reaction of the body after an acute exercise session of 30 minutes, against 90% VO_2max. It seems that to exercise against such a high intensity did not inflict any oxidative DNA damage. A physiological protection was activated during this exercise session. When first observed in both subject groups, the trail was repeated and yet again the same results were found, ruling out any experimental error.

The third and last hypothesis stated that DNA repair will be more extensive in conditioned males in the age group of 40 to 55 years than in their unconditioned counterparts. As with the second hypothesis, this hypothesis will be discarded. Repair of DNA damage in conditioned men was not significantly more than in the unconditioned counterparts. Both groups indicate almost

exactly the same damage-repair pattern. Although the higher anti-oxidant capacity in the conditioned subjects indicated a higher chance for oxidative repair, the already higher amounts of free radicals contradict this hypothesis.

4.3. STUDY LIMITATIONS

The following limitations were observed with this study:

- Small sample size due to in- and exclusive criteria.
- Statistical analyses influenced by the sample size.
- Large variation between samples analysis.
- Stress, diet, sleep patterns and job descriptions were not recorded.

4.4. RECOMMENDATIONS

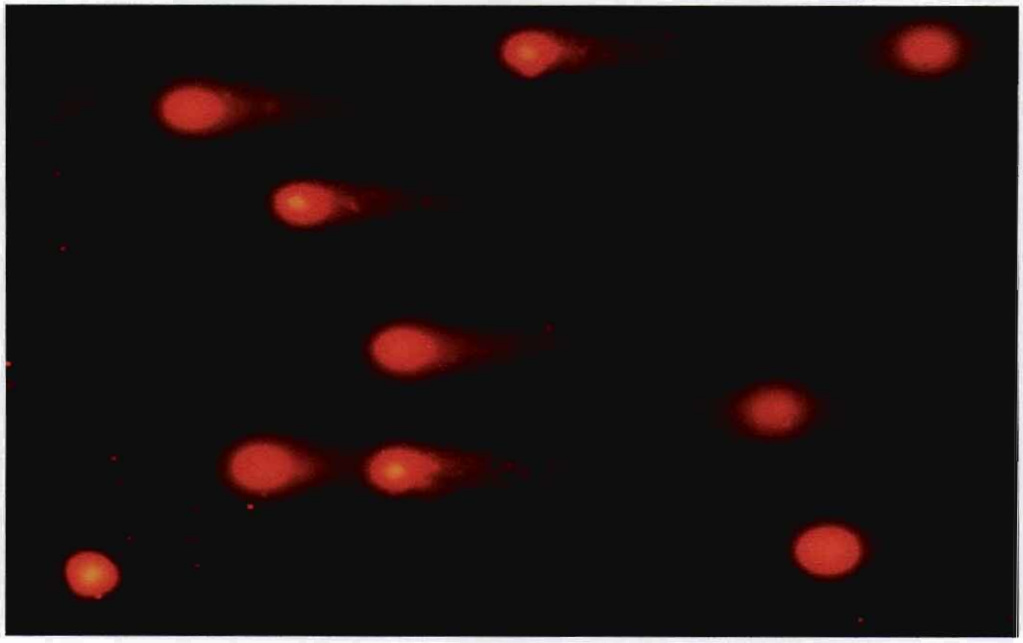
Results obtained through this study indicate that physical activity against either a low or very high intensity VO_2max will lead to the formation of oxidative DNA damage. This damage will be repaired in a bi-phasic pattern over a 72 hour period. It is stated in the literature that conditioned subject's reaction towards exercise should be better adjusted than unconditioned subjects.

Because this definite difference between the two subject groups could not be observed the following recommendations can be made for further studies:

- Large sample size to accommodate genetic differences.
- Perform additional biochemical analysis to Comet analysis.
- Follow-up post-exercise measurements limited to 48hours.

Appendices

Guidelines for authors



APPENDIX A

- AFRICAN JOURNAL FOR PHYSICAL, HEALTH EDUCATION, RECREATION AND DANCE (AJPHERD)

APPENDIX B

- INTERNATIONAL JOURNAL OF SPORT NUTRITION AND EXERCISE METABOLISM (IJSNEM)

Appendix A

AFRICAN JOURNAL FOR PHYSICAL, HEALTH EDUCATION, RECREATION AND DANCE (AJPHERD)

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Text should carry the following designated headings: Introduction, materials and methods, results, discussion, acknowledgements, references and appendices (if appropriate).

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The Introduction should start on a new page an in addition to comprehensively giving the background of the study should clearly state the problem and purpose of the study. Authors should cite relevant references to support the basis of the study. A concise but informative and critical literature review is required.

Materials and Methods

This section should provide sufficient and relevant information regarding study participants, instrumentation, research design, validity and reliability estimates, data collection procedures, statistical methods and data analysis techniques used. Qualitative research techniques are also acceptable.

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Findings should be presented precisely and clearly. Tables and figures must be presented separately or at the end of the manuscript and their appropriate locations in the text indicated. The results section should not contain materials that are appropriate for presentation under the discussion section. Formulas, units and quantities should be expressed in the *Systeme Internationale* (SI) units. Colour printings of figures and tables are expensive and could be done upon the authors' expense.

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Appendix B

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Journal Articles: Surname of first author, initials, then initials and surname of each coauthor; title of article (capitalize only the first word and proper nouns); name of journal (underlined and abbreviated according to *Index Medicus*); volume, inclusive page numbers, and year (see Example 1).

Book References: Author(s) as above; title of book (underlined and all major words capitalized); city of publication; publisher; and year (see Example 2).

Chapter in Edited Book: Same as book references but add title of chapter (capitalize first word and proper nouns); title of book; name of editor; and inclusive pages of the chapter (see Example 3).

1. Chisolm, D.J., J.D. Young, and L. Lazarus. The gastrointestinal stimulus to insulin release. *J. Clin. Invest.* 48:1453-1460, 1969.
2. Wadler, G.I., and B. Hainline. *Drugs and the Athlete.* Philadelphia: F.A. Davis, 1989.
3. Haymes, E. Proteins, vitamins, and iron. In *Ergogenic Aids in Sport*, M.H. Williams (Ed.). Champaign, IL: Human Kinetics, 1983, pp. 27-55.

Figures and Tables: Figures, not larger than 5 in. x 7 in., should be professional in appearance and have clean, crisp lines. Hand drawings and hand lettering are not acceptable. PC-generated screens (i.e., dot patterns) do not reproduce well during the printing process and should not be used to create shading in figures. Stripe patterns or solids are better choices for shading. Figures can be submitted electronically in .TIF or .PDF file formats. Submit a copy of each figure with each copy of the manuscript. On each figure indicate figure number, author's name, and top side. Authors are encouraged to submit illustrations rather than tables. When tabular material is necessary, it should not duplicate the text. Tables should be double-spaced on separate sheets and include brief titles.

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