DOCTORAL THESIS

Exercise, DNA damage and consideration for i) antioxidant supplementation in males and ii) female endocrinology

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EXERCISE, DNA DAMAGE, AND CONSIDERATIONS FOR: (i) ANTIOXIDANT SUPPLEMENTATION IN MALES AND, (ii) FEMALE ENDOCRINOLOGY

By

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Abstract

Reactive oxygen and nitrogen species (RONS) are continuously produced in three main ways: (1) intercellularly as by-products of oxidative metabolism during the generation of ATP, (2) extracellularly and in a controlled manner via specialized enzymes as part of the evolutionary process of developing the innate immune system, and (3) exogenously, via exposure to external/environmental factors. On the one hand, at physiological levels, RONS act as key signalling molecules for normal cell function (i.e., intracellular signalling, and immune function). On the other hand, in an oxidative stress state, whereby redox homeostasis is altered (excessive RONS production and impaired antioxidant systems), they can inevitably cause oxidative damage to DNA, lipids and proteins.

The experimental studies presented in this thesis investigated oxidative stress in both sexes, in response to strenuous aerobic exercise, with a particular focus on DNA damage in white blood cells. Furthermore, the use of an antioxidant effect was explored on the grounds of its effectiveness against exercise-induced oxidative stress and the DNA damage response. In study 1, the systematic review and meta-analysis (data from 35 studies), a significant increase in DNA damage was demonstrated immediately following an acute exercise bout, as well as following 4 hours and up to 1-day post-exercise, whereas this was not evident following 5-28 days.

In study 2, twenty healthy males (33 ± 12 years) cycled at a medium exercise intensity (67 % of $\dot{V}O_{2max}$) for one hour. Results demonstrate an increase in DNA damage as a function of exercise (*baseline vs. post-exercise*, $P < 0.001$), which decreased back to baseline levels following 3 hours post-exercise. Furthermore, lipid hydroperoxides decreased as a function of exercise (*baseline vs. 3 hours post-exercise*, $P < 0.05$). Although 10g of ascorbic acid (AA) supplementation had no effect on DNA damage (*time x group*, $P > 0.05$), AA markedly increased ascorbyl free radical concentration following exercise (AA group; *baseline vs post-supplementation/post-exercise/3 hours post-exercise*, $P < 0.05$).

In study 3, seven healthy females (35 ± 5 years) performed two bouts of treadmill exercise (average time 12 ± 2 mins) to exhaustion on day 26-28 and 11-13 of their menstrual cycle corresponding to the late luteal phase

(LL) and the late follicular phase (LF) ($\dot{V}O_{2max}$: 43.5 ± 5.9 and 42.3 ± 10.1 mL kg^{-1} min^{-1}, respectively). Results demonstrate no difference between phases (*LF vs LL*) or an interaction effect of *time x phase* ($p > 0.05$) but an increase in DNA damage as a function of exercise (*pre-exercise vs post-exercise*, $P < 0.05$) by 357% and 200% in the LF and LL phases, respectively. Additionally, following exercise, lipid hydroperoxides increased by 10% (*pre-exercise vs post-exercise*, $P < 0.05$). There was no change in oestradiol concentration between phases and no antioxidant oestradiol effect on reducing DNA damage was observed ($P > 0.05$). Lastly, oestradiol was not correlated with DNA single strand breaks, but there was a positive strong correlation during the LL phase with DNA double strand breaks at baseline ($r = 0.79$, $P < 0.05$).

In study 4, four healthy males (26 ± 2.6 years) performed a 1-hour steady-state moderate-intensity treadmill run in either hypoxia or normoxia ($FiO_2 = 0.16$ and $FiO_2 = 0.21$, respectively). Gene expression of GPx7 (associated with antioxidant capacity) was downregulated and NCF2 (associated with RONS production) was upregulated in both conditions (*3 hours post-exercise vs baseline*, $P < 0.05$). Results of these pilot data warrant further research regarding specific gene expression responses to hypoxic exercise.

In conclusion, medium and/or high-intensity aerobic exercise can induce temporal DNA damage which may underpin mechanisms of RONS/exercise-mediated adaptation. The data demonstrates that, neither a chronic high ingested dose of AA in males nor the phase of the female menstrual cycle seem to affect or blunt this temporary observed exercise/DNA damage response.

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Date: ……

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Abbreviations

Ground-state diatomic oxygen or dioxygen	$^3\Sigma g^- O_2$ or O_2
17β-oestradiol	E2
4-hydroxynonenal	4-HNE
8-hydroxy-2'-deoxyguanosine	8-OHdG
8-hydroxyguanine	8-OH-Gua
8-hydroxyguanosine	8-OHG
8-iso-PGF2α	F_2-Isoprostanes
Abasic site	AP site
Adenosine triphosphate	ATP
Alkoxyl radical	$RO^\bullet$
Alkoxyl radical	$LO^\bullet$
Apoptosis signal-regulating kinase 1	ASK1
Arbitrary unit	AU
Ascorbic acid or L-ascorbic acid or vitamin C	AA
Base excision repair	BER
Beats per minute	bpm
Body mass index	BMI
Branched-chain amino acids	BCAAs
Butylated hydroxytoluene	BHT
Carbon dioxide	CO_2
Catalase	CAT
Centigrade	°C
Chlorine	Cl^-
c-Jun NH_2-terminal kinase	JNK
Coefficient of variation	CV
Complementary DNA	cDNA
Complementary RNA	cRNA
Confidence intervals	CI
Copper	Cu
Cupric ion	Cu^{2+}
Dehydroascorbate	DHA
Deoxyribonucleic acid	DNA
Dihydrolipoic acid	DHLA
Dimethyl sulfoxide	DMSO
Di-potassium ethylene diamine tetra-acetic acid	EDTA
Double distilled water	ddH_2O

Double strand breaks	DSBs
Early follicular	EF
Early luteal	EL
Electron	e^-
Electron paramagnetic resonance	EPR
Electron spin resonance	ESR
Endoplasmic reticulum	ER
Endothelial nitric oxide synthase	eNOS
Enzyme-linked immunosorbent assay	ELISA
Estimated marginal means	EMM
Estriol	E3
Estrone	E1
Extensor digitorum longus	EDL
Extracellular signal-regulated kinase	ERK
Extracellular superoxide dismutase	ecSOD
Ferric iron	Fe^{3+}
Ferric-induced antioxidant potential	FRAP
Ferrous iron	Fe^{2+}
Fetal calf serum	FCS
Follicle-stimulating hormone	FSH
Formamidopyrimidine DNA glycosylase	FPG
Fraction of inspired oxygen	FiO_2
Free radical theory of aging	FRTA
Gas chromatography-mass spectrometry	GC-MS
Gigahertz	GHz
Glutathione	GSH
Glutathione disulfide or oxidized glutathione	GSSG
Glutathione peroxidase	GPx
Gram	g
Haematocrit	HCT
Haemoglobin	Hb
Heart rate	HR
High-performance liquid chromatography	HPLC
High-performance liquid chromatography with tandem mass spectrometry	HPLC-MS
High-performance liquid chromatography-electrochemical detection	HPLC-ECD
Homologous recombination	HR

Hour	h
Human 8-oxoguanine DNA glycosylase	hOGG1
Hydrogen peroxide	H_2O_2
Hydrogen peroxide	H_2O_2
Hydroperoxide	ROOH
Hydroxyl radical	$OH^{\bullet}$
Hypoclorous acid	HOCI
Inducible nitric oxide synthase	iNOS
International unit	IU
IκB kinase	IKK
Kilogram	kg
Kilohertz	kHz
Kilometer	km
Late follicular	LF
Late luteal	LL
Level of significance (probability)	p
Lipid hydroperoxides	LOOH
Lipid radical	$R^{\bullet}$
Lipid soluble antioxidants	LSA
Lipoic acid or α-lipoic acid	ALA
Litre	L
Low-density lipoprotein	LDL
Luteinizing hormone	LH
Malonaldehyde	MDA
Manganese	Mn
Maximal oxygen uptake	$\dot{V}O_{2max}$
Mean age	MA
Menstrual cycle	MC
Messenger RNA	mRNA
Meter	m
Microlitre	µL
Micromolar	µM
Micromole	µmol
Microwave power	mW
Mid follicular	MF
Mid luteal	ML
Miles per hour	mph
Milliampere or milliamp	mA

Milligram	mg
Millilitre(s)	mL
Millimeter	mm
Millimolar	mM
Millimole	mmol
Minute(s)	min(s)
Mismatch repair	MMR
Mitochondrial DNA	mtDNA
Mitochondrial free radical theory of aging	MFRTA
Mitogen-activated protein kinase	MAPK
N-acetylcysteine	NAC
NADPH oxidase	NOX
Nanogram	ng
Nanomole	nmol
Neuronal nitric oxide synthase	nNOS
Neutrophil cytosol factor 2	NCF2
Nicotinamide adenine dinucleotide (oxidized form)	NAD^+
Nicotinamide adenine dinucleotide (reduced form)	NADH
Nicotinamide adenine dinucleotide phosphate	NADPH
Nicotinamide adenine dinucleotide phosphate hydrogen	NADPH
Nitric oxide synthase	NOS
Nitrogen	N_2
Nitrogen monoxide or nitrogen oxide or nitric oxide	$NO^\bullet$
Nitroso peroxocarboxylate	$ONOOCO_2^-$
Non-homologous end joining	NHEJ
Nuclear factor kappa B	NF-κB
Nucleotide excision repair	NER
Oestrogen receptors	Ers
Oestrogen replacement therapy	ERT
Oxygen saturation	SaO_2
Ozone	O_3
Packed cell volume	PVC
Partial pressure of atmospheric oxygen	PO_2
Percentage	%
Perhydroxyl radical	$HOO^\bullet$
Peripheral blood mononuclear cells	PBMC
Peroxiredoxin	Prx

Peroxisome proliferator-activated receptor gamma co-activator 1-alpha/beta	PGC1-α/β
Peroxyl radical	$ROO^{\bullet}$
Peroxynitrite	$ONOO^{-}$
Peroxynitrous acid	ONOOH
Phosphate buffered saline	PBS
Phospholipase A2-dependent processes	PLA_2
Phosphorylated histone 2AX	γH2AX
Picogram	pg
Picomole	pmol
Polyunsaturated fatty acids	PUFA
Preferred reporting items for systematic reviews and meta-analyses	PRISMA
Prostaglandin H_2	PGH_2
Protein carbonyl groups	PC
Protein carbonyls	PC
Rate of perceived exertion	RPE
Reactive nitrogen species	RNS
Reactive oxygen and nitrogen species	RONS
Reactive oxygen species	ROS
Reduced nicotinamide adenine dinucleotide phosphate	$NADP^{+}$
Relative weight	RW
Respiratory exchange ratio	RER
Resting intracellular oxygen tension	iPO_2
Reverse transcription-quantitative polymerase chain reaction	RT-qPCR
Revolutions per minute	rpm
Ribonucleic Acid	RNA
Sample number	n
Sarcoplasmic reticulum	SR
Selenenic acid	E-SeOH
Selenenyl sulfide adduct	E-Se-SG
Selenium	Se
Selenol	E-SeH
Semidehydroascorbate	SDA
Serum separation tube	SST
Single cell gel electrophoresis	SCGE
Single strand breaks	SSBs
Singlet oxygen	$^1(\Delta_g)O_2$

Sodium chloride	NaCl
Sodium hydroxide	NaOH
Standard deviation	SD
Standard error of the mean	SEM
Standardised mean differences	SMD
Strand breaks	SBs
Succinate	$FADH_2$
Superoxide anion radical	$O_2^{\bullet-}$
Superoxide dismutase	SOD
Telomere length	TL
Thiobarbituric acid	TBA
Thiobarbituric acid reacting substances	TBARS
Thioredoxin	Trx
Thromboxane A_2	TXA_2
Time to exhaustion	TTE
Tme-point	TP
Total antioxidant capacity	TAC
Tris-EDTA	TE
Tumour necrosis factor	TNF
Volt	V
Water	H_2O
Week	wk
White blood cells	WBCs
Xanthine oxidase	XO
Years	yrs
Years old	y/o
Zinc	Zn

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Research Communications

Publications

Tryfidou D. V., McClean C., Nikolaidis M.G. and Davison G.W. (2020). DNA Damage Following Acute Aerobic Exercise: A Systematic Review and Meta-analysis. *Sport Med* **50:** 103–127.

Conference Presentations

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CHAPTER ONE

CHAPTER 1: INTRODUCTION

1.1 Introduction

The evolutionary process of oxygen-forming photosynthesis in plants, allows animals and mammalian species to survive. However, oxygen can be toxic, forming the backbone of the "oxygen paradox" (Eze, 2006). The "oxygen paradox" states that molecular oxygen is essential for all aerobic multicellular eukaryotic life forms, but is simultaneously inherently toxic to those very same forms of life (Davies *et al.*, 2017). The chemistry of oxygen and its derivates are directly involved in its toxicity. Ground-state diatomic oxygen ($^3\Sigma g^- O_2$ or O_2) is only moderately reactive due to its two unpaired electrons which are located in different molecular orbitals and possess "parallel spins". Consequently, O_2 accepts electrons from other free radicals one at a time (Beckman & Ames, 1998). A free radical is defined as any molecular species, capable of existing independently, containing one or more unpaired electrons in its outer shell (Valko *et al.*, 2007; Lobo *et al.*, 2010). Consequently, one- and two-electron reduction of O_2 forms the superoxide anion radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) respectively, and in the presence of free transition metal ions (specifically iron and copper), $O_2^{\bullet -}$ and H_2O_2 form the highly reactive hydroxyl radical ($OH^{\bullet}$) (Beckman & Ames, 1998).

As presented in **Figure 1.1**, there are several key publications of pioneering work by several international authors which have shaped the current field of oxidative stress research. The original definition of oxidative stress was first published by Sies & Cadenas in 1985, described as "a disturbance in the prooxidant and antioxidant balance in favour of the former" which was later updated by Sies & Jones in 2007 to: "an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage".

Firstly, Harman (1956) postulated that aging and ultimately death, are a consequence of the accumulation of oxidative damage induced by free radicals generated during aerobic respiration. As a result, the free radical theory of aging (FRTA) was born (Chance *et al.*, 1979; Davies, 1995).

Almost 20 years later, the FRTA was revised by Harman (1972) into the mitochondrial free radical theory of aging (MFRTA) proposing that mitochondria, as a primary source of free radical generation during normal metabolism, can lead to oxidative damage. In turn, oxidative damage drives the ageing process. A few years prior to the seminal work of Harman (1956), early experiments using electron spin resonance (ESR) spectroscopy detected the presence of the hydroxyl radical ($OH^{\bullet}$) in biological materials (Commoner *et al.*, 1954). This led to the hypothesis that endogenous oxygen free radical generation *in vivo* is a by-product of enzymatic redox chemistry (Harman, 1956). Further support of this hypothesis, was gleaned from the discovery of superoxide dismutase (SOD) by McCord & Fridovich in (1969), thus providing the first convincing evidence of *in vivo* formation of $O_2^{\bullet -}$ and further clarification of the complex defence systems against oxidative threats (Yu, 1994).

Moreover, oxidative stress is also described as an interruption of homeostasis resulting in injury to the biological system, disrupting the normal functioning of the human's body (Kassahn *et al.*, 2009). The term "homeostasis" was initially proposed by physiologist Walter Bradford Cannon in 1926 from the ancient Greek ὅμος (hómos, "similar") + ιστημι (histēmi, "standing still")/*stasis* (from *στάσις*) into a Modern Latin form to invent the term *homeostasis* (Davies, 2016). Cannon highlighted that homeostasis does not refer to something immobile or set, but rather a condition which may change, and remain relatively constant (Davies, 2016). Lastly, Nikolaidis *et al. (*2012*b*) attempted to elucidate the term oxidative stress by proposing an alternative definition of 'alterations in redox homeostasis'. The authors explain that since the term "oxidative stress" is not associated with a specific type of exercise, tissue, or species, it rather constitutes a ubiquitous fundamental biological response to the alteration of redox homeostasis induced by exercise. Reactive oxygen and nitrogen species (RONS), generated either endogenously during normal metabolism (such as via mitochondria, NADPH (nicotinamide adenine dinucleotide phosphate) oxidases, cytochrome P450 reductases) or via exogenous processes (such as via radiation, xenobiotics, drugs), can constantly challenge cellular homeostasis (Davies *et al.*, 2017).

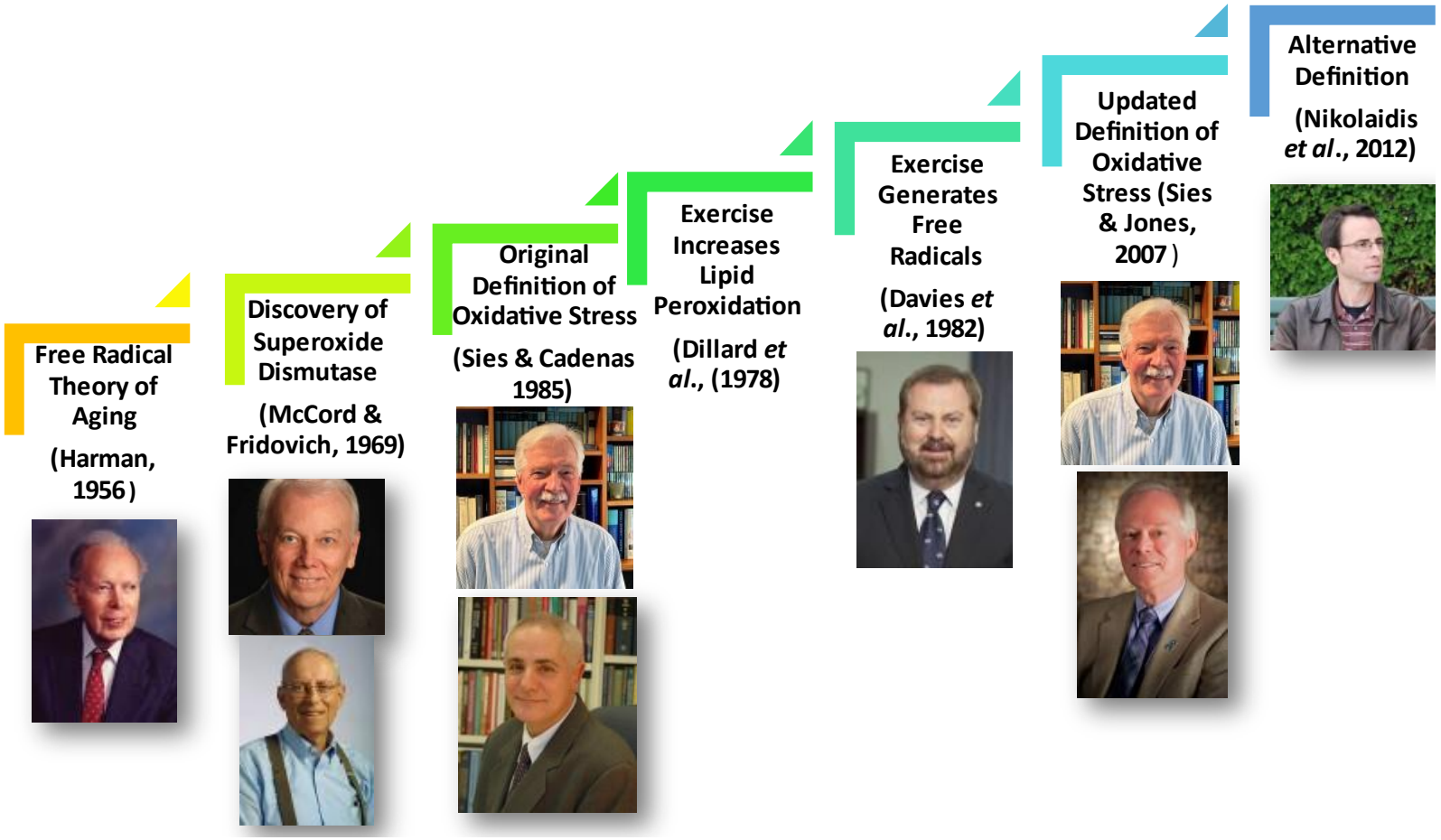


Figure 1.1 Key publications in the oxidative stress field of research presented in chronological order.

The hormesis framework, initially used in toxicology, has been successfully applied to the field of biology and medicine and extended to RONS, which describes: "'a process in which exposure to a low dose of a chemical agent or environmental factor that is damaging at higher doses, induces an adaptive beneficial effect on the cell or organism" (Calabrese & Baldwin, 2001; Mattson, 2008; Radák *et al.*, 2017). Fortunately, this seemingly paradoxical phenomenon can be mechanistically explained through 'adaptive homeostasis.' This term refers to the cells' ability to initiate a non-destructive process. In this process, a toxic agent (such as RONS) can trigger signalling pathways. These pathways, in turn, increase the expression of protective genes through mechanisms distinctly different from those that cause toxicity at high concentrations (Davies *et al.*, 2017). Therefore, single or repeated exposure to mild stress which occurs upon exposure to agents at nanomolar or picomolar levels, is beneficial against higher damaging/toxic levels in the millimolar range (Davies, 2016). Direct evidence of exercise-induced free radical generation was first provided when Davies *et al.* (1982) demonstrated a two-threefold increase in free radical concentration (measured by EPR) in muscle and liver of male rats following an exercise test to exhaustion. Additionally, the authors observed that exercise decreased mitochondrial respiratory control, increased lipid peroxidation products, as well as a loss of sarcoplasmic reticulum and endoplasmic reticulum integrity.

Ever since the first report linking exercise to a marker of oxidative stress, numerous subsequent reports have provided robust evidence supporting the phenomenon of exercise-induced oxidative stress. This occurs because exercise exerts pro-oxidant activity through the production of free radical species, which can then damage several biomolecules, including DNA, lipids, and proteins (Fisher-Wellman & Bloomer, 2009). The first report that high-intensity exercise leads to 1.8 fold increase in exhaled pentane levels, a marker of lipid peroxidation, was pioneered by Dillard *et al.* in 1978.

Although the method of exhale pentane to measure lipid peroxidation requires specialist equipment and is subject to artifacts (Devasagayam *et al.*, 2003), early reports observed similar findings (Leaf *et al.*, 1997). In the following years, Hartmann *et al.* (1995) reported a link between increased lipid peroxidation following maximal aerobic treadmill exercise, using another widely known biomarker, malonaldehyde (MDA), which is an end product formed during the breakdown of unsaturated fatty acids (Abeyrathne *et al.*, 2021). On the other hand, Niess *et al.* (1996) reported unchanged MDA following incremental maximal treadmill exercise in both untrained/trained subjects. Additionally, Bouzid *et al.* (2014) observed no change in young, but increased MDA in the older group following an incremental exhaustive cycle ergometer test. Moreover, lipid hydroperoxides (LOOH), which are the primary products of the lipid peroxidation process, have been widely used as useful indices to measure oxidative stress in tissues (Ayala *et al.*, 2014). Multiple authors have reported an increase in LOOH following various exercise tests, including a maximal cycle ergometer test (Davison *et al.*, 2002), a maximal treadmill test (Williamson *et al.*, 2018), one hour of treadmill running at 75% of $\dot{V}O_{2max}$ (McClean *et al.*, 2015) two separate acute resistance and aerobic exercise sessions (Vincent *et al.*, 2004), and 100 maximal isolated and continuous knee extensions (Fogarty *et al.*, 2013*a*). On the other hand, there have been several reports indicating no change in LOOH following one hour of 60% of maximum heart rate cycle ergometer test (McClean *et al.*, 2011) or one hour of treadmill running at 55% or 100% of $\dot{V}O_{2max}$ for 30 and 5 mins, respectively (McClean *et al.*, 2015).

Along with lipid peroxidation, protein oxidation can also be generated by excess RONS production, and this is most commonly assessed by measuring protein carbonyls (PC) (Dalle-Donne *et al.*, 2003). Several reports have been published supporting a link between elevated PC following exercise. Alessio *et al.* (2000) observed a 67% increase in PC immediately following a treadmill test to exhaustion in men and women. Moreover, PC concentration was elevated after 30, 60 or 120 mins of cycling at 70% of $\dot{V}O_{2max}$ in aerobically trained men and women, in a dose-dependent manner (Bloomer *et al.*, 2007*c*). During a 4-day super-marathon race, serum PC increased after day one (93km) reaching a plateau on the second (120 km), third (56 km) and forth (59 km) days of competition (Radák *et al.*, 2003*b*).

In contrast, unchanged PC have been reported after ultramarathon swimming (Kabasakalis *et al.*, 2011), short maximal anaerobic or moderate-intensity aerobic exercise (Shi *et al.*, 2007), as well as treadmill exercise to exhaustion (Fogarty *et al.*, 2013*b*).

Since DNA damage is heavily implicated in human disease aetiology and progression, the relationship between DNA damage and exercise has been a hot topic in oxidative stress research over the past three decades. Understanding the mechanisms and biological ramifications of DNA lesions that may result in genetic mutations, which may ultimately become carcinogenic, is therefore a major focus of the current research in the subject of DNA damage. The most studied biomarker of DNA damage, 8-hydroxy-2'-deoxyguanosine (8-OHdG), is an end-product derived from damage to 2'-deoxyguanosine. Due to its mutagenic ability, it is considered a sensitive biomarker for oxidative DNA damage and oxidative stress (Qing *et al.*, 2019). One of the first reports investigating exercise and DNA damage using this biomarker observed that, 8-OHdG measured in human lymphocytes, decreased after swimming or running (Inoue *et al.*, 1993). On the other hand, Okamura *et al.* (1997) observed increased urinary 8-OHdG during an 8-day training camp (consisting of an average of 30km per day of running) but unchanged 8-OHdG in lymphocytes before and after the training camp. Moreover, increased urinary 8-OHdG has been reported following 30 days of vigorous long distance exercise training (Poulsen *et al.*, 1996) and a 4-day super-marathon (Radák *et al.*, 2000). Another popular and sensitive method used to detect single strand breaks in DNA is the comet assay (or else known as single cell gel electrophoresis), which Hartmann *et al.* (1994) firstly applied in the exercise oxidative stress field, reporting increased DNA migration in lymphocytes following an exhaustive treadmill test. Many authors using various exercise protocols and implementing different modalities and durations/intensities have supported similar findings (Niess *et al.*, 1996; Hartmann *et al.*, 1998; Tsai *et al.*, 2001; Zhang *et al.*, 2004; Davison *et al.*, 2005; Reichhold *et al.*, 2009*b*; Wagner *et al.*, 2010).

In summary, it has been established that oxidative stress is now implicated in several disease mechanisms (as either a cause or consequence) leading to impaired health and premature ageing. It is therefore clear that excessive or unregulated generation of RONS, occurring in an exercise-induced oxidative stress state, may pose a physiological threat to normal cell function and healthy aging. This threat manifests through damage to critical molecules, including lipids, proteins, and DNA; the latter is the primary focus of investigation in this thesis.

1.2 Experimental Aims and Objectives

The main aim of the work outlined in this thesis is to explore the relationship between high-intensity aerobic exercise and DNA damage in blood of healthy male and female volunteers.

The primary objectives are outlined below:

Systematic Review and Meta-analysis

(1) To systematically investigate DNA damage following acute aerobic exercise and perform a meta-analysis to examine the overall effect from these studies.

(2) To further consider the physiological consequences of exercise-induced DNA damage in line with a new proposed multi-dimensional model.

Experimental Study One

(1) To measure exercise-induced oxidative DNA damage in human lymphocytes following high-dose ascorbic acid supplementation in men.

Experimental Study Two

(1) To assess the potential effects of exercise during the menstrual cycle in relation to DNA damage (quantifying both double and single strand breaks) in lymphocytes of menstruating women.

(2) To further explore the effects on oxidative stress biomarkers of the natural variations in endogenous oestrogen production during the two main phases of the menstrual cycle.

Experimental Pilot Study

(1) To measure changes in gene expression as a result of high-intensity exercise in normobaric hypoxia compared to normoxia.

(2) To further investigate the expression of genes associated with antioxidant capacity and RONS formation.

CHAPTER TWO

CHAPTER 2: LITERATURE REVIEW

2.1 History of Oxygen Toxicity and Free Radicals

The metabolic reaction of molecular oxygen (O_2) reduced to water, requires four electrons, giving rise to the partially reduced metabolites of O_2 superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (Fridovich, 1998; Gladyshev, 2014). These dangerously reactive intermediates, collectively termed *Reactive Oxygen Species* (ROS), are responsible for the toxicity of O_2 and the first report to impute oxygen poisoning due to the formation of free radicals was by Gerschman in 1954 (Gerschman *et al.*, 1954). Since free radicals contain one or more unpaired electrons, the spin of the unpaired electron is not compensated for and therefore, all free radical species are paramagnetic (Rozantsev & Loshadkin, 2001).

In 1945, the physicist Zavoisky, discovered electron spin resonance (ESR or EPR) absorption which then led to the development of ESR spectrometers (Commoner *et al.*, 1957). A spectrometer records the ESR signal, which is the absorption of an electromagnetic wave with a set frequency. This absorption occurs from the interaction between the magnetic moment of an unpaired electron and the external magnetic field (Commoner *et al.*, 1957). Following its discovery, this technique was first applied to biological materials in 1954, successfully detecting a small signal across a wide variety of lyophilized tissues (Commoner *et al.*, 1954). Using a more sensitive spectrometer, Commoner et al. (1957) showed that free radicals are intermediates of living cells and oxidation-reduction systems. Later in 1956, Denham Harman proposed the Free Radical theory of aging which propositioned that aging occurs due to accumulation of oxidative damage (Harman, 1956). After 31 years of its initial isolation, McCord and Fridovich discovered that the enzyme superoxide dismutase catalyses the dismutation of O_2^- to O_2 and H_2O_2 (McCord & Fridovich, 1969).

2.2 RONS and Oxidative Stress

ROS is a term used to describe a family of reactive molecules and free radical species derived from molecular oxygen (Turrens, 2003), while *Reactive Nitrogen Species* (RNS) are derived from both oxygen and nitrogen (Powers & Jackson, 2008). RONS is also used to collectively describe both ROS and RNS including both free and non-free radical intermediates (Powers &

Jackson, 2008). Free radicals contain one or more unpaired electrons and can exist independently (Powers & Jackson, 2008). ROS are generated endogenously in aerobic systems by the incomplete reduction of oxygen, mainly via the mitochondrial electron transport chain during cellular respiration (Ray *et al.*, 2012). **Table 2.1** overviews RONS and their characteristics.

In 1985, Sies and Cadenas firstly defined the term oxidative stress as "a disturbance in the pro-oxidant-antioxidant balance in favour of the former"(Sies & Cadenas, 1985). However, due to the complexity of cellular oxidation-reduction reactions, the simplicity of this definition being 'pro-oxidant versus antioxidant' was argued. As such, Sies & Jones (2007) proposed to redefine it as "a disruption of redox signalling and control". The updated definition refers to oxidative stress as "an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage".

When RONS are produced, they can overwhelm the endogenous antioxidant system and damage molecules such as DNA, proteins and lipids, causing a state of oxidative stress, potentially leading to disease, rapid ageing, mutagenesis, and ultimately carcinogenesis (**Figure 2.1**) (Davison *et al.*, 2005; Cobley *et al.*, 2015).

2.2.1 RONS and Carcinogenesis

In comparison to healthy cells, cancer cells have been shown to contain higher levels of RONS which can stimulate the activation of oncogenes, tumour activators and mutation of many genes. For example this includes the activation of oncogenes such as Fox and Jun genes (Pisoschi & Pop, 2015). The first step of carcinogenesis, and in turn mutagenesis and aging, involves permanent modifications of genetic material in the cell due to the oxidative damage caused from excess RONS (Valko *et al.*, 2004). DNA damage can either be in the form of single- and/or double-strand breaks, pyrimidine, purine or deoxyribose modifications and DNA cross links (Hrycay & Bandiera, 2015). If mis-repaired, DNA damage can lead to replication errors or mutations in genes, while strand breakage can result in chromosomal rearrangement (Manda *et al.*, 2009).

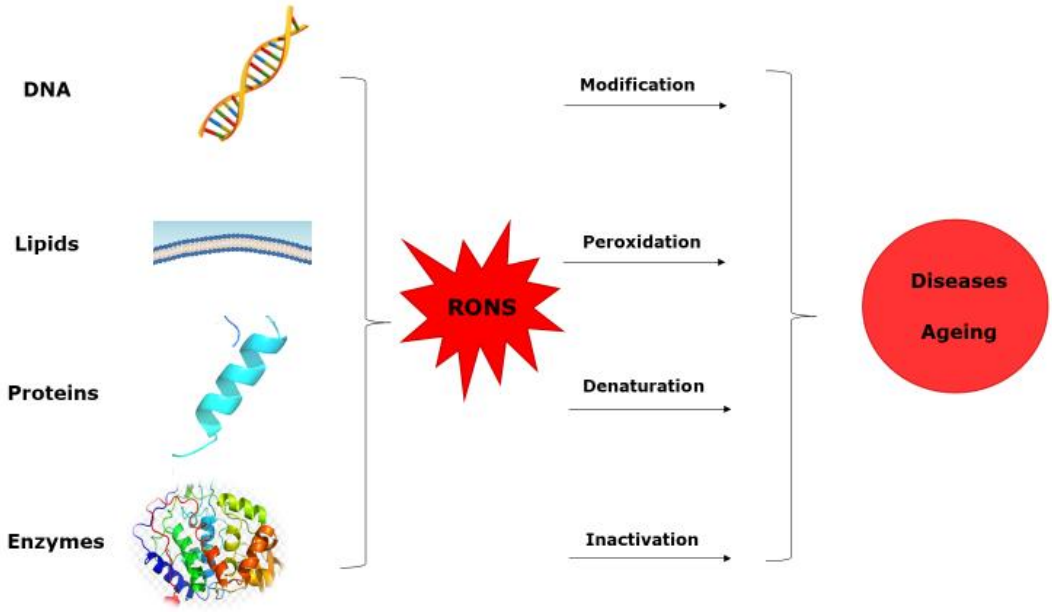


Figure 2.1 Oxidative modification of molecules due to excess RONS production. Adapted from Radák (2000).

Therefore, oxidants have the potential to contribute to carcinogenesis following a DNA base substitution or a single nucleotide deletion, activation of oncogenes, inactivation or tumour suppressor genes, genomic instability (Manda *et al.*, 2009). Moreover, and the higher amount of DNA lesions that are mis-repaired, the more potential for mutagenesis to occur (Valko *et al.*, 2004; Hrycay & Bandiera, 2015).

Another mechanism by which oxidative damage can contribute to carcinogenesis is by modulating gene expression and stimulating growth signals and proliferation (Valko et al., 2004). Errors in repair of DNA strand breaks can cause the chromosome to be rearranged and thus contribute to genetic amplifications as well as alterations in gene expression and tumour promotion (Valko et al., 2004).

Furthermore, tumour cells can generate an excess of RONS through the NADPH oxidase pathway regulated by GTPase Rac1, which belongs to the Rho subfamily of GTPases of the Ras superfamily of enzymes and is positioned downstream to the proto-oncogene Ras (Manda et al., 2009; Tebar et al., 2018). Additionally, p21Ras participates in the generation of RONS by nonphagocytic cells stimulated by growth factors or cytokines but the pathway by which Ras modulates RONS production is not well understood. Furthermore, expression of NOX1, an isoform of NADPH

oxidase, in ovarian and breast tumour cells correlates positively with the expression of cytochrome c oxidase encoded by mitochondrial DNA, which may indicate a possible cross-link between NADPH oxidase and mitochondria (Desouki et al., 2005).

2.2.2 Oxidative stress, Aging and Disease

Aging constitutes the process whereby physiological functions gradually decline over time, especially following the reproductive phase of life (Valko *et al.*, 2007). In 1956, Harman firstly proposed the free radical theory of aging, suggesting that free radicals play a role in the process of aging by exerting deleterious effects on cells through accumulation of irreversible oxidative damage affecting the lifespan (Harman, 1956; Lagouge & Larsson, 2013).

There are generally two main theories describing the process of aging in association with free radicals, damage-accumulation and the genetic related hypothesis (Valko *et al.*, 2007). Damage accumulation theory is based on the fact that RONS cause damage to DNA, proteins and lipids which accumulates over time. As mentioned in 2.3.1.1, electrons leak from the electron transport chain and when they react with oxygen (about 0.12-2%), superoxide radicals are generated in the mitochondria. Although DNA damage can be repaired, mitochondrial DNA (mtDNA) cannot be repaired easily (Valko *et al.*, 2007). This causes a cycle of accumulated mtDNA damage that negatively affects the function of the respiratory chain. This, in turn, leads to further formation of RONS, which can evidently cause cell death and aging (Lagouge & Larsson, 2013). Thus, the mitochondrial free radical damage theory is associated with four basic observations: (1) the level of RONS and RONS-related oxidative damage may increase with age; (2) during aging the function of the mitochondria diminishes; (3) the dysfunction in the mitochondria cause increased RONS generation; and (4) numerous age-related diseases are associated with higher oxidative stress (Hekimi *et al.*, 2011).

On the other hand, there are some observations which may not fully the support this theory: (1) there is no evidence showing an increase in lifespan by decreasing RONS production; and (2) high RONS production has been linked to higher longevity (Hekimi *et al.*, 2011). Therefore, Hekimi and colleagues have proposed that RONS may not be a direct cause of aging per se; instead, RONS generation represents a stress signal in response to age-

dependent damage, and as result, it gradually increases with chronologically age.

The implication of oxidative stress in numerous pathological conditions such as cancer, diabetes, cardiovascular disease, neurological disorders and inflammatory diseases is evident, as free radical oxidative damage has been shown to contribute to their pathogenesis and pathophysiology (Valko *et al.*, 2007).

2.2.3 Mechanistic Insight into DNA damage and Repair

Nearly 70 years ago, in a landmark publication, authors Watson and Crick proposed the DNA double helix structure. Ever since, DNA sequencing and genomic profiling have been associated with many unequivocal topics of biological importance, such as DNA damage, repair, genomic instability, genetic diseases, and cancer (Watson & Crick, 1953).

It is a fundamental priority of all cells to preserve genomic sequence information for the continuation of survival and reproduction. However, DNA is highly reactive and susceptible to alterations of its sequence by either exogenous or endogenous stressors/agents (Chatterjee & Walker, 2017). These stressors/agents can damage the DNA directly by causing its chemical bonds to break, thereby changing its structure, or indirectly by interacting with other endogenous agents/by-products of normal cell metabolism, such as RONS. These interactions can also damage DNA and affect its genomic stability.

Types of endogenous DNA damage, which can result as an outcome of normal cell metabolism, include:

(1) base alterations due to hydrolysis of nitrogenous DNA bases; an example of such an event includes deamination of cytosine, which produces uracil and guanine producing xanthine (Chakarov *et al.*, 2014).

(2) base loss due to hydrolysis of nitrogenous DNA bases; a common example of such an event, which can occur in a human cell approximately 10,000-20,000 per day, is DNA depurination and depyrimidination. These processes result in apurinic/apyrimidinic lesions, commonly referred to as abasic sites (AP site). An AP site occurs when the N-glycosylic bond between the nitrogenous base and the deoxyribose sugar is cleaved. This cleavage can happen due to spontaneous base hydrolysis/loss, other types of base

damage, or enzymatically via DNA glycosylases (Chakarov *et al.*, 2014; Thompson & Cortez, 2020).

(3) base modifications due to base alkylation (addition of alkyl groups) of a nitrogen or oxygen atom in a nitrogenous DNA base, which can lead to further base modification and abnormal base pairing, or DNA mismatch (Chakarov *et al.*, 2014).

(4) oxidation of nitrogenous DNA bases; all four bases are subject to oxidative damage. The oxidation of purines (A,G) results in oxidation products such as 7,8-dihydro-8-oxoguanine, 7,8-dihydro-8-oxoadenine as well as formamidopyrimidines. Meanwhile, the oxidation of pyrimidines (C,T) results in products such as 5-hydroxycytosine, 5-hydroxyuracil, thymine glycol and uracil glycol. Lastly, strand break damage can arise from base oxidative damage caused by RONS (Chakarov *et al.*, 2014).

(5) DNA single strand breaks (SSBs) and double strand breaks (DSBs); approximately 70 000 DNA lesions occur in a human cell, with the most common being SSBs. These result from the disruption of one of the two phosphodiester bonds of the DNA duplex. In contrast, DSBs arise when the phosphodiester backbone in both strands of the same DNA duplex breaks, and the broken ends become separated (Chakarov *et al.*, 2014; Zilio & Ulrich, 2020).

On the other hand, examples of exogenous sources of DNA damage include: (1) electromagnetic radiation causing dimerization; (2) bulky adduct formation; (3) alkylation caused by alkylating agents, such as haloalkanes; (4) damage caused by free radical species of environmental origin; (5) SSBs and DSBs caused by exogenous agents/factors, such as environmental UV and ionizing radiation (gamma, X-rays), and chemical agents; (6) thermal damage due to infrared energy, which can cause SSBs or base hydrolysis; and (7) microwave and radiofrequency electromagnetic radiation (Chakarov *et al.*, 2014).

Fortunately, cells are equipped with five major DNA damage repair pathways: (1) base excision repair (BER); (2) nucleotide excision repair (NER); (3) mismatch repair (MMR); (4) homologous recombination (HR); and (5) non-homologous end joining (NHEJ) (Chatterjee & Walker, 2017). BER refers to the removal of modified or mismatched bases due to oxidation, alkylation, or deamination. This process is facilitated by a family of enzymes

known as DNA glycosylases (Krokan & Bjoras, 2013). NER refers to the process of excision and repair of nucleotides that have been modified by bulky adducts into bulky DNA lesions, such as pyrimidine dimers and some alkylated bases (Schärer, 2013). MMR refers to the process of correcting mismatched nucleotide lesions, such as base-base mismatches (G-T instead of G-C) and small insertion/deletion mispairs, due to errors caused during DNA replication, repair, or recombination (Li, 2008). Cells employ mainly the last two pathways, HR and NHEJ, to repair the most deleterious lesions, DNA DSBs (Ceccaldi *et al.*, 2016).

Table 2.1 Characteristics of RONS.

Reactive Oxygen Species	Molecular Formula	Half-Life	Description	Reactivity/Notes	Formation Reaction
Free Radical Species					
Superoxide anion	$O_2^{\bullet-}$	10^{-6} s	One-electron reduction state of ground state molecular oxygen	Relatively unreactive; but can react rapidly with other radicals such as $NO^{\bullet}$ and iron-sulphur proteins	$O_2 + e^- \rightarrow O_2^{\bullet-}$
Hydroxyl radical	$OH^{\bullet}$	10^{-10} s	Three-electron reduction of ground state molecular oxygen	Extremely reactive	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$ (Fenton reaction) $O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^-$ (Haber-Weiss reaction)
Nitrogen monoxide	$NO^{\bullet}$	s	One-electron reduction of nitrite	Relatively unreactive; but reaction with metals or other radicals, including oxygen, is very rapid	L- Arginine + O_2 + NADPH → L – Citrulline + $NO^{\bullet}$ + $NADP^+$
Alkoxyl and Peroxyl radicals	$RO^{\bullet}$ and $ROO^{\bullet}$	10^{-6} and 17 s	One-electron reduction of and one-electron oxidation of hydroperoxide respectively	Alkoxyl radicals are more reactive than peroxyl radicals	$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$
Non-Radical Species					
Singlet oxygen	$^1\Delta_g O_2$	10^{-6} s	Excitation of ground-state molecular oxygen	Highly reactive	$HOCl + H_2O_2 \rightarrow {}^1O_2 + H_2O + Cl^-$
Hydrogen peroxide	H_2O_2	Stable	Two-electron reduction state of ground molecular oxygen	Not very reactive; can easily diffuse across membranes	$O_2 + O_2^{\bullet-} + 2H_2O \rightarrow H_2O_2 + O_2$
Hydroperoxide	ROOH	Stable	Auto-oxidation and singlet oxygen oxidation of unsaturated compounds;	Not very reactive; main product of lipid peroxidation	$ROO^{\bullet} + RH \rightarrow ROOH$
Peroxynitrite	$ONOO^-$	10^{-3} s	Formed in the reaction of nitrogen monoxide with the superoxide radical	Highly reactive/toxic; can directly react with CO_2 to form highly reactive nitroso peroxocarboxylate ($ONOOCO_2^-$) or peroxynitrous acid (ONOOH)	$NO^{\bullet} + O_2^{\bullet-} \rightarrow ONOO^-$
Hypoclorous acid	HOCI	Stable (mins)	Hydrolysis of molecular chlorine	Highly reactive	$H_2O_2 + Cl^- \rightarrow HOCl + OH^-$
Ozone	O_3	s	Oxidation of ground state molecular oxygen with atomic oxygen formed by photolysis of ground state molecular oxygen	Very Reactive	$3\ O_3 \rightarrow 2\ O_3$

Abbreviations: mins, minutes; s, seconds. Adapted from(Radák, 2000; Finaud & Biologie, 2006; Phaniendra & Babu, 2015)

2.3 Biochemistry of RONS

2.3.1 Superoxide Anion Radical ($O_2^{\bullet -}$)

Dioxygen (O_2) can receive two electrons during oxygen metabolism, however it prefers to receive one at a time, thus forming the superoxide anion (equation 1, **Table 2.1**) (Finaud & Biologie, 2006). Superoxide is also formed through the enzymatic process of auto-oxidation (equation 2) (Phaniendra & Babu, 2015). The main enzymes that produce $O_2^{\bullet -}$ are respiratory complexes I (NADH) and II (ubisemiquinone), xanthine oxidase (XO), lipoxygenase, cyclooxygenase (Phaniendra & Babu, 2015; Patlevič *et al.*, 2016).

$$O_2 + e^- \rightarrow O_2^{\bullet -} \text{ (Equation 1)}$$

$$O_2 + Fe^{+2} \rightarrow Fe^{+3} + O_2^{\bullet -} \text{ (Equation 2)}$$

$$O_2 + O_2^{\bullet -} + 2H_2O \xrightarrow{Cu,Zn,Mn\text{-}SOD} H_2O_2 + O_2 \text{ (Equation 3)}$$

It is relatively unreactive with other biomolecules and is mainly produced within the mitochondria, acting as a reducing molecule forming other free radicals. For example, it can react with nitrogen monoxide ($NO^{\bullet}$) rapidly, thus forming peroxynitrite ($ONOO^-$), which is highly toxic (**Table 2.1**) (Radák, 2000; Phaniendra & Babu, 2015).

2.3.1.1 Scavenging of $O_2^{\bullet -}$

Superoxide dismutases (SOD) are a family of scavenger enzymes that regulate the intracellular level of $O_2^{\bullet -}$. SODs are classified into four groups: iron SOD (Fe-SOD), found in the chloroplasts of eukaryotic cells; manganese SOD (Mn-SOD), found mostly in mitochondria but also in peroxisomes; copper–zinc SOD (Cu/Zn-SOD), the most abundant group, found in the cytosol, and the extracellular space; and nickel SOD (Ni-SOD), which has been isolated from Streptomyces bacteria and cyanobacteria (Patlevič *et al.*, 2016). Peroxynitrite can be formed from the reaction of $NO^{\bullet}$ with $O_2^{\bullet -}$, but this can be prevented through dismutation of $O_2^{\bullet -}$, catalysed by Cu/Zn-SOD (equation 3), where one radical is reduced to hydrogen peroxide (H_2O_2) and the other is oxidized to oxygen (Phaniendra & Babu, 2015). The Cu/Zn-SOD contains two subunits, each containing a copper (Cu) and zinc (Zn) , and the reaction occurs in two stages: firstly, the oxidized Cu^{2+} is reduced by $O_2^{\bullet -}$, releasing O_2 (equation 4); and secondly, the reduced Cu^{2+} is oxidized by an adjacent $O_2^{\bullet -}$ and two protons, forming hydrogen peroxide (H_2O_2) (equation 5) (Patlevič *et al.*, 2016).

$$O_2^{\bullet-} + Cu^{2+}/Zn\text{-}SOD \rightarrow Cu^{+}/Zn\text{-}SOD + O_2 \text{ (equation 4)}$$

$$O_2^{\bullet-} + Cu^{+}/Zn\text{-}SOD + 2H^{+} \rightarrow H_2O_2 + Cu^{2+}/Zn\text{-}SOD \text{ (equation 5)}$$

2.3.2 Hydroxyl Radical ($OH^{\bullet}$)

In biological systems, the hydroxyl radical ($OH^{\bullet}$) is generated in the presence of H_2O_2 and iron ions in a two-step process through the Haber–Weiss and Fenton reactions. Firstly, the ferric ion state (Fe^{+3}) is reduced into ferrous iron (Fe^{2+}) in the presence of $O_2^{\bullet-}$ (equation 6), reacting with H_2O_2 to generate the hydroxyl radical; this is termed the Haber-Weiss reaction (equation 7) (Finaud & Biologie, 2006; Patlevič *et al.*, 2016). Secondly, $OH^{\bullet}$ radicals are formed via the Fenton reaction, where H_2O_2 reacts with metal ions (Fe^{2+}) (equation 8) (Phaniendra & Babu, 2015).

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2 \text{ (equation 6)}$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^{-} \text{ (equation 7)}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \text{ (equation 8)}$$

$OH^{\bullet}$ is a highly reactive molecule with a half-life of 10^{-11} seconds and can react strongly with all biomolecules including lipids, proteins and DNA (Finaud & Biologie, 2006). Since there is no known enzymatic scavenging mechanism to target the $OH^{\bullet}$ radical, it has the ability to cause DNA damage, lipid peroxidation and protein oxidation, and if in excess, cell death (Patlevič *et al.*, 2016).

2.3.3 Hydrogen Peroxide (H_2O_2)

Although hydrogen peroxide is not a free radical, as it contains no unpaired electrons, it is still considered a ROS due to its high reactivity and ability to easily diffuse through biological membranes (Finaud & Biologie, 2006). It does not directly damage macromolecules; however, as mentioned, it can lead to the generation of the highly reactive hydroxyl radical· This occurs by reacting with a superoxide anion through the Haber-Weiss reaction (equation 7), which in turn cause severe damage to DNA, proteins, and lipids (Phaniendra & Babu, 2015).

2.3.3.1 Scavenging of H_2O_2

Two enzymes that can remove hydrogen peroxide from cells by converting it into H_2O are: catalase (CAT) and glutathione peroxidases (GPx) (Fang *et al.*, 2017). There are four known subunits of GPx, all of which contain

selenium at their active site (Patlevič *et al.*, 2016). GPx1 is a tetrameric protein consisting of four identical subunits that contain selenocysteine, found in the cytosol of most cells, including red blood cells. GPX-2 is also found in cytosol, primarily in the liver and large intestine. GPx3, a glycoprotein, is found predominantly in epithelial kidney cells. GPx4 is located in mitochondria, and unlike other GPX isoforms, has a monomer structure, which can bind to a wide range of substrates such as hydrogen peroxide, alongside phospholipid and lipid hydroperoxides (Arthur, 2001).

At their enzyme active site, selenol (E-SeH), the active form of selenocysteine, is responsible for catalysing the reduction of H_2O_2, using glutathione (GSH) as the reducing substrate (equation 9). Selenol is oxidized to selenenic acid (E-SeOH), which reacts with GSH to form a selenenyl sulphide adduct (E-Se-SG) (equation 10). A second GSH then regenerates and uses E-Se-SG to form oxidized glutathione (GSSG) (equation 11) and therefore two GSH are oxidized to H_2O, and two sulphide adducts and H_2O_2 are reduced to the corresponding alcohol, selenol (Deponte, 2013; Patlevič *et al.*, 2016).

$$\text{E-SeH} + H_2O_2 \rightarrow \text{E-SeOH} + H_2O \text{ (equation 9)}$$

$$\text{E-SeOH} + \text{GSH} \rightarrow \text{E-Se-SG} + H_2O \text{ (equation 10)}$$

$$\text{E-Se-SG} + \text{GSH} \rightarrow \text{E-SeH} + \text{GSSG} \text{ (equation 11)}$$

Catalase, a heme-containing peroxisomal enzyme, is a potent scavenger of H_2O_2. There are three main forms of CAT: monofunctional, which are mammalian-type tetramers with no peroxidase activity; bifunctional, which act both as a catalase and peroxidase and are active as dimers or tetramers; and pseudo catalases, which are a group of non-heme manganese-containing catalases (Patlevič *et al.*, 2016). CAT can function in two ways, "catalytic" or "peroxidatic" (Lardinois & Rouxhet, 1996). In the catalytic mechanism, the heme Fe^{3+} reduces hydrogen peroxide to water, generating Compound I., This compound is a covalent Fe^{4+}= O species with a porphyrin π-cation radical (equation 12). Compound I then oxidizes another hydrogen peroxide molecule to the ferric oxygen species as water (equation 13) (Lardinois & Rouxhet, 1996; Putnam *et al.*, 2000). In the peroxidatic mechanism, CAT uses hydrogen peroxide to oxidize various compounds (Lardinois & Rouxhet, 1996).

$$\text{Catalase-Fe}^{3+} + H_2O_2 \rightarrow \text{Compound I} + H_2O \text{ (equation 12)}$$

$$H_2O_2 + \text{Compound I} \rightarrow \text{Catalase-Fe}^{3+} + H_2O + O_2 \text{ (equation 13)}$$

2.3.4 Nitric Monoxide ($NO^{\bullet}$)

Nitric monoxide or nitric oxide is a small stable radical containing an unpaired electron in an antibonding $2\pi^*_y$ orbital, and can easily diffuse across the plasma membrane and cytoplasm due to its aquatic and lipid soluble nature (Radák, 2000; Phaniendra & Babu, 2015). It can be generated through various metabolic pathways in mitochondria and the surrounding cell, and there are three known isoforms (NOS) that can lead to its formation: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Lacza *et al.*, 2009; Phaniendra & Babu, 2015). $NO^{\bullet}$ is primarily formed in the cell from the conversion of L-Arginine to L–Citrulline by the three NOS isoforms (equation 14) (Lacza *et al.*, 2009).

$$\text{L-Arginine} + O_2 + \text{NADPH} \rightarrow \text{L–Citrulline} + NO^{\bullet} + NADP^{+} \text{ (equation 14)}$$

Although relatively unreactive, it can react with metals and other radicals such as superoxide to generate peroxynitrite ($ONOO^-$) (equation 15) and oxygen, which form nitrate and nitrite ions (Lacza *et al.*, 2009; Phaniendra & Babu, 2015). In case of an excessive production of reactive nitrogen species such as $NO^{\bullet}$, a state of nitrosative stress can occur potentially leading to the inhibition of normal protein function (Valko *et al.*, 2007).

$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^- \text{ (equation 15)}$$

2.3.5 Peroxynitrite ($ONOO^-$)

The peroxynitrite anion is a highly toxic oxidizing agent generated from the reaction between $NO^{\bullet}$ and $O_2^{\bullet -}$ (equation 15), which can cause DNA breakage and lipid oxidation (Valko *et al.*, 2007). It can also react with carbon dioxide to form other highly reactive molecules such as nitroso peroxocarboxylate ($ONOOCO_2^-$) or peroxynitrous acid (ONOOH), which can be further homolysed to form NO_2 and $OH^{\bullet}$, and thus cause cellular damage (Phaniendra & Babu, 2015).

2.3.6 Peroxyl Radical ($ROO^{\bullet}$)

The peroxyl radical is derived from oxygen, and its simplest form is the perhydroxyl radical ($HOO^{\bullet}$) (equation 16). This radical is the protonated

form of $O_2^{\bullet -}$ and initiates lipid peroxidation (Radák, 2000; Phaniendra & Babu, 2015).

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \text{ (equation 16)}$$

2.3.7 Hydroperoxide (ROOH)

Hydroperoxide is primarily the main product of lipid peroxidation, produced through the auto-oxidation and singlet oxygen oxidation of unsaturated compounds (Radák, 2000).

$$ROO^{\bullet} + RH \rightarrow ROOH \text{ (equation 17)}$$

2.3.8 Hypochlorous Acid (HOCL)

Hypochlorous Acid is generated through the conversion of $\xrightarrow{\text{myeloperoxidase}}$ chloride to HOCL by myeloperoxidase (equation 18) (Phaniendra & Babu, 2015)

$$H_2O_2 + Cl^- \rightarrow HOCl + OH^- \text{ (equation 18)}$$

Myeloperoxidase, a heme enzyme found in neutrophils, is known to generate hydrogen peroxide and superoxide at sites of inflammation. In turn, myeloperoxidase uses hydrogen peroxide to oxidize various substrates, including chloride, to form HOCL (Winterbourn, 2002). Due to its high reactivity, HOCL is involved in both chlorination and oxidation reactions. It can oxidize thiols as well as ascorbate, tryptophan and urate, while its chlorination reactions occur with (1) amines to produce chloramines; (2) tyrosyl residues to produce ring chlorinated products and (3) pyridine nucleotides, unsaturated lipids and cholesterol to produce chlorohydrins (Winterbourn, 2002; Phaniendra & Babu, 2015).

2.3.9 Ozone (O_3)

Ozone is formed from the oxidation of ground state molecular oxygen by solar radiation in the upper atmosphere with atomic oxygen formed by photolysis of ground state molecular oxygen (equation 19) (Radák, 2000).

$$3\ O_3 \rightarrow 2\ O_3 \text{ (equation 19)}$$

Although a non-radical species, it is a very powerful oxidant that can react with different unsaturated compounds, thereby generating free radicals and other intermediates. It can oxidize various functional groups present in proteins and nucleic acids such as amines, alcohols, aldehydes, and it can cause lipid peroxidation (Radák, 2000; Phaniendra & Babu, 2015).

2.4 Sources of RONS Formation

2.4.1 Endogenous Sources

Intracellularly, superoxide anion and other free radicals can be formed endogenously inside the cell through several sources as shown is **Figure 2.2**.

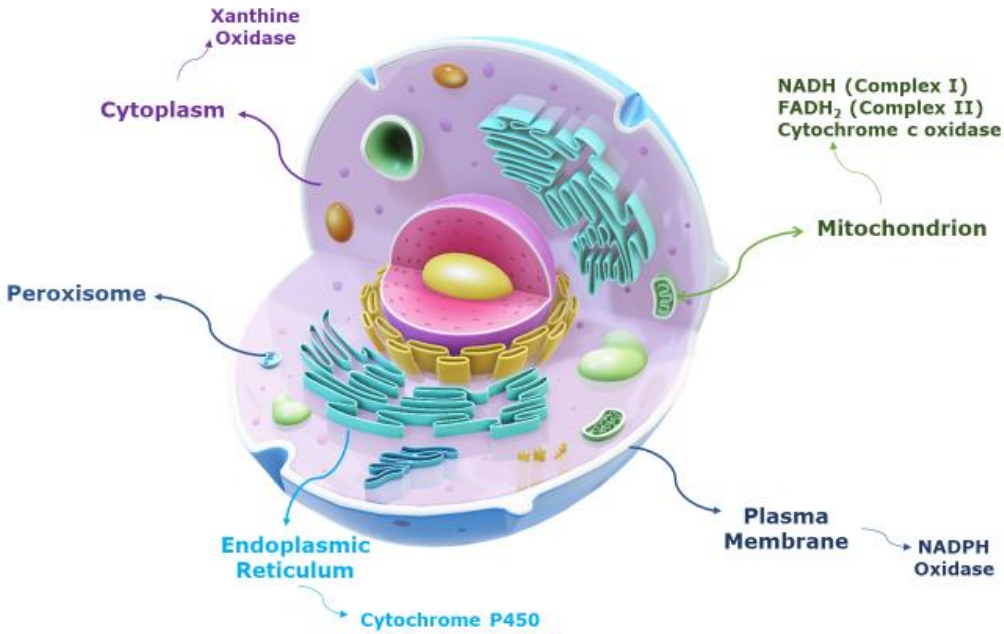


Figure 2.2 Potential endogenous sources of free radical generation.

2.4.1.1 Mitochondria – Electron Transport Chain

The respiratory chain, comprised of four complexes (I, II, III and IV), transfers electrons step by step across the four complexes until they reduce O_2 to form H_2O (Lagouge & Larsson, 2013). This process results in the accumulation of protons, creating an electrochemical proton gradient across the inner mitochondrial membrane. This gradient drives ATP synthesis through complex V (**Figure 2.3a**) (Lagouge & Larsson, 2013).

During oxygen metabolism, the predominant source of free radical production occurs in the mitochondria, a normal consequence of oxidative phosphorylation. This occurs at two major sites in the electron transport

respiratory chain: complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase), where superoxide radicals are generated (Lagouge & Larsson, 2013; Phaniendra & Babu, 2015).

As the by-product of a process named "electron leakage", electrons derived from NADH (complex I) and succinate ($FADH_2$) (complex II) may escape and react with O_2 ,thus generating free radicals (Lagouge & Larsson, 2013). Due to electrons escaping, a reduced form of coenzyme Q10 is formed, QH_2. This regenerates coenzyme Q10 through an unstable intermediate semiquinone anion ($^{\bullet}Q^{-}$), which can form the superoxide anion (equation 20) (Phaniendra & Babu, 2015).

In turn, the reduction of superoxide anion by one electron can generate both the hydroxyl radical and hydrogen peroxide through the two mitochondrial superoxide dismutases (MnSOD and Cu-ZnSOD). Subsequently, hydrogen peroxide can then be converted into H_2O through the actions of GPx and CAT (**Figure 2.3b**) (Lagouge & Larsson, 2013; Phaniendra & Babu, 2015). The hydrogen peroxide that is generated can also be modulated through the intramitochondrial concentration of nitric oxide (Cadenas, 2004).

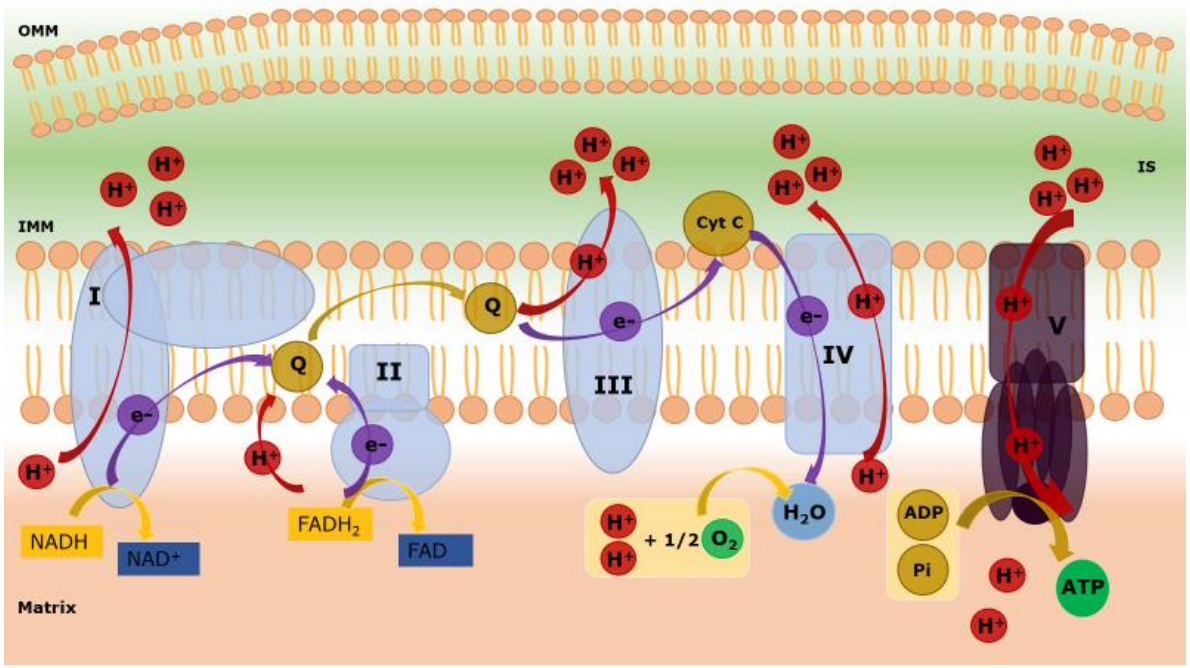
$$QH^2 + O_2 \rightarrow QH^{\bullet} + O_2^{\bullet -}$$

$$QH^{\bullet} + O_2 \rightarrow Q + H^{+} + O_2^{\bullet -}$$

(equation 20)

During normal respiration *in vitro*, the amount of oxygen that is reduced to superoxide, and then further to hydrogen peroxide, is understood to be between 0.12-2%, whereas *in vivo* the % of total oxygen consumption during mitochondrial RONS production is estimated to be much lower (Boveris *et al.*, 1972; Chance *et al.*, 1979).

(a)



(b)

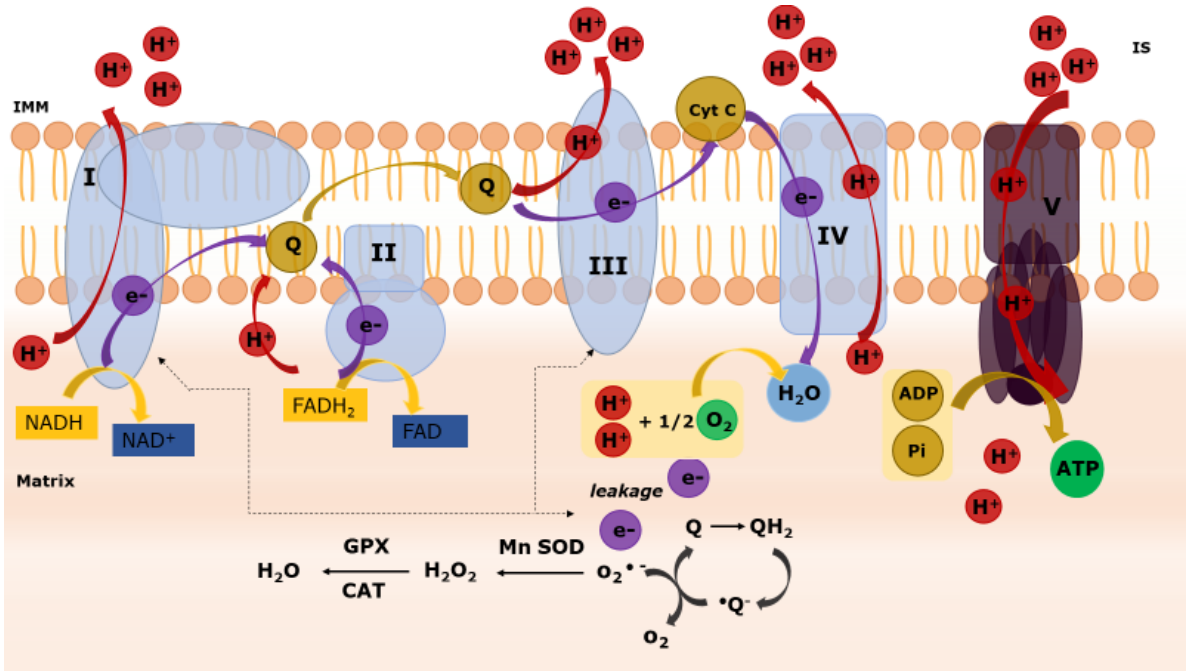


Figure 2.3 (a) The protein complexes of mitochondrial respiratory chain. Electron transport complexes I (NADH dehydrogenase), II (succinate dehydrogenase), III (ubiquinone cytochrome c reductase), IV (cytochrome c oxidase), V (ATP synthase). **(b)** Electrons escape from complexes I and III, leading to the formation of $^{\bullet}Q^{-}$ which reacts with $O_2^{\bullet -}$ to generate H_2O_2,

and in turn, converted to H_2O through the action of GPx and CAT. Adapted from (Lestienne & Rossignol, 2009; Kühlbrandt, 2015).

Abbreviations: IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; IS, intermembrane space; e^-, electron; Q, coenzyme Q10; $^{\bullet}Q^-$, semiquinone anion; QH_2, reduced coenzyme Q_{10}; MnSOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Pi, inorganic phosphate; $O_2^{\bullet-}$, superoxide anion; H_2O_2, hydrogen peroxide; H_2O, water.

2.4.1.2 Peroxisomes

Peroxisomes contain enzymes, such as acyl CoA oxidases, D-amino acid oxidase, L-α-hydroxy oxidase, urate oxidase, xanthine oxidase and D-aspartate oxidase, that oxidize molecules inside cells, and thus generate hydrogen peroxide and other free radical species such as the hydroxyl radical, superoxide anion and nitric monoxide (**Table 2.2**) (Bonetta, 2005; Phaniendra & Babu, 2015).

Table 2.2 Peroxisome enzymes producing RONS.

Enzyme	Molecule	RONS
Acyl CoA oxidases (β-oxidation enzymes)	Fatty acids	H_2O_2
D-amino acid oxidase	D-proline	H_2O_2
L-α-hydroxy oxidase	Glycolate	H_2O_2
Urate oxidase	Uric acid	H_2O_2
D-aspartate oxidase	D-aspartate	H_2O_2
Xanthine oxidase	Xanthine	H_2O_2 , $O_2^{\bullet-}$

Source from (Phaniendra & Babu, 2015).

2.4.1.3 NAPDH (nicotinamide adenine dinucleotide phosphate) oxidase

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme first discovered in phagocytic cells such as neutrophils, undergoes activation during a respiratory burst. this process is essential for bacterial destruction, generating $O_2^{\bullet-}$ (Genestra, 2007; Valko *et al.*, 2007). In an inflammatory environment, this oxidative burst involving neutrophils, leads to an enhanced production of superoxide radicals and other RONS (Valko *et al.*, 2007). NAPDH exists as an enzyme complex containing two membrane-bound polypeptides $p22^{phox}$ (22 kDa) and $gp91^{phox}$ (91 kDa), which together, comprise the centre of the complex, cytochrome b558. This enables electrons to be transferred from the cytosol across the membrane, to

molecular oxygen (Genestra, 2007). There are other cytoplasmic polypeptides that are part of the enzyme complex, $p47^{phox}$ (47kDa), $p67^{phox}$ (67 kDa) and $p40^{phox}$ (40 kDa) as well the small G coupled proteins Rac and Rap1A, all of which when stimulated, translocate to the inner plasma membrane to form a fully active enzyme complex (Genestra, 2007; Valko *et al.*, 2007).

There are other types of non-phagocytic cells, known to modulate intracellular signalling pathways, which generate free radicals through NADPH oxidase such as endothelial cells, fibroblasts, vascular smooth muscle cells and cardiac myocytes. Nonphagocytic cells produce $O_2^{\bullet-}$ at a rate of about one third compared to neutrophils. In contrast to neutrophils, fibroblasts, vascular smooth muscle cells and endothelial cells generate $O_2^{\bullet-}$ and H_2O_2 intracellularly (Genestra, 2007; Valko *et al.*, 2007). Moreover, in vascular cells, the NADPH oxidase isoforms generate $O_2^{\bullet-}$ and other RONS upon the activation of certain growth factors and cytokines (Valko *et al.*, 2007). For example, Angiotensin II drives the generation of NADPH-mediated $O_2^{\bullet-}$ vascular smooth and fibroblasts. Whereas thrombin—a platelet-derived growth factor— and tumour necrosis factor-α (TNF-α) drive the formation of $O_2^{\bullet-}$ in vascular smooth muscle cells. Finally, interleukin-1, TNF-α, and platelet-activating factor all enhance the formation of $O_2^{\bullet-}$ in fibroblasts (Genestra, 2007; Valko *et al.*, 2007).

2.4.1.4 Ischaemia Reperfusion

An ischaemic reperfusion injury occurs usually following surgical interventions, or during high-intensity exercise, when blood flow is restricted in active sites such as skeletal muscles (Finaud & Biologie, 2006). Following exercise or after a surgery, the restricted blood flow to the deprived-oxygen tissues is then restored, and this reintroduction of oxygen generates an excessive pool of RONS, which can subsequently damage surrounding tissue (Valko *et al.*, 2007). During this ischaemia reperfusion mechanism, and in hypoxic tissues, higher ATP consumption leads to the accumulation of hypoxanthine and xanthine which are metabolised into XO, generating $O_2^{\bullet-}$ and H_2O_2 (Valko *et al.*, 2007).

2.4.1.5 Xanthine Oxidase (XO)

XO is found inside the cytoplasm of epithelial cells, belongs to a group of enzymes, known as molybdenum iron–sulphur flavin hydroxy-lases. These enzymes catalyse the hydroxylation of purines, specifically the reaction of hypoxanthine to xanthine, during which molecular oxygen is reduced

forming $O_2^{\bullet-}$, and xanthine to uric acid, in which H_2O_2 is formed (Valko *et al.*, 2004).

2.4.1.6 Haemoglobin and Myoglobin Auto-oxidation

Approximately 3% of total haemoglobin (about 750 g) in the human body undergoes auto-oxidation. This process increases during exercise and releases methaemoglobin and $O_2^{\bullet-}$. Similarly, myoglobin can be oxidized via auto-oxidation or free radicals during ischaemia reperfusion, generating H_2O_2. This, in turn, can react with the myoglobin and form other radicals such as peroxyl or ferryl radicals (Finaud & Biologie, 2006).

2.4.1.7 Cytochrome P450

Cytochrome P450 are a group of oxygenase enzymes, found predominantly in the endoplasmic reticulum (ER) of liver cells and have been shown to play a crucial role in the efficient elimination of foreign chemical agents (*i.e.*, toxins, drugs and heavy metals) from the body (Yue *et al.*, 2018). They are haemoproteins consisting of approximately 500 amino acids, classified into 18 families, and can partially contribute to formation of intracellular $O_2^{\bullet-}$ and H_2O_2, leading to oxidative damage (Nair *et al.*, 2016).

In the catalytic cycle of P450, NADPH-P450 reductase provides electrons that are needed in the activation of atmospheric oxygen (monooxygenase activity). They can also utilise H_2O_2 and other peroxides in the one-electron oxidation of substrates (peroxidase activity), as well as donate oxygen atoms from H_2O_2 and other hydroperoxides to oxygenate substrates (peroxygenase activity) (Zangar *et al.*, 2004; Hrycay & Bandiera, 2015). In addition, cytochrome b5, a small membrane-bound haemoprotein, may also provide electrons for P450 but may be less effective (Zangar *et al.*, 2004).

2.4.2 Exogenous Sources

Externally RONS generated sources are summarized in **Table 2.3** below.

Table 2.3 List of exogenous sources generating RONS.

Exogenous sources	Exposure
Environmental pollution	Water, air
Smoke	Tobacco
Alcohol	Any drinking alcohol
Transition metals	Cd, Hg, Pb, As
Heavy metals	Fe, Cu, Co, Cr
Pesticides, herbicides	Food
Industrial solvents	CCl_4
Ionizing radiation	Environmental, medical
Ultraviolet light	Skin
Cooking	PU fat in vegetable oils, smoked meat
Drugs	Halothane, Paracetamol, Bleomycin, Doxorubicin, Metronidazole, Ethanol

Adapted from (Lobo *et al.*, 2010; Phaniendra & Babu, 2015).

Abbreviations: Cd, cadmium; Hg, mercury; Pb, lead; As, arsenic; Fe, iron; Cu, copper; Co, cobalt; Cr, chromium; CCl_4, tetrachloromethane; PU, polyunsaturated.

2.5 RONS and Oxidative Damage to Biomolecules

It is now well established, that free radicals damage DNA, proteins and lipids, and to a lesser extent, RNA. This oxidative stress can lead to a dysfunctional and compromised cell state. There are numerous biomarkers that have been developed to detect oxidative damage as shown in **Table 2.4**.

2.5.1 Damage to DNA

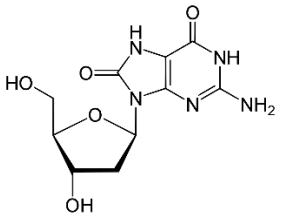
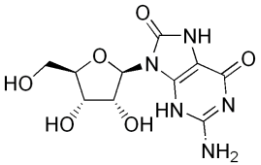
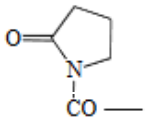
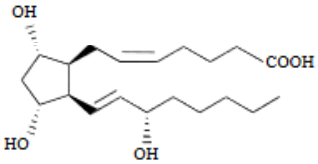
All components of DNA, such as nucleic acid bases and the deoxyribose sugar, are vulnerable to oxidative damage. The highly reactive hydroxyl radical is primarily responsible for causing damage to nucleic acids by either adding or abstracting a hydrogen atom, for example, from the methyl group of thymine and each of the five carbon atoms of 2'-deoxyribose (Dizdaroglu *et al.*, 2002; Finaud & Biologie, 2006). Hydroxyl radicals add a hydrogen atom to the double bonds of the C5- and C6-positions of thymine and cytosine, yielding C5-OH- and C6-OH-adduct radicals in DNA bases.

Whereas, abstraction reactions form the allyl radical of thymine and carbon-centred sugar radicals (Dizdaroglu *et al.*, 2002).

For thymine, the distribution of the addition of hydrogen at the C5 position is 60%, and at C6 at 30%, whereas for cytosine it is 87% and ~10%, respectively, since the C5 position is more electronically dense than C6 (Dizdaroglu & Jaruga, 2012). Furthermore, in the presence of oxygen, peroxyl radicals are formed, as oxygen binds to hydrogen to form C5-OH-adducts, yielding 5-hydroxy-6-peroxyl radicals (Dizdaroglu *et al.*, 2002).Further reactions of the carbon-centred sugar and base radicals can lead to single- and/or double-strand breaks, tandem lesions, and DNA protein cross-links (Dizdaroglu *et al.*, 2002). When oxygen is absent, 5-OH- and 6-OH-adduct radicals of pyrimidines are reduced, forming 5-hydroxy-6-hydro- and 6-hydroxy-5-hydropyrimidines (Dizdaroglu *et al.*, 2002). Moreover, the hydroxyl radical adds hydrogen to purines forming C4-OH-, C5-OH-, and C8-OH-adduct radicals, which upon undergoing dehydration, oxidised purine radicals are formed (Dizdaroglu *et al.*, 2002). The one-electron oxidation of the C8-OH-adduct radical leads to 8-hydroxyguanine (8-OH-Gua), and guanine has the lowest reduction potential amongst the four DNA bases (Dizdaroglu & Jaruga, 2012).

The reduction potential of adenine is greater compared to guanine and therefore it is not as readily oxidized. Hydroxyl radicals add to the double bonds of adenine, just as with guanine, to C4,C8, C5 and C2 distributed at 50%, 37%, 5%, 2%, forming the C4-OH–, C8-OH–, C5-OH– and C2-OH adduct radicals, respectively (Dizdaroglu & Jaruga, 2012).

Table 2.4 Biomarkers of Oxidative Damage and their detection methods in serum, plasma or urine.

Target Molecules of Oxidative damage		Chemical Structure	Detection Methods			
			Molecular	Fluorescence	Chemiluminescence	Analytical
DNA	8-hydroxy-2'-deoxyguanosine (8-OHdG)		·PCR ·qPCR ·LMPCR ·ICPCR ·Ku, XRCC1 (RP) ·γH2AX	·Comet assay (N) ·Comet assay(A) ·Lesion Enzymes ·Comet-FISH ·Halo assay ·TUNEL assay ·DBD-FISH ·RIA	·ELISA ·IHC assay ·IM assay	·HPLC-MS ·GC-MS ·EM
RNA	8-hydroxyguanosine (8-OHG)			·Northwestern blot analysis	·ICH assay ·IP	·HPLC-ECD
Proteins (carbonyl groups)	2-pyrrolidone		·DNPH assay	·Electrophoresis (1 and 2 dimensional)	·IM assay	·MS
Lipids	Malonaldehyde		·TBARS assay ·TBA assay			· HPLC-MS ·GC-MS
	F_2-isoprostanes (8-iso-PGF2α)		·EPR spectroscopy ·FOX-1 assay		·IM assay	·LC-MS ·HPLC-MS ·GC-MS

Adapted from (Manda *et al.*, 2009; Kong & Lin, 2010; Figueroa-González & Pérez-Plasencia, 2017).

Abbreviations: PCR, polymerase chain reaction and agarose gel electrophoresis; qPCR, quantitative PCR; LMPCR, ligation-mediated PCR ; ICPCR, immuno-coupled PCR ; XRCC1, x-ray repair cross complementing 1; RP, repair protein; γH2AX, phosphorylated histone γ2AX protein; Comet assay, single-cell gel electrophoresis; N, neutral; A, alkaline; Comet-FISH, bromodeoxyuridine-labelled DNA-comet fluorescence in situ hybridization; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labelling; DBD-FISH, DNA breakage detection- fluorescence in situ hybridization; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; DNPH assay, 2,4-dinitrophenylhydrazine spectrophotometric assay; IHC, immunohistochemical; IM, immunological; ICH, immunocytochemical; IP, immunoprecipitation; LC, liquid chromatography-mass spectrometry; HPLC-MS, high performance liquid chromatography-electrospray tandem mass spectrometry; HPLC-ECD, high-performance liquid chromatography-electrochemical detection; MS, mass spectrometry; GC-MS, gas chromatography-mass spectrometry; EM, electrochemical methods; EPR, electron paramagnetic resonance; TBARS, thiobarbituric acid reactive substance; TBA, thiobarbituric acid.

2.5.1.1 Biomarkers of oxidative DNA damage

There is a variety of techniques developed for detecting and quantifying DNA damage, as shown in **Table 2.4**. This part of the review will focus on the most commonly used techniques, especially in the field of oxidative stress and exercise.

2.5.1.1.1 Fluorescence

Single-cell gel electrophoresis, or comet assay, is commonly used to detect single or double DNA strand breaks. The concept of electrophoresis was firstly reported in 1984 as a way to measure SSBs introduced by ionizing radiation from single cells under an electric field (Collins *et al.*, 2008). Many years later, a modified version using alkaline conditions was produced, with added advantage of visualising the migration of DNA strands from agarose-embedded cells (Olive & Banáth, 2006).

Inside cells, DNA winds around histone cores, creating (negative) supercoils, which remains supercoiled after lysis(Collins *et al.*, 2008). When breaks are present in negatively charged DNA, the supercoils are relaxed and broken ends can migrate toward the anode under the electrophoretic field. But, if the DNA is not damaged, there is no migration due to large DNA fragments with no free ends (Olive & Banáth, 2006). After electrophoresis, the DNA is

stained with a fluorescent dye and can be seen under microscope. As the broken DNA migrates outside of nucleus, it resembles a comet (**Figure 2.4b**) consisting of a bright fluorescent head (nucleus) and a tail (fragmented DNA) (Dmitrieva & Burg, 2007). The amount of the migrated DNA (size of the tail) provides the measurement of DNA damage in an individual cell (Olive & Banáth, 2006). Since the 1990s, migrating fragments were measured using digitize images using specific software (Olive & Banáth, 2006).

The alkaline comet assay utilizes high pH buffers (pH=13) and causes denaturation, which can uncoil/unwind both single- and double-strand breaks of DNA. That is maybe why that neutral and alkaline comet tails differ in detail, where the DNA in the tail of an alkaline comet with fragments present, may appear more granular, compared to the neutral assay (Collins *et al.*, 2008). It has been claimed that, under neutral pH (<10) buffers, detection of DSB is preferred since the DNA in not unwound. However, this may not be the case as both SSBs and DSBs are identified at neutral pH, since the relaxed supercoils and broken ends can still appear in the tail regardless of pH (Collins *et al.*, 2008). To make that distinction, a modification was suggested where the lysed cells are additionally treated in the agarose solution at 50° with proteinase K. This may disrupt the ''loop'' structure of the DNA and facilitate the release of double-stranded pieces of DNA (Dmitrieva & Burg, 2007).

In addition to strand breaks, the comet assay can be used with specific lesion-enzymes in the detection of specific oxidized base DNA damage or pyrimidine dimers (Figueroa-González & Pérez-Plasencia, 2017). Specifically, endonuclease III is used to detect oxidized pyrimidines, whereas human 8-oxoguanine DNA glycosylase (hOGG1) or formamidopyrimidine DNA glycosylases (FPG) can detect oxidized purines; which can detect the most common guanine oxidation product 8-oxo-7,8-dihydroguanine (8-oxoG, 8-oxo-dG or 8-OHdG) (Collins *et al.*, 1993, 1996).

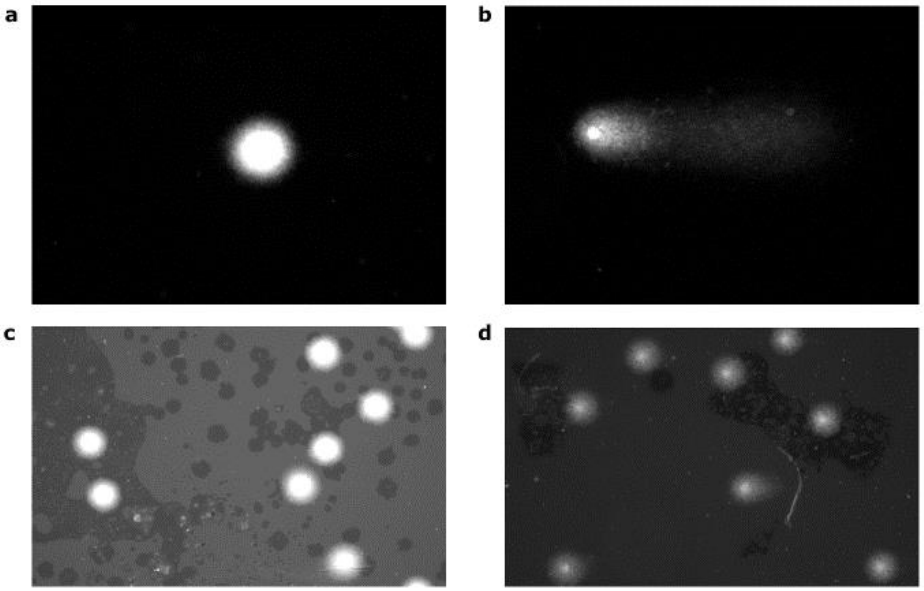


Figure 2.4 Healthy male human lymphocytes at baseline **(a)** undamaged cell (no comet), **(b)** damaged cell (comet tail) at acute aerobic post-exercise, **(c)** healthy female human lymphocytes using alkaline comet assay at baseline, **(d)** cells using neutral comet assay at baseline.

2.5.1.1.2 Molecular

For quantifying DNA damage within the mitochondrial genome, real-time or qPCR are commonly used. These methods can quantify the amount of DNA damage on both strands in a specific gene and expressed mathematically in lesions per kb (Figueroa-González & Pérez-Plasencia, 2017).

Phosphorylated histone 2AX (γH2AX) protein is another common biomarker of double strand breaks (DSB). In detail, γH2AX is a histone variant of the H2A family in mammalian cells (Firsanov *et al.*, 2016). When DSB are formed, H2AX molecules in the chromatin surrounding the DSB site become quickly phosphorylated at serine 139 by the phosphatidylinositol 3-kinase family (PI3) and this leads to the formation of γH2AX foci, which can be observed as small discrete spots following specific immunofluorescence staining (Reddig *et al.*, 2018). The amount of serine 139-γH2AX loci is associated with the quantity of DNA damage (Figueroa-González & Pérez-Plasencia, 2017).

2.5.1.1.3 Chemiluminescence/Analytical

The most common techniques used to quantify 8-OHdG (the most studied biomarker of DNA base oxidation) are: enzyme-linked immunosorbent assay (ELISA); and high-performance liquid chromatography with tandem mass

spectrometry (HPLC-MS). ELISA involves placing an unknown quantity of antigen and antibody to a surface so that the antibody binds to the antigen, which can be quantified by adding a fluorescent/coloured substrate (Figueroa-González & Pérez-Plasencia, 2017). Finally, HPLC-MS is an analytical method commonly preferred for detection of oxidised base products and it may also provide information on the location and quantity of DNA damage (Figueroa-González & Pérez-Plasencia, 2017).

2.5.2 Damage to RNA

When the highly reactive hydroxyl radical is in close proximity to RNA, it can be easily modified (Kong & Lin, 2010). The most common oxidized RNA base product of hydroxyl radical-induced modification is 8-hydroxyguanosine (8-OHG). The hydroxyl radical adds to guanine on the C8 position to form a C8-OH adduct radical and from the loss of an electron (e^-) and a proton (H^+) 8-OHG, an oxidized RNA nucleoside is formed (Kong & Lin, 2010).

2.5.2.1 Biomarkers of oxidative RNA damage

One common technique which is used to measure 8-OHG in serum, plasma and tissues, is high-performance liquid chromatography-electrochemical detection (HPLC-ECD), as shown in **Table 2.4**. Concentrations of 8-OHG were measured in the cerebrospinal fluid (CBF) and serum of patients suffering from Alzheimer's disease, where it was found to be five times greater compared to matched controls and also illness duration dependent, since 8-OHG decreased as duration of the illness decreased (Abe *et al.*, 2002). Similar results were observed in patients with Parkinson's disease, where the concentration of 8-OHG in cerebrospinal fluid was found to decrease significantly with the duration of the disease. Additionally, it was increased three-fold compared to controls. However, this increase was not mirrored in serum samples, suggesting that the 8-OHG concentration reflects changes in brain tissue rather than serum. This could indicate the involvement of oxidized RNA in the early stages of Parkinson's disease development (Abe *et al.*, 2003).

Immunocytochemistry techniques and the use of antibodies have been utilized to detect 8-OHG levels and show the involvement of RNA oxidation in a variety of neurological disorders, such as Alzheimer's, Parkinson's, down syndrome and prion diseases (Kong & Lin, 2010).

Another method of detecting RNA oxidation products is through northwestern immunoblot analysis. A study used this technique to measure

RNA oxidation (using an antibody which recognized 8-OHG) within the ribosome complexes, using ribosomal RNA from individuals with Alzheimer's disease and mild cognitive impairment (Ding *et al.*, 2006). They observed elevated levels of 8-OHG in the fractions of precursor and mature ribosome fractions in the Alzheimer's disease subjects, while in the mild cognitive impairment subjects this increase was observed only in the precursor ribosome fractions. This suggests that Alzheimer's disease subjects may exhibit maximal levels of oxidative RNA damage (Ding *et al.*, 2006).

Finally, immunoprecipitation has also been used via the use of antibodies to separate oxidized RNAs from non-oxidized RNAs and thereby quantifying oxidized RNA levels and identifying oxidized RNA species. This has been done using Alzheimer's disease and amyotrophic lateral sclerosis post-mortem tissues (Shan & Lin, 2006; Chang *et al.*, 2008).

2.5.3 Damage to Lipids

Lipids are classified into two groups, apolar and polar; triglycerides, which belong to the apolar group, are the primary form of energy storage in mammals and are stored in various cells, but mainly in adipose (fat) tissue (Ayala *et al.*, 2014). Polar lipids are the structural components of cellular membranes that are responsible for the formation of lipid bilayers controlling cell barrier permeability, and subcellular organelle structure The most important lipid present for defining the bilayer is the glycerol-based phospholipid (Ayala *et al.*, 2014).

Lipid peroxidation is the damage caused by the formation of lipid peroxidation products. This process is essentially a chain reaction initiated by the hydrogen addition or abstraction of an oxygen radical. This leads to polyunsaturated fatty acids (PUFA) being oxidatively damaged in three steps: initiation, propagation and termination, as shown in **Figure 2.5** (Repetto *et al.*, 2012)**.**

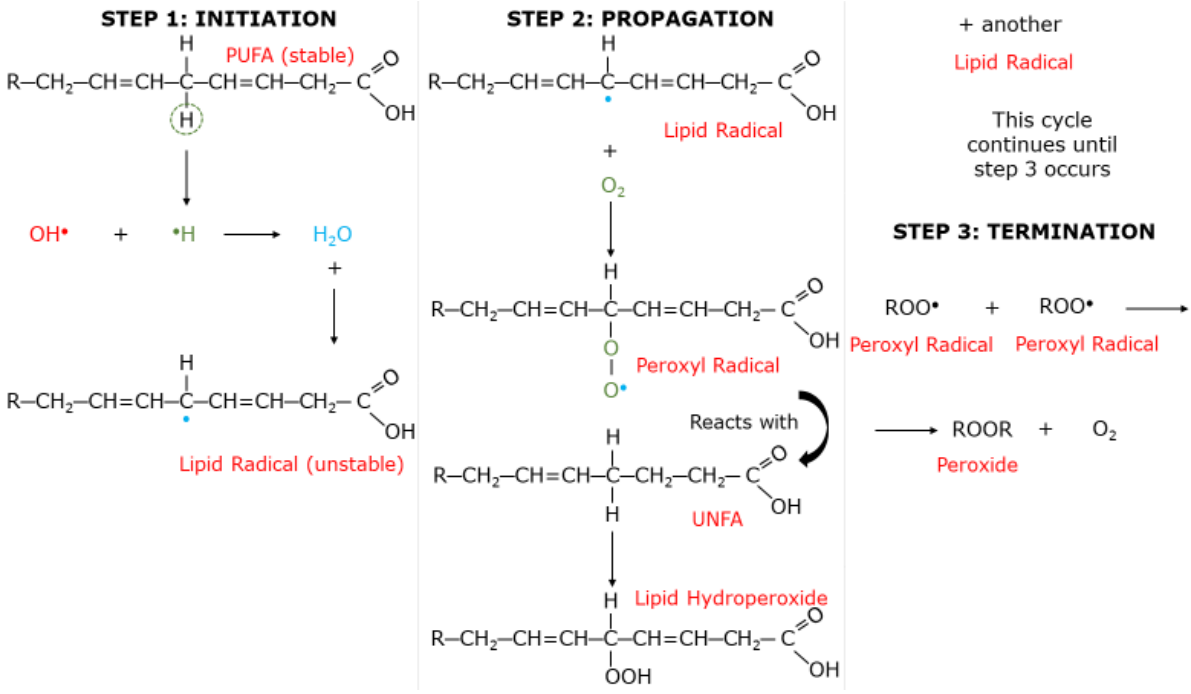


Figure 2.5. The three distinct steps of lipid peroxidation: initiation, propagation and termination. Adapted from (Ayala *et al.*, 2014). Abbreviations: PUFA, polyunsaturated fatty acid; UNFA, unsaturated fatty acid.

As shown in **Figure 2.5**, the activated methylene bridge of the PUFA is a sensitive target for damage, as the presence of the double bonds adjacent to a methylene group makes the C-H bond weaker, and thus more susceptible to abstraction. This leads to an unpaired electron on the carbon, thereby forming a carbon-centred radical, which is described as the initiation step (El-Aal, 2012; Repetto *et al.*, 2012). Following this, in the second step of lipid peroxidation, known as propagation, the formed unstable lipid radical ($R^{\bullet}$) reacts with O_2 to form a peroxyl radical ($ROO^{\bullet}$). This can then react with other unsaturated fatty acid to form organic hydroperoxides (ROOH) (Ayala *et al.*, 2014). The formation of ROOH reacts with an additional PUFA by hydrogen abstraction, and therefore continues a cycle of the same propagation chain reactions, resulting in numerous PUFA being converted to lipid hydroperoxides (Repetto *et al.*, 2012; Ayala *et al.*, 2014). This cycle is terminated when two $ROO^{\bullet}$ react together and termination products are produced, such as cytotoxic aldehydes and hydrocarbon gases such as ethane and pentane. However, the chain can also terminate when an

antioxidant, such as vitamin E, donates a hydrogen atom to $ROO^{\bullet}$ forming a vitamin E radical to generate a nonradical product (Repetto *et al.*, 2012; Ayala *et al.*, 2014).

The primary product of the lipid peroxidation cascade is lipid hydroperoxides, which are relatively stable molecules. When measured in blood, lipid hydroperoxides can predict the level of oxidative stress (Ayala *et al.*, 2014). In the presence of transition metals, such as reduced iron ions, lipid hydroperoxides can be converted to alkoxyl radicals ($LO^{\bullet}$), whereas in the presence of oxidized metals such as Fe^{3+}, they can generate peroxyl radicals, both of which can react to adjacent lipids to continue the propagation of lipid peroxidation (Ayala *et al.*, 2014).

However, secondary products can be formed including multiple aldehydes, which are produced from the fragmentation of peroxides: malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE). While MDA is considered the most mutagenic product of lipid peroxidation, it is generally stable and has a good membrane-permeability, while 4-HNE is considered the most toxic (Valko *et al.*, 2004; Ayala *et al.*, 2014). The latter is due to the ability of 4-HNE to cause the inhibition of gene expression, which in excessive amounts, can induce cell death via irreversible damage and apoptosis (Ayala *et al.*, 2014). MDA and 4-HNE are both end-products generated from the degradation of arachidonic acid or larger PUFAs via enzymatic and non-enzymatic processes (Ayala *et al.*, 2014).

During the decomposition of arachidonic acid, cyclooxygenases act on AA to form prostaglandin endoperoxide, or prostaglandin H_2 (PGH_2). The enzyme thromboxane A_2 (TXA_2) synthase is involved in the biosynthesis of TXA_2, an active metabolite produced through the decomposition of AA by acting on PGH_2. As a result of this process, MDA is generated as a side product (Ayala *et al.*, 2014). Moreover, MDA is also generated through non-enzymatic process by bicyclic endoperoxides produced during lipid peroxidation. Once formed, it can be metabolized enzymatically or react with cellular/intracellular proteins or DNA to form adducts, which cause damage to biomolecules (Ayala *et al.*, 2014).

4-HNE is generated as an end-product of lipid peroxidation through the 15-lipoxygenases enzymes. This transform *n*-6 PUFAs, such as arachidonic acid and linoleic acid, but 4-HNE can also be formed non-enzymatically via various oxygen radical-dependent pathways that involve the formation of

hydroperoxides, alkoxyl radicals, epoxides, and fatty acyl crosslinking reactions (Ayala *et al.*, 2014). 4-HNE may act as second messenger molecules as well as a toxic product of lipid peroxidation, which is evident in the pathogenesis of diabetes mellitus (Jaganjac *et al.*, 2013). Furthermore, 4-HNE may induce protein oxidation and thereby play a role in the pathogenesis of neurodegenerative diseases (Perluigi *et al.*, 2012).

2.5.3.1 Biomarkers of oxidative lipid damage

There are various analytical techniques to measure lipid peroxidation quantitatively or qualitatively. These include the measurements of primary or secondary products as well as fatty acid losses (Desai *et al.*, 2014). A widely used biomarker of lipid peroxidation is the MDA biomarker. MDA reacts with thiobarbituric acid (TBA) to yield an intensely coloured chromogen fluorescent red adduct and thus the thiobarbituric acid reacting substances (TBARS) assay was developed. However, this assay has been widely criticized for its low specificity (Repetto *et al.*, 2012; Ayala *et al.*, 2014; Cobley *et al.*, 2017). Other methods have therefore been developed to determine the concentration of free and total MDA, such as gas chromatography-mass spectrometry (GC-MS) and HPLC-MS, as displayed in **Table 2.4** (Ayala *et al.*, 2014). Furthermore, since LOOH are the primary product of lipid peroxidation, they can be used to detect lipid peroxidation directly via HPLC, GC, MS and spectrophotometric assays (Desai *et al.*, 2014) and lipid-derived peroxyl radicals via Electron Paramagnetic Resonance (EPR) Spectroscopy (North *et al.*, 1992).

Lastly, F_2-Isoprostanes (8-iso-PGF2α) is widely utilized as a biomarker due to its properties as a prostaglandin-like compound generated through the non-enzymatic degradation of arachidonic acid during lipid peroxidation. This biomarker is generally regarded as an accurate and reliable method to assess *in vivo* oxidative stress that stems from lipid peroxidation (Milne *et al.*, 2005).

2.5.4 Damage to Proteins

$OH^{\bullet}$ can abstract a hydrogen atom from the protein polypeptide backbone, forming a carbon-centred radical (equation 21). $OH^{\bullet}$ reacts with O_2 under aerobic conditions, to yield peroxyl radicals (equation 22), which then combine with the protonated form of superoxide ($HO_2^{\bullet}$) to form alkyl peroxide (equation 23). Furthermore, the alkyl peroxide reacts with $HO_2^{\bullet}$ to generate an alkoxyl radical (equation 24),which in turn reacts with $HO_2^{\bullet}$ to form a hydroxyl derivative (equation 25) (Stadtman, 2004).

$$\sim NHCRHCO\sim + OH^{\bullet} \rightarrow \sim NHC^{\bullet}RCO\sim + H_2O \text{ (equation 21)}$$

$$\sim NHC^{\bullet}RCO\sim + O_2 \rightarrow \sim NHCOO^{\bullet}RCO\sim \text{ (equation 22)}$$

$$\sim NHCOO^{\bullet}RCO\sim + HO_2^{\bullet} \rightarrow \sim NHCOOHRCO\sim + O_2 \text{ (equation 23)}$$

$$\sim NHCOOHRCO\sim + HO_2^{\bullet} \rightarrow \sim NHCO^{\bullet}RCO\sim + H_2O + O_2 \text{ (equation 24)}$$

$$\sim NHCO^{\bullet}RCO\sim + HO_2^{\bullet} \rightarrow \sim NHCOHRCO\sim + O_2 \text{ (equation 25)}$$

Additionally, the alkoxyl radical derivatives of proteins may undergo peptide bond cleavage by either of two mechanisms: the diamide pathway or the α-amidation pathway, which both lead into two peptides. Last but not least, hydroxyl radical can also cause peptide bond cleavage by attacking glutamic acid and proline protein residues to form a mixture of products, such as 2-pyrrolidone, which are all protein carbonyl derivatives (Stadtman, 2004).

RONS can oxidize amino acids that are a part of the main structure of proteins and can consequently cause denaturation, loss of function, protein–protein cross linkages, loss of enzyme activity, as well as loss of function of receptors and transport proteins (Phaniendra & Babu, 2015). In particular, the residues of the sulphur-containing amino acids, methionine and cysteine, are quite prone to being oxidized by RONS as their oxidation can lead to the formation of disulphides and methionine sulfoxide respectively, which can both be repaired (Stadtman, 2004; Valko *et al.*, 2007; Phaniendra & Babu, 2015).

An attack by RONS on amino acids can lead to the formation of various oxidation products and amino acid residues, including lysine, proline, threonine, and arginine, all of which can yield carbonyl groups. As detailed in **Table 2.5**, an increase in these carbonyl groups has been associated with multiple pathological disorders, such as Alzheimer's Disease, Parkinson's Disease, Rheumatoid arthritis, muscular dystrophy, progeria, diabetes, atherosclerosis, Werner’s syndrome, and is also involved in the aging process (Valko *et al.*, 2007; Phaniendra & Babu, 2015).

Table 2.5. Amino acid residues and their oxidation products formed.

Amino Acid Residue	Oxidation Products
Arginine	Glutamic semialdehyde
Cysteine	Disulfides: Cys-S-S-Cys, Cys-S-S-R, CysSOH, CysSOOH, $CysSO_2H$
Glutamate	4-Hydroxyglutamate, pyruvate, ketoglutarate
Histidine	2-Oxohistidine, aspartic acid, asparagine
Leucine	3- and 4-hydroxy-leucine
Lysine	2-aminoadipic-semialdehyde, 3-,4-, and 5-hydroxy-lysine
Methionine	Methionine sulfoxide, methionine sulfone
Phenylalanine	2-,3-, and 4-hydroxy-phenylanine, 2-,3-dihydroxy-phenylanine
Proline	Glutamic semialdehyde, pyroglutamic acid, 2-Pyrrolidone, 4-,5-hydroxy-proline
Threonine	2-amino-3-ketobutyric acid
Tryptophan	N-formyl-kynurenine, kynurenine, 2- ,4- ,5-, 6, and 7- hydroxy-tryptophan, nitrotryptophan
Tyrosine	3-4-dihydroxy-phenylanine, Tyr-Tyr cross-linked proteins, 3-nitrotyrosine, 3,5-dichloro-tyrosine
Valine	3-, 4–hydroxy-valine

Source from (Sitte, 2003; Stadtman, 2004).

In addition to the hydroxyl radical, proteins can be altered by peroxynitrite and the mechanism that this occurs is through the nitric oxide. Nitric oxide is produced in the metabolism of the amino acid arginine, as it reacts quickly with $O_2^{\bullet -}$, forming the highly toxic $ONOO^-$ (equation 15). Specifically, $ONOO^-$ has the ability to oxidize methionine and cysteine residues but its ability to take part in these reactions is influenced by the presence of CO_2. Thus, when $ONOO^-$reacts with CO_2, it produces the highly reactive nitroso $ONOOCO_2^-$ (equation 26).

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \text{ (equation 26)}$$

However, the ability of $ONOO^-$ to oxidize methionine and cysteine residues, is inhibited by physiological concentrations of CO_2 (Stadtman, 2004).

2.5.4.1 Biomarkers of oxidative protein damage

Protein carbonyl groups can be generated via multiple mechanisms and their concentration is widely used as a marker of protein oxidation. Several sensitive assays, such as the 2,4-dinitrophenylhydrazinespectrophotometric assay, have been developed to measure the concentration of carbonyl groups during protein oxidation (Mesquita *et al.*, 2014). Another method of measuring protein oxidative damage is 1- or 2-dimensional gel electrophoretic detection of mitochondrial or non-mitochondrial protein carbonyls derivatized by biotin-hydrazide (Gallagher, 2012; Wu *et al.*, 2016).

2.6 Antioxidants

Antioxidants are molecules that can prevent or reduce the oxidation of other molecules, including free radicals, by accepting or donating electrons (s), thus neutralizing them and stabilizing the molecule (Lü *et al.*, 2010). Antioxidants can be categorized/characterized in various ways: (1) according to whether they are produced endogenously or exogenously; (2) according to their activity they can be enzymatic or nonenzymatic; (3) according to their solubility they can be water- or lipid-soluble; (4) their size, low or high molecular weight antioxidants; and (5), as natural or synthetic (Nimse & Pal, 2015; Aziz *et al.*, 2019). There are nine classifications of antioxidant molecules, which are shown in **Table 2.6,** and a detailed classification of antioxidants is shown in **Figure 6a** and **6b**.

Table 2.6. Antioxidant classifications, categories and examples.

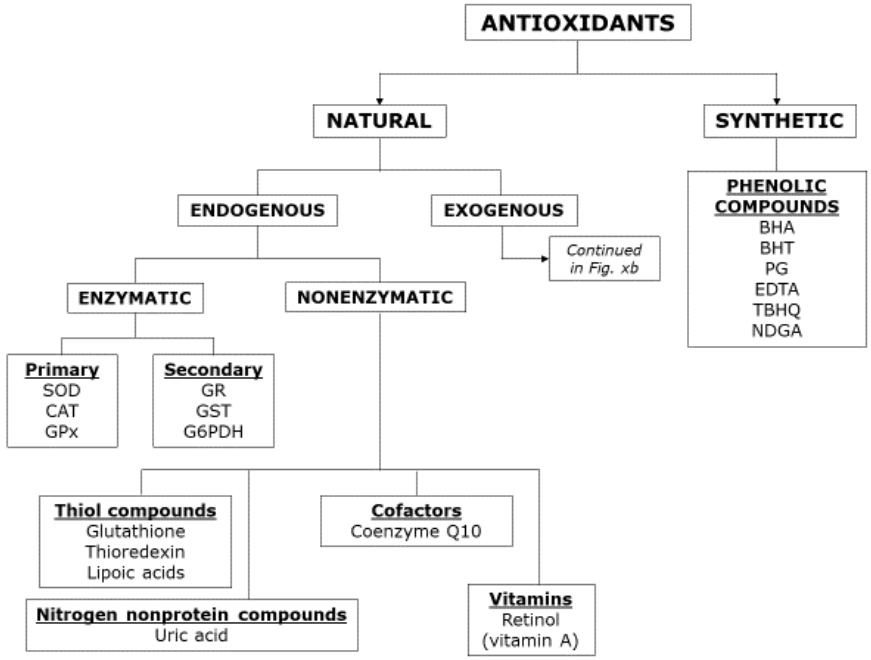
Classification	Category	Examples
Antioxidant C	Carotenoids	β-carotene, Lycopene, Lutein
Antioxidant E	Enzymes	SOD, Catalase, GPx
Antioxidant G	Glutathione	Glutathione
Antioxidant H	Hormones	Melatonin, Oestrogen
Antioxidant L	Lipid associated chemicals	Ubiquinol-10, N-acetyl cysteine, lipoic acid
Antioxidant M	Minerals	Zinc, Selenium, Copper
Antioxidant P	Phenolics	Quercetin, Catechin
Antioxidant S	Saponines, Steroids	Cortisone, Oestradiol, Estriol
Antioxidant V	Vitamins	α-tocopherol, Ascorbic acid

Source from (Flora, 2009).

2.6.1 Endogenous Enzymatic Antioxidants

Natural-occurring antioxidants can be characterized as enzymatic or nonenzymatic; enzymatic antioxidants are the cell's best internal defence, as their main role is to catalyse reactions to neutralize RONS (Aziz *et al.*, 2019). Amongst these enzymatic antioxidants, the primary enzymes produced in the cell that provide immediate defence are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Whereas, secondary enzymatic antioxidants include glutathione peroxidase (GPx), Thioredoxin and peroxiredoxin (Lü *et al.*, 2010). Most of these enzymes require micronutrient cofactors, such as copper (Cu), zinc (Zn), manganese (Mn), iron (Fe) and selenium (Se), in order for their catalytic activity to work optimally (Aziz *et al.*, 2019).

(a)



(b)

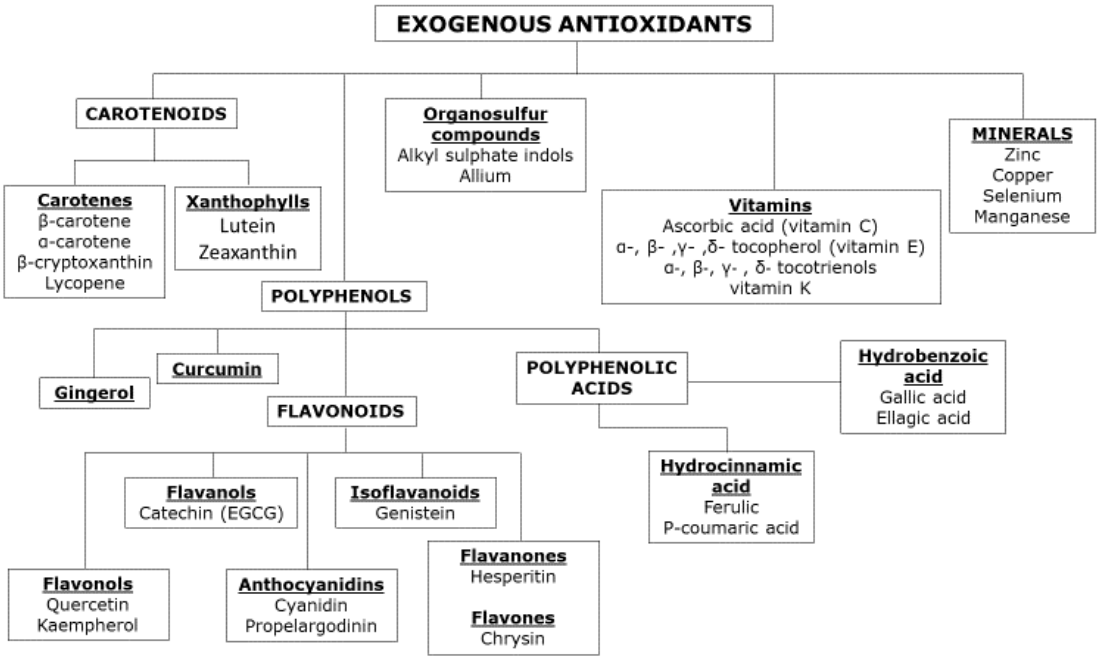


Figure 2.6 (a) and (b). Classification and subclassification of antioxidants. Adapted from (Anwar *et al.*, 2018).

2.6.1.1 Superoxide Dismutase (SOD)

Perhaps one of the most effective antioxidants enzymes is SOD, which belongs to a group of enzymes called oxidoreductases and was discovered in 1967 by Irwin Fridovitch and Joe McCord (Aziz *et al.*, 2019). In mammalian cells, there are three forms of SOD enzymes, each characterized by their metal cofactors, containing one or two atoms of different transition metals: cytosolic SOD, extracellular (Cu/Zn-SOD) and mitochondrial SOD (Mn-SOD)(Lü *et al.*, 2010; Nimse & Pal, 2015). The SOD enzymes convert two $O_2^{\bullet-}$ into O_2 and H_2O_2 (equation 4 & 5; section 2.3.1.1). The Cu/Zn-SOD consists of two identical subunits (32 kDa), each one containing a metal cluster, an active site, and a Cu and Zn atom bridged by a histamine residue. The Mn-SOD is a homotetramer (96 kDa) with each of its subunits containing one Mn atom that cycles from Mn^{3+} to Mn^{2+} and back during the oxidation and reduction of $O_2^{\bullet-}$ (Aziz *et al.*, 2019). Extracellular SOD is a tetrameric protein consisting of Cu and Zn, and as the name suggests, exists mainly in the extracellular membrane and (in lesser amounts) in extracellular fluids; its protective role acts by inactivating NO released from the endothelium through $O_2^{\bullet-}$ diffusion to smooth muscle (Aziz *et al.*, 2019). Thus, studies suggest it can play an integral part in diseases such as hypertension, ischemia reperfusion injury, and lung injury, where endothelial function can be compromised (Aziz *et al.*, 2019).

2.6.1.2 Catalase (CAT)

As mentioned in section 2.3.3.1, CAT is one of the main enzymes responsible for scavenging H_2O_2. Human CAT is a tetrameric structure containing four identical subunits of 60 kDa, and each subunit contains one heme group with a molecular mass of 240 kDa (Aziz *et al.*, 2019). In the presence of a high H_2O_2 concentration, it acts in a catalytic manner by converting H_2O_2 to H_2O and O_2. However, in low H_2O_2 concentrations, and in the presence of a hydrogen donor such as methanol, ethanol, it acts peroxidically by removing H_2O_2 and oxidizing its substrate (Nandi *et al.*, 2019).

2.6.1.3 Glutathione Peroxidase (GPx)

GPx was firstly described in 1957 as an erythrocyte enzyme that protects haemoglobin from oxidative breakdown (Mills, 1957). There are 5 GPx isoenzymes known to exist in mammals but GPx1 is thought to be the main enzyme that is found in the cytosol, mitochondria and peroxisome compartments that is responsible for removing H_2O_2 from cells (Li *et al.*, 2000; Aziz *et al.*, 2019).

GPx, another essential primary antioxidant enzyme, is a selenocysteine protein containing four identical subunits integral for scavenging H_2O_2, converting it into H_2O as part of an intricate antioxidant defence system as shown in **Figure 2.7** (Aziz *et al.*, 2019). GPx requires secondary enzymes such as GR and G6PDH, as well as cofactors such as GSH and NADPH, which enable its efficient function (Li *et al.*, 2000).

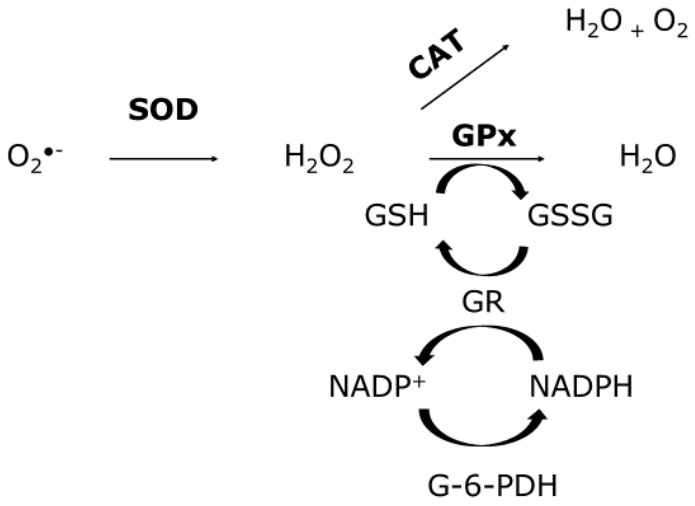


Figure 2.7. Antioxidant defence system involving primary, secondary enzymes and cofactors. Sourced from (Li *et al.*, 2000).
Abbreviations: SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH; reduced $NADP^+$.

2.6.2 Endogenous Nonenzymatic Antioxidants

There are various compounds which function as nonenzymatic antioxidants through free radical scavenging inside cells.

2.6.2.1 Glutathione

Glutathione (γ-glutamyl-cysteinyl-glycine) is a tripeptide synthesized from glutamate, cysteine and glycine by γ-glutamylcysteine synthetase and glutathione synthetase (Ginter *et al.*, 2014; Aziz *et al.*, 2019). (Ginter *et al.*, 2014; Aziz *et al.*, 2019). It is the most abundant intracellular antioxidant defending cells against RONS, and its main role is to act as a substrate for RONS scavenging enzymes (Hrycay & Bandiera, 2015; Aziz *et al.*, 2019). It exists mainly in mammalian cells in its reduced form (GSH), and in small

amounts in its oxidized form (GSSG), acting as a potent reducing agent and cofactor for GPX, GR and GST (Hrycay & Bandiera, 2015). However, it can also directly scavenge free radical species (Young & Woodside, 2001). Finally, it has a vital role in the regeneration of vitamin C and E that generally contributes to the overall cellular redox balance (Hrycay & Bandiera, 2015).

2.6.2.2 Thioredoxin

Thioredoxin (Trx), an oxidoreductase protein, is found in a plethora of organisms, from archae to mammals. First discovered in 1964 in Escherichia coli as an electron donor for ribonucleotide reductase, which is an enzyme required for DNA synthesis (Moore *et al.*, 1964; Collet & Messens, 2010). Trx plays multiple roles in the cell, including: acting as a reductase unit in redox control mechanisms; modulating stress caused by sources such as RONS and peroxynitrite; protecting proteins against oxidative damage/inactivation; and regulating cell apoptosis through protein denitrosylation (Collet & Messens, 2010).

2.6.2.3 Lipoic acid

Lipoic acid, also known as α-lipoic acid (ALA), is a short-chain fatty acid and a potent antioxidant; it scavenges free radicals such as the hydroxyl radical and singlet oxygen radicals, and supresses the formation of new free radicals by bonding with metals like iron and copper (El Barky *et al.*, 2017). There are two forms, the R form, which is the main active form, and the S form, which can be chemically synthesized and is not biologically active (El Barky *et al.*, 2017). ALA is reduced to dihydrolipoic acid (DHLA), which is also a free radical scavenger (Aziz *et al.*, 2019). Both ALA and DHLA are generally characterized by: their free radical scavenging properties; their metal chelating ability; their capacity to regenerate endogenous antioxidants such as vitamin C, E, GSH; as well as their ability to repair oxidized proteins (Biewenga *et al.*, 1997).

2.6.2.4 Uric acid

Uric acid is the most abundant aqueous antioxidant found in human blood, estimated to contribute to approximately 60% of free radical scavenging capacity in plasma (Nimse & Pal, 2015; Aziz *et al.*, 2019). It is produced from xanthine and it can prevent the formation of RONS during the action of XO in the catalysis of hypoxanthine to xanthine (Ginter *et al.*, 2014; Aziz *et al.*, 2019). Although it cannot scavenge $O_2^{\bullet -}$, it is a highly efficient scavenger of peroxynitrite (ONOO-) in the extracellular fluid (Nimse & Pal,

2015). Furthermore, it important to note that in order to scavenge ONOO-, it requires the presence of AA and thiols, as neither of these antioxidants can act alone to efficiently scavenge ONOO-. Thus, uric acid is a crucial component of ONOO- scavenging (Nimse & Pal, 2015). Since uric acid is adequately produced *in vivo*, it is not required to be sourced from exogenous intake (Ginter *et al.*, 2014).

2.6.2.5 Coenzyme Q10

In humans, coenzyme Q10 (also known as coenzyme Q, CoQ10, CoQ, ubiquinone, ubiquinone-Q10 or vitamin Q10) is a naturally occurring lipid-soluble benzoquinone ring structure, which as the name suggests, has 10 isoprenyl units in its side chain. Although, in other species this number varies, and as mentioned in section 2.4.1.1, it is a key part of the mitochondrial transport chain for ATP synthesis (Saini, 2011; Liu *et al.*, 2016). It was first isolated in the mitochondria of bovine heart tissue in 1957 and is located in the inner membrane of mitochondria, other membranes and in plasma lipoproteins; the amount present in membranes depends on membrane type (Prakash *et al.*, 2010). It can inhibit lipid peroxidation by preventing the generation of lipid peroxyl radicals during the initiation process and interfere with the propagation step of lipid and protein oxidation, thus it is a very effective antioxidant (Bentinger *et al.*, 2007).

2.6.2.6 Oestrogen

Amongst the four major groups of naturally occurring lipid-soluble antioxidants carotenoids, tocopherols and coenzyme Q10, there are oestrogens (Bentinger *et al.*, 2007). There are three major oestrogens: 17β-oestradiol (E2), estrone (E1) and estriol (E3). E2 is the most potent during the reproductive years, whereas E1 and E3 are present at lower levels (Melmed *et al.*, 2011). The beneficial effects of oestradiol have been documented as having a cardioprotective role and a strong *in vitro* antioxidant effect (Viña *et al.*, 2006).

However, the possible antioxidant action of oestrogen *in vivo* is unlikely to be due to its chemical phenolic structure, as physiological levels are not potent to exert an antioxidant effect (Viña *et al.*, 2006). In fact, the antioxidant effect may occur due to the upregulation and expression of antioxidant genes (Viña *et al.*, 2006; Borrás *et al.*, 2010). This may be a receptor mediated effect as low/physiological concentrations of oestradiol (equivalent dose to circulating levels in menstrual women) act through oestrogen receptors to reduce hydrogen peroxide levels in mammary gland

tumour cells (MCF-7 cells), thereby exerting an antioxidant effect, while an oestrogen receptor antagonist blocks this effect (Borrás *et al.*, 2005; Viña *et al.*, 2006). Furthermore, oestrogens have been shown to activate MAP kinases through an increase in ERK1 and ERK2 (MAPK) after incubation of MCF-7 cells with E2, and in turn this activates the translocation of nuclear factor kappa B (NF-κB) (Borrás *et al.*, 2005). In addition, co-incubation with inhibitors of both MAPK (MEK phosphorylation inhibitor UO126) and NF-κB (pyrrolidine dithiocarbamate; PDTC) blocks the antioxidant effect of E2 (Borrás *et al.*, 2005). Therefore, receptor activation via oestrogen can lead to activation of MAPK and NF-κB signalling pathways, which in turn, may drive the upregulation of MnSOD and GPx, as their expression was shown to be increased 3- and 2-fold respectively; this increase was prevented after cells were co-incubated with UO126 and PDTC inhibitors (Borrás *et al.*, 2005). Thus, physiological levels of oestrogen may have a non-genomic antioxidant effect by upregulating antioxidant enzymes via activation of MAPK and NF-κB signalling pathways.

As a result of this antioxidant effect, mitochondria in females may exhibit increased antioxidant gene expression compared to males, which has been shown in rats (Borrás *et al.*, 2003). In more detail, liver mitochondria from female rats has been shown to generate 50% less peroxides than male, GSH levels are also higher in female compared to male, and finally mitochondrial DNA damage in male rats is 4 times higher than in females (Borrás *et al.*, 2003). As females live approximately 10% more than males in most species, including humans, and in the context of the mitochondrial theory of aging, oestrogens may provide an added advantage to females by protecting against ageing free radical oxidative damage via the upregulation of antioxidant genes (Viña *et al.*, 2006).

2.6.3 Exogenous Antioxidants

2.6.3.1 Ascorbic acid (Vitamin C)

Ascorbic acid (or L-ascorbic acid; AA) is a water-soluble vitamin required for numerous biological functions, and is one of the most salient antioxidants as it has the capability of penetrating into cell to scavenge free radicals such as superoxide, thus preventing oxidative damage (Tariq, 2007). Some of its other functions include: its role as an essential enzyme co-factor for collagen synthesis; biosynthesis of carnitine; conversion of the neurotransmitter

dopamine to norepinephrine and in tyrosine metabolism; as well as its role in iron absorption (Pehlivan, 2017).

2.6.3.1.1 Biosynthesis

AA is synthesised from D-glucose but via various pathways in animals and plants. The reason why humans are not capable of synthesising AA is due to a single gene defect that arose during the course of evolution; some species such as the guinea pig, some fruit-eating bats, fish, birds and most primates (including humans) have the inability to express the terminal enzyme of the biosynthetic pathway, L -gulonolactone oxidase (Chatterjee, 1998; Johnston *et al.*, 2007). The biosynthesis of L-ascorbic acid in animals and plants is shown in **Figure 2.8**.

2.6.3.1.2 Redox Recycling and Antioxidant Properties

There are three redox states of AA, ascorbate (fully reduced form), semidehydroascorbate (SDA; mono-oxidized form) and dehydroascorbate (DHA; fully oxidized form). As ascorbate acts as an antioxidant or enzyme co-factor, it reacts with highly reactive oxidizing species by losing/donating an electron, and is converted to the SDA radical which is less reactive, stable and enzymatically recyclable (Linster & Van Schaftingen, 2007). Furthermore, disproportionation of SDA (2 SDA → ascorbate + DHA) leads to the formation of DHA, but SDA and DHA can be reduced back to ascorbate via various enzymatic processes (Linster & Van Schaftingen, 2007). In more detail, the enzymes NADH (NADH-cytochrome b reductase) or NADPH (thioredoxin reductase) drive the one-electron step reduction of SDA to ascorbate, while DHA can be reduced in a two-electron step back to ascorbate either nonenzymatically or enzymatically (Linster & Van Schaftingen, 2007). The former occurs via spontaneous reaction with GSH, while the latter involves enzymes such as: omega class glutathione *S*-transferases 1 and 2 (GSTO1, GSTO2); thioredoxin reductase; and 3α-hydroxysteroid dehydrogenase (Linster & Van Schaftingen, 2007).

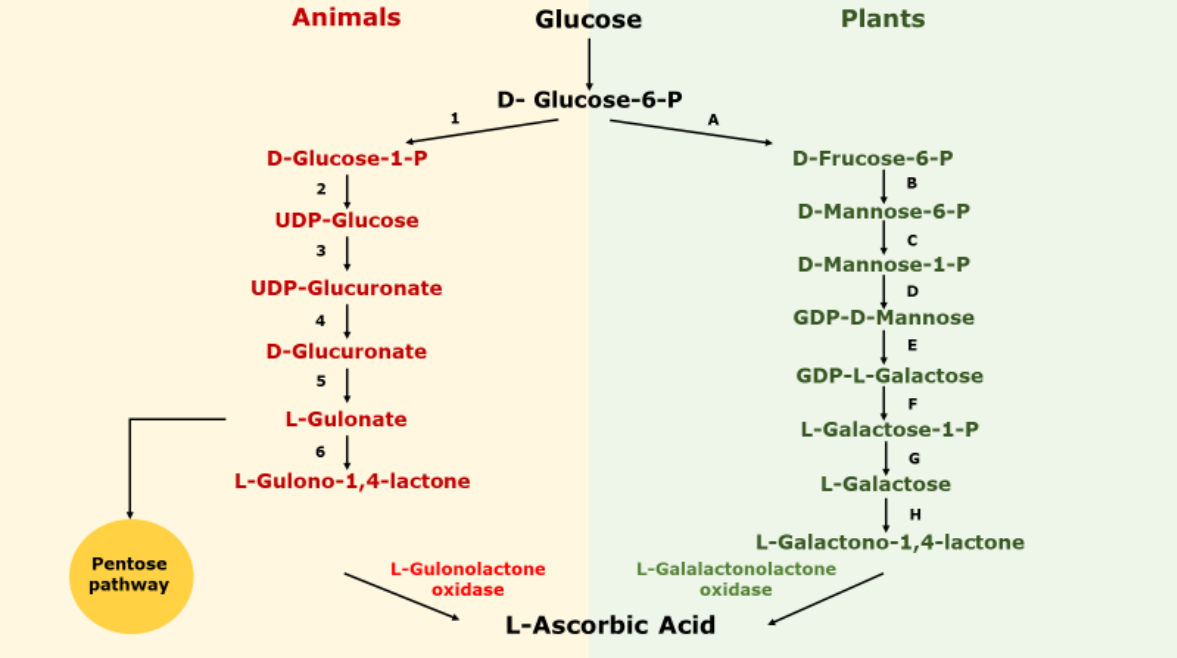


Figure 2.8. Biosynthesis of L-ascorbic acid in animals and plants. Abbreviations: 1, Phosphoglucomutase; 2, UDP-glucose pyrophosphorylase; 3, UDP-glucose dehydrogenase; 4, Glucuronate-1-phosphate uridylyltransferase and Glucurono kinase; 5, Glucuronatereductase; 6, Aldono-lactonase; A, Glucose-6-phosphate isomerase; B, Mannose-6-phosphate isomerase; C, Phosphomannomutase; D, GDPD-mannosepyrophosphorylase; E, GDP-D-mannose-3,5-epimerase; F, Phosphodiesterase; G, Sugar phosphatase; H, L-galactose dehydrogenase. Source from (Figueroa-Méndez & Rivas-Arancibia, 2015).

AA can regenerate vitamin E from its radical form, as it donates an electron to the tocopheroxyl radical and reduces/regenerates it to α-tocopherol. Furthermore, AA is a substrate for the redox enzyme ascorbate peroxidase, which can reduce H_2O_2 to water by using ascorbate as an electron donor (Pehlivan, 2017). Moreover, it can protect against lipid peroxidation as it helps to reduce lipid hydroperoxyl radicals through the vitamin E redox cycle (Pehlivan, 2017).

On the other hand, although AA can reduce ROS such as H_2O_2, it can act as a prooxidant by reducing metal ions and generating free radicals through the Fenton reaction, and this is mainly determined by: the absence/presence of transition metals (iron, copper); the redox potential of the cellular environment; and the local concentration of ascorbate (Akbari *et al.*, 2016).

2.6.3.1.3 Sources

AA sources include a variety of vegetables (broccoli, cabbage, avocado, cauliflower, brussel sprouts, potatoes, spinach), fruits (apples, banana, strawberry, currant, orange, tomato, kiwi, lemon, melon) and in smaller amounts in animal organs (chicken kidney, liver, ham, beef, milk) (Johnston *et al.*, 2007).

2.6.3.2 Vitamin E

Vitamin E is another major lipid-soluble chain-breaking antioxidant and it includes molecules with α-tocopherol activity (tocol and tocotrienol derivatives); there are four tocopherols and tocotrienols, each with similar structure (Johnston *et al.*, 2007).

2.6.3.2.1 Antioxidant Properties

Vitamin E mainly protects PUFAs within membrane phospholipids of biological membranes and in plasma lipoproteins, as it is a strong scavenger of peroxyl radicals, which are oxidized from lipid hydroperoxides. In the absence of vitamin E (equation 26), peroxyl radicals initiate lipid peroxidation and further autoxidation of lipids (Johnston *et al.*, 2007).

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$$

$$R^{\bullet} + O2 \rightarrow ROO^{\bullet}$$

(equation 26)

However, in the presence of vitamin E peroxyl radicals react and form a chromanol hydroxyl group (Vit E-OH), which then reacts with a peroxyl radical to form a hydroperoxide and the chromanoxyl radical (Vit E-O$^{\bullet}$). This terminates the chain of lipid peroxidation, and prevents further autoxidation of lipids (Johnston *et al.*, 2007; Nimse & Pal, 2015).

$$ROO^{\bullet} + \text{Vit E-OH} \rightarrow ROOH + \text{Vit E-O}^{\bullet} \quad \text{(equation 27)}$$

Ascorbate, as well as glutathione, can react with the chromanoxyl radical and regenerate it back to its reduced form, and thus other antioxidants can recycle/restore vitamin E (Johnston *et al.*, 2007).

2.6.3.2.2 Sources

Only plants can synthesize vitamin E, thus the major sources of vitamin E are edible vegetable oils. α-tocopherol is high in sunflower, wheat germ and safflower oil; γ-tocopherol is high in corn and soybean oil; palm oil is high in both α- and γ-tocopherol as well as in α- and γ-tocotrienol. A reduced blood concentration of α-tocopherol can be obtained through foods such as

cereals (fortified), almonds, sunflower seeds, hazelnuts, carrot juice, beet greens, potato chips, broccoli and red peppers (Johnston *et al.*, 2007).

2.6.3.3 Carotenoids

Vitamin A is ingested from the diet in two main forms: (1) preformed vitamin A (retinyl esters and retinol) and provitamin A carotenoids (β-carotene, α-carotene, and β-cryptoxanthin) (Johnston *et al.*, 2007). Approximately 600 carotenoids have been identified from natural sources, but only about one-tenth of them exist in the human diet, and only 20 are detected in blood and tissues. Based on their composition, carotenoids are divided in two classes: carotenes, which contain only carbon and hydrogen atoms; and oxocarotenoids (xanthophylls), which carry at least one oxygen atom (Stahl & Sies, 2003). Five prominent and important carotenoids include: β-carotene, α-carotene, lycopene, lutein and β-cryptoxanthin (Johnston *et al.*, 2007).

2.6.3.3.1 Structure and Antioxidant Activity

The structural base of a carotenoid is a polyene backbone, consisting of a system of conjugated double C=C bonds, which gives carotenoids the ability to delocalize unpaired electrons and interact with free radicals and singlet oxygen (Young & Lowe, 2018). The pattern of their conjugated bonds can influence their antioxidant properties and thus, if this pattern is modified, so is their reactivity (Young & Lowe, 2018).

Carotenoids are mostly involved in the scavenging of two specific reactive oxygen species: singlet molecular oxygen ($^1O_2$) and peroxyl radicals. The way they interact with $^1O_2$ involves energy transfer between the two molecules and as the energy is transferred to the carotenoid molecule, ground state oxygen and a triplet excited carotene (^{3}CAR) is yielded through this physical quenching (equation 28) (Truscott, 2001).

$$^1O_2 + CAR \rightarrow {}^3O_2 + {}^3CAR \text{ (equation 28)}$$

This prevents any further chemical reactions, as the carotenoid dissipates its energy and returns to its ground state (Stahl & Sies, 2003). In fact, since they remain intact during this process, they can be reused for several quenching cycles. The number of the conjugated double bonds determine their efficacy for physical quenching and β-carotene, zeaxanthin, cryptoxanthin, and α-carotene are all highly active quenchers of $^1O_2$, with lycopene being the most efficient as it contributes to 30% of all carotenoids in humans (Stahl & Sies, 2003).

Furthermore, peroxyl radicals are products of lipid peroxidation and carotenoids, as lipophilic molecules, can react with them and prevent damage caused in lipophilic compartments, thus carotenoids are key for protecting cellular membranes and lipoproteins from oxidative damage (Stahl & Sies, 2003).

Finally, depending on the concertation of oxygen, carotenoids can exert pro-oxidant effects as cell protection can be diminished under zero oxygen conditions (Young & Lowe, 2018). In more detail, a study conducted in human volunteers who either took 15mg of lycopene supplement or no lycopene at all, showed that the extracted lymphoid cells that were exposed to high energy γ-radiation under atmospheric levels from the high lycopene group, were 5 times more protected compared to the no lycopene group (Boehm *et al.*, 2016). Moreover, at 0% oxygen this protection increases 50-fold, at 20% near 5-fold while at 100% there is no protection, suggesting a linear relationship between cell death and oxygen concentration in the presence of lycopene. The authors suggest that this could be due to the ability of oxygen to generate more highly reactive peroxyl radicals, and/or that the peroxyl radicals can remove lycopene, thereby counteracting its protectivity (Boehm *et al.*, 2016). β-carotene, as a strong scavenger of $^1O_2$, at low oxygen concentrations can also effectively attack lipid peroxyl radicals, but at high oxygen concertation it can display pro-oxidant activity (Ribeiro *et al.*, 2018).

2.6.3.3.2 Sources

Carotenoids are amongst the most common natural lipid-soluble pigments responsible for the yellow, orange, red and green colour of plants and fruits. β- and α-carotene are present in green vegetables (spinach, collard, turnip, broccoli, lettuce), carrots, peppers, melons, mangos, pumpkin, sweet potato; cryptoxanthin is present in orange fruits (orange, tangerine, peach), butternut squash, persimmons, hot chili peppers, papaya; lycopene is found in tomato, watermelon, grapefruit, guava; lutein and zeaxanthin are found in corn, green leafy vegetables (kale, spinach, broccoli), persimmons, mustard (Ribeiro *et al.*, 2018).

2.6.3.4 Synergistic Action

In conclusion, all three antioxidants (carotenoids, vitamin C and E) can work synergistically to protect cell membranes. Vitamin C quenches the radical cations of carotenoids, which if not removed may have a pro-oxidant effect, whereas carotenoids can repair vitamin E radical cations (Truscott, 2001).

2.7 RONS in Cellular Signalling

Cellular RONS are involved mainly in two main categories: (1) released as waste/by-products in biological processes, specifically in the mitochondrial transport chain during oxidative metabolism (explored in detail in section 2.4.1); and (2) generated as a cellular response to xenobiotics, cytokines and invasion from bacteria either in molecular synthesis or as parts of signalling transductions pathways or mechanisms of cell defence (Zhang *et al.*, 2016).

2.7.1 NF-κB Signalling Pathway

The NF-κB transcription factor is a multiprotein complex essential in DNA transcription and numerous cellular defence processes (immune, inflammatory response, cellular adhesion, differentiation, proliferation, autophagy, senescence, and apoptosis) and disorders of NF-κB have been linked to cancer, inflammation and neurodegenerative diseases (Zhang *et al.*, 2016). NF-κB consists of a heterodimer with one 50-kD (p50) and one 65-kD (p65) polypeptide (Genestra, 2007). The main activators of the NF-κB pathway are comprised of TNF-α, lipopolysaccharide (LPS), and interleukin 1 (IL-1) (Cooper *et al.*, 2001) (Lingappan, 2018).

The NF-κB is located in the cytoplasm as an inactive complex along with the inhibitor protein IκBα that inactivates it.(Genestra, 2007). However, pathogenic stimulus or stress causes the activation of the NF-κB pathway due to release of the IκB kinase (IKK), an enzyme complex which phosphorylates the inhibitor protein IκBα, and thereby freeing the NF-κB which migrates to the nucleus, binds to DNA and induces the synthesis of mRNA as well as the expression of target genes (Genestra, 2007; Zhang *et al.*, 2016). This is the canonical or classical activating pathway of NF-κB, but it can also be activated in the noncanonical pathway, which relies on IKKα through a variety of agents such as tumour necrosis factor (B cell–activating factor), cytokines (lymphotoxin β), CD40/CD27 ligand (proteins expressed on T cells), and viruses (human T-cell leukaemia and Epstein-Barr viruses) (Genestra, 2007; Zhang *et al.*, 2016).

H_2O_2 can modulate the activation of the NF-κB pathway by inhibiting the phosphorylation of IκBα and it has also been shown to inactivate IKK (Korn *et al.*, 2001; Morgan & Liu, 2011). Furthermore, NF-κB activity may influence RONS levels through increased expression of antioxidant enzymes such as MnSOD, CuZnSOD, thioredoxins 1 and 2, GPx1, which can all be targeted and up-regulated by NF-κB as an oxidative stress response, or

through intermediates such as ferritin heavy chain protein (an iron storage protein which may work with MnSOD to protect the cell by preventing the generation of highly reactive $OH^{\bullet}$ from H_2O_2) (Morgan & Liu, 2011; Lingappan, 2018). However, there are also targets of NF-κB which can promote RONS formation such as NADPH oxidase NOX2 (an inflammatory phagocytic enzyme which produces $O_2^{\bullet-}$, used for immune defence and cellular signalling) and XO (involved in generating $O_2^{\bullet-}$ and H_2O_2), both of which can be regulated/induced by NF-κB (Morgan & Liu, 2011).

2.7.2 MAPKs Signalling Pathway

Mitogen-activated protein kinase (MAPK) cascades are crucial intracellular signal transduction pathways involved in important cellular processes such as cell growth, differentiation, development, cell cycle, survival, and cell death; there are four well known MAPKs subgroups: the extracellular signal-related kinases (ERK1/2); the c-Jun NH_2-terminal kinases (JNK); the p38 kinase (p38); and the big MAP kinase 1 (BMK1/ERK5) pathway (Genestra, 2007; Zhang *et al.*, 2016).

A MAPK cascade is composed of a three-kinase signalling module: MAPK, MAPK kinase (MKK), and MAPKK kinases (MAPKKK). These form the MAPK module MAPKKK-MKK-MAPK, where MAPKKK phosphorylates and activates MKK, which in turn phosphorylates and activates MAPK.This sequential cascade of phosphorylation events is what firstly activates all subgroups of MAPKs (Son *et al.*, 2011).

Studies have shown that exogenous exposure of cells to H_2O_2 can lead to activation of MAPK pathways and that prevention of H_2O_2 accumulation, through antioxidants, can stop such activation (Torres & Forman, 2003). In particular, the JNK and p38 MAPK are suggested to be activated through RONS. A possible mechanism is through activation of ASK1 (apoptosis signal-regulating kinase 1) where H_2O_2 induces oxidation of thioredoxin (Trx) leading to disrupted interaction between Trx and ASK1, resulting in the release of ASK1 and its subsequence phosphorylation of p38 MAPK and JNK activation (Torres & Forman, 2003). Additionally, the JNK pathway is known to be activated by a variety of receptors, specifically tumour necrosis factor (TNF) receptors, and this may be partly mediated by oxygen radicals, as $O_2^{\bullet-}$ and lipid peroxide scavengers inhibit JNK activation (McCubrey *et al.*, 2006). Similar to JNK, the p38 MAPK pathway is known to be activated in a number of various cell types (Jurkat, 3T3, HeLa, fibroblasts, and endothelial

cells). Moreover, H_2O_2, $^1O_2$, $NO^\bullet$ and ONOO- have all been shown to activate the p38 MAPK pathway (McCubrey *et al.*, 2006).

Furthermore, activation of the ERK pathway has been shown to be activated by $NO^\bullet$ treatment of Jurkat T lymphocytes and ONOO- in epithelial cells. The BMK1/ ERK5 pathway is also activated by cellular stress, a number of receptors such as NGF and EGF, and its activation has been shown to be stimulated by H_2O_2 human skin fibroblasts, vascular smooth muscle cells, and endothelial cells (McCubrey *et al.*, 2006). Thus, RONS intermediates have the capability to activate all four major MAPK signalling pathways and, depending on their concentration and cell type, induce cellular apoptosis and proliferation, since their role as signalling molecules is evident, activating a number of receptors and kinase pathways (McCubrey *et al.*, 2006).

2.7.3 Other Signalling Pathways

Other pathways to note in RONS-mediated signalling are: Keap1-Nrf2-ARE and phosphoinositide-3-kinase- (PI3K-) Akt pathways. The Keap1-Nrf2-ARE pathway is important to maintain redox cellular balance and consists of Kelch-like ECH-associated protein 1 (Keap1), nuclear factor erythroid 2-related factor 2 (Nrf2) and antioxidant response elements. The PI3K- Akt pathway is involved in protein synthesis, cell cycle progression, proliferation, apoptosis, autophagy, and hormone and cytokine stimulation (Zhang *et al.*, 2016).

2.8 Oxidative stress, RONS and Exercise

2.8.1 A historical overview

The first report of an association between oxidative stress and exercise in humans was published in 1978, where 1-hour of cycling at 50% of $\dot{V}O_{2max}$ led to an increased production of pentane, a marker of lipid peroxidation, and vitamin E supplementation suppressed this increase both at rest and following the exercise (Dillard *et al.*, 1978). This exercise-induced oxidative stress effect was confirmed one year later in the rat model, where exercising rats exhibited increased lipid peroxidation after swimming to exhaustion (Brady *et al.*, 1979).

In the following decade, researchers focused on looking at skeletal muscle specifically. In 1982, using EPR, it was first discovered that contracting rat skeletal muscle generates RONS and that this effect was further enhanced by vitamin E deficiency (Davies *et al.*, 1982). A similar experiment confirmed this finding (Jackson *et al.*, 1985) and numerous investigations following

these early reports have confirmed that strenuous exercise is associated with oxidative stress in both humans and animals (Powers *et al.*, 2020). Soon after the discovery that skeletal muscle generates radicals, investigators linked vitamin E deficiency in the rat model with higher oxidative stress susceptibility (Jackson *et al.*, 1983). Additionally, it was first discovered that antioxidants systems, including GR, CAT, G6PDH and Cu/Zn-SOD, were elevated in the cardiac and skeletal muscle tissues of trained rats, when compared to untrained, suggesting a skeletal muscle exercise training adaptive response (Chearskul & Srichantaap, 1994). Following this, further studies showed an increase in antioxidant enzymes in skeletal muscle as a function of exercise training (Powers *et al.*, 2016). In 1985, the term oxidative stress was defined and several years later redefined (detailed in section 2.2). During the 1990s, it was firstly reported that free radicals contribute to muscle fatigue in animals and a few years later it was observed that, during prolonged (electrically stimulated) leg exercise, oxidants contribute to muscle fatigue (Reid *et al.*, 1994). Due to this research, further reports stemmed focusing on identifying the possible mechanisms of skeletal muscle fatigue mediated by RONS (Reid, 2008). Another earlier study showed that RONS concertation increased during muscle fatigue, leading to decreased muscle force production and this effect may be prevented by exogenous antioxidant administration (Reid *et al.*, 1992, 1993). This early research indicated that an optimal redox state may be necessary for maximal muscle force production to occur, which also later led to the proposed phenomenon of the now well-mentioned hormesis "inverted U curve" (Powers *et al.*, 2016). The hormesis theory suggests that RONS, depending on their concentration, can have either a positive or negative effect and if that concertation is too high, it can reduce muscle force production (Radák & Koltai, 2018). Going back to the discovery that contracting muscle generates RONS, in 1992 it was also first reported that superoxide radicals are formed through contracting rat skeletal muscle (Reid *et al.*, 1992) and a few years later this was also observed with hydroxyl radicals in cats (O'Neill *et al.*, 1996).

Furthermore, in the 1990s, there was also initial research linking increased antioxidant capacity with exercise training as a function of exercise intensity and duration. Specifically, there were numerous investigations showing elevated antioxidant enzymes, mainly SOD1, SOD2, GPx1, due to exercise training (Criswell *et al.*, 1993; Powers *et al.*, 1993, 1994*a*, 1994*b*), although reported increases of CAT remain controversial (Powers & Jackson, 2008).

Another salient discovery that was made during the '90s was that, contracting muscle generates NO and two isoforms of nitric oxide synthase (NOS) expressed by skeletal muscle (Kobzik *et al.*, 1994, 1995). These discoveries set the scene for more studies investigating the role of NO as a skeletal muscle signalling molecule. Finally, in the late 1990s with all the precedent discoveries about NO, a paradigm shift occurred with regards to the concept of viewing free radicals as solely damaging molecules. Instead, studies published in the early 2000s suggest that RONS molecules are to be considered crucial signalling molecules involved in cell homeostasis and regulation of gene expression (Dröge, 2002; Jackson, 2008; Powers *et al.*, 2016).

As it was discovered that exercise-induced radicals contribute to muscle fatigue, investigators focused on dietary antioxidant supplementation and its effect on exercise performance. Initial *in vitro* animal experiments showed that antioxidants may improve muscle performance and delay fatigue, but such an effect was not observed in human experiments (Powers & Hamilton, 1999). Early human studies, which found no improvement of antioxidants on muscle performance predominantly used vitamin E. Whereas N-acetylcysteine (NAC) administration during prolonged submaximal, but not high-intensity (60-80% vs ≥ 90% $\dot{V}O_{2max}$) human exercise, could delay muscle fatigue (Powers & Hamilton, 1999; Medved *et al.*, 2004; McKenna *et al.*, 2006).

Over the past few decades, investigators have utilised various types of exercise linked to biomarkers of oxidative stress, which include acute aerobic, prolonged endurance, anaerobic, resistance and eccentric exercise protocols, which will be discussed in more detail in the sections to follow. (Fisher-Wellman & Bloomer, 2009; Powers *et al.*, 2016).

2.8.2 RONS Sources Generated by Skeletal Muscle

Skeletal muscle generates primarily two RONS, superoxide and NO, which lead to secondary RONS; their intracellular content seems to be increased contractile activity (Jackson, 2011).

2.8.2.1 Superoxide Generation Sites

There are multiple superoxide generation sites in skeletal muscle which include: mitochondria, sarcoplasmic reticulum (SR), transverse tubules, plasma membrane, phospholipase A2-dependent processes (PLA_2) and xanthine oxidase (Powers & Jackson, 2008).

Early reports cited the mitochondria as the major source of exercise-induced generation of RONS in skeletal muscle, with complexes I and III as the main sites of superoxide production (Powers & Jackson, 2008). Precisely, in the very early reports, 2-5% of molecular oxygen consumed in mitochondria was estimated to form superoxide (Boveris *et al.*, 1972). This was later reassessed to be an overestimation and the rate of superoxide production was actually closer to around 0.15%(St-Pierre *et al.*, 2002). In fact, studies have shown that this rate during active state 3 by the mitochondria is lower compared to basal state 4 respiration, and thus mitochondria are not the predominant source of RONS generation in skeletal muscle during exercise (Powers & Jackson, 2008). In more detail, the main site of electron leakage in complex I is thought to be iron-sulphur clusters, while in complex III the enzyme Q10, and in fact, complex III releases superoxide from both sides of the inner mitochondrial membrane (Muller *et al.*, 2004; Radák *et al.*, 2013). It is suggested that, during contractile activity RONS, formation is increased as oxygen consumption demands are higher, and this increase has been reported to be between 50- and 100-fold (Urso & Clarkson, 2003).

Furthermore, NAD(P)H oxidase enzymes, generating superoxide, have been identified to be associated with the SR of both cardiac and skeletal muscle (Jackson, 2008). This NAD(P)H-dependent oxidase located in the SR of skeletal muscle generates superoxide (Xia *et al.*, 2003). Moreover, studies suggest that skeletal muscle cells located in the plasma membrane, containing a NAD(P)H oxidase complex, also release superoxide in the extracellular space (Powers & Jackson, 2008). The enzyme contains four subunits that are found in phagocytic cells ($gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$), all of which were associated with the cell membranes (Jackson, 2008). There is also data that suggests an NAD(P)H oxidase localized to the transverse tubules of skeletal muscle, which also contains some of the classical subunits found in the NADP(H) oxidase of phagocytic cells, appears to release superoxide to the cytosol of skeletal muscle cells (Espinosa *et al.*, 2006; Powers & Jackson, 2008).

PLA_2 is an enzyme responsible for cleaving membranes to release arachidonic acid, which is a substrate for lipoxygenases, RONS-generating enzymes (Zuo *et al.*, 2004). Their increased activity may lead to radical formation in muscle mitochondria and their activation can stimulate NADP(H) oxidases (Nethery *et al.*, 2000; Powers & Jackson, 2008). There is a secretory calcium-dependent (s-PLA_2) and a calcium-independent form of

PLA_2 (i-PLA_2), which both are reported to contribute to muscle RONS formation (Jackson, 2008). The i-PLA_2 has been shown modulate cytosolic oxidant activity and contractile function in mouse skeletal muscle cells, while the s-PLA_2 stimulates intracellular RONS generation during contractile activity within mitochondria (Nethery *et al.*, 2000; Gong *et al.*, 2006). A third type of cytosolic PLA_2 (c-PLA_2) in non-muscle cells has been suggested to be activated by small calcium concentrations and may also be linked to RONS formation (Adibhatla & Hatcher, 2006). It is hypothesized that i-PLA_2 is a key determinant of oxidant activity under resting conditions, while s-PLA_2 can stimulate RONS generation during contractile activity, heat stress or when intracellular calcium is elevated (Gong *et al.*, 2006).

XO has been shown to contribute to RONS production in ischemia and reperfusion. However, there is speculation with regards to skeletal muscle and contractile activity in humans since most studies have been done in mice or rats (Powers & Jackson, 2008). During rat skeletal muscle contractions, XO activity is increased leading to lipid peroxidation (Judge & Dodd, 2004). Similarly, one day after exhaustive exercise, higher XO activity was measured in the liver of rats (Radák *et al.*, 1996), which has also been shown 12 hours post downhill running (Retamoso *et al.*, 2016).

Therefore, superoxide is generated in contracting skeletal muscle and the mitochondria does not seem to be the predominant source, as originally proposed. However, it is not clear whether one of these RONS-generating sites dominates or all of them contribute equally to the increased RONS activity (Powers & Jackson, 2008).

2.8.2.2 Nitric Oxide Generation Sites

Nitric oxide is generated from the amino acid L-arginine, catalysed by NO synthase (NOS) (Dyakova *et al.*, 2015; Radák & Koltai, 2018). There are isoforms of NOSs, originating from three genes: (1) an endothelial isoform (eNOS; gene location on chromosome 7); (2) a neuronal isoform (nNOS; gene location on chromosome 12); and (3) macrophage (immune)/calcium calmodulin-independent or "inducible" isoform (iNOS; gene location on chromosome 17) (Stamler & Meissner, 2001; Dyakova *et al.*, 2015). Early research utilizing immunocytochemical techniques demonstrated that, nNOS and eNOS are expressed in skeletal muscle (Kobzik *et al.*, 1994, 1995) and although nNOS is the predominant isoform, all three are expressed in skeletal muscle (Radák & Koltai, 2018). Cultured muscle cells have been shown to release higher amounts of NO during contractile activity and nNOS

seems to be the main source of NO released from skeletal muscle (Hirschfield *et al.*, 2000; Patwell *et al.*, 2004).

2.8.3 Oxidative Stress Biomarkers, RONS and Antioxidant Supplementation in Various Types of Exercise

The extent of RONS production during exercise may be influenced by the intensity, type and duration of the exercise. Studies over the past few decades have utilised various types of exercise linked to biomarkers of oxidative stress, which include acute aerobic, prolonged endurance, anaerobic, resistance and eccentric exercise protocols (Fisher-Wellman & Bloomer, 2009; Powers *et al.*, 2016). However, the majority of studies in the area of oxidative stress and acute exercise have used aerobic exercise protocols, typically submaximal or maximal effort on a treadmill or cycle ergometer.

*2.8.*3.*1 Aerobic Exercise*

During aerobic exercise, intracellular superoxide, NO and H_2O_2 are estimated to be increased by 1-3 fold during muscle contraction, with the mitochondria accounting for a small 0.15% rate of superoxide generation (Sakellariou *et al.*, 2014). Within the mitochondria however, it is suggested that complex I is the main site of superoxide production, but overall, NAD(P)H oxidases and particularly NOX2 isoform expressed in the sarcolemma of skeletal muscle, is likely to be a major source for the increased superoxide production during exercise (Sakellariou *et al.*, 2014).

2.8.3.1.1 Lipid Peroxidation

Numerous investigations have shown an increase in TBARS, a common lipid peroxidation biomarker, following submaximal (Alessio *et al.*, 1997; Laaksonen *et al.*, 1999; Vincent *et al.*, 2004; Nikolaidis *et al.*, 2007*b*), as well as maximal exercise protocols (Miyazaki *et al.*, 2001; Nikolaidis *et al.*, 2006; Michailidis *et al.*, 2007; Steinberg *et al.*, 2007).Although, a few reports have shown no increase (Vasankari *et al.*, 1995; Goldfarb *et al.*, 2005*b*; Morillas-Ruiz *et al.*, 2005).

Regarding MDA, another popular biomarker of lipid peroxidation, most studies seem to have reported no increase in both submaximal (Buczynski *et al.*, 1991; Chung *et al.*, 1999; Akova *et al.*, 2001; Bloomer *et al.*, 2005, 2006*b*) and maximal exercise protocols (Niess *et al.*, 1996; Leaf *et al.*, 1997; Bloomer *et al.*, 2007*b*) with generally fewer reports of an MDA increase post-exercise (Kanter *et al.*, 1993; Ashton *et al.*, 1998; Bryant *et al.*, 2003;

Goldfarb *et al.*, 2007). Although generally, those studies showing an increase in MDA lipid peroxidation, included near maximal or maximal exercise protocols, which may indicate that high-intensity could contribute to MDA formation.

Furthermore, a couple of reports using F_2-isoprostanes, which is considered a reliable method of measuring lipid peroxidation, have shown elevated concentrations of F_2-isoprostanes (Waring *et al.*, 2003; Goto *et al.*, 2007), which may be correlated with high-intensity exercise (Goto *et al.*, 2007). Finally, one study observed no difference, possibly attributed to the moderate exercise intensity protocol (Rush & Sandiford, 2003). Another study performed in trained athletes who followed a high antioxidant diet 2 weeks prior to the exercise test reported decreased F_2-isoprostanes after 30 mins of submaximal exercise (60% $\dot{V}O_{2max}$), followed by a significant increase only after the incremental test to exhaustion (Watson *et al.*, 2005). Interestingly, after a restricted antioxidant diet, F_2-isoprostanes were elevated throughout both the submaximal and exhaustive exercise compared to the high antioxidant diet (38 and 45% respectively), which may indicate a role of training status and/or "protection" from RONS due to antioxidants (Watson *et al.*, 2005).

2.8.3.1.2 DNA Oxidation

As previously discussed, the most commonly used markers of DNA oxidation are: 8-OHdG, the most common guanine oxidation product; and the comet assay, which measures DNA strand breaks as a % of damage in the tail. As a general consensus, it is clear that acute aerobic exercise can cause DNA damage (Tryfidou *et al.*, 2020). However, there is a lot of discrepancy between the two most common methods of measuring DNA oxidation. The majority of studies utilizing 8-OHdG show no change following various protocols (Inoue *et al.*, 1993; Sumida *et al.*, 1997; Asami *et al.*, 1998; Bloomer *et al.*, 2005, 2006*b*, 2007*b*; Goldfarb *et al.*, 2007), while only a few reports show an increase in 8-OHdG (Orhan *et al.*, 2004; Morillas-Ruiz *et al.*, 2005). On the other hand, most studies using the comet assay have shown an increase in DNA damage following acute aerobic exercise (Tryfidou *et al.*, 2020). In fact, this is specifically explored in extensive detail in Chapter 4 with a systematic review and meta-analysis on DNA damage after an acute aerobic exercise bout, comparing these two methods of assessing DNA oxidation (Tryfidou *et al.*, 2020). The difference in results can be due

to various factors including, but not limited to: duration/intensity of the exercise, training status, gender and interlaboratory differences.

With regards to the recent γ-H2AX assay as a way to quantify DNA damage, there have only a couple of studies using this assay to measure DNA damage following acute aerobic exercise, both showing an increase in DNA damage after long distance running, suggesting a link between duration/intensity and the extent of damage (Lippi *et al.*, 2016, 2018).

2.8.3.1.3 Protein Oxidation

Protein carbonyl groups (PC) accumulation has been documented by numerous studies (Goldfarb *et al.*, 2005*b*, 2007; Bloomer *et al.*, 2006*b*, 2007*c*; Michailidis *et al.*, 2007; Nikolaidis *et al.*, 2007*b*). In fact, one study suggests that PC may increase in a duration-dependent manner as cycling at 70% $\dot{V}O_{2max}$ for 2 hours resulted in higher PC than exercising for 30 and 60 mins at the same intensity (Bloomer *et al.*, 2007*c*). Another study, sampling PC multiple times, showed that PC concentration increased post-exercise by 32%, peaked after 4 hours by 96%, and remained elevated at 8 hours by 55%, compared to rest (Michailidis *et al.*, 2007). A few studies reported no increase in measured PC, which could be due to limited sampling points using only pre- and post-exercise samples (Miyazaki *et al.*, 2001). Whereas another study reported a small but nonsignificant 14% increase in PC, which may have been due to the trained status of the participants (Morillas-Ruiz *et al.*, 2005).

2.8.3.1.4 Miscellaneous Markers

There is a variety of other markers used to measure oxidative stress. These typically include: total antioxidant capacity (TAC), measured before and following exercise; measurement in specific antioxidant enzymes (such as SOD, GPx, CAT); and purine metabolism intermediates (xanthine/hypoxanthine). Specifically, for antioxidant capacity some studies reported a temporary decrease following exercise (Tozzi-Ciancarelli *et al.*, 2002; Di Massimo *et al.*, 2004; Steinberg *et al.*, 2006), while most studies have shown that TAC typically increases, especially during the recovery following exercise (Maxwell *et al.*, 1993; Alessio *et al.*, 2000; Nikolaidis *et al.*, 2006, 2007*b*; Steinberg *et al.*, 2006; Michailidis *et al.*, 2007). On the other hand, a few studies which only measured TAC immediately post-exercise showed no change (Alessio *et al.*, 1997; Ashton *et al.*, 1998; Vincent *et al.*, 2004) and one study which measured post- and 20 mins post-exercise also reported no difference in TAC (Waring *et al.*, 2003).

In terms of enzymatic activity, some reports have shown increases in SOD (Buczynski *et al.*, 1991), GPx (Buczynski *et al.*, 1991; Laaksonen *et al.*, 1999) and CAT (Buczynski *et al.*, 1991; Vider *et al.*, 2001; Michailidis *et al.*, 2007; Nikolaidis *et al.*, 2007*b*) following exercise, while some have noted decreases in GPx and SOD (Akova *et al.*, 2001). On the other hand, numerous studies have reported no change in SOD (Laaksonen *et al.*, 1999; Miyazaki *et al.*, 2001; Vider *et al.*, 2001; Tauler *et al.*, 2006*a*), GPx (Miyazaki *et al.*, 2001; Vider *et al.*, 2001; Rush & Sandiford, 2003; Tauler *et al.*, 2006*a*), CAT (Laaksonen *et al.*, 1999; Miyazaki *et al.*, 2001; Tauler *et al.*, 2006*a*). As these results are quite contradictory, this may be due to difference in sampling timing points, duration, and intensity of the exercise. Finally, some studies have noted an increase in xanthine/hypoxanthine after acute exercise (Hellsten *et al.*, 1988; Sahlin *et al.*, 1991; Inoue *et al.*, 1993; Sumida *et al.*, 1997).

*2.8.*3.*2 Aerobic Exercise: Impact of Antioxidant Supplementation*

Typical antioxidant interventions include vitamin E, C, β-carotene either alone or in combination, administrated either acutely (1-2 days pre-exercise) or chronically (several weeks pre-exercise). Other interventions include coenzyme Q10, NAC and uric acid.

Several investigations have reported a decrease in exercise-induced oxidative stress after chronic supplementation of vitamin C (Alessio *et al.*, 1997; Goldfarb *et al.*, 2005*b*), vitamin E (Dillard *et al.*, 1978; Sumida *et al.*, 1989; Hartmann *et al.*, 1995) and β-carotene (Sumida *et al.*, 1997) when administered alone. However, this decrease was not observed for all measured biomarkers (Hartmann *et al.*, 1995; Alessio *et al.*, 1997; Goldfarb *et al.*, 2005*b*). On the other hand, some studies observed no changes on exercise-induced oxidative stress of single administration of vitamin C (Bryant *et al.*, 2003) and E (Akova *et al.*, 2001; Bryant *et al.*, 2003). Reasons for discrepancies in the literature could be due to the dose and duration of the supplemented antioxidant. For example, one study noted decreased protein oxidation after vitamin C in response to 30 mins of running at 75% of $\dot{V}O_{2max}$ but no effect on lipid peroxidation (Goldfarb *et al.*, 2005*b*). A time-course study on subjects with increased oxidative stress (biomarker used: F_2-isoprotanes) linked to hypercholesterolemia, supplemented with different doses of vitamin E, showed a clear linear relationship between dose of vitamin E and suppression of oxidative stress, irrespective of exercise (Jackson Roberts II *et al.*, 2007). Additionally, a

meta-analysis on vitamin E and exercise-induced oxidative stress found no added protection against lipid peroxidation, and thus as the authors also suggest, the different doses and durations of the supplementation could impact the study outcomes (Stepanyan *et al.*, 2014). Finally, two studies using 8 week administration of coenzyme Q10 have shown no benefits of reducing exercise-induced oxidative stress markers (Braun *et al.*, 1991; Östman *et al.*, 2012).

In terms of acute antioxidant supplementation, numerous studies have reported reduction in various oxidative stress biomarkers, which include decreased: PC and 8-OHdG after drinking an antioxidant rich beverage (Morillas-Ruiz *et al.*, 2005); DNA damage following vitamin E supplementation (Hartmann *et al.*, 1995); lipid peroxidation after vitamin C supplementation (Alessio *et al.*, 1997; Ashton *et al.*, 1998); F_2-isoprotanes following uric acid supplementation (Waring *et al.*, 2003); and finally increased antioxidant capacity after supplementation with NAC (Sen *et al.*, 1994) and uric acid (Waring *et al.*, 2003).

*2.8.*3.*3 Anaerobic Exercise*

As it has been established, the extent of RONS production can depend on the intensity, duration and type of exercise. The intensity of aerobic exercise is described as % of $\dot{V}O_{2max}$ performed in a timed manner, while the intensity of anaerobic exercise is usually described by the % of repetition maximum performed in a number of sets/repetitions, or by repeating a set number of short bursts of maximal exercise (He *et al.*, 2016). The redox mechanisms of anaerobic exercise have been explored using various protocols including sprinting, dynamic resistance, isometric and eccentric exercises.

2.8.3.3.1 Eccentric Exercise

The majority of eccentric based protocols typically include eccentric contractions of the knee extensor (Saxton *et al.*, 1994; Child *et al.*, 1999; Radák *et al.*, 1999; Nikolaidis *et al.*, 2007*a*; Paschalis *et al.*, 2007) or elbow or arm flexor muscles (Saxton *et al.*, 1994; Childs *et al.*, 2001; Lee *et al.*, 2002; Goldfarb *et al.*, 2005*a*; Bryer & Goldfarb, 2006). Following eccentric muscle contractions, increased lipid peroxidation (Childs *et al.*, 2001; Goldfarb *et al.*, 2005*a*; Nikolaidis *et al.*, 2007*a*; Paschalis *et al.*, 2007), PC (Lee *et al.*, 2002; Goldfarb *et al.*, 2005*a*; Nikolaidis *et al.*, 2007*a*; Paschalis *et al.*, 2007) and DNA oxidation (Radák *et al.*, 1999; Fogarty *et al.*, 2013*a*; Gray *et al.*, 2014) have been reported. In addition, changes in glutathione redox status were also reported (Goldfarb *et al.*, 2005*a*; Bryer & Goldfarb,

2006; Nikolaidis *et al.*, 2007*a*; Paschalis *et al.*, 2007). On the other hand, a few investigations reported no difference in lipid peroxidation (Saxton *et al.*, 1994; Child *et al.*, 1999; Gray *et al.*, 2014), PC (Saxton *et al.*, 1994; Gray *et al.*, 2014) and glutathione status (Lee *et al.*, 2002).

In regards to antioxidant supplementation and eccentric exercise, a reduction in oxidative stress markers have been shown with administration of a combination vitamin C, E and selenium (Goldfarb *et al.*, 2005*a*) as well as vitamin C alone (Bryer & Goldfarb, 2006). Additionally, 3g of fish oil supplementation resulted in increased PC, reduced lipid peroxidation and H_2O_2-induced lymphocyte DNA damage compared to the control group post-exercise (Gray *et al.*, 2014). While no benefit was reported after supplementation with vitamin C and NAC (Childs *et al.*, 2001) or a mixture of vitamin C and E (Bloomer *et al.*, 2007*a*).

2.8.3.3.2 Isometric Exercise

Isometric protocols typically involve handgrip exercise with (Steinberg *et al.*, 2006) or without thumb adduction (Alessio *et al.*, 2000; Steinberg *et al.*, 2002; Rodriguez *et al.*, 2003; Matuszczak *et al.*, 2005) at 50–100% of maximal voluntary contraction until exhaustion, or for a set amount of time as well as static knee extensions (Sahlin *et al.*, 2006). Most of these studies have reported: higher lipid peroxidation after exercise (Alessio *et al.*, 2000; Steinberg *et al.*, 2002, 2006); changes in glutathione redox status (Steinberg *et al.*, 2002, 2006; Matuszczak *et al.*, 2005); and decreased antioxidant capacity (Steinberg *et al.*, 2006). Although a few report no changes (Alessio *et al.*, 2000; Sahlin *et al.*, 2006). Lastly, in terms of antioxidant supplementation, one study supplemented participants with NAC and noted a reduction in oxidized glutathione following handgrip exercise (Matuszczak *et al.*, 2005).

2.8.3.3.3 Sprinting/Jumping

Studies have utilized sprinting at maximal effort to elicit fatigue on a cycle ergometer (Groussard *et al.*, 2003; Baker *et al.*, 2004; Bloomer *et al.*, 2006*a*, 2007*d*; Thomas *et al.*, 2007) or running surface (Schiffl *et al.*, 1997) as well as protocols such as intermittent shuttle-running exercise (Thompson *et al.*, 2001, 2003) and jumping (Ortenblad *et al.*, 1997). Some of these studies have reported higher lipid peroxidation (Thompson *et al.*, 2001, 2003; Groussard *et al.*, 2003; Baker *et al.*, 2004), protein oxidation (Bloomer *et al.*, 2007*d*) and DNA damage (Schiffl *et al.*, 1997), while others showed no changes in lipid (Bloomer *et al.*, 2006*a*, 2007*d*; Thomas *et al.*,

2007), protein (Bloomer *et al.*, 2006*a*) and DNA oxidation (Bloomer *et al.*, 2007*d*).

2.8.3.3.4 Resistance Exercise

There have also been studies exploring oxidative stress induced through resistance exercise, which typically include protocols utilizing multiple joint exercises, usually performed in a circuit for ≥3 sets of 60-95% 1 repetition maximum (Viña *et al.*, 2000; Avery *et al.*, 2003; Ramel *et al.*, 2004; Viitala *et al.*, 2004; Liu *et al.*, 2005; McAnulty *et al.*, 2005*b*; Güzel *et al.*, 2007; Rietjens *et al.*, 2007). Other studies have used single dynamic resistance movement protocols, such as squats (Bloomer *et al.*, 2005, 2006*b*, 2007*d*) or single-leg isokinetic knee extension (Bailey *et al.*, 2004, 2007). Most of these investigations reported higher oxidative stress by increased lipid peroxidation (Avery *et al.*, 2003; Bailey *et al.*, 2004, 2007; Ramel *et al.*, 2004; Viitala *et al.*, 2004; Liu *et al.*, 2005; Güzel *et al.*, 2007), protein oxidation (Viitala *et al.*, 2004; Bloomer *et al.*, 2005) and glutathione redox status changes (Viña *et al.*, 2000; Bloomer *et al.*, 2005). No changes in DNA oxidation were reported (Bloomer *et al.*, 2005, 2007*d*). In terms of antioxidant intervention, studies reported no effect of a carbohydrate drink (McAnulty *et al.*, 2005*b*) or vitamin E on reducing oxidative stress biomarkers (Avery *et al.*, 2003; Viitala *et al.*, 2004).

*2.8.*3.*4 Long-Duration Exercise*

There have been studies utilizing long duration exercise protocols, which typically include running a marathon (Inayama *et al.*, 1996; Liu *et al.*, 1999; Tsai *et al.*, 2001; Radák *et al.*, 2003*a*; Mastaloudis *et al.*, 2004*b*, 2004*a*), half marathon (Duthie *et al.*, 1990; Child *et al.*, 1998, 2000; Case *et al.*, 1999), ultramarathon (Radák *et al.*, 2000; Mastaloudis *et al.*, 2001; Nieman *et al.*, 2002; Palmer *et al.*, 2003) and triathlon (Margaritis *et al.*, 1997; Hartmann *et al.*, 1998; Ginsburg *et al.*, 2001; Nieman *et al.*, 2004; McAnulty *et al.*, 2005*a*; Knez *et al.*, 2007). Moreover, other long duration exercise protocols include: long duration cycle ride (Davison *et al.*, 2007), run (Dawson *et al.*, 2002; Steensberg *et al.*, 2002; McAnulty *et al.*, 2003), bike race (Almar *et al.*, 2002; Aguiló *et al.*, 2005; Tauler *et al.*, 2006*b*), duathlon race (Margaritis *et al.*, 2003; Tauler *et al.*, 2003; Palazzetti *et al.*, 2004) and march (Chevion *et al.*, 2003). Lastly, some studies have used overtraining protocols (Poulsen *et al.*, 1996; Okamura *et al.*, 1997; Palazzetti *et al.*, 2003).

These long duration aerobic exercise protocol studies have observed increases in lipid peroxidation using MDA (Child *et al.*, 1998, 2000; Tsai *et al.*, 2001; Tauler *et al.*, 2006*b*; Knez *et al.*, 2007), TBARS (Margaritis *et al.*, 2003; Palazzetti *et al.*, 2004), F_2-isoprostanes (Mastaloudis *et al.*, 2001, 2004*a*; Nieman *et al.*, 2002, 2004; Steensberg *et al.*, 2002; McAnulty *et al.*, 2003, 2005*a*; Palmer *et al.*, 2003), lipid hydroperoxides (Nieman *et al.*, 2002; McAnulty *et al.*, 2003; Palmer *et al.*, 2003), LDL oxidation susceptibility (Liu *et al.*, 1999; Kaikkonen *et al.*, 2002), protein oxidation (Tauler *et al.*, 2006*b*), DNA damage with the 8-OHdG biomarker (Radák *et al.*, 2000; Tsai *et al.*, 2001; Almar *et al.*, 2002) and the comet assay (Tsai *et al.*, 2001; Mastaloudis *et al.*, 2004*b*). Reduction in GSH (Duthie *et al.*, 1990; Margaritis *et al.*, 2003; Palazzetti *et al.*, 2003, 2004) and increased GSSG (Duthie *et al.*, 1990; Margaritis *et al.*, 2003; Palazzetti *et al.*, 2003, 2004; Aguiló *et al.*, 2005; Tauler *et al.*, 2006*b*) have also been observed.

Some studies, however, have reported no differences in lipid peroxidation, the majority of which have used TBARS (Duthie *et al.*, 1990; Vasankari *et al.*, 1995; Inayama *et al.*, 1996; Margaritis *et al.*, 1997; Palazzetti *et al.*, 2003) and only a few using MDA (Child *et al.*, 2000; Dawson *et al.*, 2002) or measuring LDL oxidation susceptibility (Case *et al.*, 1999), 8-OHdG (Hartmann *et al.*, 1998; Nieman *et al.*, 2004), protein oxidation (Chevion *et al.*, 2003) and glutathione redox status (Margaritis *et al.*, 1997).

Moreover, in terms of measuring antioxidant defences, many studies reported increases in antioxidant capacity following the race (Child *et al.*, 1998, 2000; Liu *et al.*, 1999; Kaikkonen *et al.*, 2002; McAnulty *et al.*, 2003, 2005*a*; Palazzetti *et al.*, 2003). Specifically, there has been both a noted increase in GPx (Dawson *et al.*, 2002; Tauler *et al.*, 2003), SOD (Tauler *et al.*, 2006*b*), CAT (Aguiló *et al.*, 2005), GR (Tauler *et al.*, 2003, 2006*b*; Aguiló *et al.*, 2005), vitamin C (Duthie *et al.*, 1990; Kaikkonen *et al.*, 2002; Mastaloudis *et al.*, 2004*a*), vitamin E (Mastaloudis *et al.*, 2001; Aguiló *et al.*, 2005), vitamin A (Duthie *et al.*, 1990) as well as a decrease in GPx/vitamin E (Mastaloudis *et al.*, 2001, 2004*a*), SOD/CAT (Knez *et al.*, 2007), and vitamin C (Liu *et al.*, 1999; Mastaloudis *et al.*, 2001). No changes post-exercise have also been reported in levels of GPx (Duthie *et al.*, 1990), SOD (Duthie *et al.*, 1990; Palazzetti *et al.*, 2003; Tauler *et al.*, 2003, 2006*b*), CAT (Duthie *et al.*, 1990; Tauler *et al.*, 2003, 2006*b*), vitamin C (Liu *et al.*, 1999), vitamin E (Duthie *et al.*, 1990; Liu *et al.*, 1999; Kaikkonen *et al.*, 2002; Tauler *et al.*, 2006*b*) and vitamin A (Kaikkonen *et al.*, 2002; Aguiló

et al., 2005; Tauler *et al.*, 2006*b*). All these studies had trained participants who exercised between 20-30 hours weekly, which may explain some of the observed increased antioxidant defences. It is suggested that while the duration of such events/protocols is long, the intensity is likely low to moderate and thus enough to counter free radical production due to the "highly trained" level participants, which might explain why some reports report decreased or unchanged oxidative stress biomarkers (Margaritis *et al.*, 1997).

Furthermore, studies have implemented antioxidant supplementation before such long duration protocols, typically consisting of single administration (Nieman *et al.*, 2002, 2004; Tauler *et al.*, 2003; McAnulty *et al.*, 2005*a*) or mixtures of vitamin C, E and A (Dawson *et al.*, 2002; Margaritis *et al.*, 2003; Mastaloudis *et al.*, 2004*b*; Palazzetti *et al.*, 2004; Davison *et al.*, 2007). Lastly, studies have used a carbohydrate beverage with (Palmer *et al.*, 2003) or without the addition of vitamin C (McAnulty *et al.*, 2003). Studies have generally observed no effect of supplementation on oxidative stress biomarkers (Nieman *et al.*, 2002; Margaritis *et al.*, 2003; McAnulty *et al.*, 2003). However, with the exception of few reports observing reductions in TBARS (Davison *et al.*, 2007), F_2-isoprostanes (Mastaloudis *et al.*, 2004*a*) and DNA damage (Mastaloudis *et al.*, 2004*b*), following supplementation with a combination of vitamin C and E. Such results may vary due to training status, timing and dosage of supplementation and it is likely that after exhaustive and long duration exercise. The increase in RONS may result in favour of prooxidants versus antioxidants and that the dose/duration of supplementation is not sufficient to produce added protection (Jackson Roberts II *et al.*, 2007).

2.8.4 RONS, Muscle Adaptation to Exercise Training and Antioxidant Supplementation

RONS are produced in contracting skeletal muscle and also play a crucial role as signalling molecules, specifically involved in various signalling pathways important for exercise muscle adaptation (Powers *et al.*, 2010). Specifically, there is evidence suggesting that a moderate increase in production of RONS in skeletal muscle during a short time period (within mins) can lead to activation of pathways important for cell adaptation, whereas higher levels of RONS over long periods of time (within hours) may result in continuous activation of pathways involved in cellular proteolysis and potentially apoptosis (Ji *et al.*, 2006). Some of the most important

pathways that RONS regulate, as mentioned in section 2.7, are the MAPKs pathway involving the JNK and the p38 kinases, as well as the NF-κB pathway. RONS-induced activation of p38 may play a role in muscle atrophy, JNK is involved in RONS-mediated apoptosis (Kramer & Goodyear, 2007; Powers *et al.*, 2010) and the NF-κB pathway is involved in skeletal muscle adaptation to exercise as well as inactivity-induced atrophy (Kandarian & Jackman, 2006; Li *et al.*, 2008). Moreover, chronic training in animals has been shown to result in less oxidative damage due to upregulation of endogenous antioxidant enzymes such as GPx and MnSOD (Gomez-Cabrera *et al.*, 2008*b*). Specifically, exercise in rats has been shown to activate MAPKs (p38 and ERK1/ERK2), in turn activating NF-κB, which led to increased expression of cell defence enzymes (MnSOD, GPx) and exercise adaptation enzymes (iNOS, eNOS), suggesting that "moderate exercise is an antioxidant" (Gomez-Cabrera *et al.*, 2005, 2008*b*).

Furthermore, 5 consecutive days of training (running at 65% of maximal oxygen uptake) in rats has shown to improve diaphragm oxidative stress capacity by decreasing lipid peroxidation by 30%, as well as enhancing antioxidant capacity by increasing activity of CAT and SOD, compared to untrained matched control rats (Vincent *et al.*, 2000). These enzymes have been shown to contain NF-κB binding sites in their gene promoter regions, which suggests that they can be targeted for exercise-activated upregulation via the NF-κB pathway (Hollander *et al.*, 1999).

Exercise training is known to be associated with enhanced muscle oxidative capacity and mitochondrial biogenesis, both of which the peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC1-α) is involved in (Kramer & Goodyear, 2007). Specifically, activation of the p38MAPK pathway has been shown to stimulate PGC1-α promoter activity in cultured myocytes through engaging the downstream transcription activating factor-2 in response to exercise (Akimoto *et al.*, 2005). It is therefore clear that, both MAPKs and NF-κB signalling pathways are activated by exercise and RONS are necessary intermediate signalling molecules (Kramer & Goodyear, 2007).

Moreover, there has been some evidence suggesting that supplementation of antioxidants may hamper training-induced muscle adaptations. In fact, a randomized double-blinded study specifically investigated the daily administration of 1g of vitamin C in males during 8 weeks of training, which consisted of static bicycle exercise 3 days per week, ranging between 65-

80% of $\dot{V}O_{2max}$ over the course of the 8 weeks (Gomez-Cabrera *et al.*, 2008*a*). With regard to endurance capacity, the $\dot{V}O_{2max}$ of the non-supplemented group after 8 weeks of training increased by 22%, compared to 11% in the supplemented group. This study also performed a rat experiment, where rats trained on the treadmill 5 days/week at 75% $\dot{V}O_{2max}$ for 3 and 6 weeks and were given 500mg/kg of bodyweight of vitamin C daily. Whereas the untrained group exercised for 10 mins every 3 days. The trained rats had increased maximal running time compared to the untrained (284 vs 99 mins) and this increase was prevented by daily vitamin C supplementation (Gomez-Cabrera *et al.*, 2008*a*). Specifically, after 3 weeks of training, the rats had higher mRNA skeletal muscle concentrations of Mn-SOD and GPx, which was decreased by supplementing with vitamin C, as well as increased skeletal muscle protein concentrations of PGC1-α, which was not observed in the vitamin C trained group (Gomez-Cabrera *et al.*, 2008*a*). Another study has demonstrated similar negative effects of vitamin E and C supplementation on exercise adaptation (Ristow *et al.*, 2009), whereas six other investigations reported non-beneficial outcomes and only two rat studies reported ergogenic effects (Nikolaidis *et al.*, 2012*a*). Therefore, supplementation of vitamin C and/or E is not recommended to healthy individuals as there is not enough evidence promoting beneficial effects (Nikolaidis *et al.*, 2012*a*).

In conclusion, a recent review of the current evidence on various antioxidant compounds (Vitamin C, E, C & E, A, catechins, curcumin, astaxanthin, anthocyanins, quercetin, resveratrol, α-lipoic acid, coenzyme Q10, melatonin, selenium, zinc and NAC) and endurance exercise training concludes that there is insufficient supporting evidence for supplementation of these antioxidant compounds to be recommended to athletes (Mason *et al.*, 2020).

2.9 Oxidative stress, RONS and Hypoxic Exercise

Oxygen is a central part in the generation of RNOS. Hypoxia can be defined as an imbalance between supply and demand for oxygen. In the state of cellular hypoxia, the insufficient O_2 that is available to be reduced in the mitochondria transport chain can lead to accumulation of the reducing equivalents (NADH and $FADH_2$), and thus RONS are more likely to be formed (Clanton, 2007). Thus, cellular hypoxia has been characterized by being in a more reductive state and described as a form of reductive stress (increased mitochondrial $NADH/NAD^+$), with the mitochondria suggested as

a possible source of free radicals involved in hypoxia-induced oxidative stress (Magalhães *et al.*, 2005). There is clear evidence of both acute and long-term hypoxic exposure associated with high-altitude and higher oxidative stress (Debevec *et al.*, 2017).

2.9.1 Exercise in Hypoxia and Oxidative Stress

In skeletal muscle, resting intracellular oxygen tension (iPO_2) is about 34 mmHg, however near maximal exercise it drops to 2-5 mmHg and $NADH/NAD^+$ redox status can be used to assess the availability of intracellular oxygen (Clanton, 2007). NADH content has been shown to decrease after 10 mins of 40% of steady work load in human muscle, whereas at 75 and 100% of maximal exercise it increases compared to resting levels (Sahlin *et al.*, 1987). This increase may not represent local hypoxia, but regardless, it is necessary for RONS production (Clanton, 2007).

In nature, "intermittent" hypoxia is the common form of hypoxia where transient decreases in oxygen occur over time during exercise in hypoxic conditions, altitude exposure (with or without exercise), or even in a variety of disease states (Clanton & Klawitter, 2001). During transition to hypoxia, RONS have been shown to be formed in skeletal rat muscle and peak over a short time, while NADH rapidly increase and iPO_2 decrease (Zuo & Clanton, 2005). This can explain the oxidative stress that has been shown to be associated with intermittent hypoxia, however the mechanisms of hypoxia-induced RONS formation in skeletal muscle are not fully understood (Clanton, 2007). Neuronal NO synthase (nNOS), one of the most important sources of NO in skeletal muscle, has low affinity for oxygen. Decreases in local iPO_2 can reduce nNOS activity, which can lead to the shift of NO/ $O_2^{\bullet-}$ balance towards $O_2^{\bullet-}$ (Clanton, 2007). NADPH oxidase is a much less likely source of RONS formation in hypoxia, as it is localized in the sarcolemma and likely exposed to higher iPO_2 compared to deep in the cell in the mitochondria (Clanton, 2007). However, RONS formation in hypoxia could play an important role in signalling adaptive responses and antioxidant treatment could enhance such responses. Specifically, rat diaphragm muscles treated with antioxidants scavenging $O_2^{\bullet-}$ during hypoxic exposure, can retain 30% more contractile force, compared to no treatment (Wright *et al.*, 2005). There are numerous human exercise investigations indicating that hypoxic exercise can lead to overproduction of RONS and increased oxidative stress. A study showed that runners who exercised for 6 weeks (2

running session/week) in hypoxia (3000m simulated altitude) had higher plasma MDA by 56% and protein oxidation products by 44% compared to normoxic conditions (Pialoux *et al.*, 2006). Similarly, acute maximal bicycle exercise in hypoxia (4559m for 3 days) resulted in higher urinary 8-oxodG, as well as increased DNA strand breaks, ENDO III- and FPG-sensitive sites, compared to normoxic exercise, indicating increased level of oxidative stress (Møller *et al.*, 2001). Specific to DNA damage and acute exercise in hypoxia, there are very limited reports, however a recent study utilized γ-H2AX as well as the comet assay to assess single- and double strand-breaks after 30 mins of cycling at 80-85% of $\dot{V}O_{2max}$ in hypoxia versus normoxia (12% FiO_2 vs 20.9% FiO_2) (Williamson *et al.*, 2020*a*). The results showed that hypoxic exercise increased both single- and double DNA strand-breaks and FPG-sensitive sites, which were all repaired within 4 hours post-exercise as well as within 48 hours *in vitro*. The authors also reported marked increases in lipid hydroperoxides and ascorbyl free radical concertation (Williamson *et al.*, 2020*a*). Such evidence may indicate that strenuous exercise in hypoxia may result in even greater oxidative damage in response to RONS formation. Similarly, urinary 8-OHdG has also been shown to increase as a function of exercise as higher levels were reported 1 hour post maximal cycle hypoxic exercise compared to rest, and interestingly, a 5-day tart cherry supplementation protocol attenuated this increase compared to placebo (Horiuchi *et al.*, 2023).

Another study assessed oxidative stress markers in nine males who climbed up and down mount Rainer (peak altitude at 4393m) over a 2-day period for a total of 14 hours of steady-pace climbing including rest periods (Miller *et al.*, 2013). Ferric-induced antioxidant potential (FRAP) was increased at 3000m (8 and 11% at ascent and decent respectively from pre) compared to pre and post values. Antioxidant capacity also increased by 8 and 10% at 3000m decent and post climbing compared to pre values. PC were elevated at 3000m (increase of 194% in ascent and 138% in decent) and lastly lipid hydroperoxides were increased post climbing by 257% compared to pre (Miller *et al.*, 2013).

Collectively, evidence shows that plasma antioxidant capacity is altered as a result of high altitude exercise (Quindry *et al.*, 2016). Studies at high altitude have shown an increase in uric acid following exercise, which is likely to be a product of purine metabolism in skeletal muscle (Sinha *et al.*, 2009; Peters *et al.*, 2016). Following an ultra-marathon at peak altitude of 3000m,

elevated uric acid concentration was correlated with elevated FRAP (Quindry *et al.*, 2008). Therefore, the observed increases in antioxidant capacity may be due to the acute changes of water soluble antioxidants like uric acid and vitamin C (Sinha *et al.*, 2009; Miller *et al.*, 2013; Peters *et al.*, 2016; Quindry *et al.*, 2016).

Furthermore, concentrations of circulating endogenous antioxidants may also be acutely elevated in response to hypoxic exercise. After 3 days of trekking in the Alps, CAT activity has been shown to increase, and the ratio SOD / CAT to decrease (Krzeszowiak *et al.*, 2014). Additionally, after 10 days of continuous hypoxic exposure and exercise, SOD and CAT were elevated, as well as FRAP and GPx (Debevec *et al.*, 2014). Another interesting study looked at exercise recovery at either normoxia or hypoxia (simulated altitude 5000m), where participants performed normoxic interval cycle ergometer exercise for 20 mins and muscle biopsies were obtained pre and 6 hours post recovery (Ballmann *et al.*, 2014). The authors reported increased gene expression of SOD2 by 42% in normoxic recovery compared to hypoxic recovery, as well as a 20% increase of nuclear factor (euthyroid-derived 2)-like factor (NFE2L2), which may indicate that hypoxic exercise recovery may exhibit alterations of exercise adaptations to oxidative stress (Ballmann *et al.*, 2014).

2.10 The Menstrual cycle, exercise, and oxidative stress

The female reproductive system is a vital part of the human reproduction process, designed to carry out several important functions. Its primary function is to produce egg cells required for reproduction. As an integral part of the female reproductive system, the menstrual cycle regulates various physical and hormonal changes. A healthy normal menstrual cycle lasts 28 days on average, but normal cycle length can vary from 22-36 days (Lamina *et al.*, 2013). The two major hormones involved in the menstrual cycle are oestrogen and progesterone, and their levels fluctuate throughout the monthly cycle. The menstrual cycle may be divided into three main phases: the menses; the follicular phase; and the luteal phases (Lamina *et al.*, 2013; Draper *et al.*, 2018). As depicted in **Figure 2.9**, phases can be further divided into the early, mid- and late follicular (EF, MF, LF); and early, mid- and late luteal phases (EL, ML and LL). LL phase can also be referred to as the pre-menstrual phase, as it is the phase prior to the following menstrual cycle (Draper *et al.*, 2018).

The follicular phase begins at the onset of menstruation lasting until the day prior to the onset of ovulation, while the luteal phase begins at the onset of ovulation, which lasts until the last day of the menstrual cycle (**Figure 2.9**). In the follicular phase, the uterine lining breaks down and sheds during menses (EF). After the last day of the menses, the body prepares for ovulation and a mature egg is released; this phase is oestrogen dominant (LF). Following this, ovulation takes place, where the mature egg is released and travels to the uterus to be fertilised by the sperm and, if contact is made, potential pregnancy occurs. Finally, in the last stage, known as the luteal phase, occurs during the second half of the cycle, where progesterone is dominant. This phase begins as the egg starts traveling through the fallopian tube (ML) and ends before the next menses begins (LL). Progesterone thickens the lining of the uterus so that a fertilized egg can implant. If the egg is not fertilized, oestrogen and progesterone levels start to drop, the uterine lining will again shed and the entire cycle will repeat (Melmed *et al.*, 2011).

Oestrogens are naturally occurring steroid molecules consisting of 18 carbons that are derived from cholesterol (Gruber *et al.*, 2002; Enns & Tiidus, 2010). The three major oestrogens are 17β-oestradiol (E2), which is the most potent during the reproductive years; estrone (E1) and estriol (E3) are also present at lower levels (Melmed *et al.*, 2011). Aromatase is an important enzyme (cytochrome P450 aromatase; CYP19a1) that synthesizes oestrogens from androgen precursors such as testosterone, 16-hydroxytestosterone and androstenedione, therefore its expression is crucial in regulating a normal reproductive cycle in females (Stocco, 2012). Aromatase is expressed in the theca cells, differentiated preovulatory granulosa cells, and luteal cells of the ovaries, making these the primary sources of E_2 in women (Gruber *et al.*, 2002; Stocco, 2012).

2.10.1 Antioxidant Properties of Oestrogen

Oestrogens act through oestrogen receptors (ERs), that exist in two forms, ERα and ERβ, which are widely expressed in reproductive organs as well as numerous tissues (Arias-Loza *et al.*, 2013). Phenol species contain one or more hydroxyl groups, which gives them the ability to reduce electrons (McKinley-Barnard & Willoughby, 2015).

Research suggests that oestrogen can have a high antioxidant capacity and can exert protective effects on cardiac, smooth and skeletal muscle in the animal model (Enns & Tiidus, 2010). Due to oestrogen's phenol-originating

carbon-ring structure, it contains one or more hydroxyl groups which have the ability to reduce electrons (Kendall & Eston, 2002). Similar to vitamin E, a well-known potent antioxidant, the hydroxyl group is located on the same position of their phenolic ring (Kendall & Eston, 2002). Skeletal muscle rat studies have shown female rodents, as well as male rodents, supplemented with oestrogen exhibit decreased inflammation and myofiber injury following exercise-induced muscle injury (Enns & Tiidus, 2010). Oestrogen supplementation in male rats has also been shown to greatly increase the number of muscle satellite cells following exercise, and this mechanism may be mediated through oestrogen receptors. Therefore, oestrogen may influence skeletal damage repair processes (Enns *et al.*, 2008).

Oestrogen can donate a H^+ atom to a peroxyl radical and exert free radical scavenging properties (Ayres *et al.*, 1998*a*). Thus, it was initially hypothesized that antioxidant properties could exist due the carbon-ring phenolic structure of E_2. However, this hypothesis has been deemed unlikely since plasma E_2 concentrations are too low to cause a meaningful effect via direct chemistry (Viña *et al.*, 2013). Instead, research suggests that binding to ERs stimulates gene expression of antioxidant defence enzymes by activating the MAPK and NF-kB pathways (López-Grueso *et al.*, 2014; Zárate *et al.*, 2017). Specifically, evidence has shown that undergoing surgical menopause (hysterectomy) in healthy females leads to impaired redox balance, as seen by an increased GSSG/GSH ratio and reduced mRNA expression of SOD and GPx. However, this imbalance was restored through oestrogen replacement therapy (ERT) consisting of 30 days of oestrogen administration (Bellanti *et al.*, 2013). Thus, increased oxidative stress and altered redox balance may be negatively associated with serum E_2 levels in a linear manner. Furthermore, E_2 can act as a signalling molecule in the upregulation of antioxidant gene expression, thus modulating redox status. Animal studies have indicated similar results, where mitochondria from oestrogen deprived ovariectomized rats have reduced levels of GSH, similar to male rats. In contrast, female rats have naturally higher GSH levels and oestrogen therapy can reverse this. Additionally, 8-OHdG mitochondrial oxidative damage is four times higher in males, compared to female rats, explained through higher expression of MnSOD and GPx, along with lower levels of peroxide in female rats (Borrás *et al.*, 2003). Further evidence supporting these findings in the rat model shows higher peroxide production in ovariectomized rats compared to controls. Notably, when ERT was applied immediately following ovariectomy, it offered significant protection against

this increase. However, this beneficial effect was not observed when ERT was initiated 3 or 6 weeks after the ovariectomy (López-Grueso *et al.*, 2014). In addition, ovariectomy resulted in higher MDA formation, indicating increased lipid peroxidation and protein oxidation, measured by oxidized protein levels in the brain. GPx and CAT were also shown to be decreased in the ovariectomized rats but upregulated with ERT applied immediately after ovariectomy (López-Grueso *et al.*, 2014).

Increased oxidative stress markers have been reported in young men, compared to young premenopausal women (Ide *et al.*, 2002). Immediately following as well as 3 hours post an 80% maximal heart rate treadmill run, increased MDA levels were observed in men. Whereas, this increase was observed in women only in the 3 hours post-exercise (Baghaiee *et al.*, 2016). Furthermore, there was an inverse correlation, observed only in the male group, between MDA and both MnSOD and Cu/ZnSOD, where measured MDA levels increased, gene expression of MnSOD & Cu/ZnSOD decreased. On the other hand, MDA levels, gene expression of Cu/ZnSOD as well as TAC (total antioxidant capacity) were all positively correlated (increased) in women. Additionally, at all measured time points, women had higher levels of gene expression of antioxidant enzymes as well as TAC (Baghaiee *et al.*, 2016). In cultured human epithelial cells, treatment with 17β-oestradiol has shown to trigger a rapid increase in intracellular mitochondrial ROS, accompanied by a transient rise of MnSOD activity at the 90 min incubation time point, but not beyond 6 hours of incubation (Gottipati & Cammarata, 2008).

Other animal studies have observed similar findings of greater MnSOD measured in the liver mitochondria of female compared to male rats (Ademoglu *et al.*, 2013). Furthermore, skeletal muscle of mice that were treated with 17β-oestradiol 48h after ovariectomy exhibited greater gene expression of GPx3, GPx2, Nox4, Txnip, and GPx1, compared to placebo (Baltgalvis *et al.*, 2010). However, after 3 weeks of ovariectomy, GPx3 was significantly higher —3.9-, 3.1-, and 3.4-fold, respectively —in the soleus, extensor digitorum longus (EDL) and tibialis anterior muscles of 17β-oestradiol-treated mice compared to ovariectomized mice.This effect was found to be mediated by ERα. Gpx1 and Txnip expression was also slightly greater, but only in the EDL muscle (Baltgalvis *et al.*, 2010). Lastly, expression of MnSOD and ecSOD are enhanced by oestrogens via

transcriptional pathways, mediated by oestrogen receptor activation in vascular smooth muscle rat cells (Strehlow *et al.*, 2003).

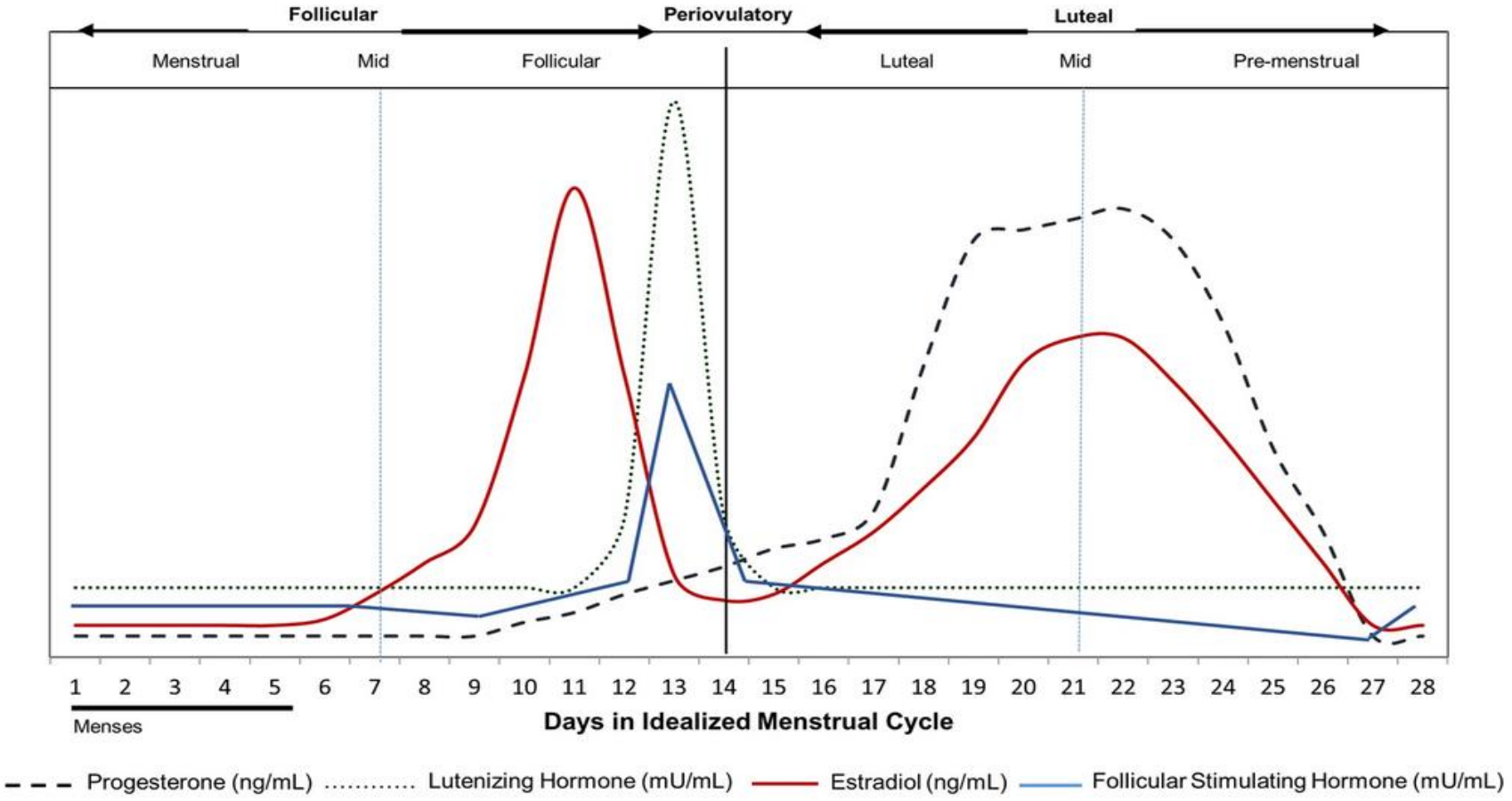


Figure 2.9 Idealized theoretical pattern of oestradiol, progesterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) changing concentrations according to the main five menstrual cycle phases (menses, follicular, periovulatory, luteal and pre-menstrual). Source from Draper *et al.* (2018).

CHAPTER THREE

CHAPTER 3: METHODOLOGY

3.1 Introduction

This chapter outlines the general methodology used for all experimental studies. The methodologies used in a single chapter alone will be outlined within the method section of that specific chapter.

3.2 Ethical Approval

Ethical approval for all studies involving human participants was obtained from Ulster University Research Committee (UUREC).

3.3 Participant Consent

Following recruitment, all participants who met the inclusion criteria of each study provided informed consent and completed a health history questionnaire. They were also informed they could withdraw from the study at any time without giving any reason.

3.4 Inclusion Criteria

3.4.1 Study 1 (experimental chapter 5)

Participants were recruited from the University population (staff and students) as well as the public using verbal, email communication and flyer/posters distributed in Jordanstown and Belfast campuses as well as in local gym clubs in the Belfast area. Individuals were recruited if they were: healthy males; aged 18-65 years old; non-smokers; recreationally fit (exercising 2-4 times per week); not taking any medication; and not taking any supplemental antioxidants like multivitamins or any other supplements such as creatine, BCAAs, pre-workout, fish oils/omega-3s at least 3 weeks prior to and for the whole duration of the study.

3.4.2 Study 2 (experimental chapter 6)

Participants were recruited from the University population (staff and students) using verbal, email communication and flyer/posters distributed in Jordanstown campus. Individuals were recruited if they were: healthy males; aged 18-35 years old; non-smokers; endurance trained (defined as partaking in at least 30 mins of moderate-intensity exercise 5 or more times weekly); and individuals who were not altitude trained.

3.4.3 Study 3 (experimental chapter 7)

Participants were recruited from the University population (staff and students) using verbal, email communication and flyer/posters distributed in Jordanstown campus. Individuals were recruited if they were: healthy females, aged 18-40 years old, non-smoking.

3.5 Anthropometry

Body weight and height were assessed using a calibrated scale (Seca, Hamburg, Germany) and a free-standing Harpenden stadiometer (Holtain Ltd, UK) to the nearest 0.1kg and 0.1 cm respectively. This was completed prior to exercise testing in the morning hours, following an overnight 8 hour fast. Participants were asked to wear comfortable clothing (shorts, loose t-shirt) for all exercise testing (footwear was removed for height/weight measurements).

3.6 Exercise Measurements and RPE

3.6.1 Maximum Oxygen Uptake ($\dot{V}O_{2max}$)

Breath-by breath gas exchange was measured using a Quark CPET Metabolic Cart (Quark CPET, COSMED s.r.l, Rome, Italy). The software used for gas analysis was PFT Ergo Version 1.6.5 (COSMED, Italy). Participants breathed through a sterilised rubber mouthpiece, which was attached to a two-way non-rebreathing valve and connected to a plastic hose for the transfer of expired air to the mixing chamber. Calibration was performed before all exercise testing (Calibration gas: 5% CO_2, 16% O_2, Balance N_2). The following criteria were used to determine $\dot{V}O_{2max}$: an increase in $\dot{V}O_2$ of less than 2 $mL.min^{-1}.kg^{-1}$ despite an increase in work rate; Rate of Perceived Exertion (RPE) ≥20; a Respiratory Exchange Ratio (RER) >1.15; and the attainment of 90% of age predicted max HR (max HR=220-age). Detailed descriptions of each study's $\dot{V}O_{2max}$ protocols are outlined in their corresponding chapters.

3.6.2 Heart Rate

Heart rate (beats per minute) was measured during all exercise testing using Polar Electro RS400. An adjustable heart rate monitor was placed around the chest of the individual, which measured the electric signals generated by the heart. The measured beats per min were transmitted to the Polar wristwatch and were recorded every 1 min for the $\dot{V}O_{2max}$ testing and every 5 mins of all other exercise testing.

3.6.3 Rate of Perceived Exertion

Rate of perceived exertion was measured using Borg's scale (Borg, 1982). A range of 6-20 was presented to participants every 1 min during $\dot{V}O_{2max}$ testing and every 5 mins for all other exercise testing. Participants were asked to describe their rate of perceived exertion ranging from "*very very light*" to "*maximum*".

3.6.4 Temperature and Humidity

During all exercise testing, room temperature was set at 18°C and humidity at 45% using an air conditioning system (Sanyo, Watford, UK).

3.7 Haematology

All blood sampling was performed by a qualified and experienced phlebotomist. All participants undertook an overnight fast (8-10 hours) prior to each experimental testing.

3.7.1 Venous Blood Sampling

The Vacutainer™ (Becton, Dickinson, Oxford, UK) method was used to collect all venous blood samples. After participants rested in a supine position, a tourniquet was fixed above the distal region of the bicep. A prominent vein was then selected from the antecubital region of the forearm and the area was cleaned with a 70% isopropyl alcohol swab. Blood was then collected using serum separation (SST) and di-potassium ethylene diamine tetra-acetic acid (EDTA) blood tubes (Greiner Bio-One, Austria). Following blood collection, EDTA tubes were immediately placed on ice whilst SST tubes were allowed to clot for 10 mins before placing on ice. Blood tubes were then centrifuged for 10 mins at 4000 rpm (Hettich, Germany). Plasma and serum were extracted using a 1 mL pipette (Gilson Medical Electronics, USA) and transferred to a 1.5 mL Eppendorf tube (Thermo Fisher Scientific, Waltham, MA, USA). Aliquots were immediately frozen and stored at -80°C prior to analysis.

For study 1, blood was collected at baseline, post-supplementation, post- and 3 hours post-exercise for a total of 4 time points. For study 2, blood was collected pre-exercise and 3 hours post-exercise for a total of 2 time points. Finally, for study 3, blood was collected pre-exercise, post- and 3 hours post-exercise for a total of 3 time points.

3.7.2 Packed Cell Volume (PVC) / Haematocrit (HCT)

After every sample was collected, 75 µL of whole blood was collected into a Hawksley heparinised capillary tube (Cat No 01603). One end of the capillary tube was sealed with a Hawksley Cristaseal. The heparinised samples were then centrifuged for 5 mins at 4000 rpm in a Micro Haematocrit centrifuge (Hawksley & Sons Ltd, UK). The samples were then removed, and packed erythrocytes were measured on a Hawksley micro-haematocrit reader (Hawksley & Sons Ltd, UK). Concentrations were expressed as a percentage of whole blood.

3.7.3 Haemoglobin (Hb)

With every collected sample, whole blood was placed into a micro-cuvette to determine the total amount of haemoglobin in whole blood. The micro-cuvette contains dried reagents. Sodium deoxycholate disintegrates the erythrocyte membranes, releasing the Hb. Sodium nitrite then converts the Hb iron from ferrous to the ferric state to form methaemoglobin, which then combines with azide to form azidemaethemoglobin. The micro-cuvette is then placed in the HemoCue Hb 201 DM analyser (Sweden) where the absorbance is measured (at 570 nm and 880 nm to compensate for turbidity) and the Hb level is calculated as $g \cdot L^{-1}$. The intra/inter assay CV was 5%.

3.7.4 Plasma Volume Changes

As exercise causes plasma volume loss, the intramuscular content of metabolites increases, and in turn, the extravascular osmolarity increases (Komka *et al.*, 2022). This, alongside increased arterial pressure and the activation of the sympathetic nervous system, leads to the enhanced filtration of plasma into the interstitial space known as the haemoconcentration phenomenon (Alis *et al.*, 2016). The Dill and Costill equations were used to estimate this phenomenon from changes in HCT and Hb. Thus, in order to estimate plasma volume loss, Hb and HCT were collected before and following exercise (Dill & Costill, 1974).

Blood volume $BV_{post} = BV_{pre} (Hb_{pre}/Hb_{post})$

Red cell volume $CV_{post} = BV_{post} (HCT_{post}/100)$

Plasma volume $PV_{post} = BV_{post} - CV_{post}$

Plasma volume change PVC (%) $= 100 (PV_{post} - PV_{pre}) / PV_{pre}$

3.8 Biochemical Analysis

3.8.1 Lipid Hydroperoxides (LOOH)

The FOX-1 assay was used to measure concentrations of lipid hydroperoxide. As described by Wolf, The FOX-1 reagent was prepared using 100 µM of xylenol orange (XO), 250 µM of ammonium ferrous sulphate [$(NH_4^+)_2Fe(SO_4)_2$], 100 mM of sorbitol and 25 mM of sulphuric acid (H_2SO_4) (Wolff, 1994). After mixing thoroughly, 90 µL of serum was added to 900 µL of the FOX-1 reagent and incubated in the dark for 30 mins. The absorbance is subsequently read at 560 nm with a UV mini-1240 UV-VIS spectrophotometer (Ireland), prior to which samples are centrifuged to remove any flocculant material, and the signal is read against a standard H_2O_2 linear curve (0-5 µM concentration) (Wolff, 1994). Under acidic conditions, Fe^{2+} can be oxidized by H_2O_2 to Fe^{3+}, which reacts with xylenol orange to generate a colour (purple) complex (Fe^{3+}-XO) (Li, 2019).

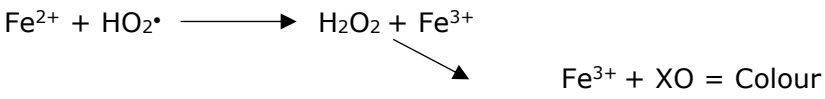


Figure 3.1 Hydroperoxyl-mediated Fe^{2+} oxidation and reaction with XO.

Abbreviations: HO_2•, hydroperoxyl radical; Fe^{2+}/Fe^{3+}, iron ion; XO, xylenol orange; H_2O_2, hydrogen peroxide.

3.8.2 Lipid Soluble Antioxidants

Serum samples were analysed for the measurements of γ-tocopherol, α-tocopherol, retinol, lycopene, α-carotene and β-carotene using High Performance Liquid Chromatography, as described by Thurnham *et al.*, (1988). 300 µL of serum was added to a glass test tube, along with quality control samples for each batch. The internal standard used was tocopherol acetate. A stock solution of 50mg tocopherol acetate was added to 10 mL of ethanol (containing 0.25% BHT). Following this, 1 mL of stock solution was diluted in 99 mL of ethanol with a final working solution of 1:100. 250 µL of internal standard was then added to each sample, followed by 500 µL of heptane. Tubes were then placed in a shaker for 7 mins and centrifuged at 3000 rpm for 5 mins. The resulting heptane layer (350 µL) was then added to an identically labelled glass tube. A further 500 µL of heptane was added to the original sample/tube and the above process was repeated. The combined heptane layers (700 µL) were evaporated to dryness in a

centrifugal evaporator under vacuum for approximately 90 mins. 150 µL of mobile phase (methanol, acetonitrile, dichloromethane; ratio 47:47:12 plus 250mg of BHT) was added to each sample/tube and mixed thoroughly by vortexing for about 30 seconds and then pipetted into HPLC vials to run overnight. Samples were then analysed various wavelengths of 292, 325 and 450 nm. Empower software (Empower Photodiode Array Software, Waters, Dublin, Ireland) was used to interpret the results. The intra/inter-assay coefficient of variation (CV's) was <7%.

3.8.3 Electron Paramagnetic Resonance Spectroscopy

The ascorbyl free radical was measured using EPR, which can detect free radicals unambiguously, on a Bruker EMX spectrometer (Bruker Instruments Inc., Billerica, MA, USA), as described previously by Clifford and colleagues (Clifford *et al.*, 2016). Firstly, 1 mL of plasma was mixed thoroughly with 1 mL of dimethyl sulfoxide (DMSO) in a glass test tube, and 1 mL of the final solution was drawn into a sterile syringe and flushed into the cavity. The EMX spectrometer settings were: frequency (9.785 GHz); microwave power (20mW); modulation frequency (100 kHz); and modulation amplitude (1.194 G) for three scans. Using the available software Bruker Win EPR System (Version 3.2, Bruker Instruments Inc., Billerica, MA, USA), spectra parameters were identically filtered. The concentration of the ascorbyl free radical was determined by the average spectral peak-to-trough line width.

3.8.4 Deoxyribonucleic Acid (DNA) Damage

DNA damage was measured in human white blood cells (WBCs) using single cell gel electrophoresis (SCGE) known as the comet assay technique (Singh *et al.*, 1988; Collins *et al.*, 1993). The assay was performed according to the classic alkaline assay as described by Collins *et al.*, 1993. Lymphocytes were isolated by layering 3 mL of whole blood onto 3 mL of Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 4000 rpm for 30 mins at 4°C. Next, 1 mL of WBCs was drawn and washed with 10 mL of PBS and centrifuged at 4000 rpm for 10 mins at 4°C. The formed pellet was then stored with 1600 µL RPMI, 200 µL FCS and 200 µL DMSO at -80°C. On the day of the comet assay, cells were thawed at room temperature and then centrifuged at 4000 rpm for 5 mins. Each pellet was then washed twice with 1 mL PBS and resuspended with 500 µL of PBS. Next, 100 µL of cells were mixed with 300 µL of 1% low melting point agarose, of which 70 µL was layered on to prepared normal agarose slides and allowed to solidify under coverslips at 4°C. After 10 mins, the coverslips were removed and placed in

lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma, 1% Triton-X, pH 10) for 1 h at 4°C. Slides were then placed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 12.5–13) for a 20-min incubation period, followed by 30 mins electrophoresis at 4°C (25 V, 300 mA). Following this, slides were washed for 5 mins with PBS and ddH_2O, respectively, and allowed to dry overnight in the dark. Slides were stained the next morning with SYBR ® Gold (Thermo Fisher Scientific, Waltham, MA, USA) diluted in TE buffer (10 mM Trizma, 1 mM EDTA, pH 8) on a rocker for 30 mins in the dark. 50 random cells were counted using the comet assay software (version 4.3.1) (Perceptive Instruments, Ltd, UK) at magnification 400× utilizing an Olympus BH-2 epifluorescence microscope. Quantification of DNA damage was based on the intensity of the comet tail fluorescence relative to the head (%). The median of the 50 random cells was used to calculate the average % of tail intensity for each time point/sample. The intra/inter-assay coefficient of variation (CV's) was <8%.

3.8.4.1 FPG Modification

A modification of the comet assay with bacterial formamidopyrimidine DNA glycosylase (FPG) was used for detecting not only strand breaks which the alkaline comet assay detects, but also specific DNA lesions and in this case, oxidized DNA bases (Azqueta & Collins, 2014). FPG removes the oxidized form of guanine, 8-oxoguanine (Prakash *et al.*, 2012). On the day of the comet assay, cells were thawed at room temperature and then centrifuged at 4000 rpm for 5 mins. Each pellet was then washed twice with 1 mL PBS and resuspended with 500 µl of PBS. Next, 100 µL of cells were mixed with 300 µL of 1% low melting point agarose, of which 70 µL was layered on to prepared normal agarose slides and allowed to solidify under coverslips at 4°C. After 10 mins, the coverslips were removed and placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma, 1% Triton-X, pH 10) for 1 h at 4°C. After cells were lysed, slides were washed three times in an enzyme reaction buffer (40 mM HEPES, 0.1 M KCL, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin, pH 8.0 with KOH) at 4°C in a tray for 5 mins each. Slides were removed after the final wash and dabbed dry to remove excess liquid. 50 µL of either the buffer alone (control) or the buffer containing the FPG enzyme was placed on the slides and covered with a 22 x 22 coverslip. The slides were then placed in a moist box and incubated for 30 mins at 37°C. Slides were then placed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 12.5–13) for a 20-min incubation period, followed by 30 mins electrophoresis at 4°C (25 V, 300 mA). Following this, slides were washed

for 5 mins with PBS and ddH_2O respectively and allowed to dry overnight in the dark. Slides were stained the next morning with SYBR ® Gold (Thermo Fisher Scientific, Waltham, MA, USA) diluted in TE buffer (10 mM Trizma, 1 mM EDTA, pH 8) on a rocker for 30 mins in the dark. 50 separate random cells were scored from control and FPG as mentioned in 3.8.4. The % of DNA tail intensity of the "Buffer" slide was subtracted from the % of DNA tail intensity of the "FPG" slide (Net FPG-sensitive sites = % FPG slide - % Buffer slide) in order to calculate the "net FPG-sensitive sites" (Azqueta & Collins, 2014).

3.8.4.2 Neutral Comet Assay

A modification of the comet assay using a neutral pH (7-10) of the electrophoresis buffer was performed according to Wojewódzka *et al.* (2002) and Fracasso *et al.* (2009). On the day of the comet assay, cells were thawed at room temperature and then centrifuged at 4000 rpm for 5 mins. Each pellet was then washed twice with 1 mL PBS and resuspended with 500 µL of PBS. Next, 100 µL of cells were mixed with 300 µL of 1% low melting point agarose, of which 70 µL was layered on to prepared normal agarose slides and allowed to solidify under coverslips at 4°C. After 10 mins, the coverslips were removed and placed in lysis buffer (2.5 M NaCl, 100 Mm EDTA, 10 Mm Tris-HCL, 1% *N*-lauroylsarcosine, pH 9.5). Immediately before use, 0.5% Triton-X and 10% DMSO were added to the buffer and mixed well for 10 mins. Following 1-2 hours of lysis at 4°C, slides were washed three times (5 minutes per wash) with electrophoresis buffer (300 mM sodium acetate, 100 mM Tris-HCL, pH 8.5) and then left in fresh buffer for 1 hour. Slides were placed to an electrophoresis tank covered with fresh buffer for 1 hour at 4°C at 14 V and 60 mA. Following this, slides were washed for 5 mins with PBS and ddH_2O respectively and allowed to dry overnight in the dark. Slides were stained the next morning with SYBR ® Gold (Thermo Fisher Scientific, Waltham, MA, USA) diluted in TE buffer (10 mM Trizma, 1 mM EDTA, pH 8) on a rocker for 30 mins in the dark. 50 separate random cells were scored as mentioned in 3.8.4.

The comet assay, also known as single-cell gel electrophoresis, was developed to determine DNA damage in agarose-embedded isolated cells. A DNA molecule is a highly supercoiled molecule, negatively charged at normal pH; after cells are lysed with a nonionic detergent and high-molarity sodium chloride, the cytoplasm, nucleoplasm, membranes, and histones are

removed, and a nucleus-shaped nucleoid is formed. As the negative supercoiled DNA breaks/unwinds, the relaxed loops/fragments expand from the nucleoid core and form a "halo" (Collins, 2004; Gagné *et al.*, 2014). The relaxed DNA loops/fragments stretch towards the anode during electrophoresis and form a structure resembling a "comet", made up of a "head" and a "tail" (Collins, 2004; Gagné *et al.*, 2014). The tail is made up of the DNA loops/fragments, as they migrate quicker than the DNA nucleoid core during the electrophoresis. Following fluorescent dye staining, the fluorescence intensity of the migrated DNA at the tail, relative to the head, is quantified to assess the extent of damage (Gagné *et al.*, 2014).

The concept of this assay, known as the "halo" assay was first introduced by Cook and Brazell in 1976 (Cook & Brazell, 1976). Later in 1984, Östling and Johansson proposed the "comet assay" using an electrophoresis buffer pH of <10 (Östling & Johanson, 1984). Even though this first version of the comet assay was performed under neutral conditions, it was not widely used, and a modified version was later developed by Singh in 1988 using an alkaline denaturing environment (Singh *et al.*, 1988). The rationale of the alkaline denaturation is that it was essential to reveal the strand breaks (SBs), however this was not the case (Collins, 2004). Instead, the alkaline treatment produces more pronounced comet tails, which extends their useful detectable range, but does not necessarily increase the sensitivity of the assay by detecting the lowest dose of damage possible (Collins, 2004). It was then suggested that an adapted neutral assay can provide improved sensitivity by detecting double SBs alone. The modifications included a 4 hour treatment of lysed cells in neutral pH solution at 50°C with the addition of proteinase K (0.5mg/mL) in the lysis solution (Olive *et al.*, 1991).

3.9 Statistical Analysis

All computerised statistical analysis was conducted using jamovi statistical software (The jamovi project, Sydney, Australia, v.1.6.8).

3.9.1 Coefficient of Variance

The coefficient of variance percentage (CV%), which measures the relative dispersion of data points in a data set in relation to the mean of the population, was calculated using the following equation:

$$CV\% = \frac{SD}{Mean}\ x\ 100$$

3.9.2 Descriptive Statistics

Normal distribution was determined using the Shapiro-Wilks test ($P > 0.05$) and all descriptive statistics data are expressed as Mean ± Standard Deviation (M ± SD).

3.9.3 Comparative Statistics

To compare variable differences between groups, a two-sample unpaired t-test (study one), a paired samples t-test (study two) and a two-tailed paired sample t-test (study 3) were used with a significant level set at $P < 0.05$. A two-way, repeated-measures ANOVA was used to measure differences between groups and within time. Following a significant interaction effect, between group and within differences were analysed using a one-way ANOVA with a *posteriori* Tukey Honestly Significant Difference test. The linear relationship between variables was assessed using Pearson's correlation coefficient (study two).

CHAPTER FOUR

CHAPTER 4: DNA DAMAGE FOLLOWING ACUTE AEROBIC EXERCISE: A SYSTEMATIC REVIEW AND META-ANALYSIS

4.1 Introduction

Exercise is widely regarded as a primary conduit to a proficient state of health, and there is now ample evidence from both observational studies and randomised trials to postulate that regular exercise is a contributing factor in the prevention of cardiovascular disease, cancer, diabetes and other chronic conditions, as well as reducing the risk of all-cause mortality (Finaud & Biologie, 2006; Warburton *et al.*, 2006).

Despite this paradigm, multiple studies have established a link between strenuous and/or exhaustive exercise, and the increased formation of reactive oxygen and nitrogen species (RONS) (Aguiló *et al.*, 2005). RONS are generated endogenously in most aerobic organisms by an incomplete reduction of oxygen, and mainly via the mitochondrial electron transport chain during cellular respiration (Ray *et al.*, 2012). It is currently well-understood that between 0.12-2% of the oxygen utilised by mitochondria during normal respiration is not converted to water (tetravalent reduction), but instead is reduced to the superoxide anion ($O_2^{\bullet -}$), which can subsequently be reduced to hydrogen peroxide (H_2O_2) and further to the more potent hydroxyl free radical ($OH^{\bullet}$) (Urso & Clarkson, 2003; Packer *et al.*, 2008). However, of note, the percentage estimation of total oxygen consumption in mitochondrial RONS production refers primarily to the *in vitro* based experiments performed by Chance and colleagues (Chance *et al.*, 1979); as such, the production of $O_2^{\bullet -}$ *in vivo*, may indeed be much less (Boveris *et al.*, 1972; Murphy, 2009; Brown & Borutaite, 2012).

RONS are often implicated in complex molecular mechanisms designed to explain the process of human ageing and associated chronic diseases states (Boccatonda *et al.*, 2016). Associated molecules such as lipid, protein and DNA, are known vulnerable targets of RONS attack, and therefore can be oxidatively modified (Reichhold *et al.*, 2009*a*). Oxidative free radical attack and subsequent damage to DNA in particular, is of prime biomedical importance and interest, as if left unrepaired, significant DNA alterations (e.g., chromosomal rearrangement, base damage, and strand breaks) may lead to mutagenesis, and ultimately carcinogenesis (Birben *et al.*, 2012; Cobley *et al.*, 2015; Davison, 2016). Paradoxically, although excessive RONS production may be implicated in the pathology of numerous diseases

(Pesta & Roden, 2017), when produced in moderate/low amounts (i.e. not inducing a state of oxidative stress defined as an 'imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage') (Sies & Jones, 2007), they act as key intracellular signalling molecules regulating a host of physiological and biological processes (Radák *et al.*, 2008; Boccatonda *et al.*, 2016). RONS are generated in skeletal muscle and play a key role in skeletal muscle adaptation to aerobic exercise training (Powers *et al.*, 2010; Nemes *et al.*, 2018). *In vitro* work has shown myotubes exposed to hydrogen peroxide (exogenous), increases the expression of peroxisome-proliferator-activated receptor (PPAR-γ), and peroxisome-gamma co-activator-1 alpha (PGC-1α), whereas further exposure to N-acetylcysteine, an antioxidant, impeded its activity (Irrcher *et al.*, 2009). PGC-1α, which is induced by AMP kinase (AMPK), is a signalling pathway involved in adaptation to endurance exercise leading to mitochondrial biogenesis (Radák *et al.*, 2008). Similarly, *in vivo* work has demonstrated that antioxidant supplementation can hinder essential training adaptation mechanisms in humans. A study administrating 1 g of vitamin C per day to humans during 8 weeks of training (3d/week at 65% to 80% $\dot{V}O_{2max}$; 5% increase every 2 weeks), resulted in decreased expression of PGC-1α and mitochondrial transcription factor A, both of which are key transcription factors involved in mitochondrial biogenesis (Gomez-Cabrera *et al.*, 2008*a*).

Mechanistically, there are several ways free radicals can be generated during exercise. While exercising, the energy requirements in the body greatly increase, leading to a substantially higher rate of oxygen uptake up to 15-fold, and in active muscle, the oxygen flux may increase to about 100-fold compared to resting values (Cooper *et al.*, 2001; Banerjee *et al.*, 2003). The primary radical species produced by the contracting skeletal muscle are $O_2^{\bullet-}$ and nitric oxide (NO) (Jackson *et al.*, 2016). When electron transfer occurs normally through the electron mitochondrial transport chain to reduce oxygen to water, about 1-3% of all electrons are leaked resulting in the formation of $O_2^{\bullet-}$ by adding one electron to molecular oxygen (Powers & Jackson, 2008; Birben *et al.*, 2012). Apart from the mitochondria, there are enzymatic sources that contribute substantially to free radical production such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the enzyme which catalyses the one electron reduction of molecular oxygen (reaction 1) upon the activation of phagocytosis (Turner *et al.*, 2011*a*; Birben *et al.*, 2012).

$H\ O_2^{\bullet-} \leftrightarrows O_2^{\bullet-} + H^+$ (reaction 1)

Central to these mechanisms is the generation of the superoxide anion ($O_2^{\bullet-}$; one-electron reduction) and subsequently, produced through the superoxide dismutases (SODs), hydrogen peroxide (H_2O_2; two-electron reduction) (Birben *et al.*, 2012). Following the production of H_2O_2, the hydroxyl radical ($OH^{\bullet}$; three-electron reduction) can be produced in the presence of transition metal catalysts, through the Haber-Weiss Fenton reaction (reaction 2) (Powers & Jackson, 2008; Birben *et al.*, 2012). $O_2^{\bullet-}$ also reacts with NO (reaction 3) to produce peroxynitrite ($ONOO^-$), a highly reactive RONS that can cause damage to DNA and nitrate proteins (Powers & Jackson, 2008).

$H_2O_2 + Fe^{2+} \leftrightarrows Fe^{3+} + OH^- + OH^{\bullet}$ (reaction 2)

$O_2^{\bullet-} + NO^{\cdot} \rightarrow ONOO^-$ (reaction 3)

As RONS accumulate in the cell, either from metabolic signalling (NADPH) pathways or external sources, they are balanced by scavenging antioxidant systems (Mittler, 2017). Under these balanced conditions, RONS are used as signalling molecules or, under unbalanced conditions, can interact with Fe^{2+} through Fenton chemistry, as mentioned above, and cause cellular damage due to hydroxyl radicals ($OH^{\bullet}$), which in turn can be attenuated by DNA repair mechanisms. In the case of over-accumulation of such DNA damage and insufficient repair, it is conceivable to suggest that rapidly dividing cells may promote a mutational profile leading to disease. However, per their signalling role, RONS and DNA damage can trigger physiological programmed cell death (apoptosis) by activating p53 to prevent mutagenesis/carcinogenesis (Mittler, 2017). Therefore, it is important to differentiate whether cell death is caused by oxidative stress (i.e. DNA damage), which can be avoided (scavenging systems, DNA repair mechanisms), or programmed cell death via RONS signalling which could be advantageous when the cell becomes compromised, as a result of DNA damage (Mittler, 2017). RONS are therefore important molecules involved in the fate of the cell's destiny as they regulate crucial processes such as growth, differentiation, and cell death (Nocella *et al.*, 2019). Once DNA is damaged, it is normally repaired by mechanisms such as base excision repair (BER), nucleotide excision repair (NER), or through a process of homologous recombination (HR) or nonhomologous end joining (NHEJ); the

type of DNA repair will depend on the mechanism and the extent of the damage (Hakem, 2008) (**Figure 4.1**).

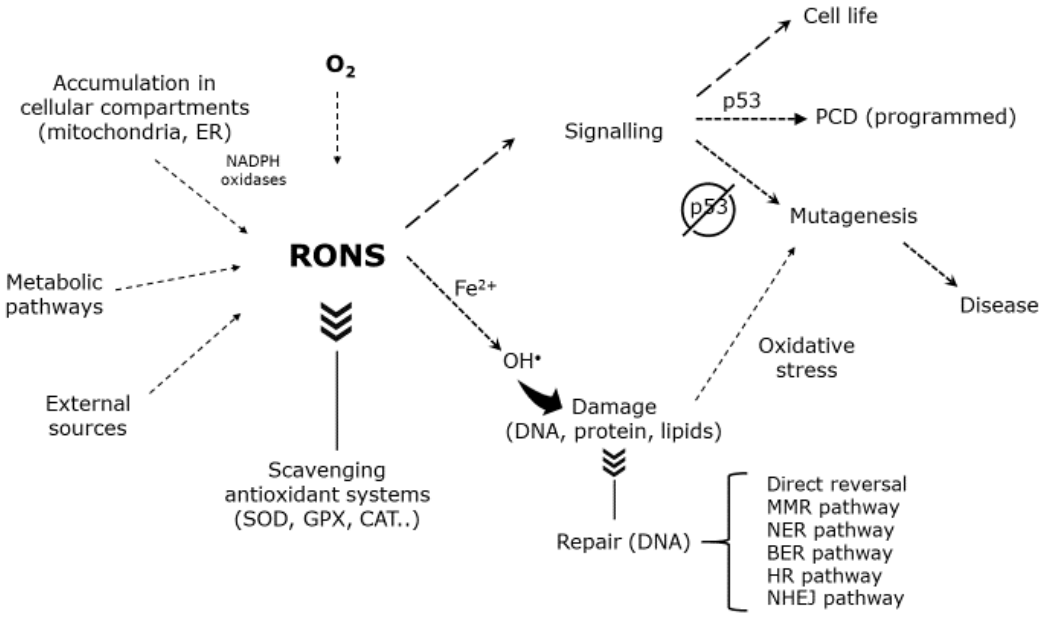


Figure 4.1. RONS production, scavenging and DNA damage repair pathways. Adapted from Mittler (2017).

Abbreviations: ER, endoplasmic reticulum; RONS, reactive oxygen and nitrogen species; NADPH, nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase; O_2, dioxygen; $OH^{\bullet}$, hydroxyl radical; PCD, physiological cell death; MMR, mismatch repair; NER, nucleotide excision repair; BER, base excision repair; HR, homologous recombination; NHEJ, non-homologous end joining.

Exercise represents an intriguing model to examine the dynamic role of RONS from both a physiological and pathological perspective. Evidence suggests that only exhaustive (long-distance) and/or strenuous exercise (high-intensity maximal exercise, marathons, triathlons and overtraining) can induce detrimental DNA alterations, if left unrepaired (Radák *et al.*, 2008; Fisher-Wellman & Bloomer, 2009). However, during low or moderate-intensity and distance exercise, the generated RONS may serve to act as signalling molecules responsible for the initiation of exercise and skeletal muscle adaptation (Sachdev & Davies, 2008; Fisher-Wellman & Bloomer, 2009; Powers *et al.*, 2010), as often conceptualised through the hormesis theory.

The aim of this work is to systematically investigate data reporting DNA damage following acute aerobic exercise and perform a meta-analysis to examine the overall effect from these studies. There are discrepancies regarding exercise intensity and that it necessarily needs to be very exhaustive/strenuous to cause oxidative damage and/or stress and this review will aim to elucidate this. Furthermore, the possible physiological and/or pathological consequences of exercise-induced DNA damage need to be considered in relation to the exercising individual in line with a new proposed multi-dimensional model. This is the first meta-analysis aimed to investigate the relationship between DNA damage and exercise.

4.2 Methods

4.2.1 Search Strategy

According to the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) guidelines (Moher *et al.*, 2009), a detailed search was conducted to identify all relevant studies (including a range of publication from 1900 – April 2019) across the following five databases: Web of Science, PubMed, MEDLINE, EMBASE, and Scopus. Searching was limited to articles published in English and the filter "in humans" was applied on PubMed, MEDLINE and EMBASE.

4.2.2 Inclusion/Exclusion Criteria

All published studies were checked for the following criteria: (1) the study was a full report published in a peer-reviewed journal; (2) the study assessed humans; and (3) the keyword combination referred to the following terms (used in all possible combinations): *exercise, exercis*, exercise training, endurance, exhaustive, exercise-induced, acute exercis*, physical activity, DNA, nucleoid DNA , deoxyribonucleic acid, 8-hydroxy-2'-deoxyguanosine, 8-hydroxy-2-deoxyguanosine, 8-oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxo-7,8-dihydro-2-deoxyguanosine, 8-hydroxy-2-deoxy guanosine, 8-hydroxydeoxyguanosine, 8-oxoguanine, 8-hydroxyguanosine, 8-oxo-2-deoxyguanosine, 8-OHdG, 8OHdG, 8-OH-dG, 8-OHG, 8-oxo-dG, 8-oxodG, 8-Oxo-dG, 8-oxo-G, damage, oxidative damage, oxidative stress*. Note that for the purposes of this review we used the term *DNA damage* to encompass DNA single strand breakage and nucleotide base oxidation.

One investigator initially reviewed records generated from all databases and applied the inclusion/exclusion criteria to identify eligible studies for inclusion; these were then agreed with at least three of the authors. The

inclusion/exclusion criteria are shown in **Table 4.1**. To note, acute exercise was defined as aerobic exercise performed over a short period of time but could also extend up to 1-3 days of a marathon event. To minimise the limitation of various biological samples, studies utilising urine, red blood and muscle cells were also excluded. Please see **Appendix G Supplementary Table S1** for information and detail of excluded studies.

Table 4.1. Inclusion/Exclusion Criteria.

Criteria	Include	Exclude
Participants	Humans	Animals
Age group	18-70	Children, elderly
Exercise protocol	Acute, aerobic	Anaerobic, training (chronic)
Sample	White blood cells (leukocytes, lymphocytes, PBMC),	Urine, red blood cells (erythrocytes), muscle
Outcome measure	SBs (%), tail damage (%), tail length, tail moment, 8-OHdG	Double SBs

Abbreviations: SBs, strand breaks; 8-OHdG, 8-hydroxy-2'–deoxyguanosine; PBMC, peripheral blood mononuclear cells.

4.2.3 Data Extraction

A general extraction form was used, once the number of included studies was finalised. Characteristics of the participants (sample size, age, and sex), the exercise protocol (distance and intensity), assayed biomarkers, and methods of DNA quantification used were extracted by one investigator. The outcome measure, DNA damage, was expressed using multiple descriptors, and with regard to the comet assay these were: DNA in the tail (%); DNA migration (μm) (otherwise known as tail length); tail moment (also known as olive tail moment) which is the product of tail (%) and tail length (Møller *et al.*, 2014).

The biomarker used was 8-OHdG. Due to variations in the analytical approach, high performance liquid chromatography (HPLC) or enzyme-linked immunosorbent assay (ELISA), 8-OHdG (pg/mL) and 8-OHdG/ 10^5 dG are also reported. The tail DNA (%), DNA migration (μm) or tail length and tail moment correspond to the comet assay and the 8-OHdG (ng/mL) or (pg/mL) and 8-OHdG/ 10^5 dG to HPLC or ELISA methods. In reference to the comet assay, where multiple image descriptors were reported by one study, the authors used tail (%), as this is regarded as the most sensitive descriptor/parameter compared to tail moment or length (Collins & Brunborg, 2017). Data were collected as means and standard deviation (SD) or standard error of the mean (SEM). Graph digitizer software (DigitizeIt, Braunschweig, Germany) and WebPlotDigitizer (Web Plot Digitizer, V.4.2. Texas, USA: Ankit Rohatgi, 2019) were used to obtain data from studies where data were only presented in a figure format. In two studies (Asami *et al.*, 1998; Roh *et al.*, 2016), data were not extractable and therefore not included in the meta-analysis.

Numerous studies included heterogeneous groups of participants: trained or untrained, young or old, sport-specific volunteers (such as swimmers, rowers, runners), physically active and sedentary participants and a few studies compared men and women. Furthermore, three studies (Niess *et al.*, 1996; Liu *et al.*, 2015; Ryu *et al.*, 2016) used more than one parameter to quantify DNA damage. Lastly, some studies measured DNA damage at only one time-point (TP) while other studies included multiple post exercise measures of DNA damage following exercise. **Table 4.2** details corresponding TPs for each investigation.

4.2.4 Data Analysis

The primary outcome was defined as DNA oxidative damage before and following exercise at TP 0 (0h) grouped by method of DNA damage quantification (1) comet assay and (2) 8-OHdG. Secondary outcomes included: (3) high-intensity (≥75% of maximum rate of oxygen consumption; $\dot{V}O_{2max}$) and (4) long-distance (≥42km) as different exercise protocols all measured, and finally (5), DNA damage at further time-points 1-11 (ranging from 15 mins-28 days).

4.2.5 Quality Assessment

In order to assess the quality of included studies, the risk of bias was assessed by one investigator using the 12 criteria (rating: yes, no, unsure) recommended by the Cochrane Back Review Group (**Table 4.3**) (Furlan *et*

al., 2009). The criteria assess risk of bias using the five following categories: selection bias; performance bias; attrition bias; reporting bias and detection bias. However, due to the inherent difficulties in blinding participants to exercise treatments, seven of the twelve criteria were not applicable, and as such not included. These were: adequate method of randomization; allocation concealment; outcome assessor blinding; participant and provider blinding; similarity or not of co-interventions and intention-to-treat analysis. In contrast, two additional sources of bias, smoking and training status, were included as criteria given their potential to influence exercise responses. Following these modifications, the maximum score that studies could gather would be seven, with the lowest scores indicating high risk of bias and higher scores indicating lower risk of bias. To establish a clearer overall assessment of bias, a high, moderate, and low risk scale was developed according to how studies scored. Therefore, the following ranges were developed: 1-3 = high risk, 4-5 = moderate and 6-7 = low risk.

4.2.6 Statistical Analysis

Assessment of effect size: Meta-analyses were calculated using Comprehensive Meta-Analysis (Version 3.3.070, NJ:USA: Biostat, Inc). A random effects model was used since it assumes statistical heterogeneity among studies and that studies represent a random sample of effect sizes that could have been observed (Borenstein *et al.*, 2009; Higgins & Green, 2011). Standardised mean differences (SMD) adjusted with Hedges' g and 95% confidence intervals (CI) were calculated as the difference in means before and after exercise divided by the pooled standard deviation (Borenstein *et al.*, 2009). Where studies did not report standard deviations, these were calculated from standard errors (Higgins & Green, 2011). The SMD measure was used to express effect size, the magnitude of which was calculated using Cohen's categories: (1) small: SMD = 0.2-0.5, (2) medium: SMD = 0.5-0.8 and (3) large: SMD > 0.8 (Cohen, 1988; Faraone, 2008). A positive SMD measure was considered to show increased DNA damage after exercise compared to rest, whereas a negative SMD measure would show greater DNA damage at rest in comparison to after exercise. The overall effect was assessed using Z scores with a set significance level of $P < 0.05$.

Assessment of heterogeneity: The chi^2 Cochran's Q test and the I^2 statistic were used for the assessment of statistical heterogeneity among studies. The chi^2 test assesses whether the observed differences in results are compatible with chance alone and a p value ≤ 0.10 was considered to display significant heterogeneity (Higgins & Green, 2011). Furthermore, the I^2 statistic was used to quantify inconsistency across studies, with (1) I^2=0-30% showing no heterogeneity, (2) I^2=P30-49% showing moderate heterogeneity, (3) I^2=50-74% showing substantial heterogeneity and (4) I^2=5-100% showing considerable heterogeneity (Higgins & Green, 2011).

Subgroup and Sensitivity Analysis: Subgroup analyses were performed for multiple time-points of DNA damage quantification after exercise grouped by different methodologies in DNA quantification (comet assay versus 8-OHdG), and according to the exercise protocol: high-intensity (≥75% $\dot{V}O_{2max}$) versus long-distance (≥42km). To assess the robustness of the significant outcome data, sensitivity analysis was planned by excluding studies with high risk of bias.

Publication Bias: Publication bias was assessed, when at least 10 studies were included in the meta-analyses, by visually analysing funnel plots. Generally, asymmetrical funnel plots were considered to indicate high risk of publication bias, while symmetrical funnels plots were considered to indicate low risk (Egger *et al.*, 1997).

4.3 Results

4.3.1 Literature Search

The number of articles identified from all electronic database searches and the selection process is shown in **Figure 4.2**. Four thousand four hundred and twenty records (4,420) were retrieved in the database search, one hundred and four (104) of which were duplicates. Four thousand one hundred and forty-one articles (4,141) were excluded after title screening, leaving one hundred and seventy-five (175) records for abstract screening. One hundred and thirteen (113) records were excluded after abstract screening and sixty-two (62) full-text articles were assessed for eligibility. Twenty-three (23) full-text articles were excluded due to various reasons (detailed in **Appendix G Supplementary Table S1**). The most common reason for study exclusion was the exercise protocol not consisting of acute and aerobic exercise. Thirty-nine studies (39) were included in the qualitative

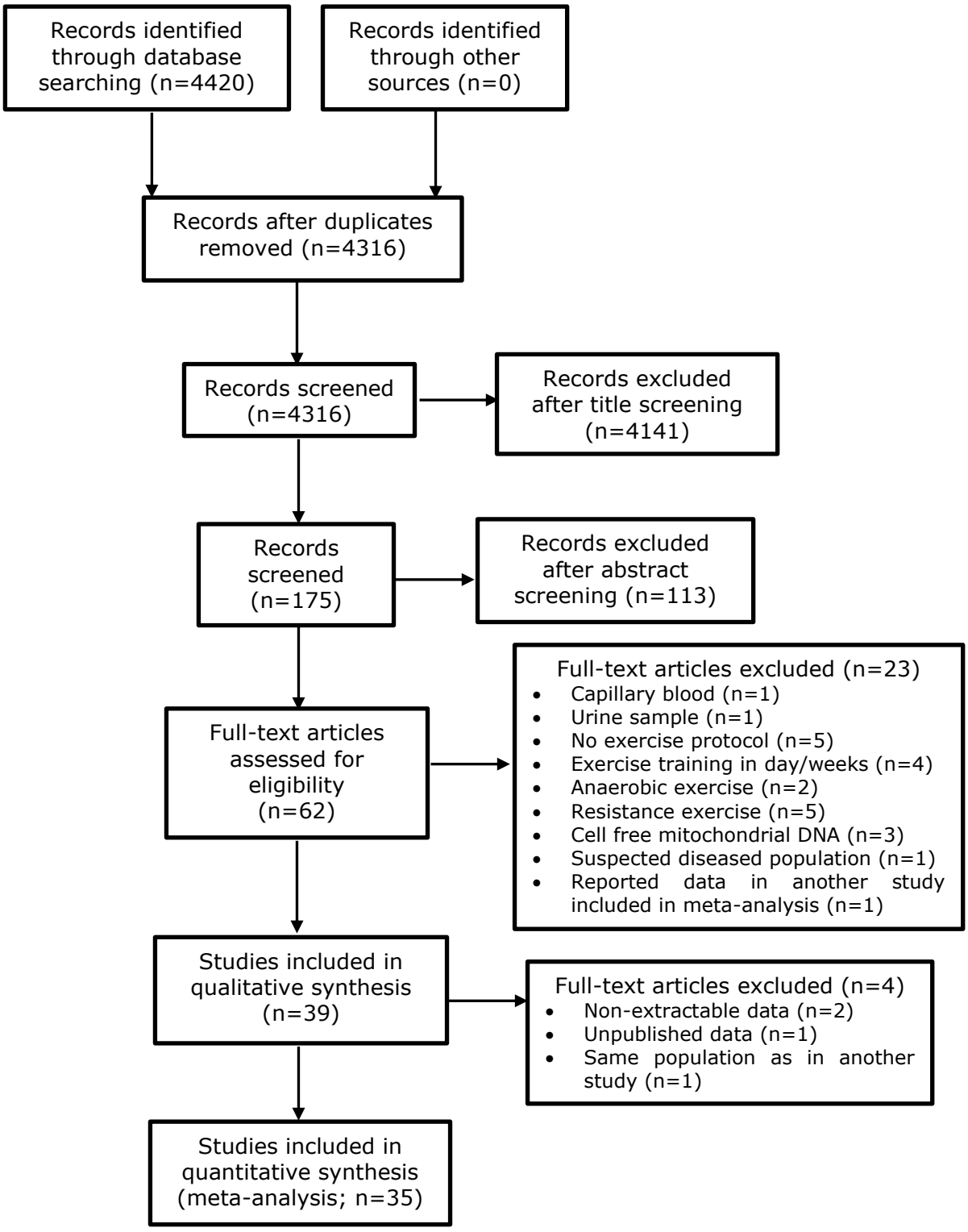


Figure 4.2. PRISMA flow Diagram displaying the electronic search and selection process.

analysis, out of which one (1) was excluded due to same sample size (Reichhold *et al.*, 2009*b*), one had unpublished data (Orlando *et al.*, 2018) and two (2) due to non-extractable data (Asami *et al.*, 1998; Roh *et al.*, 2016), therefore thirty-five (35) were included in the quantitative analysis.

4.3.2 Study Characteristics and Quality Assessment

Data extraction for individual time-points of each study are presented in **Table 4.2**. As seen in **Table 4.3***, Quality Assessment in Individual Studies*: No study scored in the high-risk bias range (1-3), eleven studies scored in the moderate risk range (4-5), and the remaining twenty-seven studies scored in the low-risk range (6-7).

The characteristics of each study including participants, exercise protocol, sample source, biomarker, quantification technique and results are presented in **Table 4.4**.

Participants: Participant age ranged from 18 to 70 years old. Five studies included both male and female participants (Møller *et al.*, 2001; Mastaloudis *et al.*, 2004*b*; Briviba *et al.*, 2005; Pittaluga *et al.*, 2013). Three studies included groups of untrained and trained subjects (Niess *et al.*, 1996; Asami *et al.*, 1998; Tanimura *et al.*, 2010), one study (Sardas *et al.*, 2012) used rowers and physical education students, while another (Inoue *et al.*, 1993) used swimmers and runners. Finally, two studies used volunteers participating in multiple running distances (Briviba *et al.*, 2005; Ryu *et al.*, 2016).

Biomarkers/Analytical Techniques: With regard to the biomarker and the techniques used to quantify DNA damage, twelve studies used 8-OHdG (Inoue *et al.*, 1993; Asami *et al.*, 1998; Sato *et al.*, 2003; Sacheck *et al.*, 2003; Bloomer *et al.*, 2006*b*; Itoh *et al.*, 2006; Shi *et al.*, 2007; Revan, 2011; Saritaş *et al.*, 2011; Harms-Ringdahl *et al.*, 2012; Pittaluga *et al.*, 2013; Meihua *et al.*, 2018) with either HPLC or ELISA. A total of twenty-seven studies used tail DNA (%) or strand breaks, tail length, tail moment with the comet assay technique (Hartmann *et al.*, 1994, 1995, 1998; Niess *et al.*, 1996, 1998; Møller *et al.*, 2001; Tsai *et al.*, 2001; Zhang *et al.*, 2004; Mastaloudis *et al.*, 2004*b*; Briviba *et al.*, 2005; Davison *et al.*, 2005; Peters *et al.*, 2006; Tanimura *et al.*, 2008, 2010; Paik *et al.*, 2009; Wagner *et al.*, 2010; Fogarty *et al.*, 2011; Turner *et al.*, 2011*b*; Fogarty *et al.*, 2013*b*; Sardas *et al.*, 2012; Liu *et al.*, 2015; Roh *et al.*, 2016; Ryu *et al.*, 2016; Orlando *et al.*, 2018; Williamson *et al.*, 2018; Kim *et al.*, 2018)**.**

Exercise Protocol: There was variation in the chosen exercise protocols, most often involving treadmill exercise and cycling whilst employing different exercise intensities (ranged from 40%-100% $\dot{V}O_{2max}$). Eight studies included marathons, half-marathons or ultramarathons (Hartmann *et al.*, 1998; Niess *et al.*, 1998; Tsai *et al.*, 2001; Mastaloudis *et al.*, 2004*b*; Briviba *et al.*, 2005; Turner *et al.*, 2011*b*; Ryu *et al.*, 2016; Kim *et al.*, 2018) and three studies (Reichhold *et al.*, 2009*b*; Wagner *et al.*, 2010; Kim *et al.*, 2018) involved a triathlon as part of the exercise protocol.

4.3.3 Analysis of Overall Effects

In summary, as seen in **Table 4.5**, a significant increase in DNA damage following exercise was observed at time-points 0 (0h), 2 (2h), 3 (3h), 4 (4-6h), 5(1d) and 7 (3d). No significances were found at time-points 1 (15mins-1h), 6 (2d), 8 (4d), 9 (5d), 10 (6-7d) and 11 (14-28d).

Overall effect of DNA damage after exercise at TP 0: For DNA damage after exercise at TP 0 (0h), data were available from 24 studies, with a total number of 312 participants. As seen in **Figure 4.3**, compared to rest, there was a significant increase in DNA damage after exercise (SMD = 0.875; 95% CI:0.5,1.25; $P < 0.05$). Heterogeneity among studies was found to be considerable ($chi^2 = 5.25$, $p = 0.02$, $I^2 = 82.12\%$).

Comet assay vs 8-OHdG at TP 0: Similarly, as shown in **Figure 4.3**, for studies utilizing only the comet assay with 203 participants, DNA damage remained significantly higher after exercise at TP 0 (0h) compared to rest (SMD = 1.14; 95% CI:0.7,1.58; $P < 0.05$). Moreover, although the number of studies using the 8-OHdG biomarker was considerably less, with 109 participants, no change in DNA damage compared to rest using this assay was observed. (Please see **Figure 4.3**; SMD = 0.15; 95% CI:-0.58, 0.88; $P = 0.68$).

High-intensity Exercise (≥75% $\dot{V}O_{2max}$): DNA damage was increased after high-intensity exercise (≥75% $\dot{V}O_{2max}$), measured at time-point 0 (0h) & 5 (1d) (**Figure 4.4a**; SMD = 1.18; 95% CI:0.71,1.65; $P < 0.05$; heterogeneity: $chi^2 = 3.1$, $P = 0.08$, $I^2 = 63.98\%$).

Long-Distance Exercise (≥42 km): As shown in **Figure 4.4b**, DNA damage was not significantly higher after long- distance (≥42 km) exercise at time-point 0 (0h) & 1 (15mins-1h) (SMD = 0.48; 95% CI:-0.16,1.03; $P = 0.15$; heterogeneity: $chi^2 = 25.84$, $P = 0.001$, $I^2 = 72.91\%$).

Table 4.2. Individual time-points (TP) of measures of DNA damage after exercise for each investigation.

Study	TP 0 (0h)	TP 1 (15m-1h)	TP 2 (2h)	TP 3 (3h)	TP 4 (4h-6h)	TP 5 (1d)	TP 6 (2d)	TP 7 (3d)	TP 8 (4d)	TP 9 (5d)	TP 10 (6d-7d)	TP 11 (14d-28d)
Bloomer *et al*. (2006*b*)	0h											
Briviba *et al*. (2005)		20mins										
Davison *et al*. (2005)	0h	1h										
Fogarty *et al*. (2011)	0h											
Fogarty *et al*. (2013*b*)	0h											
Harms-Ringdahl *et al*. (2012)		1h										
Hartmann *et al*. (1994)	0h				6h	1d	2d	3d	4d			
Hartmann *et al*. (1995)						1d						
Hartmann *et al*. (1998)	0h					1d	2d	3d	4d	5d		
Inoue *et al*. (1993)	0h											
Itoh *et al*. (2006)	0h	1h				1d						
Kim *et al*. (2018)	0h							3d				
Liu *et al*. (2015)	0h											
Mastaloudis *et al*. (2004*b*)	0h					1d	2d	3d	4d	5d	6d	
Meihua *et al*. (2018)	0h											
Møller *et al*. (2001)	0h					1d	2d					
Niess *et al*. (1996)		15mins				1d						
Niess *et al*. (1998)		1h										
Paik *et al*. (2009)	0h	1h										
Peters *et al*. (2006)	0h			3h								
Pittaluga *et al*. (2013)		30mins				1d						
Revan (2011)	0h	30mins										
Ryu *et al*. (2016)	0h							3d				
Sacheck *et al*. (2003)						1d						

Study	TP 0 (0h)	TP 1 (15m-1h)	TP 2 (2h)	TP 3 (3h)	TP 4 (4h-6h)	TP 5 (1d)	TP 6 (2d)	TP 7 (3d)	TP 8 (4d)	TP 9 (5d)	TP 10 (6d-7d)	TP 11 (14d-28d)
Sardas *et al*. (2012)						1d						
Saritaş *et al*. (2011)	0h					1d						
Sato *et al*. (2003)	0h	1h				1d	2d					
Shi *et al*. (2007)	0h			3h		1d						
Tanimura *et al*. (2008)	0h	1h	2h	3h	4h							
Tanimura *et al*. (2010)						1d						
Tsai *et al*. (2001)	0h					1d		3d			7d	14d
Turner *et al*. (2011*b*)	0h					1d					7d	28d
Wagner *et al*. (2010)		20mins				1d				5d		19d
Williamson *et al*. (2018)	0h											
Zhang *et al*. (2004)			2h		6h	1d						

Table 4.3. Quality assessment for risk of bias of the included studies using the criteria recommended by the Cochrane Back Review Group.

Study	Selection bias	Performance bias	Attrition bias	Reporting bias		Study-specific		
	Similar baseline characteristics	Acceptable compliance	Acceptable and described drop-out rate	No selective outcome reporting	Similar timing of outcome assessment	Training status reported	Smoking status reported	Total score (maximum 7)
Asami *et al.* (1998)[#]	Unsure	Yes	No	Yes	Yes	No	Yes	4
Bloomer *et al.* (2006*b*)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Briviba *et al.* (2005)	No	Yes	Yes	Yes	Yes	No	No	4
Davison *et al.* (2005)	Yes	Yes	Yes	Yes	Yes	No	Yes	6
Fogarty *et al.* (2011)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Fogarty *et al.* (2013*b*)	Yes	Yes	Yes	Yes	Yes	No	No	5
Harms-Ringdahl *et al.* (2012)	No	Yes	Yes	Yes	Yes	No	Yes	5
Hartmann *et al.* (1994)	No	Yes	Yes	Yes	Yes	Yes	Yes	6
Hartmann *et al.* (1995)	Yes	Yes	Yes	Yes	Yes	No	Yes	6
Hartmann *et al.* (1998)	No	Yes	Yes	Yes	Yes	Yes	Yes	6
Inoue *et al.* (1993)	No	Yes	Yes	Yes	Yes	Yes	Yes	6
Itoh *et al.* (2006)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Kim *et al.* (2018)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Liu *et al.* (2015)	Yes	Yes	Yes	Yes	Yes	No	Yes	6
Mastaloudis *et al.* (2004*b*)	No	Yes	Yes	Yes	Yes	No	Yes	5
Meihua *et al.* (2018)	Yes	Yes	Yes	Yes	Yes	No	Yes	6
Møller *et al.*, (2001)	No	Yes	Yes	Yes	Yes	No	No	4
Niess *et al.* (1996)	Yes	Yes	Yes	Yes	Yes	Yes	No	6
Niess *et al.* (1998)	Yes	Yes	Yes	Yes	Yes	Yes	No	6

Study	Selection Bias	Performance Bias	Attrition Bias	Reporting Bias		Study-Specific		
	Similar baseline characteristics	Acceptable compliance	Acceptable and described drop-out rate	No selective outcome reporting	Similar timing of outcome assessment	Training status reported	Smoking status reported	Total Score (maximum 7)
Orlando *et al.* (2018)[#]	Yes	Yes	Yes	No	Yes	Yes	No	5
Paik *et al.* (2009)	Yes	Yes	Yes	Yes	Yes	No	No	5
Peters *et al.* (2006)	Yes	No	No	Yes	Yes	Yes	No	4
Pittaluga *et al.* (2013)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Revan (2011)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Roh *et al.* (2016) [#]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Ryu *et al.* (2016)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Sacheck *et al.* (2003)	No	Yes	Yes	Yes	Yes	Yes	Yes	6
Sardas *et al.* (2012)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Saritaş *et al.* (2011)	Yes	Yes	Yes	Yes	Yes	No	Yes	6
Sato *et al.* (2003)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Shi *et al.* (2007)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Tanimura *et al.* (2008)	Yes	Yes	Yes	Yes	Yes	No	Yes	6
Tanimura *et al.* (2010)	No	Yes	Yes	Yes	Yes	Yes	Yes	6
Tsai *et al.* (2001)	Yes	Yes	Yes	Yes	Yes	No	No	5
Turner *et al.* (2011*b*)	Yes	No	No	Yes	Yes	No	Yes	4
Wagner *et al.* (2010)	Yes	Yes	Yes	Yes	Yes	Yes	No	6
Williamson *et al.* (2018)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7
Zhang *et al.* (2004)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7

[#]not included in meta-analyses

Table 4.4. Characteristics of the included studies and relevant outcomes.

Study	Participants	Exercise protocol	Sample	Biomarker	Technique	Findings
Møller *et al*. (2001)	12 Healthy subjects (7 males and 5 females); MA ± SD = 26.1 ± 4.9 y/o	Maximal bicycle exercise ($\dot{V}O_{2max}$ test) under normal conditions and at 4559 m (high-altitude hypoxia) for 3 days	Lymphocytes	SBs,FPG-s s, ENDO III-s s	Comet assay	Increased SBs at hypoxia on all 3 days; ENDO III-s s increased on day 3 at hypoxia; FPG-s s remained unchanged.
Fogarty *et al*. (2013*b*)	10 Healthy males; MA ± SD = 23 ± 4 y/o	Treadmill test to exhaustion	Lymphocytes	Tail DNA (%)	Comet assay	DNA damage increased 63% ($P < 0.05$) after exercise compared to rest.
Sardas *et al*. (2012)	12 Male rowers (MA ± SD = 22 ± 3.8 y/o) and 11 PE students (MA ± SD = 21.8 ± 3.8 y/o)	3-staged exercise test on a rowing (rowers) and bicycle ergometer (PE students)	Lymphocytes	Tail DNA (%)	Comet assay	DNA damage was increased 24h post exercise compared to pre-exercise in both rowers and PE students but overall pre- and post-exercise damage was significantly higher in rowers compared to PE students.
Reichhold *et al.* (2009*b*)[#]	28 Male triathletes; MA ± SD = 32.7 ± 6.3 y/o	Ironman triathlon (3.8 km swim, 180 km cycle, 42 km run)	Lymphocytes	Tail DNA (%)	Comet assay	The % of SBs decreased significantly immediately post-race, then increased 1 d post-race and declined again 5 d after the race. Between days 5-19 post-race the levels of strand breaks decreased further below initial levels.
Hartmann *et al*. (1994)	2 Males (20 and 30 y/o) and 1 female (32 y/o)	Treadmill test to exhaustion	White blood cells	DNA migration (μm)	Comet assay	No increase in DNA migration was seen immediately after the test but a significant increase in DNA migration was found after 6, 24 and 48 h.
Liu *et al*. (2015)	8 Males athletes; MA ± SD = 21.35 ± 1.04	Treadmill exercise to exhaustion	Peripheral blood cells	Tail length; Olive tail moment	Comet assay	Compared to before exercise, both tail length and olive tail moment significantly rose after exercise ($P < 0.001$).

Study	Participants	Exercise protocol	Sample	Biomarker	Technique	Findings
Inoue *et al*. (1993)	9 Swimmers (MA ± SD = 20.1 ± 1.2 y/o) and 9 distance runners (MA ± SD = 20.9 ± 2.1 y/o)	1500 m for 90 mins (swimmers) and 15 km distance running for 70 mins (runners)	Lymphocytes	8-OHdG	HPLC	8-OHdG decreased significantly after swimming, but no significant change was observed after running.
Sacheck *et al*. (2003)	8 Young (MA ± SD = 25.4 ± 2.2 y/o) and 8 older (MA ± SD = 69.3 ± 3.5 y/o)	Downhill running on a treadmill for 45 mins at 75% $\dot{V}O_{2max}$	Leukocytes	8-OHdG	HPLC	No change in 8-OHdG 24h post-exercise.
Revan (2011)	14 Healthy males; MA ± SEM = 22.3 ± 0.5 y/o	Incremental exercise test to exhaustion on a cycle ergometer	Plasma	8-OHdG	Cayman ELISA Kit	No significant difference in 8-OHdG levels before and after exercise.
Tanimura *et al*. (2010)	8 Young untrained (MA ± SD = 23.8 ± 3.2 y/o) and 8 endurance-trained (MA ± SD = 21.1 ± 3.7 y/o) men	Cycling at 75% $\dot{V}O_{2max}$ 1 h daily for three consecutive days	Lymphocytes	Tail DNA (%)	Comet assay	DNA damage at day 4 was significantly greater than that at day 1 in both groups.
Saritaş *et al*. (2011)	22 Healthy trained men; MA ± SEM = 21.45 ± 0.43 y/o	12 mins run test	Serum	8-OHdG	ELISA Kit	8–OHdG levels were not different between the time periods (before exercise, immediately after exercise and 24 h after the exercise) ($P > 0.05$).
Mastaloudis *et al*. (2004*b*)	5 Males (MA ± SEM = 39 ± 8 y/o) and 5 females (MA ± SEM = 35 ± 4 y/o)	50 km ultramarathon	Leukocytes	Tail DNA (%)	Comet assay	On average, in both placebo and experimental groups, DNA damage increased at mid-race but returned to baseline values by the end of the race.
Harms-Ringdahl *et al*. (2012)	15 Healthy untrained males and females (7 females and 8 males; MA ± SD = 30.5 ± 6.9 y/o	20 mins cycling at 80% of maximum heart rate	Serum	8-OHdG	ELISA Kit	Levels of 8-OHdG increased 42% after the exercise.

Study	Participants	Exercise protocol	Sample	Biomarker	Technique	Findings
Hartmann *et al*. (1998)	3 Male and 3 female athletes (aged between 21-33 y/o)	Short-distance triathlon competition (1.5 km swimming, 40 km cycling, 10 km running)	Lymphocytes	Tail moment (DNA migration)	Comet assay	Increase in DNA migration was seen at 24h post-exercise, whereas at 48h the values were lower compared to 24h but higher than the pre-exercise values. At 72h, the maximum increase in DNA migration was found and baseline values were still elevated after 120 h.
Fogarty *et al*. (2011)	12 Healthy males; MA ± SD = 23 ± 4 y/o)	Bike test at 40,70 and 100% of $\dot{V}O_{2max}$	Leukocytes	Tail length	Comet assay	Increase in DNA damage observed at moderate (70% $\dot{V}O_{2max}$) and high-intensity exercise (100% $\dot{V}O_{2max}$) compared to rest.
Tanimura *et al*. (2008)	15 Healthy sedentary males; MA ± SD = 23.7 ± 1.1 y/o	Cycling at 75% $\dot{V}O_{2max}$ for 1 h	Lymphocytes	Tail DNA (%)	Comet assay	There was increased DNA damage at 3h post-exercise compared to pre-exercise ($P < 0.05$).
Paik *et al*. (2009)	10 Healthy males; MA ± SD = 25.6 ± 0.8 y/o	Treadmill run to exhaustion at 80% $\dot{V}O_{2max}$	Lymphocytes	Tail DNA (%), tail length, tail moment	Comet assay	There was increased DNA damage measured just prior to the termination of exercise compared to pre-exercise ($P < 0.05$).
Ryu *et al*. (2016)	30 Male runners, 10 in each group; 10 km group (MA ± SD = 36.5 ± 10.9 y/o), 21 km group (MA ± SD = 45.0 ± 7.8 y/o), 45 km group (MA ± SD = 37.9 ± 13.6 y/o)	3 different marathon distances: 10 km,21 km and 45 km	Lymphocytes	Tail DNA (%), tail length, tail moment	Comet assay	Compared to rest and recovery, tail moment was significantly higher in all groups. Also, at 45 km tail moment was found to be higher a post-exercise compared to the 10 km and 21 km ($P < 0.05$). No differences in DNA in tail (%) or tail length was observed.
Itoh *et al*. (2006)	8 Untrained males; MA ± SD = 21.8 ± 2.1 y/o	10 km run	Plasma	8-OHdG	ELISA kit	Decreased plasma 8-OHdG levels both immediately and 1h after the 10-km run compared to resting values ($P < 0.05$).

Study	Participants	Exercise protocol	Sample	Biomarker	Technique	Findings
Tsai *et al*. (2001)	14 Male runners (median age 21, range 20–24 y/o)	42 km marathon race	Peripheral blood mononuclear cells	Tail DNA (%), FPG-s s, ENDO III-s s	Comet assay	SBs increased on day 1; increased levels of FPG-s s were observed immediately and 24 h (Day 1) after the race; ENDO III-s s levels also increased immediately post-exercise and on day 7.
Bloomer *et al*. (2006*b*)	11 & 6 Aerobically trained men and women; MA ± SD = 23.3 ± 5.2 y/o	Treadmill run for 30 mins at 80% $\dot{V}O_{2max}$	Plasma	8-OHdG	ELISA Kit	No significant difference was observed in 8-OHdG levels before and after exercise.
Turner *et al*. (2011*b*)	9 Healthy men; MA ± SD = 46.1 ± 5.3 y/o	233 km ultraendurance race	Peripheral blood mononuclear cells	Tail DNA (%), FPG	Comet assay	Increased SBs immediately and 24 h after the race, compared to baseline ($P < 0.01$); an increase in FPG-dependent oxidative DNA damage was also observed immediately after the race ($P < 0.05$).
Peters *et al*. (2006)	8 Male athletes; MA ± SD = 34.2 ± 2.44 y/o	2.5-h treadmill run at 75% $\dot{V}O_{2max}$	Lymphocytes	Tail length/DNA migration (μm)	Comet assay	There was no significant increase in DNA strand breaks before and after the exercise ($P > 0.05$).
Zhang *et al*. (2004)	11 Healthy male students aged between 18–20 y/o	Bicycle exercise to exhaustion	White blood cells	Tail length/DNA migration (mm)	Comet assay	Significantly increased DNA migration at 6h and 24h compared to pre-exercise ($P < 0.01$).
Wagner *et al*. (2010)	42 Male athletes; MA ± SD = 35.3 ± 7 y/o	Ironman triathlon (3.8km swimming, 180km cycling and 42.2km running)	Lymphocytes	SBs, Tail DNA (%), ENDO III-s s, FPG-s s	Comet assay	SBs decreased post-race; DNA migration increased 1d post-race due to SBs ($P < 0.01$), then 5d post-race returned to pre-race levels and decreased further to below the initial levels 19d post-race ($P < 0.01$).

Study	Participants	Exercise protocol	Sample	Biomarker	Technique	Findings
Sato *et al*. (2003)	15 Male subjects aged 19–29 y/o (7 physically active and 8 sedentary)	50% $\dot{V}O_{2max}$ of cycle ergometer exercise for 30 mins	Leukocytes	8-OHdG	HPLC	No change in physically active subjects but decreased in sedentary subjects.
Hartmann *et al*. (1995)	8 Healthy men (29-34 y/o)	Treadmill run to exhaustion	White blood cells	Tail moment	Comet assay	Increase in DNA damage was seen 24 h after the run (mean increase = 35.3 ± 8.3 %).
Shi *et al*. (2007)	5 Healthy males (aged 22–38 y/o)	Exercise on a cycle ergometer at 50% $\dot{V}O_{2max}$ for 10.5 ± 1.3 mins	Leukocytes	8-OHdG	HPLC	After aerobic exercise, no significant change in leukocyte 8OHdG level was seen. (However, a significant increase was detected in samples taken 24 h after anaerobic exercise).
Davison *et al*. (2005)	7 Healthy males; MA ± SD = 22.3 ± 4.1 y/o	Treadmill test to exhaustion	Peripheral blood mononuclear cells	Tail moment	Comet assay	An increase in DNA damage was observed after exercise.
Asami *et al*. (1998)[#]	23 Healthy males aged 19-50 y/o (10 untrained and 13 trained)	Maximal cycling exercise	Leukocytes	8-OHdG	HPLC	A significant decrease in 8-OHdG levels was observed in the untrained subjects only ($P < 0.05$).
Briviba *et al*. (2005)	10 Subjects for half-marathon (5 males and 5 females; MA ± SD = 43 ± 9 y/o) and 12 subjects for marathon (2 males and 10 females; MA ± SD = 45 ± 10 y/o)	Half-marathon (21.1 km) and a marathon (42.195 km)	Lymphocytes	Tail DNA (%), FPG-s s and ENDO III-s s	Comet assay	No significant changes in the levels of DNA strand breaks in lymphocytes after either race. However, a significant difference was found in the % of ENDO III s in the tail after both races ($P < 0.05$), whereas the % of FPG-s s was slightly increased but not significantly ($P > 0.05$).
Pittaluga *et al*. (2013)	7 Females; MA ± SD = 68.1 ± 2.7 y/o	Exhaustive bout on a cycle ergometer	Serum	8-OHdG	HPLC	No significant difference in 8-OHdG levels before and after exercise.

Study	Participants	Exercise protocol	Sample	Biomarker	Technique	Findings
Niess *et al*. (1996)	5 UT and 6 TR	Treadmill run test to exhaustion	Leukocytes	Tail moment/DNA migration(μm)	Comet assay	An increase in DNA migration from 2.31 ± 0.20 (TR) and 2.22 ± 0.16 (UT) at rest to 2.65 ± 0.30 (TR) and 3.00 ±0.41 tail moment (UT) was observed 24h after exercise.
Niess *et al*. (1998)	12 Male runners; MA ± SD = 27.3 ± 4.1 y/o	Half-marathon (21.1 km)	Leukocytes	DNA migration (μm)	Comet assay	DNA migration rose significantly 24h after the race, compared to rest (P< 0.01).
Williamson *et al*. (2018)	10 Recreationally males; MA ± SD = 22 ± 2 y/o	Treadmill test to exhaustion	Peripheral blood mononuclear cells	Tail DNA (%)	Comet assay	Tail intensity was increased by 18.2% post-exercise (P < 0.05).
Orlando *et al*. (2018)[#]	21 Rugby male athletes; MA ± SD = 26 ± 5 y/o	40 mins run at 85% of maximum heart rate	Peripheral blood mononuclear cells	Tail DNA (%)	Comet assay	No significances of DNA damage were observed after the exercise bout.
Meihua *et al*. (2018)	10 Male athletes; MA ± SD = 21.1 ± 1.13 y/o	$\dot{V}O_{2max}$ (Bruce protocol)	Peripheral blood cells and plasma	DNA damage index and 8-OHdG	Comet assay and HPLC	Exercise increased DNA damage index as measured by comet assay; plasma 8-OHdG levels also increased following exhaustive exercise (P < 0.01).
Roh *et al*. (2016)[#]	10 Male college athletes; MA ± SD = 18.8 ± 0.8 y/o	1-hour run at 75% of heart rate reserve	Lymphocytes	Tail DNA (%)	Comet assay	DNA tail (%) increased following exercise (P < 0.05).
Kim *et al*. (2018)	11 Amateur male triathletes; MA ± SD = 37.9 ± 6.2 y/o	2 triathlon races (O_2 and Olympic courses)	Lymphocytes	Tail DNA (%)	Comet assay	In the Olympic course DNA tail intensity (%) increased after match and decreased after 3 and 6 days of recovery; In the O2 course, tail (%) decreased after match, increased after 3 days and decreased after 6 days of recovery (P < 0.01).

Abbreviations: SBs, strand breaks; FPG-s s, formamidopyrimidine gycosylase-sensitive sites; ENDO III-s s, endonuclease III-sensitive sites; 8-OHdG, 8-hydroxy-2'–deoxyguanosine; MA, mean age; SD, standard deviation; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; PE, physical education; $\dot{V}O_{2max}$, maximum rate of oxygen consumption; untrained, UT; trained, TR; y/o, years old.
[#]not included in meta-analyses

Table 4.5. Summary of results from all meta-analyses.

Time-point	N (sample size)			Effect (hedges' g)			P-value		
	8-OHdG	Comet	Overall	8-OHdG	Comet	Overall	8-OHdG	Comet	Overall
0 (0h)	109	203	312	0.150	1.140	0.875	0.684	0.000*	0.000*
1 (15mins-1h)	59	104	163	-0.523	0.448	0.166	0.226	0.105	0.476
2 (2h)	-	25	-	-	0.556	-	-	0.047*	-
3 (3h)	5	19	24	1.154	0.968	1.008	0.066	0.003*	0.001*
4 (4-6h)	-	28	-	-	1.480	-	-	0.010*	-
5 (1d)	73	148	221	0.141	2.468	1.113	0.720	0.000*	0.000*
6 (2d)	15	28	43	-1.208	0.338	0.231	0.509	0.497	0.630
7 (3d)	-	85	-	-	0.627	-	-	0.022*	-
8 (4d)	-	19	-	-	0.691	-	-	0.380	-
9 (5d)	-	44	-	-	0.315	-	-	0.548	-
10 (6-7d)	-	33	-	-	0.359	-	-	0.491	-
11 (14-28d)	-	51	-	-	0.105	-	-	0.801	-
High-intensity[1]	33	69	102	0.718	1.571	1.179	0.044*	0.000*	0.000*
Long-distance[2]	-	89	-	-	0.437	-	-	0.151	-

[1]≥75% $\dot{V}O_{2max}$ at time-point 0 (0h) & 5 (1d)

[2]≥42 km at time-point 0 (0h) & 1 (15mins-1h)

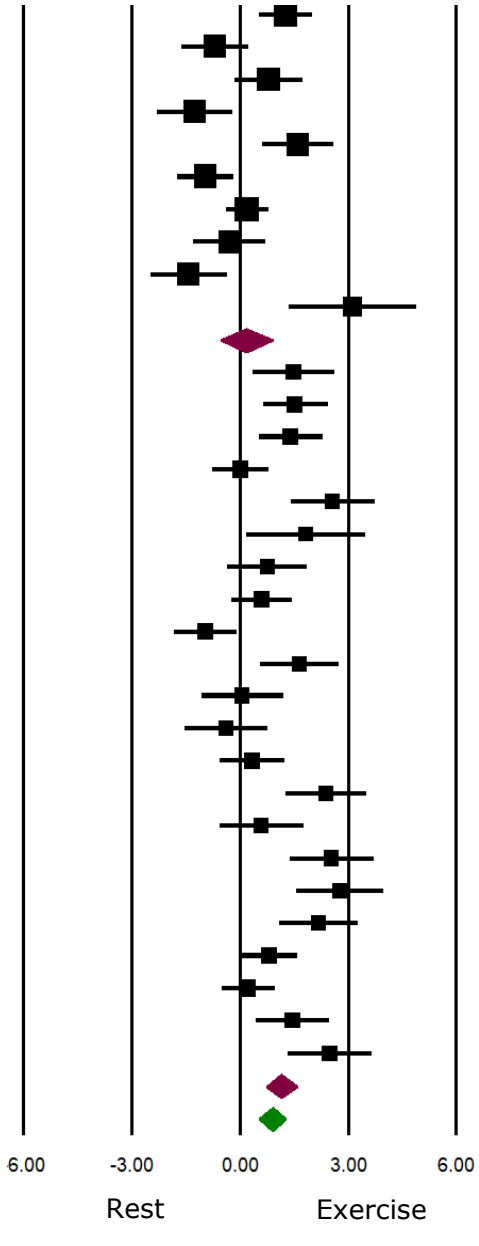
- not applicable

* denotes significance at $P < 0.05$

4.3.4 Summary of Findings

Using data from 35 studies and 312 participants, this paper quantitatively demonstrates, for the first time, that DNA damage increases immediately following acute aerobic exercise (**Figure 4.3**). Based on Cohen's classification, the effect on DNA damage was large (>0.8). No significances were seen following 1-hour post-exercise (**Figure 4.5a**); however, increased DNA damage was observed from 4 hours to 1 day following exercise (**Figure 4.5b**,**4.5c, Appendix G Supplementary Figure S2a and S2b**). Similarly, no DNA damage was observed 2 days following exercise (**Appendix G Supplementary Figure S3a**) but significantly increased 3 days post exercise (**Appendix G Supplementary Figure S3b**). Furthermore, when comparing the two methods of DNA assessment (comet assay and 8-OHdG), significance was observed only in studies using comet assay, at time-point 0 hours, 3 hours and 1 day, again with a large effect size. No differences were observed 5-28 days post-exercise (**Appendix G Supplementary Figure S4a**, **S4b** and **S4c**). Finally, when isolating protocols of high-intensity (≥75% $\dot{V}O_{2max}$) and long-distance (≥42km), greater DNA damage following exercise was observed only in the former (**Figure 4.4a** and **4.4b**). However, it should be noted that, no long-distance study in our analysis used 8-OHdG as a biomarker for oxidative damage. Whereas, in the high-intensity protocols, a mixture of both methods was utilized. As it has been suggested (Mastaloudis *et al.*, 2004*b*; Wagner *et al.*, 2010), DNA damage measured after long-distance exercise (7-10 hr race) may not be detected due to the activation of repair mechanisms and increased clearance of damaged cells initiated during the race, which would otherwise not be observed when measured after a shorter exercise protocol. Additionally, these processes could be further enhanced due to the intake of antioxidants ingested during the race.

Study	Method	n	SMD (Hedges' g)	P-Value	RW(%)
Bloomer *et al*. (2006*b*)		17	1.266	0.001	10.89
Inoue *et al*. (1993), swimmers		9	-0.702	0.130	10.24
Inoue *et al*. (1993), runners		9	0.788	0.092	10.22
Itoh *et al*. (2006)		8	-1.268	0.015	9.81
Meihua *et al*. (2018)		10	1.596	0.001	10.00
Revan (2011)		14	-0.971	0.013	10.76
Saritaş *et al*. (2011)		22	0.189	0.524	11.32
Sato *et al*. (2003), active		7	-0.299	0.553	9.96
Sato *et al*. (2003), sedentary		8	-1.431	0.008	9.72
Shi *et al*. (2007)		5	3.114	0.001	7.08
Subtotal	8-OHdG	109	0.150	0.684	100
Davison *et al*. (2005)		7	1.480	0.010	4.34
Fogarty *et al*. (2011),100%		12	1.524	0.001	4.87
Fogarty *et al*. (2011),70%		12	1.395	0.002	4.91
Fogarty *et al*. (2011),40%		12	0.000	1.000	5.12
Fogarty *et al*. (2013*b*)		10	2.569	0.000	4.27
Hartmann *et al*. (1994)		3	1.814	0.030	3.28
Hartmann *et al*. (1998)		6	0.748	0.177	4.42
Kim *et al*. (2018), olympic c		11	0.596	0.156	5.01
Kim *et al*. (2018), O2 c		11	-0.978	0.025	4.94
Liu *et al*. (2015)		8	1.637	0.003	4.43
Mastaloudis *et al*. (2004*b*), m		5	0.056	0.922	4.35
Mastaloudis *et al*. (2004*b*), f		5	-0.399	0.490	4.32
Møller *et al*. (2001)		9	0.317	0.483	4.87
Paik *et al*. (2009)		10	2.373	0.000	4.36
Peters *et al*. (2006)		5	0.583	0.320	4.29
Ryu *et al*. (2016), 10km		10	2.537	0.000	4.28
Ryu *et al*. (2016), 21km		10	2.768	0.000	4.17
Ryu *et al*. (2016), 42km		10	2.172	0.000	4.45
Tanimura *et al*. (2008)		14	0.801	0.036	5.16
Tsai *et al*. (2001)		14	0.212	0.565	5.22
Turner *et al*. (2011*b*)		9	1.451	0.004	4.62
Williamson *et al*. (2018)		10	2.482	0.000	4.31
Subtotal	Comet	203	1.140	0.000	100
Overall		312	0.875	0.000	



Heterogeneity: $Chi^2 = 5.25$, df = 31 (P = 0.02); $I^2 = 82.12\%$
Test for overall effect: Z score = 4.58 (P = 0.001)

Figure 4.3. Relative weight (RW) standardised mean difference (SMD) and 95% CI (Hedges' g adjusted) of DNA damage compared between rest and after an exercise bout at time-point 0 (0h). Values for individual trials and pooled data (Random Model) are shown and grouped by method of quantification. Abbreviations: c, course; m, males; f, females.

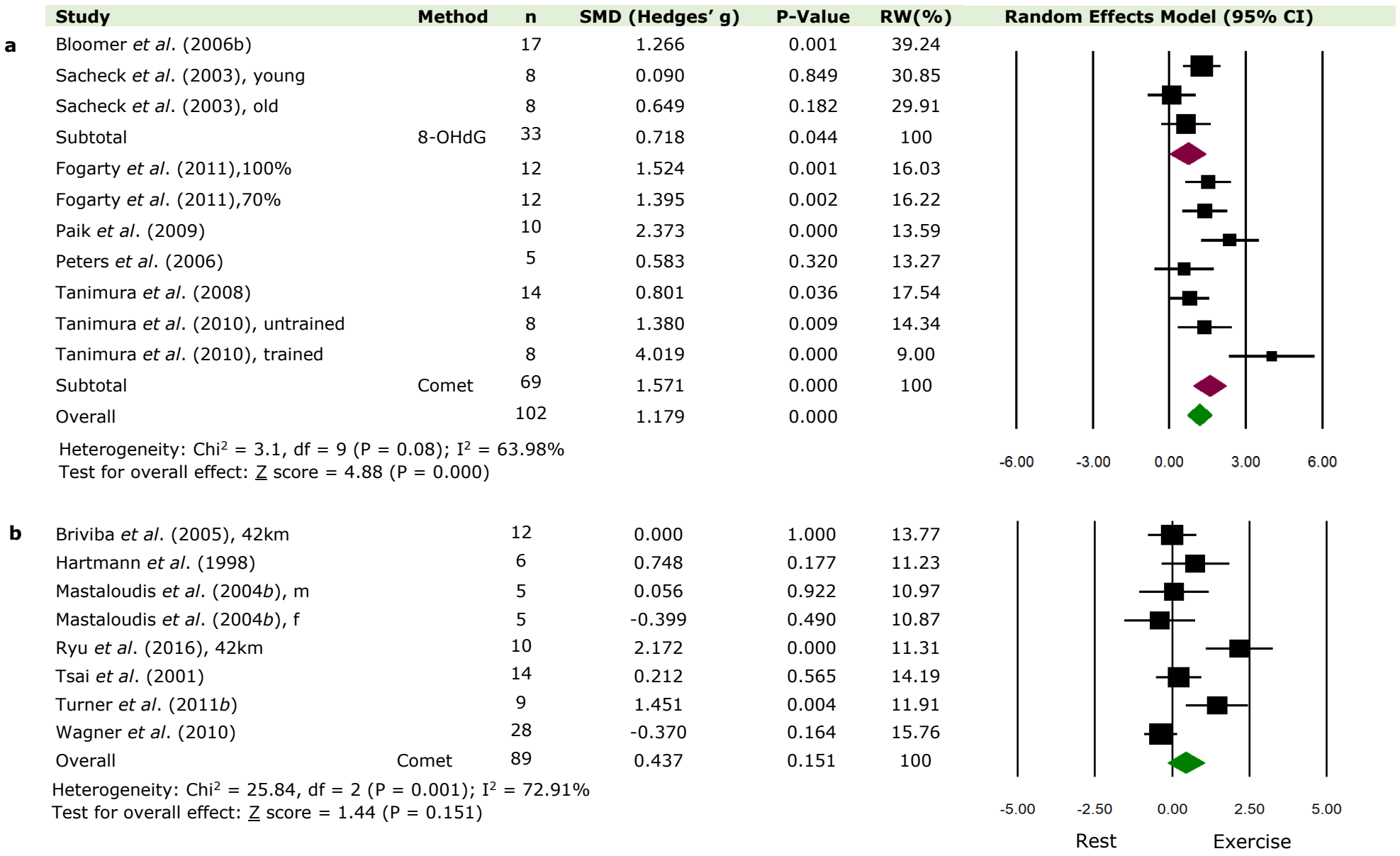


Figure 4.4. Relative weight (RW) standardised mean difference (SMD) and 95% CI (Hedges' g adjusted) of DNA damage compared between rest and after an exercise bout at **(a)** high-intensity exercise (≥75% $\dot{V}O_{2max}$) at time-point 0 (0h) & 5 (1d) and **(b)** long-distance exercise (≥42 km) at time-point 0 (0h) & 1 (15mins-1h). Values for individual trials and pooled data (Random Model) are shown and grouped by method of quantification. Abbreviations: m, males; f, females.

	Study	Method	n	SMD (Hedges' g)	P-Value	RW(%)
a	Harms-Ringdahl *et al*. (2012)		15	0.439	0.223	18.24
	Itoh *et al*. (2006)		8	-1.366	0.010	15.88
	Pittaluga *et al*. (2013)		7	0.461	0.365	16.21
	Revan (2011)		14	-1.942	0.000	17.04
	Sato *et al*. (2003), active		7	0.132	0.792	16.31
	Sato *et al*. (2003), sedentary		8	-0.930	0.063	16.32
	Subtotal	8-OHdG	59	-0.523	0.226	100
	Briviba *et al*. (2005), 21km		10	0.398	0.358	11.43
	Briviba *et al*. (2005), 42km		12	0.000	1.000	12.01
	Davison *et al*. (2005)		7	1.272	0.022	9.68
	Niess *et al*. (1996), untrained		5	0.441	0.447	9.35
	Niess *et al*. (1996), trained		6	-0.357	0.507	9.92
	Niess *et al*. (1998)		12	2.440	0.000	10.05
	Paik *et al*. (2009)		10	0.678	0.125	11.30
	Tanimura *et al*. (2008)		14	0.023	0.951	12.41
	Wagner *et al*. (2010)		28	-0.370	0.164	13.85
	Subtotal	Comet	104	0.448	0.105	100
	Overall		163	0.166	0.476	
	Heterogeneity: Chi^2 = 3.58, df = 14 (P = 0.06); I^2 = 78.06%					
	Test for overall effect: Z score = 0.71 (P = 0.476)					
b	Tamimura *et al*. (2008)		14	0.374	0.312	57.12
	Zhang *et al*. (2004)		11	0.798	0.062	42.88
	Overall	Comet	25	0.556	0.047	100
	Heterogeneity: Chi^2 = 0.56, df = 1 (P = 0.45); I^2 = 0%					
	Test for overall effect: Z score = 1.98 (P = 0.047)					
c	Shi *et al*. (2007)		5	1.154	0.066	100
	Subtotal	8-OHdG		1.154	0.066	100
	Peters *et al*. (2006)		5	1.030	0.094	28.41
	Tanimura *et al*. (2008)		14	0.944	0.015	71.59
	Subtotal	Comet	19	0.968	0.003	100
	Overall		24	1.008	0.001	
	Heterogeneity: Chi^2 = 0.07, df = 2 (P = 0.79); I^2 = 0%					
	Test for overall effect: Z score = 3.47 (P = 0.001)					

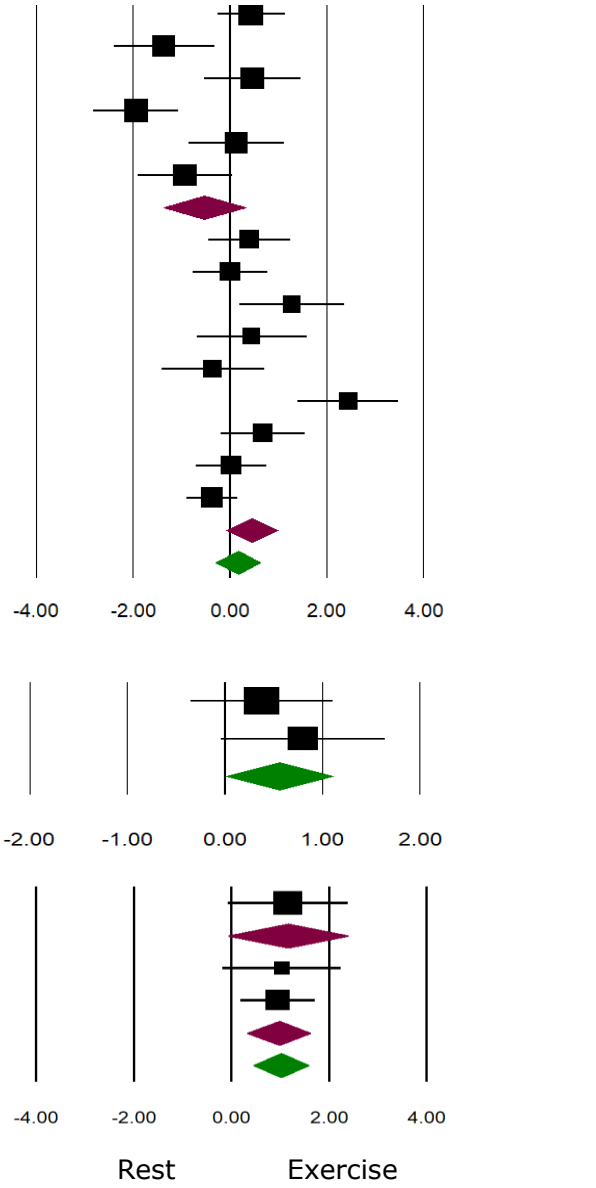


Figure 4.5. Relative weight (RW) standardised mean difference (SMD) and 95% CI (Hedges' g adjusted) of DNA damage compared between rest and after an exercise bout at **(a)** time-point 1 (15m-1h), **(b)** time-point 2 (2h) and **(c)** time-point 3 (3h). Values for individual trials and pooled data (Random Model) are shown and grouped by method of quantification.

4.4 Discussion

The main purpose of this meta-analysis was to examine the effect of acute exercise on DNA damage. These results suggest that exhaustive exercise leads to increased DNA damage. Acute aerobic exercise, regardless of intensity, seems to produce sufficient stimulus for a greater production of RONS which may evoke damaging effects to DNA. After longer distance events, such as triathlons and ultramarathons, added protection against DNA damage may be offered through the initiation of repair systems and adequate antioxidant intake from food/drinks consumed during such events; however more studies are needed to confirm this.

4.4.1 Mechanisms of Free Radical Production During Exercise

Previous work from our laboratory has shown increased DNA damage of 63% following exercise as well as a greater concentration of H_2O_2 as a function of exercise, compared to rest, indicating a possible mechanism of exercise-induced DNA damage through the increased production of H_2O_2 (Fogarty *et al.*, 2011). Moreover, the activation of inflammatory cells such as neutrophils and lymphocytes during exercise, due to muscle tissue damage, can further enhance superoxide production, which can cause direct damage to DNA (Tsai *et al.*, 2001; Williamson *et al.*, 2018). Additionally, catecholamines released during exercise can be autoxidized and lead to the production of non-radicals such as H_2O_2 (Danese *et al.*, 2017). Finally, during high-intensity aerobic exercise, tissue ischaemia occurs, resulting in an increased number of hydrogen ions which can in turn react with superoxide anions to produce further RONS (Jenkins, 1993).

4.4.2 Free Radical-Induced Damage to DNA/Repair

Although $O_2^{\bullet -}$ and NO are the primary radical species produced by contracting skeletal muscle, these do not directly cause damage to DNA (Cobley *et al.*, 2015). Instead, $OH^{\bullet}$ reacts with the different components of DNA, such as DNA bases and the deoxyribose sugar, causing damage either by hydrogen addition or abstraction, producing multiple products, as well as single- and/or double-strand breaks, tandem lesions and DNA protein cross-links (Dizdaroglu & Jaruga, 2012; Cobley *et al.*, 2015). Among the four DNA bases, guanine has the least reduction potential, and acts as an excellent electron donor and is the most prone to oxidation by $OH^{\bullet}$ (Dizdaroglu & Jaruga, 2012). For this reason, the product 8-OHdG is the most popular biomarker of DNA damage in urine and blood samples (Reichhold *et al.*,

2009*a*). Furthermore, compared to guanine, adenine has a greater reduction potential and is not oxidized to the same extent (Dizdaroglu & Jaruga, 2012). Just as with guanine, $OH^{\bullet}$ reacts with adenine by adding a hydrogen molecule to its double bonds at specific locations but in a slightly different distribution to that of guanine (Dizdaroglu & Jaruga, 2012). The base excision repair pathway is normally activated to repair DNA damage, and this occurs following the activation of a number of enzymes such as DNA glycosylase-1 (German *et al.*, 2014), endonuclease phosphodiesterase and DNA polymerase (Carter & Parsons, 2016). Repair to DNA is almost always controlled by a number of factors such as availability of said enzymes and others such as p53 and RAS (Seo & Jung, 2004; Boldogh *et al.*, 2012)**.**

4.4.3 Hormesis Theory

The relationship between exercise, RONS and DNA damage has been explained in the context of the hormesis theory (displayed in **Figure 4.6**) (Radák *et al.*, 2008). In toxicology, hormesis refers to an environmental agent's beneficial effect on a cell or organism at low doses that is otherwise harmful at high doses, creating a bell-shaped curve (Mattson, 2008). In this instance, exercise acts as the stimulus and the subsequent effects of exercise-induced RONS (physiological or pathological) are determined by the dose. Being physically inactive is a major risk factor for numerous chronic diseases and physiological disorders such as cancer, type 2 diabetes, cardiovascular disease, metabolic syndrome, hypertension and obesity (Martin *et al.*, 2009; Booth *et al.*, 2012; Knight, 2012). In 2000, physical inactivity in combination with poor diet was the second leading cause of death after tobacco in the US, contributing to 16.6% of total US deaths (Mokdad *et al.*, 2004).

Physical inactivity represents one end-point of the hormesis curve, while overtraining and strenuous unaccustomed/unindividualized exercise represents the opposite end-point; both result in a higher risk of disease and decreased physiological function and are mediated by elevated RONS production and oxidative stress (Radák *et al.*, 2017). Regular exercise can lead to adaption through up-regulation of molecular and cellular pathways, redox signalling, and antioxidant repair systems, resulting in the enhanced capacity of the organism to overcome greater stress (Boccatonda *et al.*, 2016; Radák *et al.*, 2017). In addition, exercise training can further extend that adaptive response by 'stretching' the capacity to tolerate even higher levels of RONS (Radák *et al.*, 2017). Yet, when RONS production outstrips

antioxidant defence mechanisms and there is insufficient repair of DNA double strand breaks, this can cause chromosome instability and gene mutation can occur (Ivashkevich *et al.*, 2012; Davison, 2016). However, it is unclear where the threshold limit exists between the beneficial effects of regular exercise and the point of overtraining associated with higher oxidative DNA damage and insufficient repair. This makes the concept of hormesis definitive but narrow. Defining this point is inherently complex due to the heterogeneous variations across individuals based on sex, age, fitness and exercise intensity and distance. However, along with these, there are even more complex factors that influence the degree of the damage and therefore the overall effect of the beneficial adaptations and the harmful effects of the two end-points that should be considered.

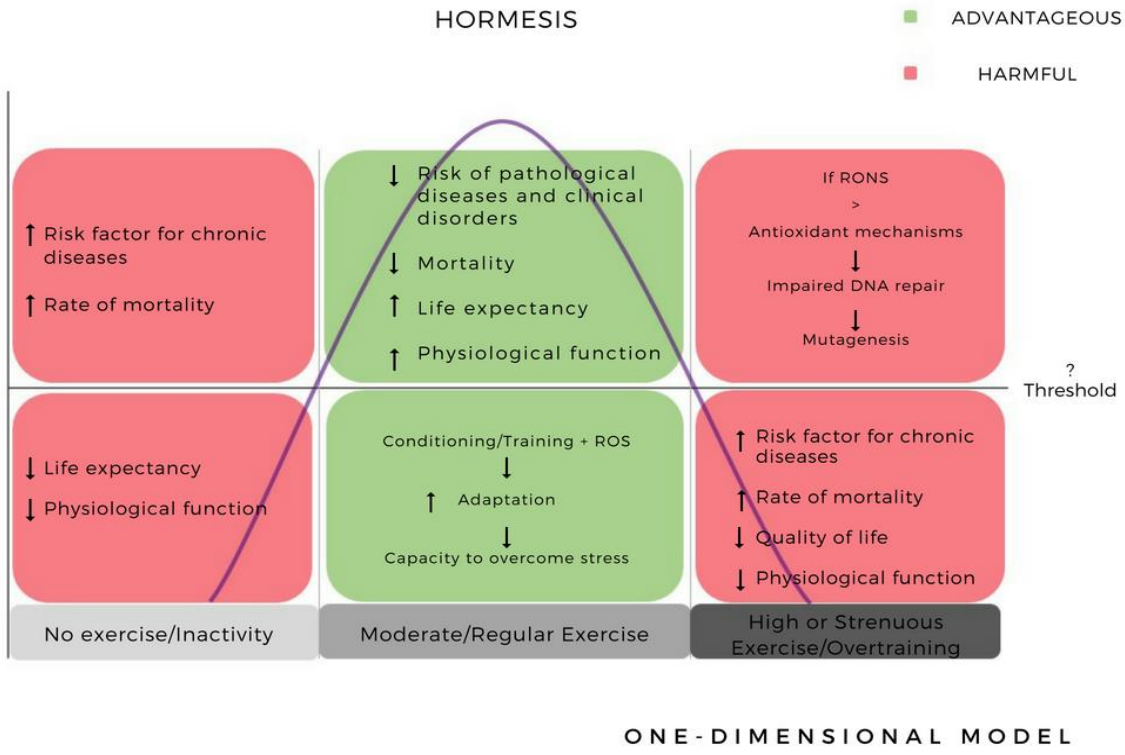


Figure 4.6. The relationship between exercise and DNA oxidation and its effects, explained by the hormesis curve (one-dimensional model). Abbreviations: RONS, reactive oxygen and nitrogen species. Adapted from Radák *et al.*, 2008.

4.4.4 One-dimensional vs Multidimensional Model

A role for RONS in exercise-mediated adaptations and responses is evident (Margaritelis *et al.*, 2016). The concept of hormesis can allow us, to some extent, to understand how the relationship between exercise and DNA oxidation can fit into a bell-shaped curve. However, it only considers levels of RONS/DNA oxidation, rendering it somewhat one-dimensional. While this may be an important factor in explaining the fundamental adaptive responses to exercise, when investigating the extent of DNA oxidative damage, there are multiple factors to consider. We propose three more basic factors instead of only levels of RONS/DNA oxidation in a more intricate and adaptable multi-dimensional model, visualised in a radar chart starting from the centre (least damaging) to the edge of the circle (most damaging) in a linear scale manner shown in **Figure 4.7**. Thus, this proposed multi-dimensional model would consist of the following four factors: (1) type of RONS, ranging from the least reactive (such as $O_2^{\bullet -}$) to the most reactive radical (such as $OH^{\bullet}$); (2) frequency of RONS attacks/episodes, ranging from one to multiple episodes; (3) type/extent of DNA damage, either single- or double-strand breaks, ranging from the least to the most damaging effect and (4) magnitude of RONS/DNA oxidation, ranging from lowest to maximum levels of DNA oxidation/RONS increase. When applying this multi-dimensional model to the exercise stimulus, there are four more specific factors to consider: (5) exercise intensity/ distance; (6) exercise frequency; (7) sufficiency of DNA repair enzymes and (8) degree of individualization (sex, age, training level, nutrition quantification method) (**Figure 4.7**).

As exercise occurs, adaptive mechanisms are stimulated and these lead to the accentuation of antioxidant enzymes, as result of training adaptation (Wagner *et al.*, 2011). However, if multiple individual sporadic bouts of acute, but not regular, exercise occur (effect of overtraining and/or excessive exercise resulting from very high-intensity and/or long-distance exercise), without sufficient rest periods in between, the repair systems most likely fail due to higher oxidative stress resulting from enhanced RONS production (Wagner *et al.*, 2011). Successful adaptations are thus unlikely and detrimental health outcomes may occur as a consequence. In contrast, individual bouts of exercise with complete recovery in between could revoke any oxidative stress via the antioxidant enzymes which are upregulated within the muscle as a function of training, suggesting exercise itself can

exert an antioxidant effect (Gomez-Cabrera *et al.*, 2008*b*; de Sousa *et al.*, 2017). In turn, this supports the now established theory that RONS production is in fact a necessary step to stimulate the adaption of the skeletal muscle in response to exercise (Powers *et al.*, 2010). Furthermore, the severity of the damage and whether genome stability is being compromised or not, depends on the type of damage/oxidation that has occurred to the DNA – base oxidation, single or double strand breaks.

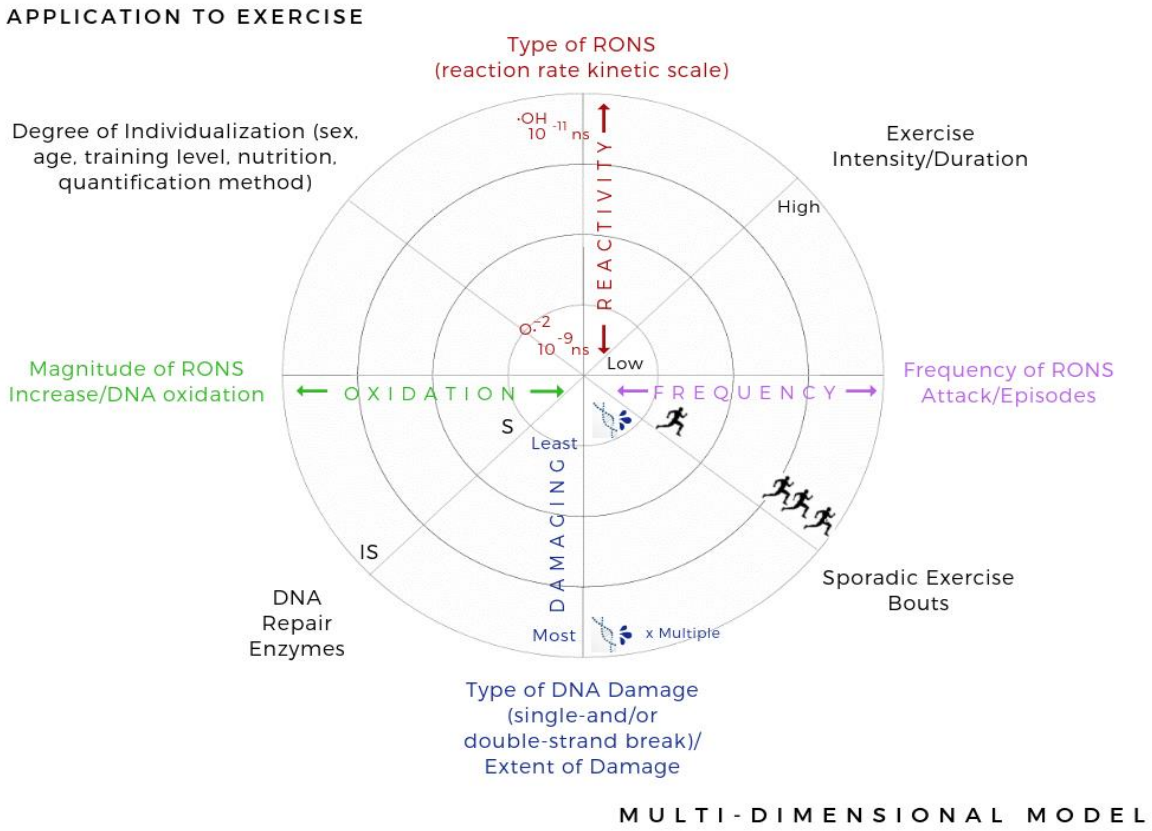


Figure 4.7. Multi-dimensional model demonstrating multiple factors to be considered when assessing the degree of oxidative damage when applied to the exercise model. Abbreviations: IS, insufficient; RONS, reactive oxygen and nitrogen species; S, sufficient.

Cumulatively, these factors can affect the degree of DNA oxidative damage by causing the least to the most amount of DNA damage and, in turn, possibly creating (individual) different individual thresholds for the end-points of physical inactivity and overtraining and the way the hormesis effect unfolds as a bell-shaped curve. Obviously, a combination of reaching the higher end of the scale in all factors (towards the circumference of the circle) would result in the most harmful kind of oxidative DNA alteration, compared

to the lower end of the scale (towards the centre of circle) where the outcome may be less harmful.

This meta-analysis suggests that aerobic exercise leads to an increase in oxidative DNA damage as measured by the comet assay. It is important to elucidate what this means in relation to health outcomes. The literature collectively suggests that a single acute bout of exercise (even of high-intensity/long-distance) is not likely able to cause any long-term and significantly harmful effects as explained under the hormesis theory. Ironman triathlon studies have shown that well-trained athletes show a large decline in DNA damage post-race. For instance, Mastaloudis *et al.* (2004*b*) reported that DNA damage decreased below baseline levels 2 days after a 50-km ultramarathon. Moreover, an 8% decrease below baseline was observed after 6 days. Similarly, Wagner *et al.* (2010) showed that levels of DNA damage after an Ironman triathlon returned to baseline values 5 days after the event, suggesting non-persistent DNA damage. This can be attributed to the up-regulation of repair mechanisms and enhanced endogenous antioxidative system which indicates that endurance training can enhance the body's ability to prevent and repair DNA damage, largely by increasing its antioxidant defenses (Wagner *et al.*, 2010). The non-trained cohorts may not have that added antioxidant protection as a function of adaptive training. Master endurance athletes are also shown to have longer telomere length (TL), a marker of biological age, than non-athlete age-matched controls (Simoes *et al.*, 2017; Sousa *et al.*, 2019). Telomeres are responsible for stopping cell division by activating DNA damage recognition systems (Corbett & Alda, 2015). TL shortening has been shown to be attenuated by long-term endurance training and thus, reduced antioxidant activity and accumulation of RONS may contribute to TL debilitation (Sousa *et al.*, 2019). Taking this into account, along with the use of different experimental designs, fitness levels and methods of DNA damage detection at various post exercise TPs, all these factors may affect the degree of the damage and the extent to which it is (efficiently) repaired.

In summary, both the advantageous and harmful effects of exercise-associated adaptations and the two end-points, physical inactivity and overtraining, are results of non-exposure or repeated exposure to the stimulus (inactivity or repeated exercise bouts) combined with a varying degree of DNA oxidative damage. Whether or not physiological or

pathological consequences occur, and to what extent, may depend on all factors mentioned in the multidimensional model.

4.4.5 Strengths and Limitations

This is the first meta-analysis available on DNA damage and exercise. DNA damage was distinguished while performing sensitivity analysis of two of the most common methods of quantification found across studies, the comet assay and 8-OHdG. The overall risk bias was low since studies scored well in the quality assessment table. Finally, PRISMA guidelines (Moher *et al.*, 2009) and Cochrane collaboration recommendations were followed (Higgins & Green, 2011).

Some limitations have been identified in the included studies. Several study data had to be manually extracted from figures due to data not being presented in the text. However, the degree of error should be minimal due to the high accuracy of the software used. Moreover, the sample size for the two quantification methods was not equal, and while this is expected given the variety of study methodologies used is nonetheless noteworthy. This may be the main reason why only studies utilising the comet assay showed significantly greater DNA damage following exercise as opposed to 8-OHdG. However, this could also result due to interlaboratory differences. In 2005, the European Standards Committee on Oxidative DNA Damage found no association between levels of oxidative DNA damage in a sample of 88 healthy males measured by the comet assay and 8-OHdG by HPLC methods in six different laboratories (Gedik *et al.*, 2005), Therefore, the validity and comparability of different methods of oxidative DNA damage across laboratories may be questioned. Similarly, the number of studies/sample size at all time-points and in the subgroup analysis (high-intensity and long-distance studies) varied and could explain the difference between observed significance and non-significance between the two protocols.

The authors chose to focus solely on studies that have quantified DNA damage assayed from blood as these represent the most frequently measured in the literature. Nevertheless, we acknowledge that DNA damage can also be determined in urine and muscle. Studies measuring DNA damage following exercise in tissues/specimens other than white blood cells (e.g., muscle and urine) support our data demonstrating that exercise induces DNA damage. Previous work from our laboratory shows an 86% increase, compared to rest, in muscle 8-OHdG concentration following 100

isolated and continuous maximal knee contractions(Fogarty *et al.*, 2013*a*). Moreover, during a four-day race, urinary 8-OHdG of five super-marathon runners were monitored where after day one (93 km) 8-OHdG increased, on day two (120 km) no further increase occurred, while on days three and four (56 and 59 km respectively) there was a decrease in 8-OHdG suggesting the likelihood of exercise adaptation and upregulation of antioxidant systems (Radák *et al.*, 2000). Similarly, after 8 days of running (30 ± 3 km/day) at a training camp, 8-OHdG measured from urine increased significantly by 26% (Okamura *et al.*, 1997). Another investigation showed that, after one hour of cycling at 70% of maximal O_2 uptake, urinary 8-OHdG was elevated, and this increase remained significant 1d post-exercise (Orhan *et al.*, 2004).

Furthermore, training status was not distinguished across studies and was only taken into account as to whether it was reported or not in the literature in the quality assessment of this review. There were a few studies using triathletes and marathons and/or triathlons as the exercise protocol but most of the investigations did not report the training status of participants. This is important as trained athletes maybe less susceptible to oxidative stress due to their enhanced expression of antioxidant enzymes and up-regulation of repair systems, acquired from previous training (Wagner *et al.*, 2010). Across studies, the time of post-exercise measurement ranged from immediately post-exercise to 28 days following exercise (**Table 4.2**). However, in most studies, DNA damage was measured immediately post-exercise. Although this was further investigated by analysis of subsequent time-points, a significant increase at some of those time-points may not have been found, due to a smaller sample size.

4.4.6 Future Research

A relatively new biomarker has been used recently, the γ-H2AX, to assess DNA double-strand breaks in cancer research (Ivashkevich *et al.*, 2012). This assay is considered a sensitive method of measuring DNA damage, due to its ability to detect very low levels of double strand breaks, which the comet assay could not otherwise detect (Heylmann & Kaina, 2016). Lippi *et al.* (2016) reported an increase in DNA injury, associated with running distance and intensity, with γ-H2AX foci analysis in lymphocytes. Amateur runners completed a 5-km, 10-km, 21-km and 42-km running trial on 4 separate occasions. The authors observed a small increase in γ-H2AX foci after both 5-km and 10-km of running, a larger increase after 21-km and

an even larger increase after 42-km, indicating a dose-dependent relationship of DNA damage with distance and intensity.

This method could represent a salient methodological approach for future research to better address the complexity of exercise and DNA damage. Similarly, although challenging, incorporating direct free radical detection in parallel studies may yield more robust results and sensitive data. Lastly, the role of antioxidant supplementation and its potential effects on DNA damage following exercise could be the next focal point of future meta-analyses. As a final practical aspect of performing subsequent meta-analyses, future authors are recommended to include all numeric values in text for easier extraction.

4.4.7 Conclusions

This systematic review and meta-analysis demonstrated a large increase in DNA damage immediately following an acute exercise bout as well as after 4 hours and up to 1-day post-exercise, while such an increase was not evident when measured between 5-28 days. Furthermore, only studies using the comet assay showed significance, compared to 8-OHdG. The analysis further showed that high-intensity exercise results in an increase in DNA damage, suggesting that greater DNA damage maybe be positively associated with increasing exercise intensity in a dose-dependent manner, while no significance was observed in the long-distance studies, possibly due to the initiation of repair systems during such events. However, due to limitations discussed and the paucity of evidence for most secondary outcomes, findings should be viewed with a degree of caution. Although an increase in DNA damage occurs after exercise, this is not necessarily a negative outcome *per se*. Such damage is most likely repaired within 3 days, or likely even sooner, as the long-distance studies may suggest, and thus may be transitory and should not confer any long-term adverse health outcomes on the individual or athlete. However, this will differ across individuals due to variation in individual thresholds since there are multiple factors to consider as explained in (but not limited to) the multi-dimensional model. The hormesis curve describes, in a somewhat one-dimensional manner, how exercise modulates any advantageous or harmful effects through RONS by increasing DNA oxidation between the two-end points of the curve, physical inactivity and overtraining. Finally, the proposed multi-dimensional model may allow for a better understanding of the complex and multi-factorial relationship between DNA damage and exercise.

CHAPTER FIVE

CHAPTER 5: HIGH DOSE ASCORBIC ACID ON EXERCISE-INDUCED DNA DAMAGE

5.1 Introduction

Exercise yields numerous physiological and metabolic changes, as it can exert a plethora of health benefits, including a reduction in the risk of several disease states, and delayed aging (Yimcharoen *et al.*, 2019). Despite this, exhaustive and/or prolonged high-intensity exercise, especially when sporadic, can inflict a degree of mechanical and metabolic stress as the higher energy demands of such exercise leads to increased tissue oxygen consumption (Taghiyar *et al.*, 2013). This results in muscle soreness, which occurs due to muscle damage caused by the mechanical stress/force of exercise (Popovic *et al.*, 2015).

Due to this contradictory phenomenon, exercise is a very commonly used example of showing that RONS are not necessarily the "enemy", despite that elevated RONS may cause repeated episodes of oxidative stress (Nikolaidis & Jamurtas, 2009). Amongst the mitochondria being an integral documented source of RONS formation, other exercise-related sources include: NADPH oxidase; xanthine oxidase (XO); exercise-induced muscle damage activated phagocytes; inflammatory processes; and damage in iron-containing proteins (Ji & Leichtweis, 1997).

As a result of strenuous exercise, the production of RONS is increased. This increase may impair cellular function, lead to higher susceptibility of tissues to oxidative stress, and potentially damage important biomolecules, such as DNA, lipids and proteins (Davison, 2016). DNA is of particular importance since its insufficient repair can lead to mutagenic and carcinogenic effects (Davison, 2016; Williamson *et al.*, 2020*a*). Superoxide anions, hydrogen peroxide, and nitric oxide are the primary RONS generated in skeletal muscle during exercise. However, these are not responsible for damaging DNA directly, as the hydroxyl radical mediates exercise-induced DNA damage through hydrogen abstraction, or by reacting with any of the four DNA bases and the sugar ribose backbone (Cobley *et al.*, 2015; Davison, 2016). RONS can therefore cause alterations in redox balance i.e., towards a pro-oxidant cellular state. However, the endogenous antioxidant defence system, including both enzymatic (e.g., as SOD, CAT, GPx, Prx) and non-

enzymatic antioxidants (e.g., ascorbic acid, α-tocopherol, β-carotene), can protect the cell from an overwhelming production of RONS (Ji, 1999).

Ascorbic acid (AA) is amongst one of the most popular investigated dietary/ supplemental antioxidants. Due to a conserved mutation in the non-functional enzyme L-gulonolactone oxidase (*Gulo*) pseudogene, humans are unable to synthesize AA *in vivo*. Therefore, it is necessary to obtain AA through dietary sources (Yimcharoen *et al.*, 2019). The average requirement of dietary AA is 90mg and 75mg in men and women respectively, and the general minimum requirement to protect against deficiency is 40-60 mg/day (Griffiths & Lunec, 2001; Levine *et al.*, 2001). This water-soluble antioxidant diffuses in and out of cells via a membrane transporter, and is a strong scavenger of superoxide anions and lipid hydroperoxyl radicals, preventing further oxidative reactions and formation of other reactive species (Arrigoni & De Tullio, 2002). In the presence of free transition metal ions (Fe^{3+} and Cu^{2+}), L-ascorbic acid can reduce Fe^{3+} to Fe^{2+} or Cu^{2+} to Cu^{+}. It can also reduce oxygen to the superoxide anion and hydrogen peroxide. This process leads to the formation of RONS and thus exert pro-oxidant activity (Villacorta *et al.*, 2007).

Acute aerobic exercise of high-intensity induces oxidative stress, including DNA, lipid and protein oxidation and increased exercise-induced DNA damage (as measured via the comet assay) following acute exercise (Tryfidou *et al.*, 2020). To counteract this observed exercise-induced oxidative stress, antioxidant-related studies have utilised either low-dose acute or chronic supplementation of AA alone, or in combination with other antioxidants to investigative their effect on oxidative stress biomarkers. While investigations have shown a notable decrease in markers of exercise-induced oxidative stress (e.g., from lipid origins) following chronic supplementation with low-dose AA (Alessio *et al.*, 1997; Goldfarb *et al.*, 2005*b*), others observed no differences from a single administration of low-dose AA (Bryant *et al.*, 2003). As such, it is difficult to reach a consensus on the efficacy of AA due to the small number of investigations, differences in administered doses, and the concurrent co-ingestion of other antioxidants in the related literature. Moreover, there is no study that has specifically used high dose AA to determine the potential effects on exercise-induced oxidative stress. Therefore, the aim of this study was to assess the effect of a high dose of AA (10g/day) administered chronically for 4 weeks on DNA and lipid oxidation following acute aerobic exercise.

5.2 Methods

5.2.1 Participants

Participants were healthy, non-smokers and recreationally active males (defined as taking part in exercise 2-4 times per week and baseline $\dot{V}O_{2max}$ <50 $mL \cdot kg^{-1} \cdot min^{-1}$) who provided written informed consent as well as a health history questionnaire. All participants were free from any medication or antioxidant supplementation at least 3 weeks prior to, and during the study. Prior to all experimental testing (between 9:00-11:00am), all participants were instructed to fast overnight for at least 8 hours while water was allowed *ad libitum*; this approach was used to standardise all blood biochemistry (Simundic *et al.*, 2014). The study was conducted in accordance with the Declaration of Helsinki and approved by a local University Ethics Committee (REC/17/0043).

5.2.2 Randomisation and Exercise testing

Twenty-four hours prior to all exercise testing, participants were requested to abstain from alcohol consumption and exercise. Preliminary baseline and anthropometric data were recorded (Table 1). Following this, all participants performed a maximal exercise test ($\dot{V}O_{2max}$) by cycling at a cadence of 60rpmon a cycle ergometer (Wattbike Ltd, Nottingham) to produce a power output of 100W. The workload was increased by 50W every 3 mins as was the resistance every 3 mins until participants could no longer maintain the required work rate. Confirmation of $\dot{V}O_{2max}$ occurred when plateau of $\dot{V}O_2$ was/were attained, the respiratory exchange ratio (RER) was greater than 1.15 arbitrary units, rate of perceived exertion (RPE) reached 20 on the Borg Scale and/or 90% of age predicted maximum heart rate (HR).

Table 5.1 Participant Characteristics (*n*=20). All values are expressed as Mean ± Standard Deviation.

Participant Characteristics	**Baseline**	
	Placebo (*n*=9)	**AA (*n*=11) ^**
Age (yrs)	33.1 ± 12	32.5 ± 11
Height (m)	1.79 ± 0.1	1.77 ± 0.1
Weight (kg)	86.3 ± 7	83.9 ± 13
BMI (kg/m^2)	27 ± 2	26.7 ± 4
Maximum HR (bpm)	178 ± 13	183 ± 12
$\dot{V}O_{2max}$ (mL·kg^{-1}·min^{-1})	41 ± 6	41 ± 8
Compliance (%)	94 ± 8	94 ± 6

Abbreviations: bpm, beats per minute; HR, heart rate; kg, kilograms; m, meters; $\dot{V}O_{2max}$, maximum oxygen uptake; yrs, years

^ P > 0.05 compared to placebo

Using a randomised, double-blinded, placebo-controlled design, participants were allocated to one of two groups using a web calculator (GraphPad Software, 2018). Participants allocated to the placebo (*n*=9) group consumed two dextrose-containing gelatine capsules per day (<2g dextrose, MyProtein, UK). Whereas participants allocated to the AA group (*n*=11) consumed a total of 10g of pure AA powder per day (Holland & Barrett, UK), in two 5g batches consumed with water at breakfast and dinner meals for 28 days. The supplementation was provided to the participants in a clear zip lock bag labelled with the number of tubes/capsules allocated for the duration of the supplementation period. Participants were instructed to keep all non-ingested tubes/capsules in the zip lock bag and return the bag to the laboratory to account for the number of non-ingested tubes/capsules to measure compliance. The morning following the last day of supplementation, participants attended the laboratory for an experimental exercise trial. Participants cycled as part of a warm-up (*circa* 5 mins) to reach approximately 70% of their predetermined $\dot{V}O_{2max}$ and then cycled for 1 hour aiming to maintain 70-80% of $\dot{V}O_{2max}$. All blood biochemistry and exercise testing took place in the fasted state between 09.00 and 11.00 hours to standardize and avoid circadian rhythm effects. An overview of the experimental design is outlined in **Figure 5.1**.

5.2.3 Blood Sampling

Venous blood was extracted from a prominent antecubital forearm vein at baseline, post-supplementation (pre-exercise), immediately post-exercise and 3 hours post-exercise. Samples were collected in di-potassium ethylene diamine tetra-acetic acid (EDTA) (2×5 mL) and serum vacutainers (2×4 mL). EDTA tubes were initially placed on ice, while serum tubes were allowed to clot for 10 mins and then placed on ice until experimental testing was completed. All blood was centrifuged, aliquoted, and stored at − 80˚C prior to biochemical analysis. Plasma volume changes were calculated using the Dill and Costill equations (Dill & Costill, 1974).

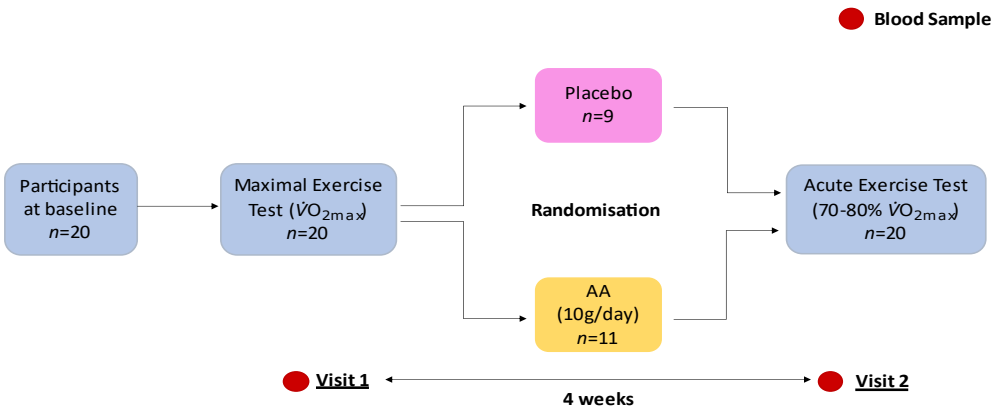


Figure 5.1 Schematic overview of the experimental protocol.

5.2.4 Ascorbic acid

Plasma samples were pre-treated by diluting 9:10 in 5% metaphosphoric acid (MPA) in saline. Furthermore, AA concentrations were measured and compared to standards pre-treated by diluting 1:3 in 10% MPA.

5.2.5 Deoxyribonucleic Acid (DNA) damage

DNA damage was measured in peripheral blood mononuclear cells (PBMCs) using the comet assay technique as detailed previously (Singh *et al.*, 1988; Collins *et al.*, 1993). See 3.8.4. A modification of the comet assay, using *FPG*, was also used to quantify specific DNA lesions (see 3.8.4.1). However,

due to unreliable data, the results are omitted from this chapter (**Appendix G Supplementary Figure S5**).

5.2.6 Lipid Hydroperoxides (LOOH)

Serum LOOH were measured using the hydroperoxide assay containing Xylenol Orange (FOX-1 assay) according to the method by Wolff (1994). See 3.8.1.

5.2.7 Lipid Soluble Antioxidants (LSA)

The following LSA: γ-tocopherol, α-tocopherol, retinol, lycopene, α-carotene, β-carotene were determined simultaneously using the high-performance liquid chromatography (HPLC) method as described by Thurnham and colleagues (Thurnham *et al.*, 1988). See 3.8.2.

5.2.8 Electron Paramagnetic Resonance (EPR) Spectroscopy

The ascorbyl free radical was measured using EPR on a Bruker EMX spectrometer (Bruker, MA, USA) as described previously by Williamson *et al.* (2020*a*). See 3.8.3

5.2.9 Statistical Analysis

As there were no published studies with a similar AA dose supplementation protocol to draw upon for a power calculation to accurately determine the sample size, previous work using a low dose (1g) AA protocol was utilised (Davison *et al.*, 2008). All data were analysed using jamovi statistical software (The jamovi project, Sydney, Australia, v.1.6.8) and data normality was determined using the Shapiro-Wilks test ($P > 0.05$). All descriptive data are expressed as Mean ± Standard Deviation (M ± SD). Equal variance two-sample unpaired t-test was used to compare trial exercise intensity and baseline participant characteristics between two groups ($P < 0.05$). A two-way, repeated-measures ANOVA was used to identify differences within groups and across time, using the Greenhouse-Geisser correction. Following a significant interaction effect (time × group, $P < 0.05$), between group and within time differences were analysed for multiple pairwise post hoc comparisons with a Tukey correction. Statistical significance was set at $P < 0.05$. For post hoc comparisons, results are reported with estimated marginal means (EMM), as they adjust for any other variables in the model, with 95% Confidence Intervals (CI).

5.3 Results

One hour exercise intensity did not differ between the two groups (64 vs 69 % of $\dot{V}O_{2max}$ for placebo and AA groups, respectively, $P > 0.05$). It should also be noted that the targeted intensity, set out as 70-80% $\dot{V}O_{2max}$ in the exercise protocol, was not met. The analysed data showed that the average intensity participants maintained throughout ranged from 64-69% of $\dot{V}O_{2max}$ between groups.

5.3.1 Ascorbic Acid

The participants reported a 94.0 ± 8 and 94.1 ± 6 % compliance in both the placebo (n=9) and AA (n=11) groups, respectively (**Table 5.1)**. Compliance was not 100% as participants reported forgetting to take the supplement and thereby missing some doses. Participants reported no serious adverse side effects for either supplementation; however, some participants reported an unpleasant taste and minor GI distress with the AA supplement. To objectively assess compliance, circulating AA levels were measured across both groups.

A main effect for group was observed (F (1,17) = 8.68, P = 0.009, η^2_p = 0.388). Furthermore, there was an interaction effect for time x group (F (3,51) = 10.7, $P < 0.001$, $\eta^2_p = 0.386$). Post hoc comparisons showed that, following the 4 weeks supplementation period (with either placebo or 10g of AA), the placebo group had significantly lower AA levels (EMM = 16.6 $\mu mol \cdot L^{-1}$, 95% CI: 11.2-21.9) compared to the supplemented group (EMM = 34.9 $\mu mol \cdot L^{-1}$, 95% CI: 29.6-40.2) (M_{diff} = -18.3 $\mu mol \cdot L^{-1}$, t (38.5) = -4.93, $P_{tukey} < 0.001$). This indicates that AA concentration increased by Δ110% following 10g/day of AA supplementation, compared to placebo. Moreover, this interaction remained significant post exercise (M_{diff} = -11.9 $\mu mol \cdot L^{-1}$, t (38.5) = -3.2, $P_{tukey} = 0.05$) but not 3 hours post exercise (M_{diff} = -6.41 $\mu mol \cdot L^{-1}$, t (38.5) = -1.73, $P_{tukey} = 0.67$). Following the acute exercise bout, the supplemented group had higher AA levels (EMM = 33.9 $\mu mol \cdot L^{-1}$, 95% CI: 28.6-39.1) compared to placebo (EMM = 22.0 $\mu mol \cdot L^{-1}$, 95% CI: 16.6-27.3) (M_{diff} = -11.9 $\mu mol \cdot L^{-1}$, t (38.5) = -3.2, $P_{tukey} = 0.05$) (**Figure 5.2**).

A main effect for time was also observed: baseline vs post supplementation (M_{diff} = -8.26 $\mu mol \cdot L^{-1}$, t (51) = -4.47, $P_{tukey} < 0.001$), baseline vs post exercise (M_{diff} = -10.45 $\mu mol \cdot L^{-1}$, t (51) = -5.66, $P_{tukey} < 0.001$) and baseline

vs 3-hour post exercise (M_{diff} = -8.97 μmol·L^{-1}, t (51) = -4.86, P_{tukey} < 0.001).

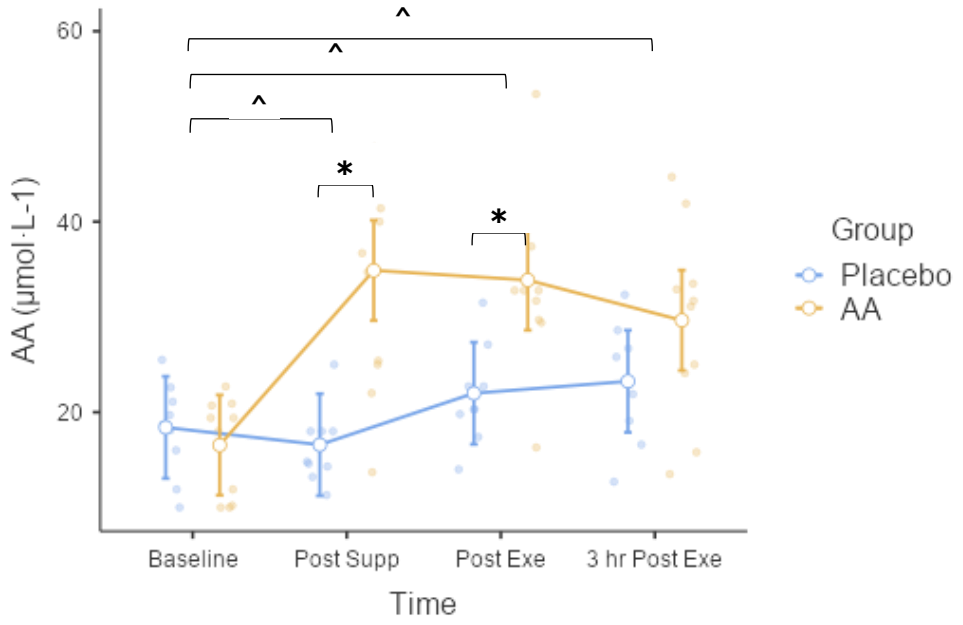


Figure 5.2 AA (μmol·L^{-1}) at baseline, post-supplementation, post-exercise and 3 hours post-exercise across placebo (n = 9) and AA groups (n = 10). Data expressed as EMM (95% CI).

^ represents a significant main effect for time

***** represents a significant interaction for time x group

5.3.2 DNA Damage

Although no interaction effect (F (3,51) = 0.187, P = 0.867, η^2_p = 0.011) was observed, there was a main effect for time (F (3,51) = 22.84, P < 0.001, η^2_p = 0.573). Post hoc analysis showed in the placebo group, that DNA damage (% tail intensity) increased from baseline (EMM = 5.73 %, 95% CI: 2.6-8.86) to post-exercise (EMM = 17.29 %, 95% CI: 14.16-20.42) (M_{diff} = -11.56 %, t (51) = -5.3, P_{tukey} < 0.001) by Δ202%. Similarly, in the AA group, DNA damage increased from baseline (EMM = 6.29 %, 95% CI: 3.48-9.09) to post-exercise (EMM = 16.32 %, 95% CI: 13.52-19.12) (M_{diff} = -10.03 %, t (51) = -5.39, P_{tukey} < 0.001) by Δ159.5%, however no difference between groups was observed (F (1,17) = 0.078, P = 0.078, η^2_p = 0.005) (**Figure 5.3**).

Similar observations were seen post-supplementation/pre-exercise compared to post-exercise within but not between groups. In the placebo group, DNA damage increased from post-supplementation/pre-exercise

(EMM = 6.76 %, 95% CI: 3.63-9.89) to post-exercise (EMM = 17.29 %, 95% CI: 14.16-20.42) (M_{diff} = -10.53 %, t (51) = -4.83, P_{tukey} < 0.001) by Δ156%. In the AA group, DNA damage increased from post-supplementation/pre-exercise (EMM = 7.59 %, 95% CI: 4.79-9.89) to post-exercise (EMM = 16.32 %, 95% CI: 13.52-19.12) (M_{diff} = -8.73 %, t (51) = -4.69, P_{tukey} < 0.001) by Δ115%. Lastly, in the placebo group, DNA damage decreased from post-exercise (EMM = 17.29 %, 95% CI: 14.16-20.42) to 3 hours post-exercise (EMM = 9.95 %, 95% CI: 6.82-13.07) (M_{diff} = 7.34 %, t (51) = 3.37, P_{tukey} = 0.03) by Δ42.5%. Although, a decrease of Δ33.6% was observed in the AA group, it was not significant (M_{diff} = 5.48 %, t (51) = 2.95 P_{tukey} = 0.08) (**Figure 5.3**).

For the main effect of time, data analysis showed that DNA damage increased from baseline to post-exercise (M_{diff} = -10.8 %, t (51) = -7.53, P_{tukey} < 0.001) by Δ180% and from post-supplementation/pre-exercise to post-exercise (M_{diff} = -9.63 %, t (51) = -6.72, P_{tukey} < 0.001) by Δ134%. Additionally, this increase gradually decreased across time but remained elevated up to 3 hours post-exercise (M_{diff} = -4.38 %, t (51) = -3.06, P_{tukey} = 0.02) by Δ73%. Finally, there was a reduction in DNA damage 3 hours post exercise (M_{diff} = 6.41 %, t (51) = -4.47, P_{tukey}< 0.001) by Δ38%.

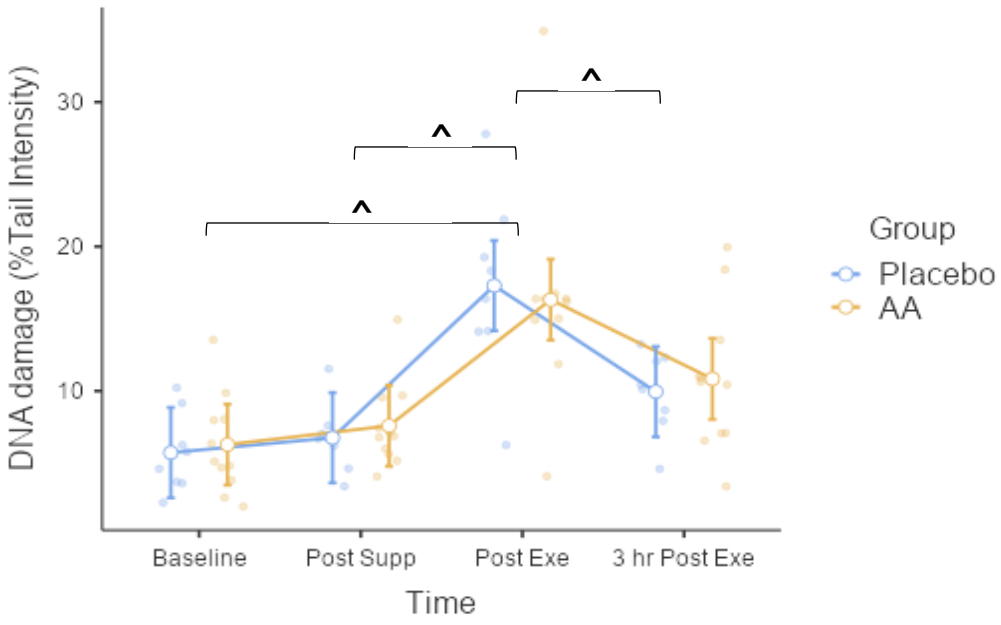


Figure 5.3 DNA damage (% tail intensity) at baseline, post-supplementation, post-exercise and 3 hours post-exercise across placebo (n = 8) and AA groups (n = 10). Data expressed as EMM (95% CI).

^ represents a significant main effect for time

5.3.3 Lipid Hydroperoxides

There was a main effect for time (F (3,48) = 3.08, P = 0.054, η^2_p = 0.161), with post hoc analysis demonstrating a difference between baseline (EMM = 1.24 µmol·L^{-1}, 95% CI: 1.12-1.36) vs. 3 hours post exercise (EMM = 1.01 µmol·L^{-1}, 95% CI: 0.89-1.13) (M_{diff} = 0.232 µmol·L^{-1}, t (48) = 3.01, P_{tukey} = 0.02), indicating that lipid hydroperoxides decreased by 18.7% 3 hours following exercise compared to baseline. There was no main effect for group (F (1,16) = 0.347, P = 0.564, η^2_p = 0.021) or interaction effect (F (3,48) = 0.87, P = 0.437, η^2_p = 0.052) (**Figure 5.4**).

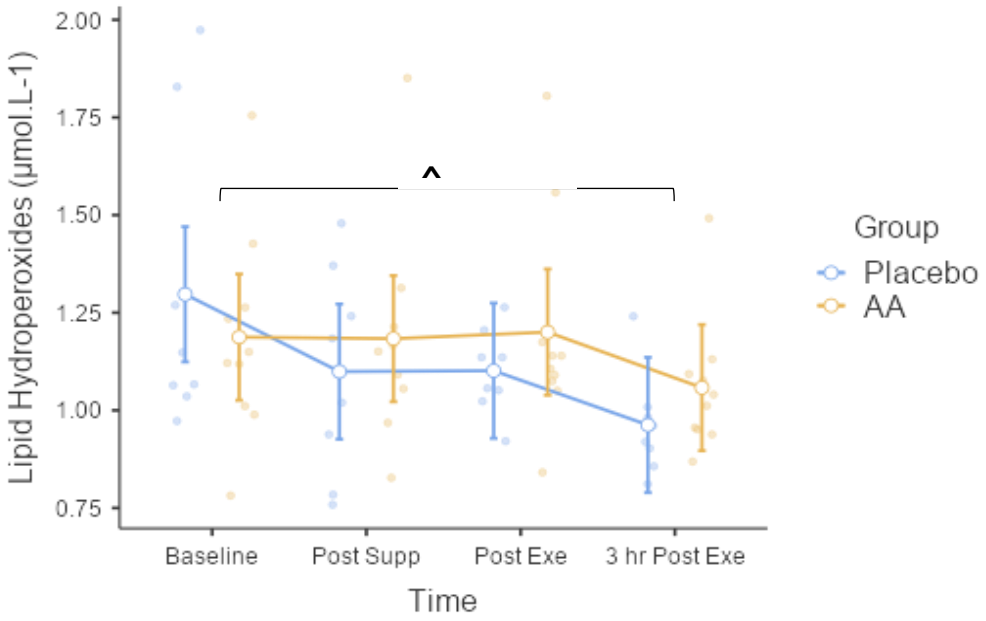


Figure 5.4 Lipid hydroperoxides (µmol·L^{-1}) at baseline, post-supplementation, post-exercise, and 3 hours post-exercise across placebo (n = 8) and AA groups (n = 10). Data expressed as EMM (95% CI).

^ represents a significant main effect for time

5.3.4 Lipid Soluble Antioxidants

There was neither an interaction effect nor main effect for group (P > 0.05) in any of the lipid soluble antioxidant concentrations as presented in **Table 5.2**. However, α-tocopherol increased as a main effect of time from baseline to post-supplementation (pooled data across groups; M_{diff} = -4.27 µmol·L^{-1}, t (54) = -6.4, P_{tukey} = 0.05) by Δ34%.

5.3.5 Ascorbyl Free Radical

A significant interaction effect was observed (F (3,54) = 6.82, P = 0.003, η^2_p = 0.275) with post hoc comparisons showing that ascorbyl free radicals

were lower in the AA group at baseline (EMM = 140838 AU, 95% CI: 29426-252252) than post-supplementation (EMM = 341957 AU, 95% CI: 230545-453369) (M_{diff} = -201119 AU, t (54) = -3.82, P_{tukey} = 0.008), post-exercise (EMM = 330717 AU, 95% CI: 219305-442129) (M_{diff} = -189879 AU, t (54) = -3.61, P_{tukey} = 0.01), as well as 3 hours post-exercise (EMM = 331220 AU, 95% CI: 219808-442632) (M_{diff} = -190382 AU, t (54) = -3.62, P_{tukey} = 0.01) (**Figure 5.5**).

Table 5.2. Lipid soluble antioxidants at baseline, pre-exercise (post-supplementation), post-exercise and following 3 hours post-exercise for placebo and AA groups. All values are expressed EMM (95% CI: lower, upper) and expressed as $mmol \cdot L^{-1}$. Exe = Exercise.

Lipid Soluble Antioxidants	Baseline	Post-Sup	Post-Exe	3 hours Post-Exe
γ-Tocopherol				
Placebo	0.93 (0.32,1.43)	1.45 (0.95,1.95)	1.17 (0.67,1.67)	1.16 (0.66,1.65)
AA	1.01 (0.53,1.49)	1.17 (0.64,1.59)	0.75 (0.27,1.23)	0.91 (0.43,1.39)
α-Tocopherol^				
Placebo	9.7 (5.13,14.2)	16.3 (11.7,20.9)	13.3 (8.71,17.8)	14.1 (9.54,18.7)
AA	11.08 (6.67,15.5)	13.0 (8.63,17.4)	10.2 (5.76,14.6)	11.8 (7.39,16.2)
Retinol				
Placebo	2.44 (1.8,3.09)	2.38 (1.74,3.02)	2.64 (2.0,3.29)	2.53 (1.88,3.17)
AA	3.14 (2.52,3.77)	2.93 (2.31,3.55)	3.08 (2.46,3.70)	3.08 (2.46,3.71)
Lycopene				
Placebo	0.30 (0.17,0.43)	0.33 (0.20,0.46)	0.25 (0.12,0.38)	0.32 (0.19,0.45)
AA	0.35 (0.23,0.48)	0.33 (0.20,0.46)	0.18 (0.06,0.31)	0.27 (0.14,0.39)
α-Carotene				
Placebo	0.03 (-0.004,0.06)	0.03 (0.00,0.06)	0.03 (-0.007,0.06)	0.03 (0.00,0.0.6)
AA	0.04 (0.01,0.07)	0.06 (0.03,0.09)	0.03 (0.00,0.06)	0.05 (0.02,0.08)
β-Carotene				
Placebo	0.17 (0.01,0.32)	0.27 (0.11,0.43)	0.20 (0.05,0.36)	0.25 (0.09,0.41)
AA	0.25 (0.1,0.41)	0.27 (0.11,0.42)	0.18 (0.02,0.33)	0.26 (0.10,0.41)

^ represents a significant main effect for time

This indicates that in the AA group, ascorbyl free radicals were elevated by Δ142.8, Δ134.8 and Δ135.2% at post-supplementation, post-exercise and 3 hours post-exercise, respectively, when compared to baseline. Lastly, there were no main effects for time (F (3,54) = 1.09, P = 0.345, η^2_p = 0.057) or group (F (1,18) = 1.61, P = 0.22, η^2_p = 0.082).

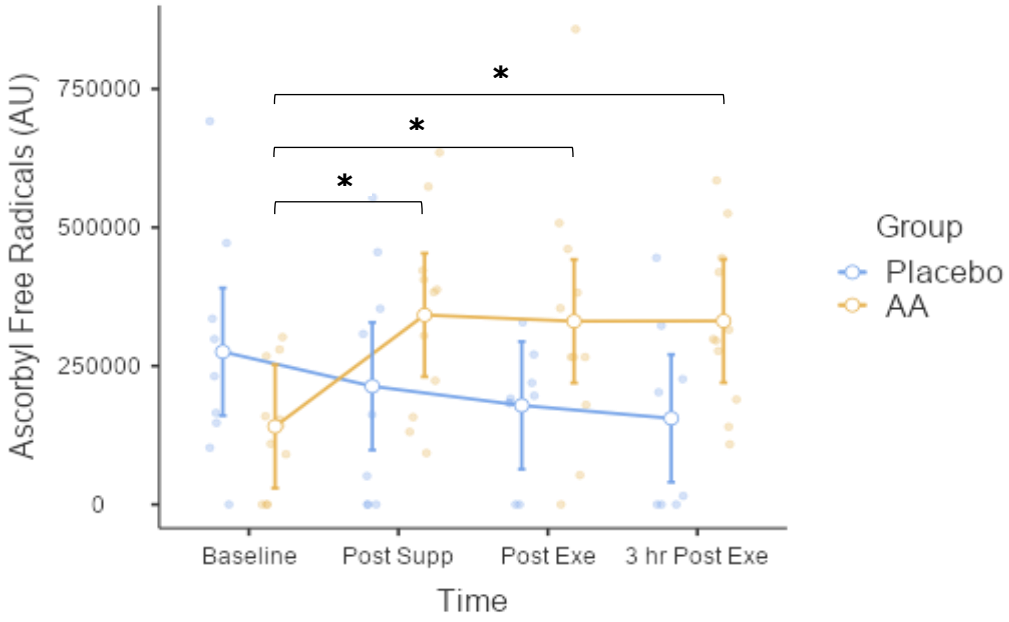


Figure 5.5 Ascorbyl free radical concentration (arbitrary units) at baseline, post-supplementation, post-exercise, and 3 hours post-exercise across placebo (n = 9) and AA groups (n = 11). Data expressed as EMM (95% CI).
***** represents a significant interaction for time x group

5.4 Discussion

The aim of this research was to assess the effect of a high dose of chronic AA supplementation (10 grams/day for 4 weeks) on DNA damage measured by the comet assay, as well as other biomarkers of oxidative stress, before and following an acute aerobic exercise bout (1-hour cycling at 65-70% of $\dot{V}O_{2max}$).

5.4.1 DNA Damage

This investigation demonstrated that DNA damage, as measured by the comet assay, increased 3-fold in the placebo group and 2.6-fold in the AA group immediately post-exercise, but no differences between groups were observed. This finding is in line with other investigations from our laboratory. For example, a higher exercise-induced DNA damage following maximal exercise (Fogarty *et al.*, 2013*b*; Williamson *et al.*, 2018); high-intensity aerobic exercise at 80-85% of $\dot{V}O_{2max}$ (Williamson *et al.*, 2020*a*); 70 & 100% of $\dot{V}O_{2max}$ aerobic exercise (Fogarty *et al.*, 2011); or 100 isolated maximal knee extensions (Fogarty *et al.*, 2013*a*) has been observed previously. In further support of these findings, a recent systematic review and meta-analysis (using data from 35 studies) reported an increase in DNA damage

from studies using the comet assay immediately and up to 1 day post-exercise (n = 203), while no difference was observed in guanine oxidation (i.e., 8-OHdG) (n = 109) (Tryfidou *et al.*, 2020).

Although the direct mechanisms of DNA damage observed in cells following exercise were not investigated in this study, their relevance is important for the context of this discussion. Research has linked this phenomenon to exercise-related generation of RONS (Packer *et al.*, 2008). Mitochondria are considered as a predominant source of RONS generation during exercise (He *et al.*, 2016). It is well established that both resting and contractile skeletal muscle generate superoxide and nitric oxide (NO), during which their production is augmented, however neither these nor H_2O_2 are said to directly damage DNA (Dizdaroglu & Jaruga, 2012; Sakellariou *et al.*, 2013). Instead, following H_2O_2 generation, the hydroxyl radical ($OH^{\bullet}$), generated via the Haber–Weiss Fenton reactions of H_2O_2 with transition metals (reaction 1 & 2), directly reacts and damages DNA bases and the deoxyribose sugar moiety (Cobley *et al.*, 2015; Tryfidou *et al.*, 2020). However, increased production of H_2O_2, a by-product of aerobic exercise, may be a possible contributing mechanism to oxidative damage, as *in vivo* evidence has directly linked an increase of exercise-induced DNA damage to greater H_2O_2 production (Rhee, 2006; Fogarty *et al.*, 2013*b*)

Reaction 1: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{-}OH + OH^{\bullet}$

Reaction 2: $H_2O_2 + Cu^{+} \rightarrow Cu^{2+} + {}^{-}OH + OH^{\bullet}$

Complexes I (NADH dehydrogenase), II (succinate dehydrogenase) and III (coenzyme Q and cytochrome C oxidoreductase) of the electron transport chain have all been identified as major sites of mitochondrial superoxide/ H_2O_2 formation (Barja, 1999; Perevoshchikova *et al.*, 2013). Apart from skeletal muscle superoxide mitochondrial generation, other endogenous sources contributing to free radical production during exercise include NAPDH oxidases (NOXs), XO and nitric oxide synthases (NOS) (Trinity *et al.*, 2016). During skeletal muscle contractions, NOXs contribute largely to cytosolic superoxide production, greater than what the mitochondria produce, as cited in early reports, which is now estimated to be approximately 0.15% (St-Pierre *et al.*, 2002; Xia *et al.*, 2003). Furthermore, there is the ischemia–reperfusion phenomenon, which occurs during intense exercise when blood flow is shifted from organs and tissues to the active skeletal muscles, causing them to become hypoxic (Nishino *et al.*, 2005;

Mohd Sukri, 2021). Consequently, they accumulate an excess amount of oxygen which activates the conversion of xanthine dehydrogenase to XO is activated in the cytoplasm of myocytes as well as in endothelial cells. This, in turn, contributes to the generation of extracellular superoxide during isometric contractions (Powers & Jackson, 2008; He *et al.*, 2016). Additionally, in contractile muscle, phospholipase A_2 (PLA_2) is an enzyme that is involved in intra- and extracellular RONS generation as it activates NOX2 (Benipal *et al.*, 2015). PLA_2 is also associated with arachidonic acid membrane metabolism, and is thus capable of causing further RONS formation and lipid peroxidation damage (Zuo *et al.*, 2004; Steinbacher & Eckl, 2015).

In the context of experimental studies investigating exercise-induced DNA damage and AA supplementation alone, data are currently lacking. This is due to most reports having focused predominantly on: exercise performance and muscle soreness; using other oxidative stress biomarkers instead of the comet assay (i.e., 8-OHdG); and/or using a combination/mixture of ingested antioxidants. To the author's knowledge, this is the only study investigating a high dose of AA on exercise-induced DNA damage measured via the comet assay, ultimately showing no protection of DNA damage immediately following acute aerobic exercise.

Collectively, the evidence so far on whether AA offers any protection against DNA damage (exercise-induced or otherwise) seem somewhat confounding. This is based on the premise that AA has likely both antioxidant and pro-oxidant properties *in vivo*, since it has been shown to exert pro-oxidant properties *in vitro* (depending on the availability of Fe) (Lutsenko *et al.*, 2002). The earliest *in vivo* report investigating the effect of AA (35mg/kg) supplemented with breakfast on steady-state DNA damage reported a reduction in DNA tail damage of freshly isolated cells, as measured via the comet assay (Green *et al.*, 1994). On the other hand, 2-wk supplementation with either 60mg or 6g of AA per day, resulted in unchanged DNA tail moment in healthy female/male cohort (Anderson *et al.*, 1997). Furthermore, endogenous basal levels of single strand breaks (SSBs) from peripheral blood lymphocytes, isolated from a healthy male/female cohort, remained unchanged following AA supplementation (1g/day for 6wks) (Brennan *et al.*, 2000). However, following supplementation of AA and H_2O_2 (200μM)-induced oxidative DNA damage, SSBs significantly decreased showcasing a reduction effect of AA on H_2O_2 sensitivity (Brennan *et al.*,

2000). In cells incubated with 500 μM of dehydroascorbic acid (DHA, the oxidized form of AA) for 1 hour followed by H_2O_2 treatment to induce oxidative stress, a reduction of 8-OHdG was observed. This further indicates the potential of a preventive/protective role of AA in oxidative stress induced DNA mutation (Lutsenko *et al.*, 2002). In contrast, *in vivo* evidence has shown an increase in urinary and serum 8-OHdG following supplementation with AA (500 mg/day) for 6 weeks in healthy volunteers (Cooke *et al.*, 1998).

Regarding exercise as the stressor inducing DNA damage and whether AA has been found to offer any protection, previous work has shown that a mixture of antioxidants (including 600 mg of AA) did not attenuate DNA tail intensity following exercise, compared to the placebo group (Davison *et al.*, 2005). Similarly, no changes were observed in 8-OHdG before and after exercise in a group supplemented with 400 IU of vitamin E and 1 g of AA (Bloomer *et al.*, 2006*b*). Another study reported no difference in urinary 8-OHdG measured pre- and post-exercise in the antioxidant group (consumed a rich in AA beverage containing black grape (81 g/l), raspberry (93 g/l) and red currant (39 g/l) concentrates), however in the placebo group, 8-OHdG was 19% higher post-exercise, which may indicate some protection (Morillas-Ruiz *et al.*, 2005). To the author's knowledge, no experimental *in vivo* study has investigated the effects of AA alone on exercise-initiated DNA damage with the use of the comet assay. However, in an ultramarathon study, a 6-wk supplementation of 1000 mg AA and 400 IU RRR-α-tocopherol acetate resulted in 62% reduction in DNA tail intensity. Interestingly, this reduction was observed 1 day post-race only in women (Mastaloudis *et al.*, 2004*b*), highlighting a need for sex-inclusive studies of this nature, which our investigation lacked.

5.4.2 Lipid Peroxidation

Regarding lipid peroxidation, this study showed no changes in lipid hydroperoxides (LOOH) before and immediately following exercise between groups. Contradictory to this, there is evidence suggesting that LOOH increase as a function of exercise (Fogarty *et al.*, 2011, 2013*b*; Williamson *et al.*, 2018, 2020*a*). Although salivary LOOH was significantly decreased following aerobic exercise (González *et al.*, 2008) as well as resistance exercise lowering lipid peroxidation following aerobic exercise (Vincent *et al.*, 2002). However, in this investigation, regardless of group and as a main effect for time, LOOH decreased by 18.7% from baseline to 3 hours post-

exercise (pooled data baseline vs 3 hours post-exercise). Another report observed a reduction in F_2-isoprostane concentration 3 hours following resistance exercise in both trained and untrained individuals (Rahimi, 2017). It is well established that following exercise, fatty acid oxidation is increased via adipose tissue lipolysis, stimulated predominantly by circulating catecholamines, which increase under a stressor such as exercise (Laurens *et al.*, 2020). Specifically, during exercise of moderate-intensity and long duration, muscle cells rely more on fatty acid mobilization rather than glucose (as observed in short- and high-intensity exercise) through lipolysis of triacylglycerols stored in adipose tissue (Lundsgaard *et al.*, 2020); this may partially explain the observed decrease in LOOH 3 hours post-exercise especially when the average exercise was of moderate intensity.

Lastly, it is plausible to assume that this reduction in damaged lipid macromolecules may indicate enhanced antioxidant capacity as a function of exercise training adaptation in trained subjects (Jenkins *et al.*, 1984; Robertson *et al.*, 1991; Toskulkao & Glinsukon, 1996). Having said that, this study investigated acute rather than chronic exercise/training. In addition, participants training status was neither defined as "untrained" nor "trained athletes" nor was the training load of the participants during the duration of the study measured, thus the term "training" adaptation is rather vague than definitive, as used in this context.

5.4.3 Ascorbyl Free Radical

Ascorbyl free radicals are long-lived species which, detected by EPR spectroscopy, can be applied to assess oxidative status of biological systems. Ascorbate can be oxidized by any RONS (such as hydroxyl radicals, peroxyntitritre, peroxyl radicals) and metals (iron, copper) to produce ascorbyl free radicals (Spasojević, 2011). In healthy human plasma, exogenous AA increase ascorbyl free radicals (increased EPR signal intensity) in a dose dependent manner (Shyu *et al.*, 2014). In this investigation, ascorbyl free radicals increased compared to baseline but only in the AA group. Due to the high supplemented dose of AA, and consequently the high availability of ascorbic acid circulating in the blood to be oxidized, this most likely resulted in increased ascorbyl free radical concentration. High-intensity aerobic exercise may increase oxidation of ascorbic acid and elevate the formation of ascorbyl free radicals (Williamson *et al.*, 2020*a*). In this study, as this increase was observed across all time points and not in

the placebo group, it seems plausible that the rise of ascorbyl free radicals resulted as a function of both exercise and a high supplemented dose of AA.

5.4.4 Limitations and Future Research

No research is unflawed, and, in this investigation, there are some limitations that need to be considered. The concentration of AA in the plasma can depend on multiple factors such as: smoking, health status, bodyweight, lifestyle, antioxidant supplementation, season, and age. Of course, with setting our inclusion/exclusion criteria we aimed to control most of these factors. However, our participants' age ranged from young to older adults (21-61 years old). It has been reported that age is inversely associated with plasma and lymphocyte levels of AA, with a 10-20% decrease reported with increasing age, as well as 38% higher levels in the summer compared to the winter season (Lenton *et al.*, 2000). Furthermore, it is reported that at a daily intake of 1g of AA or more, only 50% or less is absorbed while the rest is excreted through the urine, compared to an intake of 30-180mg/day where 70-90% gets absorbed (Jacob & Sotoudeh, 2002). Additionally, intravenous versus orally administered doses may result in higher peak plasma concentrations; in a depletion-repletion study design, 2.5g of AA (1.25g bioavailable AA) oral dose resulted in 134.8 $\mu mol \cdot L^{-1}$ compared to 885 $\mu mol \cdot L^{-1}$ when administered intravenously (Padayatty *et al.*, 2004). Interestingly, in this study, post-supplementation level of the AA group, which received 10 g of AA orally, was 34.8 $\mu mol \cdot L^{-1}$. This poses the question of bioavailability of the chosen supplement source, as well as concerns about the veracity of the compliance data. Moreover, the concept of inter-individual redox variability has been proposed and supported with evidence from several investigations, both at rest and in response to exercise (Margaritelis *et al.*, 2014, 2018; Paschalis *et al.*, 2016). To address this, in a future study approach, the protocol could consist of groups categorized by their baseline plasma AA levels and divided into high vs low plasma groups, alongside with prescribing multiple incremental doses of AA.

Although, participant compliance was estimated at 94%, some volunteers reported a quite unpleasant sour taste and smell of the AA supplement, which raises the question of whether all 94% of volunteers followed through with the protocol and ingested all prescribed doses. Although, the plasma levels of AA following the supplementation period reflect that compliance was accurate. At high doses (2-6g/day), although it is generally non-toxic, it has been shown to cause gastrointestinal distress or diarrhoea (Naidu,

2003). Moreover, intracellular concentration of AA was not measured in this study. It is likely that this was not achieved in the AA group, which could explain the lack of treatment effect. It has been shown that to achieve saturation levels of AA in leukocytes, about 100 mg/day of AA is required, alongside plasma levels of 50-60 $\mu mol \cdot L^{-1}$ (Levine *et al.*, 1996), which the AA group did not reach despite the very high dose of 10g/day. In addition, AA is sensitive to light (Naidu, 2003) and participants did not receive specific instructions for storing their supplements away from light, although the rate of oxidation from light exposure should be negligible.

Antioxidant daily dietary intake of AA was not monitored, as individual diets throughout the study were not recorded and participants were instructed to consume their diet as normal. Having said that, baseline levels of AA did not differ between groups, thus it seems unlikely to be a confounding factor. To add to this, there were no differences in the LSA concentrations between groups pre- and post-supplementation, but pooled data for the effect of time showed a 34% rise of α-tocopherol (vitamin E) pre- to post-supplementation, although this change was non-significant ($P = 0.052$). At such a high dose of AA, we would expect an interaction effect with vitamin E, as increasing plasma AA has been shown to increase plasma vitamin E both *in vivo* and *in vitro* (Hamilton *et al.*, 2000). Lastly, there have been reports of circadian variations in some oxidative stress markers, including DNA and lipid peroxidation (Kanabrocki *et al.*, 2002; Wilking *et al.*, 2013), although in this study all sampling took place in the fasted state between 09.00 and 11.00 hours to avoid any circadian rhythm effects.

Although this study was not designed to determine the effect of AA on cell adaption, there has been emerging evidence suggesting that antioxidant supplementation could hinder exercise training adaptations. This challenges the notion of whether supplementing with antioxidants should be recommended in healthy people, especially when an individual's daily food intake is already rich in fruit and vegetables achieving the recommended amount of daily antioxidants. Supplementation of AA and vitamin E (1000 mg and 400 IU per day respectively) for 4 weeks prevented the increased expression of PGC1α, PGC1β, PPARγ, SOD1 and GPx1 induced by exercise training (which this study did not investigate). This suggests a hampering effect on cellular training adaptation and endogenous antioxidant systems (Ristow *et al.*, 2009) which is supported by similar findings (Morrison *et al.*, 2015). Therefore, future works could include the measurement of

endogenous antioxidants such as SOD, GPx, CAT or Prx in relation exercise-induced DNA damage. Lastly, the effect of AA on their specific DNA repair gene expression levels, such as hOGG1, needs further investigation to provide a more comprehensive picture and robust results.

5.4.5 Conclusions

This investigation adds to the existing literature that, acute aerobic exercise, mediated by a rise in RONS, increases DNA damage in healthy recreationally active males. However, the damage does not appear to be sustained, but rather transient, as it was shown to decrease 3 hours post-exercise by 42.5% in the placebo group, compared to immediately post-exercise, as well as returning close to baseline levels. A plausible explanation of this phenomenon is an increased DNA repair capacity (Danese *et al.*, 2017; Tryfidou *et al.*, 2020), however further examination of this hypothesis is warranted.

Furthermore, a high dose of AA did not provide enhanced protection immediately following exercise-induced DNA damage. Additionally, although at 3 hours post-exercise a 33% reduction was observed in the supplemented group, compared to immediately post-exercise, this was not significant. Thus, based on our findings and the literature, the consensus to be drawn is that high dose AA supplementation intended for protection against exercise-induced DNA damage cannot be supported, due to a lack of an antioxidant effect. In conclusion, a high dose of AA supplementation is not recommended to healthy males who exercise recreationally and have a balanced diet with sufficient intake of foods rich in antioxidants and more research is required to help elucidate this further.

Future investigations of similar supplementation protocols in this area of research may benefit from implementing the concept of a tailored intervention approach based on phenotype and/or genotype (Margaritelis *et al.*, 2018). Lastly, it may also be of interest to quantify DNA repair gene expression and endogenous antioxidants in relation to the effects of AA on exercise-initiated DNA damage.

CHAPTER SIX

CHAPTER 6: THE IMPACT OF THE MENSTRUAL CYCLE ON EXERCISE-INDUCED DNA DAMAGE

6.1 Introduction

Regular physical activity exerts several positive health effects including a lower risk of all-cause mortality, cardiovascular disease, and a select number of cancers (Jurdana, 2021). Reactive oxygen and nitrogen species (RONS) are produced as a consequence of normal physiological processes in most mammalian cells. Strenuous and sporadic bouts of exercise can further increase RONS generation, which, if generated in excess (and not counterbalanced by sufficient antioxidants), may lead to a state of oxidative stress whereby modifications to lipids, proteins and DNA may ensue. If the latter, i.e., DNA damage, is left unrepaired, it may lead to a state of genomic instability and possible carcinogenesis (Simioni *et al.*, 2018).

The main endogenous site of RONS production during exercise is contracting skeletal muscle (via several sources i.e., mitochondria, NADPH oxidase, and XO activity) and the magnitude of RONS production may be dependent upon factors such as the intensity, type of exercise and/or duration (He *et al.*, 2016; Al-Horani, 2022). The physiological response to oxidative stress is determined by both modifiable (exercise type/intensity/duration, training status, nutrition) and non-modifiable factors (age, biological sex, species, genetics), which together result in differential individual responses and adaptations to training (Viecelli & Ewald, 2022). Specifically, and with regards to biological sex, there is currently a dearth of female research in the broad domain of sport and exercise physiology (McNulty *et al.*, 2020). This has been highlighted by the work of Costello *et al.* (2014) where they report a under-represented collection of investigations including females (39% compared to 61% male participation). Additionally, another important consideration is that female data sets have often been compared to male-originated 'reference' data sets, rather than to female-female comparisons (Beltz *et al.*, 2019; O'Bryan *et al.*, 2022). Several human and animal investigations have reported sex-related differences in exercise-induced oxidative stress biomarkers (Tiidus, 2000; Ginsburg *et al.*, 2001; Goldfarb *et al.*, 2007; Nikolaidis *et al.*, 2007*b*). However, most investigations exploring DNA damage and exercise *per se* have been performed predominately in males, and, as such, there is currently limited data investigating this response in healthy females. Consequently, it is unclear

whether sex differences exist for exercise-induced DNA damage or whether this causes any associated and subsequent molecular impact.

The menstrual cycle (MC) may be divided in three main phases: the menses; the follicular phase and; the luteal phases (Lamina *et al.*, 2013; Draper *et al.*, 2018). These phases can be further divided into the early, mid- and late follicular (EF, MF, LF) and early, mid- and late luteal phases (EL, ML and LL), respectively (Draper *et al.*, 2018). As well as differences in biochemical profiles between the biological sexes, female exercise performance may also be affected by hormonal fluctuations during the stages of the MC, (Emmonds *et al.*, 2019). Consequently, the majority of studies exclude female participants, and as far this author understands, no research to date has specifically explored the relationship between the different phases of the MC on exercise-induced DNA damage (single-stranded DNA breakage as measured via the comet assay) with reference to blood sex hormone concentrations. Furthermore, we aimed to utilize the fact that oestrogens in females is metabolically higher than male counterparts, and as such the oestrogens may offer increased antioxidant protection against any observed exercise-induced oxidative stress (Baltgalvis *et al.*, 2010; Bellanti *et al.*, 2013; Viña *et al.*, 2013). Thus, the primary aim of this research was to assess the effect of an acute high-intensity bout of exercise on DNA damage and other biomarkers of oxidative stress in a healthy premenopausal female cohort during two different phases (LF and LL) of the MC.

6.2 Methods

6.2.1 Participants

All participants (n=7) were apparently healthy, not pregnant, and none were smokers. All volunteers were recreationally active females (aged 18-45) who provided written informed consent following screening using an appropriate health history questionnaire (See Chapter 3.4.2 for further details on recruitment). The recruited participants had a normal MC or lack of a MC due to contraception only. All participants were free from any medication or antioxidant supplementation at least 3 weeks prior to and during the study. Prior to all experimental testing, which took place in the morning hours between 9:00-11:00 am, all participants were instructed to fast overnight for at least 8 hours in order to standardize all blood biochemistry; water was allowed *ad libitum*, (Simundic *et al.*, 2014).

The study was conducted in accordance with the Declaration of Helsinki and approved by a local University Ethics Committee (REC/18/0110).

6.2.2 Monitoring of Menstrual Cycle and Preliminary Testing

Participants firstly attended the Ulster University Human Performance Lab to complete a medical history questionnaire and if eligible, provide informed consent (Visit 1). Height and weight were recorded using a free-standing stadiometer (Holtain Limited, Great Britain) and standard laboratory scales (Seca, Hamburg, Germany), respectively. Participants were asked to self-report the current day of their MC along with the dates of their last 2-3 MCs, if known, or provide approximate dates. Thereafter, the participant's next MC was monitored to assess integrity/accuracy of their MC via a digital body thermometer. Participants were instructed to record basal body temperature (BBT) orally every day before exiting bed for their next 2 consecutive MCs (Tenan *et al.*, 2013). In addition, ovulation/urine kits (Signalab, Shenzhen GLD Biotechnology Ltd, UK) were provided and participants were instructed to use them around days of suspected ovulation in each cycle to confirm days of ovulation for the same two consecutive cycles. Both the measurement of BBT and use of ovulation kits were used to track and estimate ovulation.

6.2.3 Experimental Testing

Twenty-four hours prior to all exercise testing, participants were asked to abstain from alcohol consumption and exercise. Volunteers were instructed to perform two separate $\dot{V}O_{2max}$ tests according to the Bruce protocol (Bruce *et al.*, 1963) on day 26-28 (Visit 2) and on day 11-13 (Visit 3) of their cycle. These corresponded to the late luteal phase (LL) and the late follicular phase (LF) of the MC, respectively. Briefly, the Bruce protocol consisted of a continuous, graded exercise test on a treadmill beginning at 1.7 mph and 10% grade and increasing 0.8–1.0 mph and 2% grade every 3 min until voluntary cessation (Bruce *et al.*, 1963; Pollock *et al.*, 1976). Volunteers provided venous blood immediately before, immediately following and 1-hour post-exercise tests. All blood biochemistry and exercise testing took place in the fasted state between 09.00 and 11.00 hours to standardize and avoid circadian rhythm effects. A timeline and overview of the experimental protocol is shown in **Figure 6.1**.

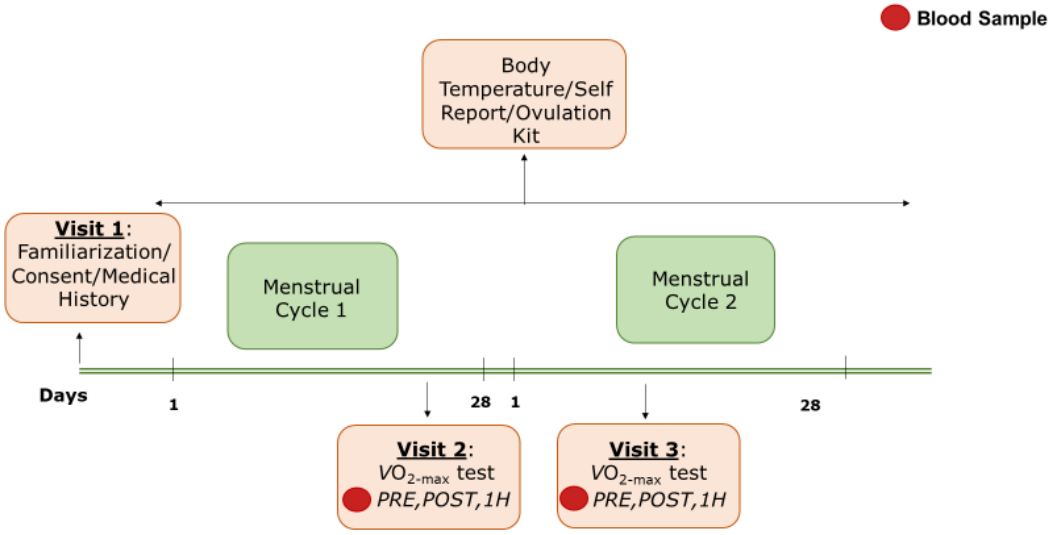


Figure 6.1 Timeline and overview of the experimental protocol.

Table 6.1 Description and rating of perceptual measures questionnaire.

Perceptual Measures Questionnaire	Rating Description
Mood	On a scale of 1 through 10 (1 having the worst mood – 10 having the best mood), rate your mood today.
Motivation	On a scale of 1 through 10 (1 feeling the lowest motivation/energy – 10 feeling the highest motivation/energy), how motivated/energized you felt today with regards to the exercise test?
Performance Expectation	On a scale of 1 through 10 (1 feeling that you performed the worst you could – 10 you performed the best), did you feel that you pushed/performed to your maximum ability with regards to the exercise test and performed the best you could?
Fatigue Post-Exercise	On a scale of 1 through 10 (1 feeling the least fatigued – 10 feeling extremely fatigued), how fatigued did you feel after the exercise test?

6.2.4 Perceptual Measures Questionnaire

Using the interval rating scale method, participants were asked to rate their experience on a scale of 1-10 during their exercise testing according to four different parameters detailed above **(Table 6.1)**.

6.2.5 Blood Sampling

Blood was extracted from a prominent antecubital forearm vein at baseline, post supplementation (pre-exercise), immediately post-exercise and 1-hour post-exercise. Samples were collected into di-potassium ethylene diamine tetra-acetic acid (EDTA) (3×5 mL) and serum vacutainers (2×4 mL). EDTA tubes were initially placed on ice, while serum tubes were allowed to clot for 10 mins and then placed on ice until experimental testing was completed. All blood was centrifuged, aliquoted, and stored at − 80◦ C prior to biochemical analysis.

6.2.6 Hormone Analysis

All hormone analyses were conducted by the Ulster Hospital Dundonald, UK. Serum oestradiol, progesterone, LH and FSH concentrations were assayed using the Roche cobas® **e**801 module which uses electrochemiluminescent technology (ECL) based on the reaction of a ruthenium complex with tripropylamine (TPA). A photomultiplier measures the emission of light that emerges from this reaction. The competitive and sandwich principle were used for the analysis of oestradiol/progesterone and LH/FSH for samples of low molecular vs higher molecular weight respectively. Evaluation and calculation of the antigen concentrations are carried out by means of a calibration curve that were established using calibrators of known antigen concentration.

6.2.7 Deoxyribonucleic Acid (DNA) damage

DNA damage was measured in human white blood cells (WBCs) using the comet assay under two buffer conditions: alkaline and neutral as detailed previously (Singh *et al.*, 1988; Collins *et al.*, 1993). The standard alkaline comet assay is used to detect global DNA damage, including both single-strand breaks (SSBs). On the other hand, the neutral comet assay, as described by Wojewódzka *et al.* (2002), predominantly detects DSBs. This method omits the high-temperature lysis and proteinase K step, which makes it less labour-intensive and more cost-effective, while still maintaining the assay's sensitivity and specificity in detecting DSBs. Details of the methods can be found in section 3.8.4. and 3.8.4.2.

6.2.8 Lipid Hydroperoxides (LOOH)

Serum LOOH was measured using the water-soluble hydroperoxide assay containing Xylenol Orange (FOX-1 assay) according to the method by Wolff (1994). More information is available in section 3.8.1.

6.2.9 Lipid Soluble Antioxidants (LSA)

γ-tocopherol, α-tocopherol, retinol, lycopene, α-carotene, β-carotene were determined simultaneously using the high-performance liquid chromatography (HPLC) method as described by Thurnham and colleagues (Thurnham *et al.*, 1988). See section 3.8.2 for more detailed information.

6.2.10 Electron Paramagnetic Resonance (EPR) Spectroscopy

The ascorbyl free radical was measured using EPR on a Bruker EMX spectrometer (Bruker Instruments Inc., Billerica, MA, USA) as described previously by Clifford and colleagues (Clifford *et al.*, 2016). Further information is detailed in section 3.8.3.

6.2.11 Statistical Analysis

Due to the study's novelty, a power calculation was not performed to determine sample size, since there have been no previously reported studies on the effects of endogenous oestradiol production on exercise-induced DNA damage with the comet assay method in females. A maximum number of 15 was determined as an appropriate number of participants to be recruited within the available time and resources. All data were analysed using jamovi statistical software (The jamovi project, Sydney, Australia, v.1.6.8) and data normality was determined using the Shapiro-Wilks test ($P > 0.05$). All descriptive statistics are expressed as Mean ± Standard Deviation (M ± SD). For baseline characteristics and exercise variables measured between phases of the MC, data were analysed using a paired samples t-test with a significance level of $P < 0.05$. For perceptual measures, data were analysed using the Wilcoxon rank paired t-test with a significant level set at $P < 0.05$. A two-way, repeated-measures factorial ANOVA was used to identify differences within phases of the MC and across time, using the Greenhouse-Geisser correction when Mauchly's W assumption of sphericity was found statistically significant ($P < 0.05$). Subsequent to a significant interaction effect (time × phase, $P < 0.05$), within phase and time differences were analysed for multiple pairwise post hoc comparisons with a Tukey correction. Tukey paired samples t-test were also used for within time differences. Statistical significance was set at $P < 0.05$. For post hoc comparisons, results

are reported with estimated marginal means (EMM), as they adjust for any other variables in the model, with 95% Confidence Intervals (CI). Pearson's correlation coefficient was used to assess the relationship between variables.

6.3 Results

6.3.1 Baseline data, exercise performance variables and perceptual measures

Participants' characteristics and exercise performance variables measured in the LF and LL phases are shown in **Table 6.2**. No differences were observed in any baseline data, exercise parameters, nor ratings of perceptual measures ($P > 0.05$).

Table 6.2 Participant, exercise characteristics and rating of perceptual parameters (n=7). All values are expressed as Mean ± Standard Deviation.

Participant Characteristics	**Baseline**	
Age (yrs)	34.6 ± 5.2	
Height (m)	1.63 ± 7.9	
Weight (kg)	56.5 ± 7.9	
Exercise Characteristics	**LF**	**LL**
Maximum HR (bpm)	180 ± 14	179 ± 16
$\dot{V}O_{2max}$ (mL kg^{-1} min^{-1})	42.3 ± 10.1	43.5 ± 5.9
TTE (mins)	11.8 ± 1.9	11.9 ± 1.8
Distance (km)	0.92 ± 0.25	0.94 ± 0.22
Perceptual Parameters Score	**LF**	**LL**
Mood	8.6 ± 0.9	8 ± 0.8
Motivation	7.7 ± 1.1	6.9 ± 1.2
Performance Expectation	7.1 ± 2.5	7.1 ± 2.6
Fatigue Post-Exercise	6.3 ± 1.9	7.7 ± 1.6

Abbreviations: bpm, beats per minute; HR, heart rate; kg, kilograms; m, meters; $\dot{V}O_{2max}$, maximum oxygen uptake; yrs, years; TTC, time to exhaustion; LF, late follicular; LL, late luteal.

6.3.2 DNA damage (Alkaline Assay)

There was no observed main effect for phase of the MC (F (1,6) = 0.29, P = 0.608, $\eta^2_p = 0.046$). Furthermore, there was no significant within subjects

interaction effect for time x phase (F (2,12) = 0.04, P = 0.961, η^2_p = 0.007). However, a main effect for time was observed (F (2,12) = 37.69, P < 0.001, η^2_p = 0.856).

Specifically, post hoc analysis showed that in the LF phase, DNA damage (% tail intensity) increased from pre- (EMM = 4.44 %, 95% CI: -2.67-11.6) to post-exercise (EMM = 20.3 %, 95% CI: 13.19-27.4) (M_{diff} = -15.86 %, t (23.2) = -3.577, P_{tukey} = 0.017) by Δ357.2%. Furthermore, DNA damage was Δ549% higher measured 1-hour post-exercise (EMM = 28.82 %, 95% CI: 21.7-35.9) (M_{diff} = -24.38 %, t (23.2) = -5.496, P_{tukey} < 0.001), compared to pre-exercise levels. Although no difference between phases was observed, similar findings were observed in the LL phase as DNA damage increased from pre- (EMM = 7.23 %, 95% CI: -0.12-14.3) to post-exercise (EMM = 21.37 %, 95% CI: 14.26-28.5) (M_{diff} = -14.14 %, t (23.2) = -3.189, P_{tukey} = 0.041) by Δ195.6%. Lastly, DNA damage was Δ314.6% higher measured 1-hour post-exercise (EMM = 29.98 %, 95% CI: 22.86-37.1) (M_{diff} = -22.75 %, t (23.2) = -5.129, P_{tukey} < 0.001), compared to pre-exercise levels. (**Figure 6.2**).

6.3.2.1 DNA damage (Neutral Assay)

There was no observed main effect for phase of the MC (F (1,6) = 0.356, P = 0.573, η^2_p = 0.056). Furthermore, there was no significant within subjects interaction effect for time x phase (F (2,12) = 0.796, P = 0.473, η^2_p = 0.117). However, a main effect for time was observed (F (2,12) = 15.98, P < 0.001, η^2_p = 0.727).

Specifically, post hoc analysis showed that in the LF phase, DNA damage (% tail intensity) increased from pre- (EMM = 10.9 %, 95% CI: 7.84-14.0) to post-exercise (EMM = 21.1 %, 95% CI: 18.0-24.1) (M_{diff} = -10.15 %, t (18.6) = -4.738, P_{tukey} = 0.002) by Δ93.6%. Similarly, in the LL phase, DNA damage increased from pre- (EMM = 10.7 %, 95% CI: 7.62-13.7) to post-exercise (EMM = 21.8 %, 95% CI: 18.73-24.9) (M_{diff} = -11.1 %, t (18.6) = -5.181, P_{tukey} < 0.001) by Δ103.7%. (**Figure 6.2**).

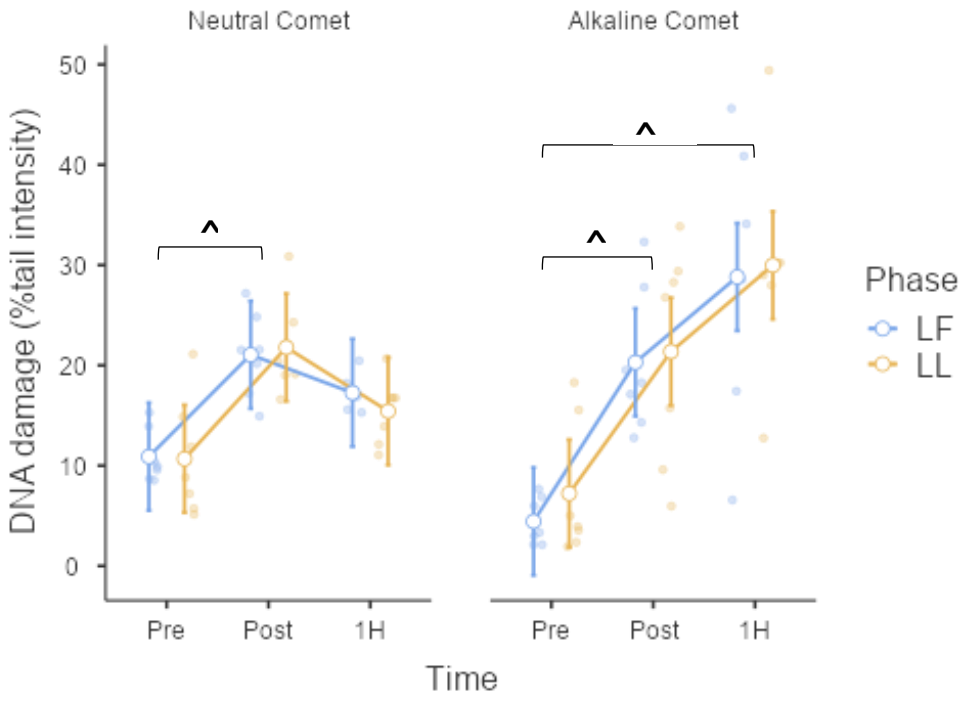


Figure 6.2 DNA damage (% tail intensity) measure by the alkaline and neutral comet assays at pre, post, post-exercise and 1-hour post-exercise across LF (n = 7) and LL phases (n = 7). Data expressed as EMM (95% CI). ^ represents a significant interaction for time

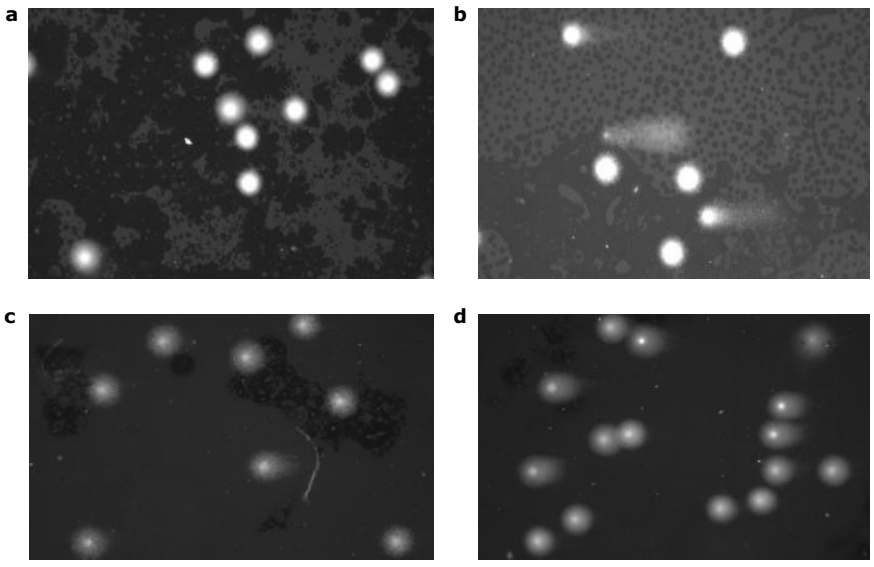


Figure 6.3 (a) Pre- and (b) post-exercise under alkaline conditions in LF phase (c) pre- and (d) post-exercise under neutral conditions in LL phase. Isolated lymphocytes from the same participant.

6.3.3 Hormone Analysis

As observed in **Table 6.3**, no significant effect was evident for oestradiol level for phase of the MC (F (1,6) = 0.06, P = 0.818, η^2_p = 0.01), time x phase (F (2,12) = 0.01, P = 0.928, η^2_p = 0.002) or time (F (2,12) = 3.94, P = 0.09, η^2_p = 0.396). However, a significant main effect for phase was shown for progesterone (F (1,6) = 10.6, P = 0.017, η^2_p = 0.638) but not for time (F (2,12) = 0.03, P = 0.884, η^2_p = 0.005) or time x phase (F (2,12) = 0.33, P = 0.723, η^2_p = 0.053). Specifically, progesterone was Δ653% higher (M_{diff} = -22.6 nmol·L^{-1}, t (6) = -3.25, P_{tukey} = 0.017) in the LL (EMM = 26.07 nmol·L^{-1}, 95% CI: 14.34-37.8) compared to the LF phase (EMM = 3.46 nmol·L^{-1}, 95% CI: -8.27-15.2). Furthermore, no significant effect was observed for LH for phase of the MC (F (1,6) = 3.32, P = 0.118, η^2_p = 0.356), time (F (2,12) = 5.74, P = 0.05, η^2_p = 0.489) or time x phase (F (2,12) = 0.13, P = 0.88, η^2_p = 0.021). Lastly, no significant main effect was seen for FSH for phase of the MC (F (1,6) = 4.93, P = 0.068, η^2_p = 0.451), time (F (2,12) = 2.3, P = 0.18, η^2_p = 0.277) or time x phase (F (2,12) = 1.1, P = 0.34, η^2_p = 0.155).

Table 6.3. Oestradiol, progesterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) measured at pre-exercise, post-exercise, and 1-hour post-exercise across LF (n = 7) and LL phases (n = 7). Data expressed as EMM (95% CI).

Hormones	Pre	Post	1H
Oestradiol (pmol·L^{-1})			
LF	508 (226,790)	599 (317,881)	445 (163,727)
LL	535 (253,817)	649 (367,931)	491 (209,773)
Progesterone (nmol·L^{-1}) $^{\#}$			
LF	3.6 (-8.5,15.8)	4.0 (-8.2,16.2)	2.8 (-9.4,14.9)
LL	25.2 (13,37.3)	25.8 (13.6,37.9)	27.3 (15.1,39.4)
LH (U·L^{-1})			
LF	10.6 (6.8,14.3)	11.2 (7.5,14.9)	7.13 (3.4,10.9)
LL	7.9 (4.2,11.7)	7.7 (3.9,11.4)	3.5 (-0.3,7.2)
FSH (U·L^{-1})			
LF	10.6 (5.3,9.1)	7.7 (5.8,9.5)	6.7 (4.8,8.6)
LL	5.0 (3.1,6.9)	5.1 (3.3,7.0)	3.7 (1.8,5.6)

All values are expressed EMM (95% CI: lower, upper). Exe = Exercise.
$^{\#}$ represents a significant effect of phase

6.3.4 Lipid Hydroperoxides (LOOH)

There was no observed main effect for MC phase (F (1,6) = 0.54, P = 0.492, η^2_p = 0.082). Similarly, there was no significant interaction effect for time x phase (F (2,12) = 0.17, P = 0.892, η^2_p = 0.019) (**Figure 6.4**). However, a main effect for time was observed (F (2,12) = 7.56, P = 0.007, η^2_p = 0.558). Specifically, post hoc analysis showed that LOOHs were increased from pre- (EMM = 1.00 μmol·L^{-1}, 95% CI: 0.93 -1.07) to post exercise (EMM = 1.10 μmol·L^{-1}, 95% CI: 1.03-1.17) (M_{diff} = -0.099 μmol·L^{-1}, t (12) = -3.31, P_{tukey} = 0.016) indicating a Δ10% increase following exercise. This increase remained significant 1-hour post-exercise (EMM = 1.10 μmol·L^{-1}, 95% CI: 1.03-1.17) (M_{diff} = -0.106 μmol·L^{-1}, t (12) = -3.42, P_{tukey} = 0.013) (**Figure 6.5**).

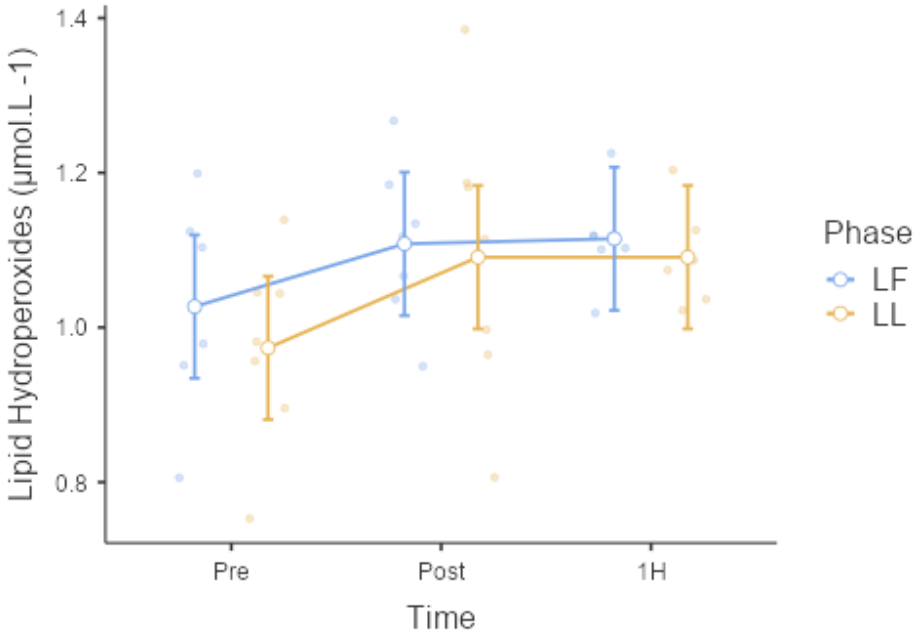


Figure 6.4 Lipid hydroperoxides (LOOH) (μmol·L^{-1}) measured at pre-exercise, post-exercise and 1-hour post-exercise across LF (n = 7) and LL phases (n = 7). Data expressed as EMM (95% CI).

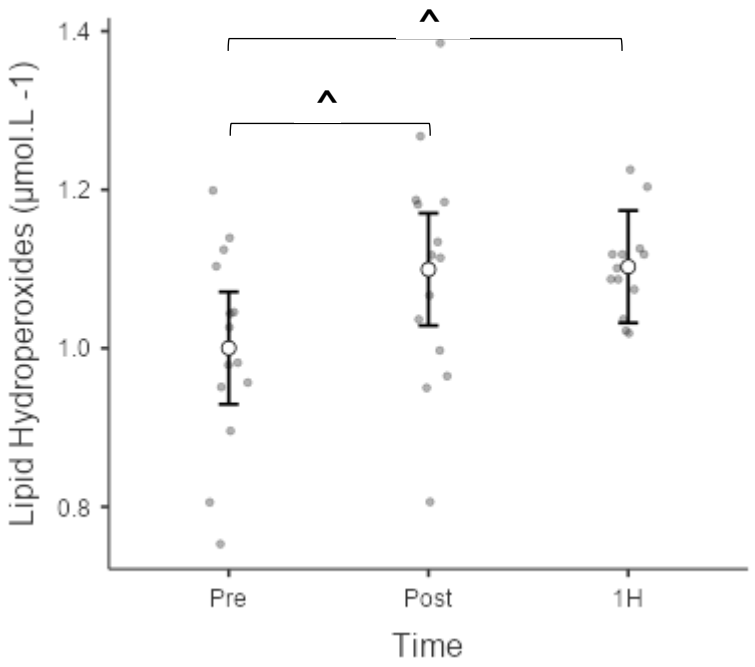


Figure 6.5 Main effect for time for lipid hydroperoxides (µmol·L^{-1}) at pre-exercise, post-exercise and 1-hour post-exercise (n = 7). Data expressed as EMM (95% CI).

^ represents a significant effect of time

6.3.5 Lipid Soluble Antioxidants

There were neither observed interaction effects of time x phase nor main effects for time or group (P > 0.05) in any of the assayed lipid soluble antioxidants as presented in **Table 6.2**.

Table 6.4. Lipid soluble antioxidants at pre-exercise, post-exercise and 1-hour post-exercise across LF (n = 7) and LL phases (n = 7). All values are expressed EMM (95% CI: lower, upper) and expressed as mmol·L^{-1}. Exe = Exercise.

Lipid Soluble Antioxidants	Pre	Post	1H
γ-Tocopherol			
LF	1.73 (1.11,2.35)	1.62 (0.99,2.24)	1.98 (1.36,2.60)
LL	2.00 (1.38,2.62)	1.24 (0.62,1.86)	1.48 (0.86,2.10)
α-Tocopherol			
LF	12.9 (10.52,15.4)	12.7 (10.23,15.1)	14.1 (11.64,16.5)
LL	13.6 (11.2,16.1)	11.2 (8.75,13.6)	11.6 (9.14,14.0)
Retinol			
LF	4.99 (3.48,6.50)	5.18 (3.67,6.69)	5.21 (3.70,6.72)
LL	3.87 (2.36,5.38)	4.93 (3.42,6.44)	4.35 (2.84,5.87)
Lycopene			
LF	0.77 (0.20,1.33)	0.63 (0.06,1.19)	0.79 (0.22,1.36)
LL	0.85 (0.28,1.41)	0.70 (0.13,1.26)	0.82 (0.25,1.39)
α-Carotene			
LF	0.08 (0.04,0.12)	0.07 (0.03,0.12)	0.08 (0.03,0.12)
LL	0.05 (0.01,0.09)	0.05 (0.01,0.09)	0.09 (0.05,0.14)
β-Carotene			
LF	0.48 (0.31,0.65)	0.45 (0.28,0.62)	0.45 (0.28,0.62)
LL	0.42 (0.25,0.59)	0.36 (0.19,0.53)	0.37 (0.19,0.54)

6.3.6 Ascorbyl Free Radical

No significant effect for phase (F (1,6) = 4.31, P = 0.083, η^2_p = 0.418) or interaction effect for time x phase was observed (F (2,12) = 3.88, P = 0.05, η^2_p = 0.393) (**Figure 6.6**).

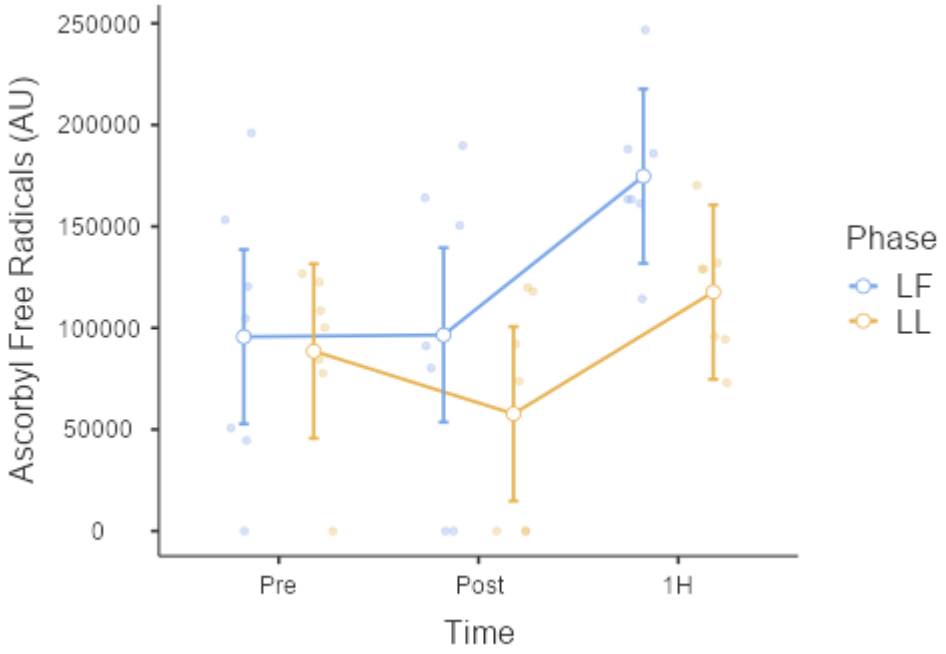


Figure 6.6 Ascorbyl free radical concentration (arbitrary units) measured at pre-exercise, post-exercise and 1-hour post-exercise across LF (n = 7) and LL phases (n = 7). Data expressed as EMM (95% CI).

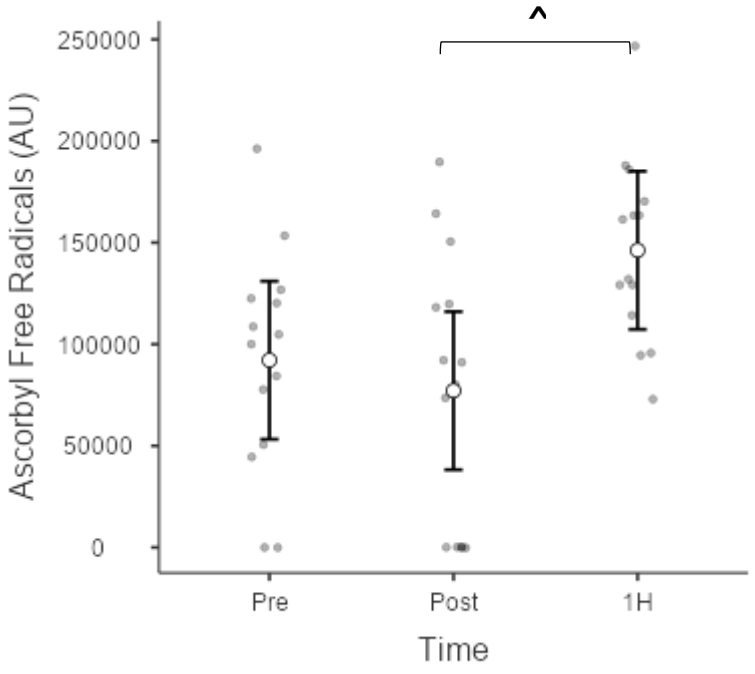


Figure 6.7 Main effect for time on Ascorbyl free radical concentration (arbitrary units) at pre-exercise, post-exercise and 1-hour post-exercise (n = 7). Data expressed as EMM (95% CI).

^ represents a significant interaction for time

There was, however, a significant main effect for time (F (2,12) = 5.29, P = 0.022, η^2_p = 0.469). Specifically, ascorbyl free radical concentration increased by Δ89.6% from post (EMM = 77128 AU, 95% CI: 38232-116024), compared to 1-hour following exercise (EMM = 146222 AU, 95% CI: 107326 -185119) (M_{diff} = -69094 AU, t (12) = -3.093, P_{tukey} = 0.023) (**Figure 6.7**).

6.3.7 Correlations

No significant correlations were observed between oestradiol or any of the other measured sex hormones and DNA SSBs. However, oestradiol showed a positive strong correlation during the LL phase with DNA DSBs at baseline (r = 0.79, P < 0.05). All observed significant correlations between all variables are presented in **Table 6.4**.

Table 6.5 Significant Pearson's correlation coefficients (r) between level of sex hormones during the MC and biomarkers of oxidative stress.

		DNA S+DSBs	DNA DSBs	LOOH	Ascorbyl Radical	γ tocopherol	α tocopherol	Retinol	Lycopene	α carotene	β carotene
MCP	**Variable**						**r**				
LF	*Oestradiol (pmol·L^{-1})*				0.80(Post)*		0.86(1H)*	-0.88(1H) **	-0.87(Pre)* -0.90(1H)**	-0.76(1H) *	-0.76(Pre)*
	Progesterone (nmol·L^{-1})		-0.82(Pre)*				-0.98 (Post)**				
	LH (U·L^{-1})		0.84(Pre)*		0.91(Pre)**		-0.92 (1H)**				
	FSH (U·L^{-1})										
							r				
LL	*Oestradiol (pmol·L^{-1})*		0.79(Pre)*								
	Progesterone (nmol·L^{-1})						-0.93(Pre)**				
	LH (U·L^{-1})		0.76(Pre)*					-0.90(1H)**			
	FSH (U·L^{-1})			0.79(1H)*		-0.86(Post)*					

Abbreviations: menstrual cycle phase, MCP

* P < 0.05 ** P < 0.01

6.4 Discussion

The primary aim of this research was to assess the effect of an acute high-intensity bout of exercise on DNA damage and other markers of oxidative stress in a healthy premenopausal female cohort during two different phases (LF and LL) of the MC. This exploratory work, while highlighting a change in DNA damage as a function of exercise per se, reports there was no difference in damage observed between the two phases of the MC. Regarding the effect of exercise on sex hormone concentrations, this study observed no significant changes in any of the four measured hormones.

Existing evidence largely supports the findings of the current study, showing no significant effect of exercise on FSH, LH, and oestradiol levels, as observed in a previous study. However, a notable decrease in progesterone was reported in response to acute exercise in a Thai female cohort during both the LF and LL phases (Chearskul & Srichantaap, 1994). In contrast, acute incremental exercise has been shown to increase total oestradiol in untrained females (Bunt *et al.*, 1987; De Crée *et al.*, 1997). Similarly, female soccer players had increased oestradiol, LH and decreased progesterone following an acute maximal aerobic shuttle run test (Otağ *et al.*, 2016).

6.4.1 DNA Damage

Exercise-related oxidative damage has been shown to occur by a multitude of research investigations (Tryfidou *et al.*, 2020). Using the alkaline comet assay, this study reports a 7- and 11-fold increase in global DNA damage (SSBs & DSBs) during the LF phase immediately post and 1-hour following exhaustive maximal exercise, respectively. Similarly, during the LL phase, there are 3.9- and 6.3-fold increases post- and 1-hour post-exercise, respectively. With regards to DSBs measured via the neutral modified comet assay, there were increases of 1.9- and 2.1-fold in DSBs immediately after exercise during the LF and LL phases, respectively. In contrast, such an increase was not observed 1-hour following exercise in either phase of the MC when using the alkaline assay. No differences were observed between the LF and LL phases in global DNA damage (SSBs & DSBs), detected by the two versions of the comet assay (**Figure 6.2**). These data reflect our meta-analysis findings, which showed DNA damage, as measured via the (alkaline) comet assay, significantly increases as a function of high-intensity aerobic exercise and can last up to 1 day post-exercise (Tryfidou *et al.*,

2020). This damage is caused predominately by an excess of hydroxyl free radical production generated from a combination of H_2O_2 and iron that are metabolically active during stages of high-intensity exercise (Tryfidou *et al.*, 2020). However, in the studies included in our meta-analysis, the majority were conducted with males, as only one third of studies included both female and male participants.

There is a lack of research investigating sex differences in oxidative DNA damage, especially as a function of exercise, and the existing evidence remains a contradicted landscape. One study reported basal (with no exercise intervention) DNA damage in lymphocytes in a healthy Indian population, and observed a significantly higher basal DNA damage in males compared to females (Bajpayee *et al.*, 2002). The authors speculate that the elevated DNA damage could be due to the possibility of males having a higher risk of cancer, which is common in India. However, it is unknown whether this higher risk of cancer results from increased DNA damage levels alone or perhaps due to other genomic and epigenomic influences. Moreover, another investigation reported higher steady-state levels of modified bases (FAPy Guanine) in males compared to females, with lower 8-OH adenine in males compared to females and no difference in 8-hydroxyguanine (Proteggente *et al.*, 2002). A separate report showed higher single SBs (measured via the comet assay), tail moment and % tail DNA intensity in young males compared to females. Interestingly, the authors reported a positive correlation, and potential confounder, of increased fruit intake and decreased DNA damage observed only in females, as females reported a higher average daily intake of fruit (Hofer *et al.*, 2006). On the other hand, two reports noted no difference of sex in baseline DNA damage levels (Møller *et al.*, 2003; Park *et al.*, 2003). Lastly, another study reported lower DNA migration (via comet assay) in females compared to males in a >65 years old Mexican cohort, as well as greater % of non-damaged cells in women >45 years old. Additionally, the authors also reported both greater SOD and GPx activity in the women aged 25-64, compared to their male counterparts, and less GPx activity in men, negatively correlated with increasing age (Mendoza-Núñez *et al.*, 2010).

Early *in vitro* work in the 1990s highlighted the potential ability of oestradiol to protect against DNA damage and lipid peroxidation. Specifically, E_2 and to a larger extent, equilin (an equine oestrogen) both decreased hydrogen peroxide-induced SSBs and DSBs, as well as damage induced by arachidonic

acid/Cu^{2+} (Tang & Subbiah, 1996). The mechanisms of action by which this protective ability exists was suggested to be through E_2 inhibiting damage; the damage was understood to be caused by a greater production of $O_2^{\bullet -}$, hydrogen peroxide and hydroperoxyl radicals, while the E_2 can act as a chain-breaking antioxidant (Ayres *et al.*, 1998*a*).

On the other hand, alkali labile sites, which produce SSBs in DNA have been previously reported to be positively correlated with oestradiol during the LF phase in healthy young females (Kapiszewska *et al.*, 2005). Likewise, in this investigation oestradiol was strongly correlated with the level of DSBs during the LL phase measured at baseline, whereas progesterone was correlated negatively with DNA DSBs in the LF phase at baseline. Mechanistically, DNA damage induced by oestradiol metabolised via the catechol pathway can occur through the generation of oxidative quinone metabolites (4-hydroxyestrone and 4-hydroxyestradiol) forming adenine- and/or guanine-binding DNA adducts, which can result in depurination and mutations *in vitro* and *in vivo* (Yue *et al.*, 2003). Additionally, through redox cycling, reduction of these oestradiol quinones back to catechols/hydroquinones may result in RONS formation accounting for DNA and/or lipid oxidative damage associated with oestradiol treatment (Nutter *et al.*, 1994; Yager & Davidson, 2006).

There is less *in vivo* work investigating the effect of sex on DNA damage, specifically exercise-mediated DNA damage, as female participants are often overlooked when research protocols are developed. This is in part, due to the apparent influence of the MC on selected biochemical, metabolic and physiological parameters. After an ultramarathon event, authors reported greater DNA damage (% tail) in females compared to males 2 days post-event, whilst the authors observed antioxidant protection 1 day post-event in females only (Mastaloudis *et al.*, 2004*b*). On the other hand, a study measuring DNA oxidation via urinary 8-OHdG and 17β-oestradiol before and following 1-hour of submaximal cycling exercise during the follicular and luteal phases (6-10 and 20-25 days after the onset of menses, respectively) of young adolescent girls, found no association between oestradiol levels and exercise-related DNA oxidation (Yasuda *et al.*, 2019).

6.4.3 Lipid Hydroperoxides

This study reported a 10% increase in lipid hydroperoxides post-exercise, regardless of MC phase, which remained at one-hour post-exercise. This is

in agreement with previous reports of increased LOOH as a function of aerobic exercise in males (Fogarty *et al.*, 2011, 2013*b*; Williamson *et al.*, 2018, 2020*a*). LOOH are formed via the abstraction of a hydrogen atom from a polyunsaturated fatty acid chain as the first by-product of lipid peroxidation (Davison *et al.*, 2005). There was no indication of oestradiol protection against lipid peroxidation contrary to suggestions arising from *in vitro* investigations (Subbiah *et al.*, 1993; Ayres *et al.*, 1998*a*). Conversely, one study demonstrated that, in comparison to eumenorrhoeic volunteers, amenorrheic female athletes had greater potential for increased lipid peroxidation following an acute bout of aerobic exercise (Ayres *et al.*, 1998*b*). This may indicate that an imbalance of female sex hormones and/or lower oestradiol levels due to lack of a MC could be associated with disrupted redox homeostasis and therefore increased RONS and oxidative stress due to higher lipid peroxidation susceptibility following exercise (Ayres *et al.*, 1998*b*; Chainy & Sahoo, 2020). Furthermore, another report conducted in postmenopausal women reported protection of exogenous E2, administered both acutely and chronically, via inhibition of low-density lipoprotein (LDL) oxidation (Sack *et al.*, 1994). Moreover, Ide *et al.* (2002) reported greater oxidative stress (lipid oxidation measured as 8-iso-PGF2α) in healthy young males compared to age-matched premenopausal women; this difference however was not mediated by any changes in plasma oestradiol concentration between the two groups.

6.4.4 Ascorbyl Free Radical

Ascorbic acid is oxidised by a plethora of free radical species, which acts as an intermediate, in a two-step oxidation process generating ascorbyl radical (Shyu *et al.*, 2014). As exhaustive exercise leads to the formation of more free radicals, ascorbate anions reduce those harmful unstable and highly reactive radicals by donating one-electron becoming (much less harmful) ascorbyl radicals (Buettner & Jurkiewicz, 1993). This investigation noted a 90% increase in ascorbyl free radical concentration as an effect of time one hour following exercise, compared to immediately post-exercise. Similar findings have been reported in male cohorts (Williamson *et al.*, 2018, 2020*a*, 2020*b*) immediately post exercise. This indicates that near maximal aerobic exercise, in both males and females, can result in ascorbic acid oxidation, by producing more ascorbyl free radicals, as evident in the plasma of a healthy menopausal female cohort across two phases of the MC. Moreover, a strong positive correlation was observed between oestradiol and ascorbyl free radicals in the LF phase only immediately following exercise. Although

not measured in this investigation, we can speculate that this observation may be related to serum/dietary ascorbic acid and endogenous oestradiol concentrations. Lastly, the difference of biological sex, training status/protocol and/or dietary/antioxidant status of the participants could explain the discrepancy in the sampling time of the reported increase in ascorbyl free radicals between the present investigation and the above-mentioned studies (1- hour post exercise vs. immediately).

6.4.5 Lipid Soluble Antioxidants

The present investigation reports no significant changes in any of the LSA between MC phases or time points. On the contrary, there have been reports of a decrease in lycopene, α-carotene α- and γ-tocopherol (Williamson *et al.*, 2020*a*) but also an increase in α- and γ-tocopherol (Williamson *et al.*, 2018) following exhaustive exercise in males. The discrepancies in these findings may be due to sex, exercise duration/intensity, dietary intake, phase of MC, and sampling in the fasted state. Regarding the phase of a MC specifically, fluctuations of plasma carotenoids have been reported in all MC phases (Forman *et al.*, 1996). This study observed a positive and negative correlation, between α-tocopherol and retinol, respectively, with oestradiol, 1-hour post exercise in the LF phase. A strong association of baseline serum antioxidants with reproductive hormones in healthy premenopausal females has been reported previously. In particular, higher oestradiol has been found to be associated with serum retinol and α-tocopherol (Mumford *et al.*, 2016).

6.4.6 Limitations and Future Research

A major limitation of this research is the absence of a male group and/or a postmenopausal group, to allow for comparison of sex-related differences and/or levels of endogenous oestradiol and associated reproductive hormones, and how they relate to the oxidative stress parameters.

Furthermore, a significant limitation was the challenging task of predicting the peak of oestrogen during the LF phase. This prediction was based on the LH surge assessed visually by the participants and investigator, and is thus, subject to error. The LH surge is initiated by a peak of oestradiol; the onset of the LH surge occurs 34-36 hours prior to ovulation (Reed & Carr, 2018). Urine strips were used to visually predict the LH surge, indicated by the darkest line on the test strip (this was assessed after several consecutive tests and followed by a "no line" or "very light line" test strip). The ideal time frame to conduct the exercise testing as closely to the predicted peak was 24-48 hours prior to the darkest test strip indicating the LH peak and

thus indirectly speculating the oestradiol peak. This was also challenging due to scheduling conflicts with participants, as if volunteers could not attend the testing in that time frame, it would then be rescheduled for the next cycle. This meant that, in some cases, all experimental testing for one participant was not conducted on the same MC. We found no difference in oestradiol concentrations between LF and LL phases, and thus, it is likely that the oestradiol peak was not predicted successfully amongst some or all participants, as its timing is highly sensitive to predict. In addition, another important limitation to consider is investigating only two phases (LF, LL) of the MC, thereby omitting the rest (menses, EF, MF, EL, ML) and their possible effects on the measured oxidative stress markers. Lastly, although we employed the monitoring of BBT, all participants were non-compliant with measuring all daily measurements across the trial and consequently, due to multiple missing data points, BBT analysis was not part of our final analyses.

Moreover, dietary intake was not monitored throughout this study and although the volunteers were all recreationally active, the training level/mode of their current exercise activity levels varied across each volunteer (0-6 hours/per week, weightlifting vs running etc.). Additionally, the occupation of our volunteers, which was not tightly screened in this investigation, is another factor to be considered. Shift workers may be more susceptible to oxidative stress through decreased antioxidant capacity following a night shift work pattern (Sharifian *et al.*, 2005). Additionally, circadian rhythm disruptions due to night shifts may cause DNA repair gene dysregulation, which may consequently lead to an increase in DNA damage (Koritala *et al.*, 2021).

Furthermore, since existing evidence has linked the antioxidant properties of oestradiol with antioxidant defence enzymes, this investigation would have provided a greater insight by perhaps quantifying the expression of enzymes such as SOD and GPx, and how these may be mediated by sex hormones. Future research in this novel area of work should also include a much larger sample size and a more accurate way of identifying the peak of oestradiol across the MC such as measuring oestradiol across 4-5 consecutive days around suspected days of oestradiol peaking.

6.4.7 Conclusions

This investigation has demonstrated a global DNA damage response to strenuous acute aerobic exercise alongside a rise in LOOH and ascorbyl free

radical concentration in a healthy female cohort. However, no significant differences were observed across the LF and LL phases, tentatively indicating that DNA damage induced by high-intensity exercise occurs to the same extent in females during both measured MC phases.

The existing evidence suggests that DNA damage increases as a function of exercise due to a greater production of RONS (Tryfidou *et al.*, 2020) and that seems to be is prevalent for either sex. However, since most studies to date have been conducted in males, this investigation adds to the limited female-included studies showing that exhaustive exercise can lead to greater oxidative stress. Furthermore, such an increase was observed across both the LF and LL phases of MC and thus our hypothesis for a potential protective effect of oestradiol is not supported by the current preliminary data. Additionally, oestradiol levels correlated positively with DNA DSBs and as such, the notion of an absence of oestradiol protection on DNA damage is further reinforced. Lastly, multiple correlations observed between LSA and the endogenous sex hormones, may showcase the complex associations and interactions between molecules.

CHAPTER SEVEN

CHAPTER 7: HYPOXIC EXERCISE AND SELECTIVE OXIDATIVE STRESS-RELATED GENES: A RT-qPCR AND MICROARRAY PILOT STUDY

7.1 Introduction

There is a plethora of evidence (as outlined in this thesis), suggesting that high-intensity exercise can increase RONS, subsequently leading to a state of oxidative stress (Tryfidou *et al.*, 2020; Williamson & Davison, 2020). Exercise performed in a different environmental condition (compared to normal sea-level barometric pressures) can also invoke an additional cellular stress that may also raise levels of oxidative stress (Debevec *et al.*, 2017). High-altitude induced hypoxia has been shown to stimulate oxidative stress due to the drop in the partial pressure of atmospheric oxygen (PO_2), leading to decreased arterial blood oxygen concentration. This results in hypoxic-related damage to cells (Askew, 2002), notably affecting lipids, protein and DNA (Møller *et al.*, 2001; Bakonyi & Radak, 2004; Miller *et al.*, 2013). In fact, 1 hour of hypoxia has been shown to increase ROS production measured by EPR in capillary blood and in addition, urinary 8-OH-dG has been reported to increase significantly after 4 hours of hypoxic exposure (F_IO_2 = 0.125) compared to normoxia (F_IO_2 = 0.21) (Mrakic-Sposta *et al.*, 2023). It is now understood that the relationship between exercise performed in hypoxia and the extent of observed cellular oxidative stress, is dose dependent (Debevec *et al.*, 2015).

A growing body of evidence demonstrates that exercise training can enhance the expression of antioxidant systems such as SOD, GPx and catalase. Moreover, RONS signalling may be important in mitochondrial adaptation, as H_2O_2 in particular, is implicated in transcription factor activation and regulation of gene expression (Ristow *et al.*, 2009; Piantadosi & Suliman, 2012). Furthermore, hypoxic exercise training, either in a normobaric hypoxic chamber or at high-altitude, may also elicit positive adaptations in skeletal muscle antioxidant capacity (Ji *et al.*, 2018). That said, there is currently a lack of research investigating gene expression in relation to exercise-induced oxidative stress in hypoxia.

The aim of this pilot work was to ascertain the magnitude of change, if any, (using RT-PCR; Study A) in a select number of oxidative stress related genes and cross-referencing any changes to a microarray transcriptome pattern. A previously obtained microarray data set (Study B) was used for the

purposes of validation and cross-comparison, where exercise was performed in hypoxia and normoxia, respectively (McGovern, 2015).

7.2 Methods

7.2.1 Participants

For Study part A (RT-PCR analysis) *n*=4 volunteers were recruited and for Study part B (microarray analysis) *n*=16 volunteers were recruited. Participants were healthy, non-smoking males, aged 18-35 and recreationally active (partaking in moderate- to high-intensity aerobic exercise 1-hour or more, 4-6 times weekly). Participants had no history of metabolic/cardiopulmonary/sleep disorders, epilepsy, nor a history of alcohol/drug abuse. Participants were excluded if they had experienced chronic or intermittent altitude exposure for more than 3-6 weeks prior to the study commencing. The study was conducted in accordance with the Declaration of Helsinki and approved by a local University Ethics Committee (REC/13/0023).

7.2.2 Experimental Testing

The study followed a single blind, randomised, cross-over design. Briefly, all participants completed a total of four visits, conducted in a simulated environment inside an environmental chamber under both normoxic ($F\text{i}O_2$ = 0.21) and hypoxic conditions ($F\text{i}O_2$ = 0.16). Each participant was randomly assigned to one environmental condition, and after completion, separated by at least a 7-day wash out/recovery period, they completed the experiment in the other condition. A simple outline of the experimental protocol is presented in **Figure 7.1**. Testing took place between 8:00 and 11:00 am. In the first visit of each condition, $\dot{V}O_{2max}$ was determined via the following protocol: running speed set at 8-10km/h with 0% gradient which was increased by 1km/h every 90 seconds until volitional exhaustion. Breath-by breath gas exchange was analysed using a Quark CPET Metabolic Cart (Quark CPET, COSMED, Rome, Italy). The second visit of each condition involved one hour of running at 75% of their pre-determined $\dot{V}O_{2max}$. To ensure this was maintained throughout the steady-state exercise, gas exchange was continuously monitored and, to maintain the desired intensity, the treadmill speed was adjusted accordingly.

Baseline measures of heart rate (Polar Electro RS400, Finland) were obtained. Venous blood samples were obtained after 30 mins of exposure in each condition and also at 3 hours following the one-hour exercise trial.

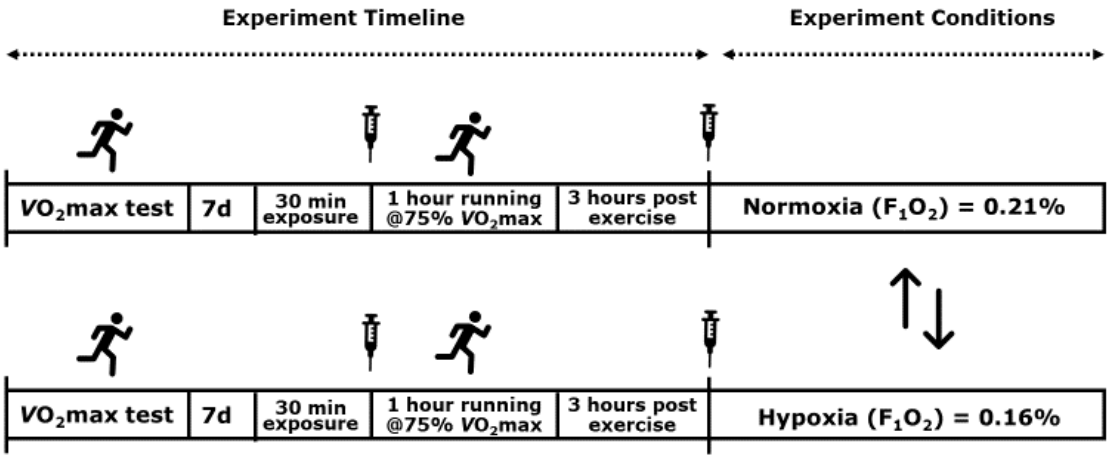


Figure 7.1 Schematic overview of the experimental protocol.

Oxygen saturation measurements, SaO_2 (Pulse Oximeter, Medscope, UK), were obtained at baseline; after 30 minutes of exposure; at 15-minute intervals during the one-hour steady-state exercise trial; and at 3 hours post exercise. Additionally, for the measurement of blood lactate, finger capillary samples were obtained using a single use lancing device (Accu-check, West Sussex, England) and analysed using a Lactate Pro Analyser (Arkray, Japan).

7.2.3 Transcriptional Analysis

7.2.3.1 Manual Purification of Total RNA from Human Blood

RNA extraction was carried out using the RNeasy® Mini Kit (Qiagen, Crawley, UK). PAXgene tubes were initially thawed at room temperature for 1-2 hours and centrifuged for 10 mins at 5000 rpm. The supernatant was removed, and 4 mL of RNase-free water was added to the pellet and vortexed until visibly dissolved. Samples were subsequently centrifuged for 10 mins at 5000 rpm and the supernatant was discarded. Thereafter, 350 µL of RLT buffer containing 1% β-mercaptoethanol was added to the pelleted cells and homogenised with a sterile 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Subsequently, 350 µL of 70% ice-cold ethanol was added to the homogenised lysate. The mixture (700 µL) was transferred to a RNeasy spin column placed in a 2 mL collection tube and centrifuged for 15 seconds at 10,000 rpm. Flow-through was discarded and

700 µL of RW1 buffer was added to the RNeasy-spin column and centrifuged again for 15 seconds at 10,000 rpm. Next, 1 µL of DNase with 70 µL RDD buffer was added to the spin column and left to incubate for 25 mins at room temperature. After incubation, 850 µL of RW1 buffer was added to the spin column and centrifuged for 15 seconds at 10,000 rpm. The spin column was transferred to a new collection tube and 500 µL of RPE buffer was then added and centrifuged for 15 seconds at 10,000 rpm. To this, 500 µL RPE buffer was added to the RNase spin column and centrifuged for 2 mins at 10,000 rpm. The spin column was placed in a new collection tube and 30 µL of RNase-free water was added directly on the spin column membrane and centrifuged for 1 min at 10,000 rpm. The final RNA yield (ng/µL) was determined for each sample by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. The extracted RNA samples were stored at -80°C for further analysis. RNA concentration and purity of the samples was analysed by spectrophotometry using the A260/A280 ratio method (NanoDrop Spectrophotometer, ThermoFisher Scientific, USA).

7.2.3.2 cDNA Synthesis

Complementary DNA (cDNA) was reverse transcribed by adding H_2O to 250 ng of total RNA to make up a combined mixture of 13.5 µL. Then, 1 µL of 0.25 µg random primers (Roche, West Sussex, UK) were added and the mixture was vortexed for 3 seconds and placed in a PCR machine (Roche, UK) for 5 mins at 65°C. Following this, 2.5 µL of 0.5 µM dNTPs (deoxyribonucleotide triphosphate; Invitrogen, Paisley, UK) were added to each tube as well as 4 µL of reverse transcriptase buffer (Fermentas, Cambridge, UK). Following this, 2 µL were removed from this mixture for negative controls, and finally 1 µL of RevertAid Reverse Transcriptase (Fermentas, Cambridge, UK) was added for a final total volume of 20 µL. Each tube containing 20 µL of this mixture was then placed in a thermocycler with the following conditions: 25 °C for 10 mins; 42 °C for 60 mins; and 70 °C for 10 mins. cDNA was then stored at – 80°C until use.

7.2.3.3 *Primer Standards*

The efficiency of primers used for RT-qPCR was determined using a LightCycler® 480 II and its associated software (LightCycler® 480 Software release 1.5.0 SP3) (Roche, UK). Firstly, cDNA was diluted 10-fold and 100-fold to generate a standard curve resulting in 3 tubes containing cDNA in no dilution, 10-fold and 100-fold dilutions, respectively. RT-qPCR was run in triplicate with each sample consisting of 1 µL cDNA, 5.5 µL LightCycler®

SYBR Green 1 Master (Roche), 3.5 µL nuclease free water (Qiagen) and 0.5 µL of 10 µM forward and reverse primers (Invitrogen).

As observed in **Table 7.1**, from the 120 µL of each primer, 27.5 µL was added to three separated tubes to make the different dilutions of cDNA containing each primer, one undiluted (1), one diluted 10-fold (0.1) and one diluted 100-fold (0.01). A total of 3 µL from the undiluted cDNA mixture was added to the first tube (1); 3 µL from the 10-fold diluted cDNA was added to the second tube (0.1); and 3 µL from the 100-fold diluted cDNA was added to the third tube (0.01). The samples were then added to a 96-well plate in order as shown in **Figure 7.2**. For each gene, 3 µL were added in triplicate across 3 well plates and for all 3 dilutions (1, 0.1, 0.01). Negative controls (no cDNA) were also added for each gene.

Table 7.1 Contents of the final solution for each of the 5 primers made in 3 different dilutions.

	cDNA Dilution					
Primers (120 µL)	Undiluted mixture (1)		10-fold mixture (0.1)		100-fold mixture (0.01)	
AKT3	27.5 µL	3 µL	27.5 µL	3 µL	27.5 µL	3 µL
HSP70	27.5 µL	3 µL	27.5 µL	3 µL	27.5 µL	3 µL
GPX7	27.5 µL	3 µL	27.5 µL	3 µL	27.5 µL	3 µL
NCF2	27.5 µL	3 µL	27.5 µL	3 µL	27.5 µL	3 µL
GAPDH	27.5 µL	3 µL	27.5 µL	3 µL	27.5 µL	3 µL

The plate was sealed, vortexed for 5 seconds and then placed in a LightCycler® 480 II. Initially, the plate was incubated at 95°C for 10 mins, followed by 60 cycles of 95°C for 10 seconds, 55°C for 10 seconds and 72°C for 10 seconds, with a final ramping of temperature to 97°C to generate a melting curve. Using the software LightCycler® 480 II, melting curves were checked for the presence of a single peak, amplified by the associated primers.

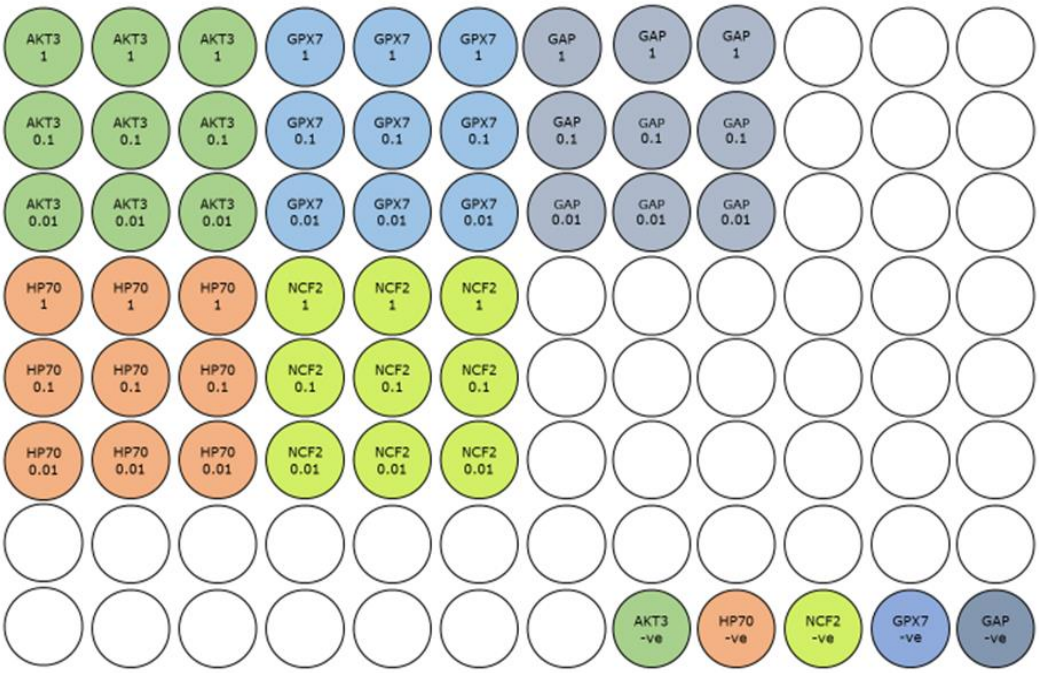


Figure 7.2 Illustration of primer standards in a 96 well plate.

The LightCycler® 480 software calculated an efficiency score for the primer set and primers with a score between 1.8-2.2 were deemed appropriate for further experiments.

7.2.3.4 RT-qPCR

Upon determining gene expression, reactions were set up in triplicate. Each reaction included: 1 µL undiluted cDNA, 5.5 µL LightCycler® 480 SYBR Green I Master (Roche), 0.5 µL of 10 µM forward and reverse primers (Invitrogen) and 3.5 µL nuclease free H_2O (Qiagen). Gene expression was normalised to GAPDH, and relative expression calculated using the $\Delta\Delta C_T$ method (Bustin, 2000). Primer sequences for all RT-qPCR primers are listed in **Table 7.2**. Only two genes, out of the four initially selected genes, were analysed in triplicate due to low sample volumes, and thus results presented include gene expression of GPx7 and NCF2.

7.2.3.5 Microarray Analysis

A detailed description of microarray analysis, as described by (McGovern, 2015), can be found in **Appendix G Supplementary Section 7.2.3.5**.

Table 7.2 RT-qPCR Primers.

Gene	Primer	Oligo sequence (5'-3')
HSPA1A	FWD	*AGC AGG TGT GTA ACC CCA TC*
	RV	*GCA GCA AAG TCC TTG AGT CC*
AKT3	FWD	*ATG GGT AGG ATG GCT GGA CT*
	RV	*GCG AGC CAT CAT CCT CAT CA*
GPX7	FWD	*AAC CAG TTT GGC CAA CAG GA*
	RV	*GCT CTC ATT GGC CAG GAG TT*
NCF2	FWD	*ACA GTT AGC ATT GGC CAC GA*
	RV	*GGA ACT AGG AGG AGC TGG GA*
GAPDH	FWD	*CGA CCA CTT TGT CAA GCT CA*
	RV	*AGG GGT CTA CAT GGC AAC TG*

Abbreviations: FWD, forward qPCR primer; RV, reverse qPCR primer.

7.2.3.6 Statistical Analysis

Each sample was run in triplicate, including a technical replicate to control for variability in the testing protocol, and a biological replicate was carried out for each gene. Means of triplicate samples were used to calculate fold change for each gene. SD was calculated from the averages of biological replicates, and SEM (shown in error bars) was calculated based on SD. Statistical significance between samples (for participant characteristic, exercise and gene transcription data) was determined with two-tailed, paired t-tests. All data are presented as Mean ± Standard Deviation.

7.3 Results

7.3.1 Baseline Data and Exercise Performance Variables

Baseline characteristics, as well as exercise variables in both conditions are shown in **Table 7.3**. As observed, there were no differences in age, height or weight between experimental conditions ($P > 0.05$, respectively). $\dot{V}O_{2max}$ and SaO_2 across conditions were lower following exercise in hypoxic compared with normoxia ($P < 0.05$, respectively). Maximum speed during the $\dot{V}O_{2max}$ test was lower in hypoxia compared to normoxia in the qPCR experiment ($P < 0.05$).

Table 7.3 Participant baseline and exercise characteristics (*n*=4) in comparison to the microarray experiment data (*n*=16).

Baseline	**Microarray data (*n*=16)**		**qPCR (*n*=4)**	
Age (yrs)	28 ± 5		26 ± 2.6	
Height (m)	1.77 ± 5.7		1.78 ± 7.7	
Weight (kg)	74 ± 7.7		75 ± 12.7	
Exercise	**Normoxia**	**Hypoxia**	**Normoxia**	**Hypoxia**
Maximum HR (bpm)	189 ± 10	185 ± 8	178 ± 7.7	174 ± 7.3
$\dot{V}O_{2max}$ (mL kg^{-1} min^{-1})	60 ± 9	45.8 ± 2^	55.7 ± 7.3	44.7 ± 3.5*****
SaO_2 (%) post-exercise	97	93^	95	86*****
Speed max (km/h)	18.5 ± 1	18.2 ± 4	11.4 ± 2.1	10.3 ± 1.8*****

Abbreviations: bpm, beats per minute; HR, heart rate; kg, kilograms; m, meters; $\dot{V}O_{2max}$, maximum oxygen uptake; yrs, years; SaO_2, oxygen saturation. Microarray data from McGovern, 2015.

^Significantly different to normoxia in the microarray experiment

*****Significantly different to normoxia in the qPCR experiment

7.3.2 RT-qPCR Gene Expression

Following steady-state high-intensity aerobic exercise, GPx7 was downregulated (**Figure 7.3A**) and NCF2 (**Figure 7.3B**) was upregulated in both normoxia (-0.31 & 5.57 fold change) and hypoxia (-0.21 & 6.93 fold change), compared to baseline ($P < 0.05$). No significant difference between conditions was observed for either gene ($P > 0.05$).

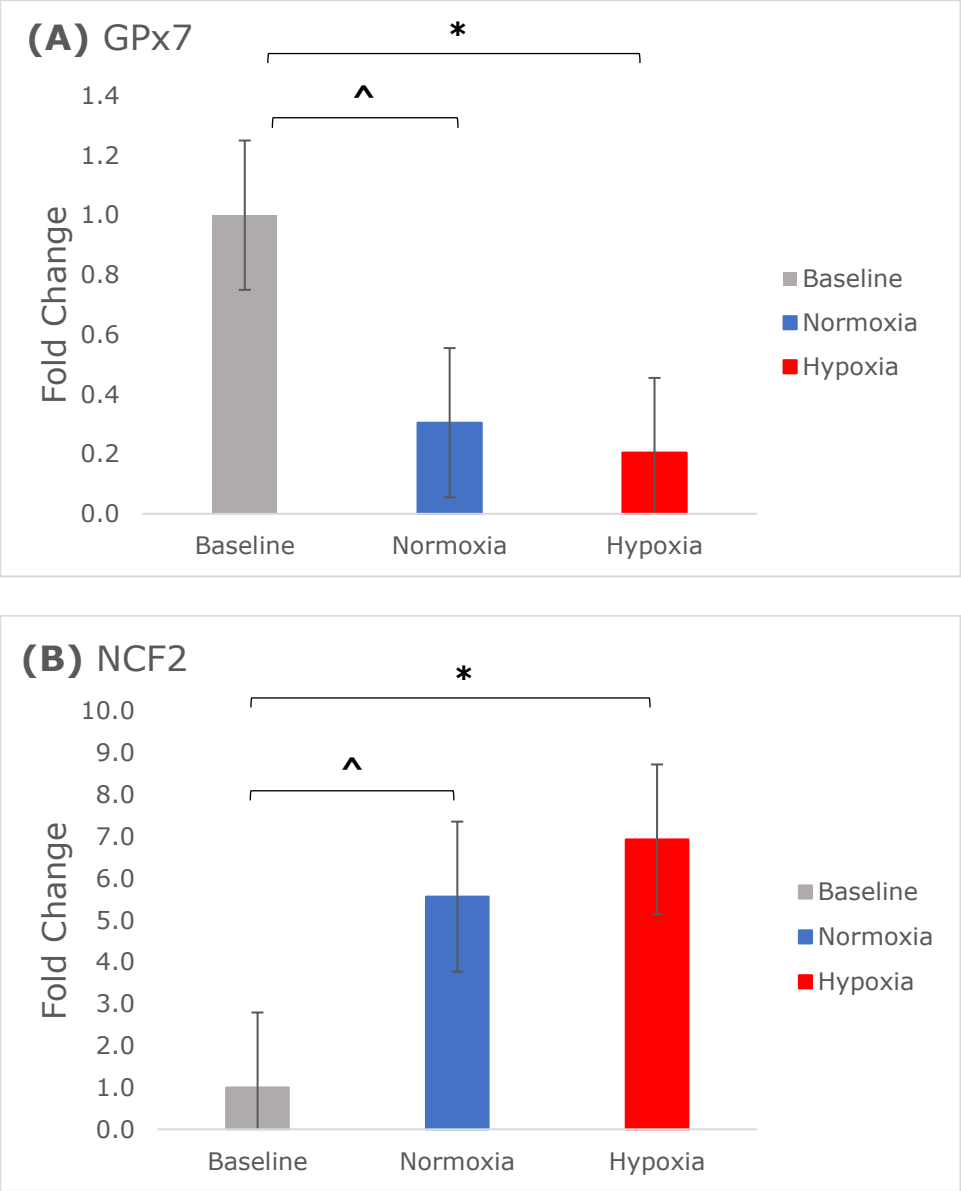


Figure 7.3 (A) Expression of *GPx7* & **(B)** Expression of *NCF2* following exercise in normoxic and hypoxic conditions.

^Significantly different to baseline in normoxia

***Significantly different to baseline in hypoxia

7.3.3 Microarray Analysis

Following steady-state high-intensity aerobic exercise, *GPx7* was downregulated and *NCF2* was upregulated in both normoxia (-1.54 & 1.98 fold change) and hypoxia (-1.71 & 1.71 fold change), respectively (**Table 7.4**) (compared to baseline; $P < 0.05$).

Both RT-qPCR and microarray analyses showcased consistent gene expression findings with the same direction of either upregulation or downregulation of the analysed genes, thereby Study A successfully validating Study B.

Table 7.4 Gene expression response following aerobic exercise in normoxia and hypoxia in comparison to the microarray experiment data (*n*=16).

	Microarray data (n=16)					
	Normoxia			**Hypoxia**		
Gene	**Direction**	**Fold change**	**P value**	**Direction**	**Fold change**	**P value**
GPx7	down	1.54**	0.00	down	1.71*	0.01
NCF2	up	1.98*	0.04	up	1.71*	0.03
HSPA1A	up	2.1**	0.00	up	2.21*	0.01
AKT3	NR	-	-	down	2.03*	0.02
	qPCR (n=4)					
GPx7	down	0.31*	0.01	down	0.21**	0.00
NCF2	up	5.57*	0.02	up	6.93	0.01

* $P < 0.05$; ** $P < 0.001$ compared to baseline; NR: not regulated

Microarray data from McGovern, 2015.

7.4 Discussion

The primary outcome of this pilot work determines that exercise *per se*, regardless of environmental condition, leads to an upregulation in the *NCF2* gene and a downregulation in the *GPx7* gene. As such, the RT-PCR data is in agreement with those data produced by DNA-microarray scanning. The use of gene expression profiling either by RT-PCR or microarray scanning is a powerful method that can offer novel understandings of the molecular mechanisms involved in the adaptations of muscle in response to exercise (Miyamoto-Mikami *et al.*, 2018). Examples of such adaptations include: mitochondrial biogenesis; adapted contractile tissue; myofiber hypertrophy; and enhanced antioxidant defences (Gomez-Cabrera *et al.*, 2021). In cells, RONS have been highlighted as key redox signalling mediators of gene transcription, capable of exerting positive stress (eustress) or cause

deleterious outcomes (distress), by regulating reversible posttranslational modifications to thiol moieties on target proteins (Niki, 2016).

The oxidative stress response to exercise has been well documented (Powers *et al.*, 2011), as well as in hypoxia at rest (Magalhães *et al.*, 2004, 2005; Debevec *et al.*, 2017), and following acute hypoxic exposure and strenuous exercise combined (Davison *et al.*, 2006; Quindry *et al.*, 2016; Debevec *et al.*, 2017). Most eukaryotic cells possess an antioxidant defence system to counteract harmful levels of RONS, with the glutathione peroxidases being one of the major antioxidant family of enzymes in RONS homeostasis and maintenance of redox balance, alongside catalase, SOD and peroxiredoxin (Halliwell, 1999).

To date, there is little to no evidence on the transcriptional response of *GPx7* and *NCF2* (the primary genes of interest of this study), in the context of exercise, especially in hypoxia related oxidative stress. GPxs can react with lipid hydroperoxides and are powerful scavengers of H_2O_2; they are responsible for keeping H_2O homeostasis, using glutathione (GSH) as a reducing substrate (except *GPx7&8*), and as H_2O_2 concentrations rise, they can rapidly react and eliminate it effectively (Brigelius-Flohé & Kipp, 2009; Chen *et al.*, 2016). Regarding GPx isoforms, *GPx1-8* have been identified thus far; *GPx1-4* and *GPx6* are classified as selenocysteine-containing, whereas *GPx5*, *GPx7&8* as non-selenocysteine (Brigelius-Flohé & Maiorino, 2013). *GPx7*, one of the 8 in the family of GPx, is an enzyme located in the endoplasmic reticulum (ER), encoded by the *GPx7* gene in humans. When oxidative stress occurs, *GPx7* acts (without binding to GSH) as an intracellular stressor/transmitter and has a key role in oxidative protein folding inside the ER and maintenance of redox homeostasis (Chen *et al.*, 2016; Hanousková *et al.*, 2021). Animal research has demonstrated increased mRNA expression following a single exercise bout (60 mins swimming) of GPx and NOX2 in mouse skeletal muscle (Henríquez-Olguín *et al.*, 2016). Furthermore, compared to untrained, chronic moderate-intensity exercise training (11 weeks of treadmill running at 45% $\dot{V}O_{2max}$ /1 hour/day) has shown to elicit 6.6-fold higher reactive oxygen metabolites production; 49% increased phagocytosis capacity; as well as enhanced protein expression of the cytosolic subunits of NADPH, p67phox and p47phox by 2.8-fold and 0.22-fold respectively in rat neutrophils (Levada-Pires *et al.*, 2007).

Moreover, after 1-hour of swimming at 75-80% of $\dot{V}O_{2max}$, increased oxidative damage was observed in neutrophils, and higher GPx activity was noted exclusively in males; however, no changes in GPx expression were detected (Ferrer *et al.*, 2009). On the other hand, exercise has shown to decrease mRNA expression of *GPx* in deep and superficial vastus lateralis muscles of untrained rats by 22% and 61%, respectively (Ji, 1999). Similarly, high-intensity interval training in a male cohort, has shown to increase antioxidant enzyme activity, including GPx, but not gene expression (Fisher *et al.*, 2011). Such findings may suggest that expression of antioxidant enzymes, and in particular GPx, may be influenced by exercise intensity and/or duration. This suggests despite an increase in enzyme activity, an abundance of mRNA *GPx* expression may not always occur in parallel. It is also possible that the observed decrease in *GPx* transcription alongside an increase in GPx protein activity is due to the properties of molecular signalling moving from RNA, to end stage protein formation, thereby exhausting (i.e., downregulating) the consumption of the RNA transcript. Further work is required to shed light on this speculation. Although this author did not quantify GPx activity *per se*, the cause of decreased *GPx* expression observed as a function of exercise, in either hypoxia or normoxia, has yet to be fully elucidated. This approach may be achieved considering the changes observed in $\dot{V}O_{2max}$ and SaO_2, as the decrease in both variables may also have a bearing on the regulation of antioxidant-related gene transcription.

Neutrophil Cytosolic Factor 2 (NCF2) is a protein composed of 526 amino acids, also known as p67phox (phox: phagocyte oxidase), which is encoded by the *NCF2* gene and is a key component of NADPH oxidase (Leto *et al.*, 1990). As it has been established, $O_2^{\bullet-}$ generating leukocyte NADPH oxidases are multi-subunit enzymes comprising, as part of their holoenzyme, their components in both the plasma membrane and the cytosol (Liu-Smith *et al.*, 2014). The component localized in the cellular membranes is flavocytochrome b_{558}, which consists of the glycoprotein cytochrome b (light) α-subunit [CYBA] (also known as p22phox) and cytochrome b (heavy) β-subunit [CYBB] (also known as gp91phox/NOX-2) (Jacob *et al.*, 2017). Upon translocating to the cellular membrane, it is activated with the help of its cytosolic protein subunits (p47phox/NCF1, p67phox/NCF2, p40phox/NCF4), interacting with the small G proteins Rac1/2) (Matute *et al.*, 2005; Liu-Smith *et al.*, 2014). Therefore, NCF2 (the "activator" of the

Nox2 complex) plays a critical role in the respiratory burst of phagocytic cells mediated by NADPH oxidases, the main non-mitochondrial sources of RONS (Okamura *et al.*, 1990). Research suggests that, moderate exercise mobilizes neutrophils into the tissues, where they can phagocytize bacteria and activate the oxidative respiratory burst (Chearskul & Srichantaap, 1994). Although, there is no current evidence directly on NCF2 gene expression in response to exercise and/or hypoxia, increased expression of NCF2 has been associated with the generation of intracellular RONS (in proliferating cells) that may protect against apoptotic cells (Italiano *et al.*, 2012). In more detail, knockdown of NCF2 led to an increased number of human colorectal carcinoma cells undergoing apoptosis, thus exerting its protective role (Italiano *et al.*, 2012). The results of this study may corroborate the notion of NCF2's critical role in regulating exercise-induced RONS (and other salient cell modifications), as its expression was upregulated after high-intensity aerobic exercise in both hypoxia/normoxia.

In conclusion, the main aim of this investigation was to validate previous microarray gene analysis with further RT-qPCR enquiry, whilst focusing on a select number of genes of interest. This work used both *GPx7* and *NCF2* gene transcripts, while observing the same directional trend of downregulation and upregulation, respectively, in both experimental conditions. With more time and resources, this exploratory experiment could be expanded upon with a much greater sample size; the inclusion of several more genes of interest; as well as accompanying oxidative stress markers such as DNA damage and lipid peroxidation. This approach would further add to the scientific enquiry and body of work contained in this thesis. Following a single exercise bout, acute and transient changes in gene transcription occur, which could cumulatively result in skeletal muscle training adaptations (Flück & Hoppeler, 2003; Hargreaves, 2015). However, although these molecular adaptive responses to exercise seem unambiguously positive, the intricate mechanism of gene transcription factor activation/regulation through exercise-mediated RONS is not well understood (Thirupathi & de Souza, 2017). Further researched is warranted, as there is a clear lack of work regarding gene expression response to hypoxic exercise, specifically regarding genes associated with RONS production and antioxidant defence and their subsequent role in the training response.

CHAPTER EIGHT

CHAPTER 8: SYNTHESIS OF FINDINGS

This chapter will address whether the null hypothesis of each experimental investigation can be accepted or rejected. Additionally, the chapter will discuss the findings of each experimental study aligned to the wider scientific themes and literature.

8.1 Testing of Null Hypothesis

Study 1 –

H_0 = An acute aerobic and exhaustive exercise bout will not change oxidative stress as measured by specific biomarkers.

Null Hypothesis Rejected

An acute bout of cycling at 70% $\dot{V}O_{2max}$ (60 mins) caused an increase in DNA damage in both placebo and supplemented groups. Furthermore, lipid hydroperoxides slightly decreased but only as a function of time regardless of group (pooled data); and ascorbyl free radical concentration increased only in the supplemented group.

H_0 = A high dose of AA will not decrease exercise-induced oxidative stress as measured by specific biomarkers.

Null Hypothesis Accepted

Although increased levels of AA were confirmed in the supplemented group, 10g of chronic administration of AA had no protective effect of exercise-induced DNA damage and lipid hydroperoxides. Ascorbyl free radical concentration increased post-exercise in the supplemented group and no changes were observed in any of the measured LSA.

Study 2 –

H_0 = Acute maximal aerobic exercise will not change oxidative stress as measured by specific biomarkers influenced by the menstrual cycle.

Null Hypothesis Rejected

Following maximal exhaustive exercise, DNA damage increased in females irrespective of phase of the menstrual cycle. Furthermore, lipid hydroperoxides and ascorbyl free radical increased as a function of time.

H_0 = Naturally occurring oestrogen will not protect against exercise-induced oxidative stress.

Null Hypothesis Accepted

There was no observed protective role of naturally occurring oestrogen against DNA damage or any other biomarkers. This may have been confounded due to lack of identifying peak oestrogen across the menstrual cycle.

Study 3 –

H_0 = Gene response will remain unchanged after exhaustive aerobic exercise in hypoxia versus normoxia.

Null Hypothesis Accepted

Gene expression remained either upregulated or downregulated in both conditions.

H_0 = Microarray gene expression results will fail to be validated against RT-qPCR gene expression.

Null Hypothesis Rejected

Selected genes of interest were expressed in the same direction measured by both microarray and RT-qPCR analysis.

8.2 Summary of Research

Fundamentally, the systematic review and meta-analysis component of this thesis demonstrated that acute aerobic exercise increases DNA damage. Further analyses revealed that DNA damage, as assessed via the comet assay, can remain elevated from 2 hours up to 1 day following exercise. However, this increase is not observed between 5 and 28 days post-exercise, indicating that the damage is transient. Notably, this effect is only seen with high-intensity exercise protocols, not with long-distance ones, suggesting the damage is not permanent.

Furthermore, the experimental components of this thesis explored the relationship between intense acute aerobic exercise and DNA damage among other quantifiable biomarkers of oxidative stress across male and female volunteers. As secondary objectives, antioxidant supplementation was investigated, as well as pilot work which briefly examined the genomic response to exercise alongside the added environmental stress of normobaric hypoxic conditions.

8.3 Summary of Experimental Work

8.3.1 High ascorbic acid supplementation administered chronically offers no protection against exercise-induced DNA damage

As presented in Chapter 5, one hour of cycling at moderate-intensity (approximately at 70% of $\dot{V}O_{2max}$) caused elevated % DNA tail intensity, accompanied with a small reduction of lipid hydroperoxides as a function of time (3 hours following exercise). Moreover, AA did not offer protection as DNA damage remained unchanged between the placebo and AA supplemented groups. AA supplementation did, however, lead to an increase in ascorbyl free radical concentration mediated by exercise.

8.3.2 Endogenous oestradiol, as a natural hormone of the female menstrual cycle, offers no protection on the global DNA damage response to maximal exercise

As presented in Chapter 6, an acute bout of treadmill running to exhaustion increased % DNA tail intensity (SSBs & DSBs), accompanied by elevated lipid hydroperoxides and ascorbyl free radicals. Moreover, endogenous oestradiol measured throughout the menstrual cycle at the LF and LL phases did not seem to offer any protection against this exercise-induced DNA damage response.

8.3.3 Selective gene response of normobaric hypoxic aerobic exercise analysed by RT-qPCR

As presented in Chapter 7, one hour of treadmill running in both normobaric hypoxia (NH) (FiO_2 = 0.16% approximately at 75% of $\dot{V}O_{2max}$) and normoxia, led to significant changes in gene expression of selective oxidative stress genes. Downregulation and upregulation of *GPx7* and *NCF2*, respectively, as measured by RT-qPCR analysis, validating the same gene response in previously conducted microarray gene analysis.

8.4 Discussion of Findings

8.4.1 Exercise Induces Oxidative Damage to DNA

The experimental investigations presented in this thesis (Chapter 5 and 6) showcased that aerobic exercise leads to oxidative DNA damage. Following a 1-hour long cycling bout of high-intensity (Chapter 5), DNA damage measured in males (by the alkaline comet assay) was 202% higher compared to baseline in the placebo group, and 159.5% in the AA supplemented group. Similarly, DNA damage measured twice in females during the menstrual cycle, was increased immediately following a maximal incremental treadmill test to exhaustion by 357.2% and 195.6% in the LF and LL phases, respectively (Chapter 6). It is important to note the difference in the exercise modes in these studies: a cycle ergometer in Chapter 5 vs treadmill running in Chapter 6. Although the mode of exercise varied across the experimental studies, it appears DNA damage occurs irrespective of exercise modality, provided the exercise reaches a sufficiently high threshold. This would translate to moderate- to high-intensity (defined as ≥60% of $\dot{V}O_{2max}$), which both protocols within this thesis achieved (60-70% of $\dot{V}O_{2max}$ vs. maximal). Thus, these findings follow the pattern of other of similar exercise protocols in the literature. For example, work by Williamson (*et al.*, 2020*a*) and Fogarty (*et al.*, 2011) reported elevated DNA % tail damage following high-intensity aerobic exercise at 80-85% and 70 & 100% of $\dot{V}O_{2max}$, respectively, as well as maximal exercise (Fogarty *et al.*, 2013*b*; Williamson *et al.*, 2018). Generally, similar observations from numerous other studies, as shown by the meta-analysis in Chapter 4 (Tryfidou *et al.*, 2020), demonstrated exercise-induced DNA damage. In addition, studies with different duration protocols of exhaustive exercise, such as long distance events at 21.1km (Niess *et al.*, 1998), 42km (Tsai *et al.*, 2001), 50km (Mastaloudis *et al.*, 2004*b*) and 51.5 km (Hartmann *et al.*, 1998) have also reported similar findings. Another systematic review and meta-analysis was conducted a couple of years following the meta-analysis in Chapter 4 (Tryfidou *et al.*, 2020) investigating only plasma levels of 8-OHdG and grouped by untrained versus trained individuals, as well as aerobic exercise duration (>30 vs <30 mins) and intensity (≥75% vs <75% $\dot{V}O_{2max}$). The authors observed: (1) no significant effect of 8-OHdG before and after aerobic exercise (Ye *et al.*, 2023). It is important to highlight a key difference between the two meta-analyses; in Chapter 4 the analysis for 8-OHdG levels only (109 participants), resulting in no change in DNA

damage (SMD = 0.15; 95% CI:-0.58, 0.88; P = 0.68), was for TP 0 only, whereas Ye *et al.* (2023) conducted the analysis with the same studies but included all time-points from all studies (230 samples) in the analysis and similarly reported no significant effect. However, when grouped by training status (which the meta-analysis in Chapter 4 did not include), the authors observed (2) a large effect of aerobic exercise on decreasing 8-OHdG levels in untrained participants (SMD = −1.16; 95% CI: −1.88 to −0.43; P< 0.01) versus a small effect of aerobic exercise on increasing 8-OHdG levels in trained participants (SMD = 0.42; 95% CI: 0.18 to 0.66; P < 0.01) (Ye *et al.*, 2023). Moreover, when looking at duration alone, they observed (3) an inverse relationship between 8-OHdG and exercise without considering exercise intensity and training status (r = −0.74, P < 0.001); specifically, when exercise duration was <30 mins, they reported increased 8-OHdG levels (SMD = 0.51; 95% CI: 0.23 to 0.80; P < 0.01), compared with decreased 8-OHdG levels with exercise >30 mins (SMD = −0.68; 95% CI: −1.24 to −0.12; P < 0.05), both with a moderated effect (Ye *et al.*, 2023). Lastly, the authors reported (4) a small overall effect of high-intensity exercise (≥75% $\dot{V}O_{2max}$) on increasing 8-OHdG levels (SMD = 0.37; 95% CI: 0.08 to 0.67; P = 0.01), a similar finding to the one reported in Chapter 4 (Tryfidou *et al.*, 2020).

Exercise is an internal stressor that can induce various types of DNA damage such as chemically altered DNA bases, and physical breaks in DNA chains. Through the actions of RONS (such as superoxide anion, hydroxyl radical and H_2O_2), which rise as a function of moderate- and/or high-intensity exercise, SSBs and DSBs can occur (Tryfidou *et al.*, 2020). Furthermore, regarding base modifications, it is well understood that DNA can be oxidatively damaged at both its sugar deoxyribose moiety (at ~70%) and nitrogenous bases (at ~30%) via reacting with the highly reactive hydroxyl radical (Cooke *et al.*, 2003; Nikitaki *et al.*, 2015). The hydroxyl radical mainly reacts with DNA via addition to the double bonds of DNA bases, giving rise to a number of adduct base radicals. For example, C5-OH and C6-OH adduct radicals from addition of the hydroxyl radical to the C5-C6 double bonds of pyrimidines (Cooke *et al.*, 2003). To a lesser extent, the attack also occurs through abstraction of an H atom from the methyl group of thymine, giving rise to the allyl radical, and each of the C-H bonds of the 2`-deoxyribose moiety (Cooke *et al.*, 2003; Al-Horani, 2022). The 5 carbons of the 2`-deoxyribose are all susceptible to H abstraction by the hydroxyl

radical, which yields DNA lesions such as 3´-phosphate, 3´-phosphoglycate, 5´-phosphate, and 5´-aldehyde (Balasubramanian *et al.*, 1998). Furthermore, purine nucleobases get attacked by hydroxyl radical addition at C4, C5, and C8 positions forming OH adduct radicals, and adenine specifically forms two C4-OH and C8-OH adduct radicals, which undergo dehydration and conversion to an oxidizing purine(-H)• radical (Cooke *et al.*, 2003). Upon one-electron oxidation, C8-OH adduct radicals generate 8-hydroxypurines. Whereas upon one-electron reduction, formamidopyrimidines are formed, specifically 6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) from guanine and 4,6-diamino-5-formamidopyrimidine (FapyAde) from adenine (Cooke *et al.*, 2003; Al-Horani, 2022). Moreover, the C8-OH adduct radical, formed upon the hydration of guanine in double-stranded DNA, leads to the generation of 8-hydroxydeoxyguanosine (8-OhdG). This compound is recognized as the predominant oxidatively altered base among the more than 100 known modified bases and is the most widely used biomarker for DNA oxidation (Cooke *et al.*, 2003; Al-Horani, 2022).

DNA damage assessed in the experimental studies of this thesis, demonstrated primarily SSBs and, to a lesser extent, DSBs. This is in accordance with others, as mentioned above. However, some reports have showcased contradicting findings. For example, no change in SSBs or FPG-sensitive sites (specific for oxidized purines like 8-oxoguanosine) were reported following a marathon or half-marathon race, but an increase in DNA % tail intensity in ENDO III sites was (specific for oxidized pyrimidines) (Briviba *et al.*, 2005). Similarly, 20 min following an ironman triathlon race (consisting a total of 225.8km of swimming, cycling and running), SSBs were actually slightly lower, however 1 day post-race they increased, compared to baseline values, respectively (Reichhold *et al.*, 2009*b*). Following the same trend, Wagner *et al.* (2011) reported a slight decrease (post-race) and then an increase (1 day post-race) and no further damage (5 and 19 days post-race) in well-trained athletes following a triathlon. It is, nonetheless, important to consider that these studies include longer duration exercise protocols compared to the experimental work in Chapter 5 and 6. Lastly, 2.5h of treadmill running at 75% of $\dot{V}O_{2max}$ increased DNA tail migration in well-trained endurance athletes 3 hours post- but not immediately following exercise (Peters *et al.*, 2006). This is contradictory to the work of others mentioned above (Hartmann *et al.*, 1998; Niess *et al.*,

1998; Tsai *et al.*, 2001). Although in these protocols, the exercise time was much longer, thus the timing of blood sampling was vastly different depending on the duration of each exercise event/race/bout. In comparison to the experimental studies in Chapters 5 and 6, the exercise protocols ranged between approximately 10 minutes (maximal) to 1-hour of acute exercise (65-70% of $\dot{V}O_{2max}$). It also may be noteworthy to point out that, in the case of the incremental maximal exercise run (Chapter 6), the total average exercise intensity workload is different compared to 1-hour of the same constant workload (i.e., 65-70% of $\dot{V}O_{2max}$ in Chapter 5). Moreover, the differences in biological sex combined with the training level of the participants (on average participants in Chapter 5 were defined as more "active" compared to those in Chapter 6), may elicit different responses. This may, to some extent, explain the greater extent of DNA damage demonstrated in Chapter 6 through higher reported % values of DNA SBs versus those in Chapter 5. As such, the discrepancies noted in the literature may also be attributed to the training status and/or current endurance capacity of the individual, since exercise training which yields greater endurance capacity has been linked to beneficial adaptive responses. This is thought to be a consequence of antioxidant defence enhancement, mediated by RONS signalling as well as redox regulation of antioxidant gene expression (Ji, 2002, 2008).

8.4.2 Lipid Peroxidation in Response to High-Intensity Exercise

In terms of lipid peroxidation, work within this thesis has demonstrated conflicting results. In the first experimental investigation (Chapter 5), LOOH concentration was attenuated by 18.7% as a function of time 3 hours post exercise. Whereas in the second experimental investigation (Chapter 6), LOOH increased by 10% following exercise which remained significant at 1-hour post exercise. Previous work reported an increase of LOOH by 12.6% immediately following maximal exercise (Williamson *et al.*, 2018) and 14% following high-intensity exercise at 80-85% of $\dot{V}O_{2max}$ (Williamson *et al.*, 2020*a*). Similarly, Fogarty (*et al.*, 2011) reported a rise in LOOH at three different exercise intensities (40, 70, 100% of $\dot{V}O_{2max}$). In addition, their work further identified the generation of alkoxyl radicals, which are lipid-derived free radicals, associated with exercise-induced DNA damage and the rise in LOOH.

On the other hand, González (*et al.*, 2008) reported decreased salivary LOOH following aerobic exercise and Vincent (*et al.*, 2002) also observed lower lipid peroxidation following resistance exercise. Lastly, although assessed via a different lipid oxidation biomarker, earlier work reported no change in serum MDA levels following maximal exercise in healthy men and women (Leaf *et al.*, 1997). Therefore, further work is required to elucidate the underlying physiological/biochemical mechanisms of a possible dampening effect of aerobic exercise on lipid peroxidation (as observed in Chapter 5) and whether exercise intensity/duration and training status can explain the discrepancies noticed in the literature and between the experimental work contained in this thesis. The link between exercise training and mitochondrial adaptations may be of relevance, since prolonged endurance training has been associated with 40-50% increase in mitochondrial volume and enhancements in their oxidative capacity and respiration (Memme *et al.*, 2021). Consequently, highly reactive lipid peroxidation products may be associated with increased RONS formation and mitochondrial dysfunction. Thus, a plausible mechanism of counteracting these negative effects may exist through endurance training via increasing the number/volume of mitochondria, resulting in greater metabolic capacity and subsequently the weakening of lipid peroxidation (Ademowo *et al.*, 2017). Lastly, although peroxyl radicals were not measured in chapter 5, another possible mechanism to be explored may be through the action of lipid-soluble chain breaking antioxidant α-tocopherol. This increased from baseline to post-supplementation by 34%, as it scavenges lipid peroxyl radicals to stop the chain propagation of lipid peroxidation, irrespective of the type of chain initiating free radical (Niki, 2021).

8.4.3 Exercise-Induced Oxidative Stress and Antioxidant Interactions

The inherent role of antioxidants in protecting against free radicals and other RONS in human health and biological systems has sparked extensive research. This work primarily explores how antioxidants interact with exercise-induced oxidative stress, with a key focus on determining whether antioxidant supplementation can mitigate some of the harmful effects of RONS. Both experimental chapters (Chapter 5 and 6) explored the interactions of a possible antioxidant effect on exercise-induced DNA damage through the actions of specific antioxidants of interest: with (1) the chronic supplementation of AA in males (Chapter 5); and (2) the

endogenous action of the naturally occurring sex hormone oestradiol in females, as part of their natural menstrual cycle (Chapter 6).

In Chapter 5, 10g of AA ingested twice a day for 4 weeks failed to exert a dampening effect on DNA damage or LOOH following one hour of exhaustive cycling at moderate- to high-intensity (approximately at 65-70% of $\dot{V}O_{2max}$), as there was no interaction effect (group x time). In support of this, Davison (*et al.*, 2005*a*) reported that 600 mg of AA (included in a mixture of other antioxidants) offered no reduction in DNA % tail intensity. In contrast, 1 day following an ultramarathon race, 6-wks of supplementing with 1000 mg AA and 400 IU RRR-α-tocopherol acetate led to 62% reduction in DNA % tail intensity, observed only in females (Mastaloudis *et al.*, 2004*b*). Unfortunately, to this author's knowledge, there is limited research investigating the effects of AA alone on exercise-initiated DNA damage with the comet assay technique. With the use of 8-OHdG biomarker, Bloomer (*et al.*, 2006*c*) reported no difference, with a mixture of 400 IU of vitamin E and 1 g of AA, in the concentration of 8-OHdG following exercise. Similarly, Morillas-Ruiz (*et al.*, 2005) observed no changes in pre- and post-exercise urinary 8-OHdG following consumption of a rich AA beverage (containing black grape (81 g/l), raspberry (93 g/l) and red currant (39 g/l) concentrates). However, in the placebo group, a 19% elevation of 8-OHdG was observed, which may indicate some protection. On the other hand, Fogarty (*et al.*, 2013*c*) reported that acute and chronic watercress supplementation, a vegetable rich in β-carotene and AA (50 mg of AA per 80g of watercress), attenuated both DNA % tail intensity and LOOH following exhaustive treadmill running.

In chapter 6, endogenous oestradiol did not differ between the LF and LL phases of the MC, nor did it lessen the observed global DNA damage (SSBs & DSBs) following maximal exercise in healthy females. To date, there is no research directly investigating the relationship between circulating oestradiol concentration and DNA damage (exercise-induced or otherwise) with the Comet Assay method. Additionally, exercise-related DNA damage female-focused studies or male-female comparisons in this specific area of research are limited. Furthermore, exercise-related DNA damage female-focused studies or male-female comparisons in this specific area of research is highly limited. Chung *et al.* (1999) assessed plasma MDA and TBARS in women during both the LF and LL phases following 30 minutes of high-intensity running (75-80% of $\dot{V}O_{2max}$) and found no differences in both

biomarkers of lipid peroxidation for either MC phase. Møller *et al.* (2001*b*) reported no change in DNA SBs in 12 healthy men (n = 7) and women (n = 5) following maximal bicycle exercise; unfortunately, no sex comparisons were included in their analyses. Moreover, Briviba *et al.* (2005) reported an increase in oxidized pyrimidines (higher DNA % tail intensity in ENDO III sites) but unchanged DNA SBs measured in the lymphocytes of 5 men and women following the completion of a marathon or half-marathon. In contrast, Mastaloudis *et al.* (2004*b*) observed higher DNA % tail intensity in females (n = 5) compared to males (n = 5) 2 days following a 50km ultramarathon. Lastly, Hartmann *et al.* (1998) reported elevated DNA migration in the lymphocytes of 3 young men and women athletes from 24 hours up to 5 days following a triathlon. In relevance to the findings of this experimental work, it is important to distinguish that the above-mentioned studies included different exercise protocols (i.e., long distance events vs acute maximal exercise) and included both sexes in their analysis. However, no female-male comparisons were made, in contrast to only female participants included in Chapter 6.

Not only are oestrogen-DNA damage related data scarce. The available data in the literature discussing its possible antioxidant properties actually suggest that, if an antioxidant effect exists, it does so indirectly by interacting with ERs and regulating antioxidant enzyme gene expression (Strehlow *et al.*, 2003; Bellanti *et al.*, 2013). In support of this notion, Borrás *et al.* (2005) observed that MCF-7 cells (mammary gland tumour cell line) incubated for 48 hours with incremental doses of 17β-oestradiol (0.02–20 nm) led to a decrease in peroxide levels across all doses. Furthermore, the authors investigated the activated mechanisms by which oestradiol exerts this antioxidant capability and proposed that this is receptor-mediated via Tamoxifen (an oestrogen receptor modulator) (**Figure 8.1**). Lastly, they reported that incubation of MCF-7 cells with physiological concentrations of oestradiol for 3 min induced the phosphorylation of the $ERK1^{[MAPK]}$ and $ERK2^{[MAPK]}$, which in turn activate the signalling pathway that involves the transcription factor nuclear factor kappa B (NF-*κ*B) (Borrás *et al.*, 2005).

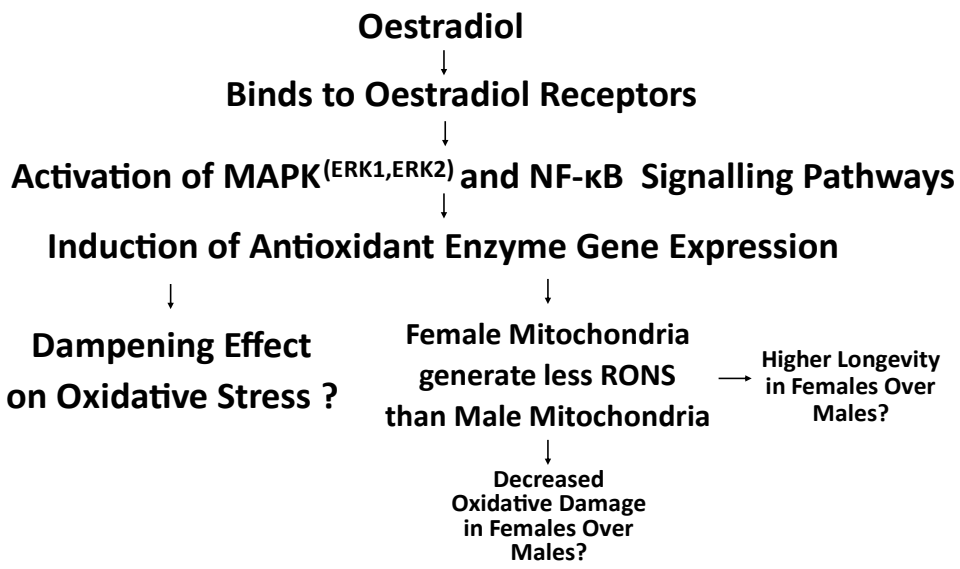


Figure 8.1 Possible mechanisms of action by oestradiol exerting an antioxidant effect. Adapted from (Viña *et al.*, 2005).

8.4.4 Gene Response to Hypoxic-Induced Stress

Eukaryotic cells have developed tightly intertwined complex mechanisms, that allow them to rapidly respond to environmental changes by altering gene expression (Kunsch & Medford, 1999). At non-deleterious intracellular levels, RONS act as key messenger molecules and regulators of gene expression, but at levels beyond a certain threshold (Pro oxidative state) they may negatively affect the survival of the cells by damaging DNA, lipids and proteins (Turpaev, 2002). Thus, RONS can induce redox-sensitive gene expression, such as GPx and SOD, to protect the cell from oxidative stress.

The experimental pilot work presented in chapter 7, determined the acute gene response through RT-qPCR, for two selective genes of interest, following exercise in NH and normoxia. The identified similar quantified gene transcription response supports the previously documented exercise response (conducted via by DNA-microarray scanning), as opposed to an environmentally specific response. The genes chosen, *GPx7* and *NCF2*, play an important role in antioxidant capacity and RONS formation, respectively. Results indicated that, in both NH and normoxia, there was an upregulation in the *NCF2* gene and a downregulation in the *GPx7* gene, following intense aerobic exercise at 75% of $\dot{V}O_{2max}$.

GPx7 functions as an intracellular stressor/transmitter (without binding to GSH) and is essential for oxidative protein folding inside the ER and the maintenance of redox equilibrium (Chen *et al.*, 2016; Hanousková *et al.*,

2021). Although studies measuring *GPx7* in relation to exercise-induced oxidative stress are limited, Ferrer *et al.* (2009) reported no changes in GPx expression following 1-hour of swimming at 75-80% $\dot{V}O_{2max}$ in males, but interestingly, higher GPx activity was observed. Likewise, higher antioxidant enzyme activity, including GPx, but not higher gene expression was reported following high-intensity interval training in a male cohort (Fisher *et al.*, 2011). These findings may imply that exercise duration, mode and/or intensity may affect the expression of antioxidant enzymes, particularly GPx. This suggests, despite an increase in enzyme activity, abundant mRNA GPx expression may not always occur simultaneously. It is also likely that an observed rise in GPx protein activity and a decrease in GPx transcription are related to molecular signalling properties that exhaust (i.e., downregulate) the consumption of the RNA transcript by transitioning from RNA to end stage protein production. However, further research is needed to clarify this hypothetical approach. Thus, despite the fact that this author did not assess GPx activity *per se*, the cause of the decrease in GPx expression seen as a function of exercise, in either normoxia or NH, has not yet been fully determined. This approach may be successful considering the changes observed in $\dot{V}O_{2max}$ and SaO_2, since the declines in both variables may also affect the regulation of antioxidant-related gene transcription.

Moreover, regarding *NCF2* gene expression, currently there is no available evidence to relate with the findings of this work in response to exercise and/or hypoxia. One report from Italiano *et al.* (2012), demonstrating increased expression of *NCF2* to be associated with the generation of intracellular RONS (in proliferating cells) that may protect against apoptotic cells; this may indicate its critical role in the regulation of RONS. In conclusion, the complex mechanism of gene transcription factor activation/regulation through exercise-mediated RONS is not well understood, despite the fact that these molecular adaptive responses to exercise may seem favourable (Thirupathi & de Souza, 2017). There is an obvious gap in the literature describing the gene expression response to hypoxic exercise, particularly with regard to the genes involved in the production of RONS and antioxidant defence.

8.4.5 Research Limitations and Future Implications

There are several limitations present throughout the experimental work which must be considered when interpreting the data of this thesis. The following common limitations which can influence physiological/metabolic

changes can be applied across all experimental studies included in this thesis, which include: (1) biological sex, (2) age, (3) training status and (4) dietary intake.

(1) Biological Sex: The experimental work outlined in this thesis included either an all-male (Chapter 5 and 7) or all female (Chapter 6) subject pool. Consequently, the main drawback from this is the lack of sex comparison analyses within the same studies, as this is key for understanding the physiological ramifications associated with biological sex in relation to oxidative stress responses to exercise since these may present differently in males and females. Greater plasma TBARS and urinary 8-iso-PGF2α were observed in young men compared to age-matched women (Ide *et al.*, 2002), although the experimental work in this thesis utilized different biomarkers in comparison. Similar findings have been presented in the animal model. For example, higher lipid peroxidation has been observed in male compared to female rats (Barp *et al.*, 2002). Furthermore, Borrás *et al.* (2003) reported 3 key findings: mitochondrial DNA damage was measured to be 4-fold higher in male than female rats; female rat mitochondria exhibit increased expression of GPx and Mn-SOD; and lastly formation of peroxide is higher in liver and brain mitochondria from male compared to mitochondria from female rats. Additionally, generation of superoxide, in the presence of NADPH, was approximately 50% lower in the cerebral arteries of female rats compared to the concentrations generated by the arteries of male rats. Interestingly, treatment with 17β-oestradiol in ovariectomized female rats lowered the generation of NADPH-stimulated superoxide (Miller *et al.*, 2007). The latter has also been demonstrated in the human *in vitro* model as 17β-oestradiol inhibited the expression of NADPH and, in turn, attenuated the NADPH-stimulated superoxide production in oestradiol-treated human endothelial cells (Wagner *et al.*, 2001). The research for sex comparisons in the context of exercise-induced stress with or without antioxidant interactions is lacking. However, one noteworthy report of 25 men and 23 women who performed 30 minutes of high-intensity running (80% of $\dot{V}O_{2max}$), supplemented with either placebo, antioxidant (A: 400 IU vitamin E + 1 g vitamin C) or a fruit and vegetable powder (FV), demonstrated: (1) 8-OHdG was lower only in men after treatment A compared to placebo or FV; (2) higher reduced glutathione (GSH) in

women; (3) lower TGSH and a higher GSSG/TGSH ratio than women, independent of treatment, or exercise; and (4) higher plasma vitamin E than men in the placebo group. Collectively the authors suggest that both sexes have similar antioxidant treatment responses associated with the decline in markers of exercise-related oxidative stress and that women have higher resting antioxidant plasma levels This may indicate enhanced protection in women in the absence of an antioxidant treatment. Similarly, although Pepe *et al.* (2009) reported no sex-time interaction in 8 untrained men and 9 untrained women who ran at three different distances (800, 1500, and 3000 m at 10 km/h), they observed similar post-exercise in changes in LOOH, SOD & CAT activity for both sexes.

(2) Age: The mean age of participants across studies 33 ± 11, 35 ± 5 and 26 ± 3, for Chapters 5, 6 and 7 respectively. Harman (1956) published the free radical theory of ageing (FRTA) to explain the aging process, which was later revised by the same author to the mitochondrial free radical theory of aging (MFRTA) (Harman, 1972). According to the FTRA, the inability of several defence mechanisms to prevent the damage caused by RONS, particularly at the mitochondria, leads to aging. Moreover, the revised MFRTA proposed that cellular damage and cell death are a consequence of mitochondrial dysfunction and increased RONS formation. Although the mean age of the participants across all experiments were not qualitatively different, it is imperative to highlight this when one interprets and relates this data across different aged populations (i.e., elderly populations including post-menopausal women).

(3) Training status: Participants' training status (acute/occasional versus chronic/regular) is generally not often reported in detail across the literature, along with other important information on lifestyle/occupation, smoking status, diet, alcohol consumption, and medication; this is important as it may influence the effects of exercise on DNA and other relative oxidative stress biomarkers. In the experiments outlined in this thesis, the best possible effort was made to define and include this information in our findings. However, while we defined the current training level of the participants, this does not necessarily reflect their "training status" including past training activity. The current training level of the participants in this thesis, at the time of recruitment, were non-sedentary individuals

engaging in exercise: (including running, cycling, weightlifting and/or other) 2-4 times/1-6+ hours per week and baseline $\dot{V}O_{2max}$ < 50 $mL \cdot kg^{-1} \cdot min^{-1}$ for Chapters 5 and 6; and moderate- to high-intensity aerobic exercise 1-hour or more, 4-6 times weekly for Chapter 7. All participants met these criteria which means that between each participant, their level of physical activity could vastly differ (i.e., someone who trains 1-2 hours versus 6+ hours per week). Chronic exercise training has been associated with decreased oxidative stress and enhanced antioxidant defence. For example, Miyazaki *et al.* (2001) demonstrated beneficial oxidative stress outcomes following a 12-week training programme (consisted of running at 80% maximal exercise heart rate for 1-hour per day) alongside an acute exhaustive exercise bout before and after the 12 weeks of training. The authors not only reported increased lipid peroxidation following the exercise bout, which was notably decreased through training, they also measured higher GPx and SOD antioxidant enzymatic activities after training but not after the exhaustive exercise bout. Similarly, six months of chronic aerobic training resulted in a 12% increase in baseline GPx activity in a cohort of obese middle-aged women, according to Shin *et al.* (2008). Immediately following two acute exercise tests at 60% and 80% VO2max, GPx activity remained unchanged in the pre-exercise training condition. However, in the post-exercise training condition, GPx activity was significantly increased after the acute exercise bouts (Shin *et al.*, 2008). Such findings may suggest that endogenous antioxidant defences may be enhanced as a consequence of chronic training adaptions. Therefore, if training status is not factored to possibly have an impact on oxidative stress biomarkers, then cautious interpretation is warranted when interpreting the findings of the presented experimental work.

(4) Diet: Dietary choices can influence an individual's susceptibility to oxidative stress since the dietary antioxidants, which are be consumed throughout one's nutritional intake primarily from fruits and vegetables, may enhance the individual's antioxidant capacity. For example, Thompson *et al.* (2005) examined the effects of a high versus low vegetable and fruit (VF) 2-week intervention on markers of DNA and lipid oxidation. Following 2 weeks of high VF consumption, the authors observed a reduction in 8-OHdG by 16.5%

and in 8-iso-PGF2α by 30.7% relative to 0.5% and 10.9% of the low VF consumption, respectively. For context, the equivalent servings of high versus low VF were 12.1 and 3.6 servings per day, respectively. In addition, the high VF intervention increased the plasma concentrations of antioxidants measured in their reports including lycopene, lutein, α- and β-carotene, in comparison to the low VF where all remained unchanged. Similarly, Pool-Zobel *et al.* (1997) investigated the effect of: a 330 mL tomato juice with 40 mg lycopene (weeks 3 and 4); 330 mL carrot juice with 22.3 mg β-carotene and 15.7 mg α-carotene (weeks 5 and 6); and 10 g dried spinach powder (in water or milk) with 11.3 mg lutein (weeks 7 and 8) on DNA SBs and oxidized bases after an initial depletion period (week 1 and 2) on the same subjects. The authors reported a reduction of DNA % tail intensity with all the three different food interventions, and a reduction on oxidized pyrimidine bases in DNA only with the carrot juice intervention. In contrast to these findings, Møller *et al.* (2003) found no difference in DNA damage following a 24-day dietary placebo-controlled intervention study in which participants were randomized into three groups receiving: an antioxidant-free basal diet/600 g of fruits and vegetables; a supplement containing the corresponding amounts of vitamins and minerals; or placebo. Interestingly, when considering specific dietary preferences, a study investigating baseline DNA damage in non-vegetarian versus vegetarian females found higher % tail DNA damage in the latter, with the least amount of damage reported in the pescetarian group and lower DNA damage in the omnivorous group compared to vegetarians (Gajski *et al.*, 2023). No diet records were taken across any of the experimental studies. This is a very tedious task for participants. Although it was initially planned to include dietary records as part of our experimental protocols, it was ultimately discarded since acquiring participants was challenging and the inclusion of this task would be an additional challenge into gathering fully compliant subjects. However, as a counterpoint, the measured LSA between groups across the experimental studies (Chapter 5 and 6) remained unchanged. Lastly, another limitation that may be considered was not measuring antioxidant capacity such as the enzymatic activity of GPx, CAT and SOD.

In summary and regarding specific limitations for each experimental study, in chapter 5 the data from the FPG comet assay were not included in the final analysis due to methodological error while conducting the assay. Furthermore, the lack of additional post-exercise sampling points (i.e., 24-, 48-, 72- hours) in the experimental design protocols, in addition to the measure of DNA repair (i.e., hOGG1) and/or activity of endogenous antioxidant enzymes, may have provided more clarity to our findings. This may be important since another possible mechanism that may explain the extent of the DNA damage response measured across the two experimental studies (Chapter 5 and 6) may be associated with DNA repair. Thus, further research is required to elaborate on the mechanisms and timing of exercise-induced DNA repair.

Moreover, the technique which was used to measure DSBs in Chapter 6 was a modified version conducted by one published report by Wojewódzka *et al.* (2002). Alternatively, the γH2AX may present as a more reliable and robust method to detect DSBs (Heylmann & Kaina, 2016). In addition, the BBT data measured in Chapter 6 was not included in the final analysis due to missing or unreported data from participants. Moreover, even though the sample size in Chapter 6 was small, the novel aspect of investigating the unique research angle of exercise/ DNA damage/ oestradiol will hopefully provide the groundwork for future investigations. Lastly, the inclusion of a male group would help to elucidate the matter of sex-related differences in association to endogenous sex hormones and exercise-induced oxidative stress variables.

Although the aim of the pilot work in Chapter 7 was to validate previously published microarray data with RT-qPCR, it would have been of great interest to further examine the relevant oxidative stress biomarkers as with the other experimental studies. Lastly, it is imperative to highlight the major limitation of RNA quality issues since this is critical for genome-wide analysis of gene expression. Following the manual purification of total RNA from the human blood samples, RNA concentration (as analysed by spectrophotometry using the A260/A280 ratio method) was not adequate for all the four genes initially selected for the RT-qPCR analysis, as each sample had to be run in triplicate and thus, limiting the analysis to half of the initially selected genes.

8.4.6 Concluding Remarks

In conclusion, it is apparent that additional research is required to gain more in-depth knowledge in the domain of free radical and oxidative stress research. However, the work presented in this thesis has hopefully provided a deeper understanding of exercise-induced DNA damage in the oxidative stress field of research. In review, the following concluding remarks can be drawn:

- High-intensity exercise of two different modalities (maximal/shorter & 65-70% $\dot{V}O_{2max}$/longer) causes oxidative damage to DNA in both females and males. The duration and/or intensity of the exercise may influence the extent of the damage response on DNA. However, the effect of exercise on lipid peroxidation has provided conflicting results.
- Maximal acute aerobic exercise produces a global DNA damage response in females, resulting in both single- and double-strand breaks.
- High dose ascorbic acid supplementation in males nor endogenous oestradiol occurring naturally during the menstrual cycle in females provide a dampening effect on exercise-induced DNA damage.
- High-intensity exercise (75% of $\dot{V}O_{2max}$) performed in both hypoxia and normoxia causes changes in gene expression of selective oxidative stress genes associated with RONS production and antioxidant defence metabolism.

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Appendix A

Ethical Approval

Memo

To: **Prof G Davison, 15C01C, School of Sport, JN**

From: Elaine Bell, Research Governance, 26A17, Jordanstown

Date: 12 June 2017 **Ref:**

Ulster University
Research Ethics Committee

Project Number: REC/17/0043

Project Title: Persistent Benefits of Exercise and Vitamin C in Ageing Mediated by Epignetics

Outcome: Approved to proceed subject to amendment to be considered by a UREC sub-committee

Please find attached the comments of the Research Ethics Committee on your recent application.

You should address these comments point by point in a covering letter and highlight or underline any revisions made to the application and associated materials. Please send your response by e-mail to e.mccormick@ulster.ac.uk. Your response will then be referred to members of a sub-committee for comment. You should note that your application does not require to be resubmitted for reconsideration at a future meeting, but you should also note that you cannot commence any research on human subjects until your response has been considered and a letter of approval has been issued.

Please note that all Ulster staff and student members of the research team must provide evidence that they have successfully completed the University's online Research Integrity course. Final approval for the study will not be confirmed until this has been provided.

If you have any queries, please contact Nick Curry or Elaine Bell.

If you do not intend to proceed with the project or if you anticipate a significant delay in responding to the concerns of the committee, please contact the Research Governance section.

I look forward to hearing from you in the near future. Please quote the **Project Number** in all correspondence.

Thank you and best wishes.

Elaine Bell
Admin Officer
Research Governance
e.mccormick@ulster.ac.uk
Ext: 66518

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Our Ref: NC:GOV

21 August 2017

Professor G Davison
Room 15C01C
School of Sport
Ulster University
Jordanstown Campus

Dear Professor Davison

Research Ethics Committee Application Number: REC/17/0043

Study Title: Persistent Benefits of Exercise and Vitamin C in Ageing Mediated by Epignetics

Thank you for your recent response to matters raised by the committee. This has been considered and the decision of the committee is that the research should proceed.

Please also note the additional documentation relating to research governance and indemnity matters, including the requirements placed upon you as Chief Investigator.

The committee's decision is valid for a period of three years from today's date (this means that the study should be completed by that date). If you require this period to be extended, please contact the Research Governance section.

1. **Please complete and return the Chief Investigator Statement of Compliance prior to commencing the study and keep a copy for your file.**

2. **Please retain all other documents.**

Further details of the University's policy along with guidance notes, procedures, terms of reference and forms are available on the Ulster University Portal.

If you need any further information or clarification of any points, please do not hesitate to contact me.

Yours sincerely

Nick Curry
Head of Research Governance
028 9036 6629
n.curry@ulster.ac.uk

Ulster University

Memo

To: **Professor G Davison**

From: Elaine Bell, Research Governance, 26A17, JN

Date: 18 April 2018 **Ref:**

Dear Professor Davison

Research Ethics Committee application number: REC/17/0043

Project Title: Persistent Benefits of Exercise and Vitamin C in Ageing Mediated by Epignetics

Amendment Number: 1

Following submission of Amendment Number 1 for ethical approval, the Research Ethics Committee is pleased to confirm that the amendment should proceed.

The period for which the committee's decision is valid remains unchanged from the original approval.

If you need any further information please do not hesitate to contact me.

Thanks and best wishes.

Elaine Bell
Research Governance Officer
Research Governance Section
Ext. 66518
e.bell2@ulster.ac.uk

Memo

To: **Professor G Davison**

From: Elaine Bell, Research Governance, 26A20, Jordanstown

Date: 19 December 2018 **Ref:** **EM/JD**

Ulster University
Research Ethics Committee

Project Number: REC/18/0110

Project Title: Role of Endogenous Oestradiol Production on Exercise-Induced DNA Damage in a Healthy Female Cohort

Outcome: Approved to proceed subject to amendment – to be reviewed by the Research Office and lead reviewer

Please find attached the comments of the Research Ethics Committee on your recent application.

You should address these comments point by point in a covering letter and highlight or underline any revisions made to the application and associated materials. Please send your response by e-mail to e.bell2@ulster.ac.uk. You should note that your application does not require to be resubmitted for reconsideration at a future meeting, but you should also note that you cannot commence any research on human subjects until your response has been considered and a letter of approval has been issued.

Please note that all Ulster staff and student members of the research team must provide evidence that they have successfully completed the University's online Research Integrity course. Final approval for the study will not be confirmed until this has been provided.

If you have any queries, please contact Nick Curry or Elaine Bell.

If you do not intend to proceed with the project or if you anticipate a significant delay in responding to the concerns of the committee, please contact the Research Governance section.

I look forward to hearing from you in the near future. Please quote the **Project Number** in all correspondence.

Thank you and best wishes.

Elaine Bell
Research Governance Officer
e.bell2@ulster.ac.uk
Ext: 66518

Ulster University

Memo

To: **Professor G Davison**

From: Elaine Bell, Research Governance, 26A20, JN

Date **6 June 2019** **Ref:**

Dear Professor Davison

Research Ethics Committee application number: REC/18/0110

Project Title: Role of Endogenous Oestradiol Production on Exercise-Induced DNA Damage in a Healthy Female Cohort

Amendment Number: 2

Following submission of Amendment Number 2 for ethical approval, the Research Ethics Committee is pleased to confirm that the amendment should proceed.

The period for which the committee's decision is valid remains unchanged from the original approval.

If you need any further information please do not hesitate to contact me.

Thanks and best wishes.

Elaine Bell
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Our Ref: NC:GOV

11 May 2017

Professor G Davison
Room 15C01C
School of Sport
Ulster University
Jordanstown Campus

Dear Professor Davison

Research Ethics Committee Application Number: REC/17/0047

Study Title: Genomic Response Following Exercise in Hypoxia

Thank you for your recent response to matters raised by the committee. This has been considered and the decision of the committee is that the research should proceed.

Please also note the additional documentation relating to research governance and indemnity matters, including the requirements placed upon you as Chief Investigator.

The committee's decision is valid for a period of three years from today's date (this means that the study should be completed by that date). If you require this period to be extended, please contact the Research Governance section.

1. **Please complete and return the Chief Investigator Statement of Compliance prior to commencing the study and keep a copy for your file.**

2. **Please retain all other documents.**

Further details of the University's policy along with guidance notes, procedures, terms of reference and forms are available on the Ulster University Portal.

If you need any further information or clarification of any points, please do not hesitate to contact me.

Yours sincerely

E Bell

Nick Curry
Senior Administrative Officer
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Appendix B

Recruitment Emails

Volunteers required for Research Study

Background

As we age, the chances of developing a disease such as type 2 diabetes increases. Exercise can be useful to decrease the risk of disease. Ageing can cause changes to our DNA, but these changes can alter following exercise training. Emerging evidence shows that vitamin C can also make a positive change to our DNA. We plan to confirm preliminary findings that exercise and vitamin C, in combination, can cause beneficial alterations to DNA.

The study is being conducted by the Sport and Exercise Sciences and the Biomedical Science Research Institutes as part of a PhD research project.

You are invited to volunteer if:

- You are healthy and aged between 18 and 65 years old
- You are a non-smoker
- You take part in regular exercise (2-4 times per week)
- Are not currently taking any supplemental antioxidants like multivitamins or any other supplements such as creatine, BCAAs, pre-workout, fish oils/omega-3s (or are willing to stop taking these prior to and for the whole duration of the study)
- Are not currently taking any form of medication

What your participation will involve:

- You will be asked to visit our sports science facilities at Ulster University, Jordanstown campus on two occasions
    - During the first visit you will be asked to complete a maximal aerobic capacity test on a bike. You are also required to give blood and a small piece of muscle tissue. Before the next visit you are asked to ingest vitamin C or a placebo for 4 weeks.
    - During the final visit, you will be asked to exercise on a bike for a total of 60 minutes, and provide further blood and muscle tissue samples.

Upon completion of the study, volunteers will receive a £50 Amazon voucher.

If you are interested in participation, or have any further questions please contact Miss Despoina Tryfidou at: Tryfidou-D@ulster.ac.uk.

This study is being completed as part of a PhD Research Studentship and has been approved by the University's Research Ethics Committee (UUREC; REC/17/0043).

We look forward to hearing from you

Despoina Tryfidou and Catherine McBride - **PhD Researchers**
Sport and Exercise Research Institute/Biomedical Sciences Research Institute

Supervisors:

Professor Gareth Davison and Colum Walsh, Drs Conor McClean and Rachelle Irwin.

Recruitment Email

Female Volunteers required for Research Study

The study is being conducted by the Sport and Exercise Sciences Research Institute as part of a PhD research project.

You are invited to volunteer if:

- You are a healthy female and aged between 18 and 40 years old
- You are not taking any hormonal contraception (currently and at least for the past 6 months; Copper IUD is allowed)
- You have a normal menstrual cycle and are not suffering from any hormone imbalances
- You are not currently pregnant or haven't been pregnant for at least a year prior to this study
- You are a non-smoker
- You take part in regular exercise (2-4 times per week)
- Are not currently taking any supplemental antioxidants like multivitamins, or any other supplements such as creatine, BCAAs, pre-workout, fish oils/omega-3s (or are willing to stop taking these for the whole duration of the study)
- You are not currently taking any form of medication

What your participation will involve:

- You will be asked to visit our exercise physiology laboratory at Ulster University, Jordanstown campus on five separate occasions:
    - During the first visit, you will be familiarised with the study protocol and be asked to complete a health history questionnaire and a consent form.
    - During the second and third visits you will be asked to complete a maximal aerobic capacity test ($VO_{2\text{-max}}$) on a treadmill.
    - During the final two visits, which will take place on two different days of your menstrual cycle, you will be asked to run on a treadmill at 80% of your pre-determined $VO_{2\text{-max}}$ until volitional exhaustion, and provide blood samples prior, immediately following and 3 hours post-exercise.

Upon completion of the study, volunteers will receive a £25 voucher of their choice (Gymshark or Amazon).

If you are interested in participation, or have any further questions, please contact Miss Despoina Tryfidou at: Tryfidou-D@ulster.ac.uk

This study is being completed as part of a PhD Research Studentship and has been approved by the University's Research Ethics Committee (UUREC).

We look forward to hearing from you,
Despoina Tryfidou PhD Researcher
Sport and Exercise Research Institute

Supervisors: Professor Gareth Davison and Dr Conor McClean.

To all staff and students

We are seeking endurance male trained participants required to take part in a study into the effects of exercise at simulated altitude. The study is being conducted by the sport and exercise sciences research institute on the Jordanstown campus as part of a PhD research project.

You are invited to volunteer if:

- You are a healthy male aged between 18 and 35 years old
- You run or take part in endurance based training sessions such as running, cycling or swimming four or more times a week
- You are a non smoker

What your participation will involve:

- You will be asked to visit our sports science facilities at the University of Ulster, Jordanstown on four occasions
    - During two of these visits you will be asked to complete tests of maximal aerobic capacity, once at sea level and once at a simulated altitude of 7000ft
    - During two of these visits you will be asked to run at 75% of your maximal capacity for 60 minutes, once at sea level and once at a simulated altitude of 7000ft
- Before, during and after the 60 minute exercise challenge various measurements including blood samples will be taken to monitor your response to exercise in each condition

If you are interested in this research project and have any further questions or would like to participate please contact Miss Despoina Tryfidou; **Tryfidou-D@ulster.ac.uk**

This research study has been reviewed and approved by the University Ethics Committee.

We look forward to hearing from you,

Despoina Tryfidou
PhD student
Sport and exercise research Institute
Ulster University

Project supervisors:
Prof Gareth Davison
Dr Conor McClean

Appendix C

Participant Information Sheets

Sport and Exercise Sciences Research Institute
Faculty of Life and Health Science

Participant Information Sheet

Persistent Benefits of Exercise and Vitamin C in Ageing Males Mediated by Epigenetics

You are being invited to take part in this research study. Before you decide whether or not to take part, it is important that you understand the purpose of the research and what you will be asked to do. You can participate if you are:

- Male
- Aged between 18-65 years old
- Non-smoker
- Not currently taking supplemental antioxidants like multivitamins or any other supplements such as creatine, BCAAs, pre-workout, fish oils/omega-3s (or willing to stop taking them a month prior to and for the whole duration of the study)
- Not currently taking any form of medication

Please read the following information and do not hesitate to ask any questions about anything that might not be clear to you. Make sure that you are happy before you decide what to do. Thank you for taking the time to consider this invitation.

1. Background and aim of this Research

As we age (aging), the chances of getting a disease such as type 2 diabetes can increase. Exercise can be useful to decrease the risk for disease. Ageing can cause changes to our DNA, and these changes can alter following exercise training, and emerging evidence shows that vitamin C (VitC) can also make a positive change to our DNA. We plan to confirm our preliminary analysis that exercise and VitC can cause long-term beneficial alterations to DNA in apparently normal male participants.

Aim: The direct aim of this work is to determine changes to DNA at baseline and following exercise and VitC supplementation in a group of male participants.

2. Procedures

Participants will be required to complete an overnight fast (circa 8 hours) prior to testing. Participants will be asked to wear appropriate footwear and clothing for exercising. This may include running trainers, a loose t-shirt/vest and a pair of comfortable shorts.

Stage One: You will attend the Ulster University Human Performance Lab exactly four weeks prior to testing to be familiarised with the study protocol and to provide informed consent; this will also include a medical history questionnaire. At this stage height and weight will be recorded. A blood and tissue sample will also be collected.

After giving blood and a small amount of tissue, you will complete one progressive incremental exercise test. You will be required to cycle at a cadence of 60rpm on a friction-braked cycle ergometer to produce a power output of 100w. The workload will be increased by 50w every 3min until you can no longer maintain the required work rate. During the test, oxygen uptake will be continuously monitored using an online gas analysis system.

Supplementation: Following stage one and 4 weeks prior to experimental testing, will ingest 2 x 5 grams of a commercially available vitamin C or placebo daily for four weeks.

Stage Two: Following 4 weeks of supplementation, you are required to return to the laboratory to provide blood and one muscle tissue sample immediately before one-hour of exercise at an intensity corresponding to 70%(lowest point)/80%(highest point) of pre-determined $VO_{2max.}$ A further blood sample immediately following and a final blood and muscle tissue sample 3 hours following the exercise will be required. All exercise testing will be performed on a stationary watt bike, while oxygen consumption and heart rate will be measured.

3. Risks & Benefits to Participants

With all scientific studies, there are a number of risks and/or hazards that could potentially occur during testing. These include muscular injury, heart complications, nausea, fainting, bacterial/viral infection, blood borne diseases and bruising and/or discomfort from blood or tissue sampling. It should be understood by the participant that the university and research team have taken the necessary actions to minimise the probability of these risks occurring. However, it should be noted that nausea/vomiting and discomfort from blood and tissue sampling may occur. It is important that if you experience any of these that you let a member of the research team know as soon as possible. There will always be one member of the research team present who is qualified in providing first aid; Dr. John Brown is the Health and Safety Coordinator. You will be asked to complete a medical history questionnaire. This information will be kept confidential and will only be used to assess whether you can participate in the study.

Note; if there are any changes in circumstances and/or medical status between completing the medical risk form and the beginning of the study, you should let a member of the research team know immediately.

4. What is something goes wrong during the study?

This study has been approved by the University of Ulster ethics committee and accordingly measures are in place to ensure there is no or minimal risk of anything going wrong during the course of this study. In the very unlikely case that something goes wrong during the study, it is important to understand that the university has procedures in place for reporting, investigating, recording and handling adverse events. Any complaints will be taken seriously, and should be made in the first instance to the Chief Investigator and/or the University. The University will provide an indemnity statement for research that has been approved through the appropriate governance and ethical review processes.

If you have any further queries on this do not hesitate to contact the University Research Governance.

5. Disclaimer

All data will be held in confidence and securely stored using a coding system rather than your name or initials. All information that may identify you will be removed prior to any publication as required under Data Protection (1998) legislation. However, in the unlikely event that during the course of the investigation any underlying health concerns are disclosed, you will be encouraged to share this information with your GP.

Data will to be stored on file for 10 years in accordance with University Policy. This means all data will be put into a secure, password-locked electronic file and stored within Ulster University. Only the investigators associated with study will have access to data. If someone was to gain access to data, all information regarding participant identity will remain anonymous.

Participant Information Sheet

(Additional information for muscle biopsy procedure and care)

Persistent Benefits of Exercise and Vitamin C in Ageing Males Mediated by Epigenetics

This sheet will provide additional information to the information already provided in the participant information sheet. It contains an overview of what a muscle biopsy is, why they are being performed in this study and most importantly, what having a biopsy will involve for you as a participant. As with the participant information sheet, it is important that before you consent to partaking in this procedure you understand and are comfortable with what is being asked of you. You are under no obligation to partake, moreover if you do give your consent you are still free to withdraw, at any point, without explanation.

What is a muscle biopsy?

A muscle biopsy is a commonly performed procedure that is used in research studies and medical diagnoses. It involves the removal of a small (no more than 150mg) piece of muscle tissue from the leg. In this study, the sample will be taken from the large muscle found at the front of your thigh.

The muscle biopsy procedure

The muscle biopsy procedure involves a small muscle sample being extracted through the skin using a sterile, hollow needle within an automated biopsy device. This allows for the biopsy to be performed with little or no discomfort.

To begin, the skin covering your thigh muscle will be carefully cleaned. A small amount of local anaesthetic (to numb the area) will be injected into and under the skin. You may experience a tingling sensation while the anaesthetic is injected. Once the area is completely numb, a small, 0.4 – 0.5 cm incision will be made in your skin in order to create an opening for the biopsy needle. You should not feel this due to the anaesthetic.

The biopsy device will then be inserted through the incision into the leg muscle which again you should be unable to feel. You will hear a clicking noise as the biopsy device is activated and you may feel a strange pulling sensation that may cause a few seconds of discomfort, this will pass very quickly. Pressure will be applied to the site to minimise any swelling or bruising deep within the muscle, this helps with the healing process. This will also stop any bleeding that may occur.

When the wound is dry, sterile strips (steri-strips) will be applied to close the incision and the area will be covered with a sterile dressing. This is to ensure that the area remains clean and to minimise the risk of infection. In the 24-48 hours after the biopsy, it is important that you keep the sterile dressing in place and dry. Therefore, it is advised that where possible you don't shower or if you do, that you apply a water tight covering to the area. If the dressing does come loose or become wet, contact the research team who can arrange to have the area re-dressed.

After the biopsy

Immediately after the biopsy you should feel no discomfort. As part of the research protocol you will be asked to exercise soon after the biopsy and again you should feel no discomfort during this period. You will be under close supervision from the research team at all times and their primary role is to ensure your safety and comfort. Following experimental exercise, you will have a second biopsy taken from the same site using the same protocol as outlined above.

After experimental exercise, you should refrain from excessive muscle (e.g. physical activity, exercise and manual labour) use for the remainder of the day. Once the anaesthetic wears off, your leg may feel a bit tight and some people have the sensation of a deep bruise. If you do experience discomfort associated with the biopsy, you may wish to use basic pain killers such as Paracetemol. It is also beneficial to periodically apply an ice pack to the biopsy site the following day, as this will help to reduce any swelling and any residual soreness. The following day your leg may feel tight and you may feel a slight discomfort when going down stairs, similar to the discomfort associated with post exercise muscle soreness experienced after a hard exercise. You should be able to comfortably exercise at normal capacity within 2 days of the biopsy.

Seven to 10 days after the biopsy you will be contacted by the research team to enquire as to how the biopsy site is healing and answer any questions you may have. You will be provided with contact details for the research team should you wish to discuss anything further in the interim.

Contact Details

Thank you for your interest in this study, and If you have any further questions or queries feel free to contact us using the following information;

PhD student: Miss Despoina Tryfidou
Email: Tryfidou-D@ulster.ac.uk

Prof. Gareth Davison – Chief Investigator
Email – gw.davison@ulster.ac.uk
Telephone – 028 90 36 6664

Sport and Exercise Sciences Research
Institute Faculty of Life and Health Science

Participant Information Sheet

Role of Endogenous Oestradiol Production on Exercise-Induced DNA Damage in a Healthy Female Cohort.

You are being invited to take part in this research study. Before you decide whether or not to take part, it is important that you understand the purpose of the research and what you will be asked to do. Please read the following information and do not hesitate to ask any questions about anything that might not be clear to you. Make sure that you are happy before you decide what to do. Thank you for taking the time to consider this invitation.

1. Background and aim of this Research

Regular physical activity is beneficial, as it improves our health, and lowers the risk of all-cause mortality, cardiovascular disease, cancer and other chronic diseases. Strenuous exercise however, has been shown to result in increased production of free radicals. These are substances, which are generated normally in most living organisms. However, excess amounts of these substances in our body that are not balanced by sufficient antioxidants can lead to a state of oxidative stress and cause damage to our DNA, which if left unrepaired, could lead to genomic instability and cancer development. There are no studies performed in females investigating the link between DNA damage following exercise and oestrogen levels at different phases of the menstrual cycle.

Aim: We plan to examine the potential antioxidant effects of oestrogen released during the normal menstrual cycle in females on DNA damage following high-intensity exercise.

2. Why have I been chosen?

You have been chosen for this study because you responded to the advertisements and completed the initial screening questionnaire. You also satisfy the admission criteria related your age, gender, health and level of activity.

3. Do I have to take part?

No, participation is completely voluntary. It is up to you to decide whether or not you want to take part. If you do decide to take part, you will be given this information sheet to keep. You will also be asked to sign a consent form. If you choose to take part, you can change your mind and withdraw from the study at any time without giving a reason.

4. Procedures

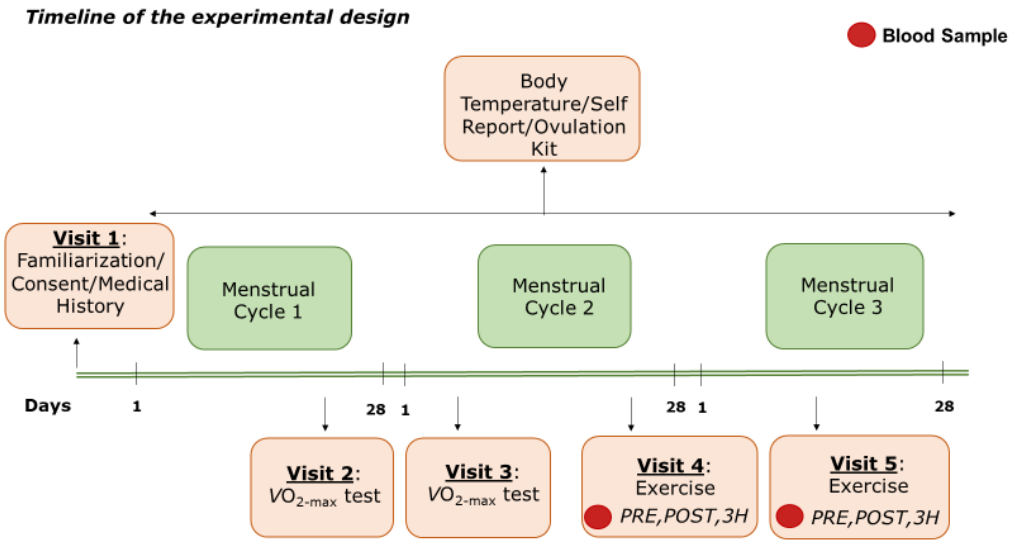
You will be required to complete an overnight fast (circa 8 hours) prior to experimental testing and asked to wear appropriate footwear and clothing for exercising. This may include running trainers, a loose t-shirt/vest and a pair of comfortable legging/shorts. Data collection for the study will last 3 months, but you will not be needed in the lab every day. You will be asked to attend Ulster University Jordanstown on 5 separate occasions across the 3 months.

Stage One - Monitoring of Menstrual Cycle and Preliminary Testing: You will attend the Ulster University Human Performance Lab to provide informed consent and complete a medical history questionnaire and to be familiarised with the study protocol (**Visit 1**). At this stage, your height and weight will be recorded. You will then be asked to self-report the current day of your menstrual cycle along with the dates of your last menstrual cycle. Your next 3 menstrual cycles will be monitored from that point on to assess integrity/accuracy of your menstrual cycle. You will be given a digital body thermometer and be instructed to record body temperature orally every day before arising for their next 3 consecutive menstrual cycles as well as an ovulation/urine kit to use around days of suspected ovulation in each cycle and this is to confirm days of ovulation for the same 3 consecutive cycles.

You will then be instructed to perform two separated maximal exercise tests ($\dot{V}O_{2\text{-max}}$ tests), one on day 26-28 (**Visit 2**) and one on day 11-13 (**Visit 3**) and of your cycle corresponding to the late luteal phase (LLP) and the late follicular phase (LFP) of the menstrual cycle respectively. The LFP is when the body prepares for ovulation and a mature egg is released; this phase is oestrogen is dominant. The LLP is progesterone dominant and it occurs when the egg starts traveling down the fallopian tube. Progesterone thickens the lining of the uterus so that a fertilized egg can implant. If the egg is not fertilized, oestrogen and progesterone levels start to drop, the uterine lining will start to shed again, and the entire cycle repeats. $\dot{V}O_{2\text{-max}}$ during running will be determined during a continuous, graded exercise test on a treadmill following the Bruce Protocol where you begin running at 1.7 mph and 10% grade and increasing 0.8**–**1.0 mph and 2% grade every 3 min until voluntary cessation. During the test, your oxygen uptake will be monitored using an online gas analysis system.

Stage Two – Experimental Testing: On the end of cycle 2 during the LLP you will be asked to run on a treadmill at 80% of your predetermined $VO_{2\text{-max}}$ until volitional exhaustion. Initially, as part of a warm up, you will exercise lightly in order to reach 80% of your $\dot{V}O_{2\text{-max}}$. As soon as this is achieved, you will then be asked to maintain that pace until volitional exhaustion and provide venous blood samples immediately before, immediately following and 3 hours post-exercise (LLP-Trial; **Visit 4**). The exact same experiment will be repeated on the middle of cycle 3 during the LFP of your menstrual cycle (LFP-Trial; **Visit 5**). You will also be asked to record a 2-day food diary before the LLP-Trial and instructed to repeat the same meals 2 days prior to the LFP-Trial to control for energy intake.

Below is a diagram visualizing the timeline of all required visits and the overall study protocol.



5. What do I have to do?

You cannot take any antioxidant supplementation such as multivitamins or any other supplements such as creatine, BCAAs, pre-workout, fish oils/omega-3s for the duration of the study.

6. Risks & Benefits to Participants

By taking part in this study you are potentially helping researchers to investigate the link between DNA damage after exercise and oestrogen levels at different phases of the menstrual cycle, which has never been assessed in females. You can also find out about your individual health and fitness levels. Upon completion of the study, you will also receive a gift card worth £25 for your time. You can choose a gift card from either Amazon OR Gymshark.

With all scientific studies, there are a number of risks and/or hazards that could potentially occur during testing. These include muscular injury, heart complications, nausea, fainting, bacterial/viral infection, blood borne diseases and bruising and/or discomfort from blood or tissue sampling. It should be understood by the participant that the university and research team have taken the necessary actions to minimise the probability of these risks occurring. However, it should be noted that nausea/vomiting and discomfort from blood and tissue sampling may occur. It is important that if you experience any of these that you let a member of the research team know as soon as possible. There will always be one member of the research team present who is qualified in providing first aid; Dr. John Brown is the Health and Safety Coordinator. You will be asked to complete a medical history
questionnaire. This information will be kept confidential and will only be used to assess whether you can participate in the study.

Note; if there are any changes in circumstances and/or medical status between completing the medical risk form and the beginning of the study, you should let a member of the research team know immediately.

7. What is something goes wrong during the study?

This study has been approved by the University of Ulster ethics committee and accordingly measures are in place to ensure there is no or minimal risk of anything going wrong during the course of this study. In the very unlikely case that something goes wrong during the study, it is important to understand that the university has procedures in place for reporting, investigating, recording and handling adverse events. Any complaints will be taken seriously and should be made in the first instance to the Chief Investigator and/or the University. The University will provide an indemnity statement for research that has been approved through the appropriate governance and ethical review processes.

If you have any further queries on this do not hesitate to contact the University Research Governance. (Nick Curry, Head of Research Governance; email: n.curry@ulster.ac.uk ; Tel: +44 28 9036 6629)

8. What happens when the study ends?

Once the study ends, you will no longer be required as a participant. If you have any questions or queries regarding the study once it is completed or if you are interested in finding out the outcome of then do not hesitate to ask the researchers involved.

9. GP Referral and Data Protection

With your given consent and due the possibility of an abnormal blood profile (i.e abnormal levels of hormones measured), we will contact you, and your GP for a follow up review. If you have not given consent for contacting your GP, the information will be sent to you only and you will be advised to contact your GP personally. All data collected will be treated with the strictest of confidence, according to the principles outlined in the General Data Protection Regulation (GDPR; 2018). All data will be held in confidence and securely stored using a coding system rather than your name or initials. However, in the unlikely event that during the course of the investigation any underlying health concerns are disclosed, you will be encouraged to share this information with your GP.

Data will be stored on file for 10 years in accordance with University Policy. This means all data will be put into a secure, password-locked electronic file and stored within Ulster University. Only the investigators associated with study will have access to data. If someone was to gain access to data, all information regarding participant identity will remain anonymous. You can find out more about how we look after your information at: https://www.ulster.ac.uk/about/governance/compliance/gdpr

If you wish to raise a complaint on how we have handled your personal data, you can contact our Data Protection Officer who will investigate the matter. If you are not satisfied with our response or believe we are processing your personal data in a way that is not lawful you can complain to the Information Commissioner's Office (ICO).

Our Data Protection Officer is Eamon Mullan; you can contact him at e.mullan@ulster.ac.uk.

10. Contact Details

Thank you for your interest in this study, and If you have any further questions or queries feel free to contact me using the following information;

Prof. Gareth Davison **–** Chief Investigator
Email: gw.davison@ulster.ac.uk
Telephone: +44 28 90 36 6664

Miss Despoina Tryfidou **–** PhD Student Researcher
Email- Tryfidou-D@ulster.ac.uk

Participant Information Sheet

The biochemical response to exercise induced oxidative stress in hypoxia and normoxia
What are the effects of exercising at altitude?

You are invited to take part in a research study which is being conducted within the Sports Academy at the University of Ulster as part of a PhD research project. This document is designed to explain the purpose of the research project as well as what would be involved for you as a participant should you decide to participate. Please read the following information clearly as it is important that you understand what the research is for and what you will be asked to do before you decide to partake. If there are any aspects that you are unclear about, please do not hesitate to ask to ensure that if you do decide to partake you are happy to do so. Thank you for taking the time to consider this invitation.

**Where muscle biopsies are mentioned, please ignore. There are NO biopsies involved in this study.*

What is the purpose of the study?
This study has been designed to investigate how exercising either with normal levels of oxygen (as seen at sea level) or with reduced oxygen (as seen at an altitude of 7000ft) affects the body's response to exercise.

During exercise your body uses more oxygen. The change in the requirements and availability of oxygen within your body during exercise can cause an exercise induced stress which may cause physiological adaptations. Accordingly, as a result of repeated exercise training people become fitter and better able to exercise for longer and at a higher intensity. Furthermore, adaptations to exercise can also help to protect against the effects of ageing and poor health, in particular exercise may aid in the prevention and moderation of cardiovascular disease, obesity and diabetes.

In exercise at altitude the body experiences a greater amount of exercise induced stress due to lower oxygen availability. The larger exercise induced stress may cause greater or different adaptations to exercise than would be seen following exercise at sea level. Moreover, it may also result in a greater ability to respond to and offset the stress imposed by ill health and disease. Conversely, altitude may impose too much stress for the body to and have a negative effect on exercise response.

Accordingly, this research project will aim to measure the physiological stress induced by exercise both at sea level and at altitude as well as any adaptive response initiated.

Why have I been asked to take part?
This study requires who are fit, healthy and endurance trained who would be able to maintain a steady running pace of moderate intensity for an hour. You have been asked because you and have good aerobic fitness and are able to exercise in this manner.

Do I have to take part?
No, this is an invitation to take part, you are under no obligation to reply to this invitation, to take part or to provide a reason if you decide not to partake. If you do decide to take part you are also free to change your mind and withdraw from the study at any time again without providing any reason for doing so.

What will happen to me if I take part?
You will be asked to complete a VO_2 peak test on a treadmill to estimate a running speed that corresponds to 75% of your maximal effort. You will be asked to return to the lab up to a week later to complete an hour running challenge at this speed. Both these tests will be completed twice, once at sea level and once at simulated moderate altitude of 7000ft. this level of altitude may reduce your exercise capacity however it is below the threshold associated with the onset of acute mountain sickness (AMS) or imposing a risk to your health. The full protocol is explained in more detail below.

Visit one: VO_2 peak test and Introduction (40 minutes- one hour and five minutes)

Introduction

You will be invited to come to our sports performance laboratory at the University of Ulster, Jordanstown to meet the researcher and discuss study participation and ensure you are happy to partake in this study. If you are happy to proceed you will be asked to complete a health history questionnaire and consent form. You will also be provided with a four day diet diary which you will be asked to complete for a four day period representative of your typical diet in between visits.

VO_2 peak test

The VO_2 peak test is a test of maximal aerobic capacity which will be completed in our environmental chamber. You will be asked to run on a treadmill at a starting speed of 8-10km/h from which the speed will be increased by 1km/h every 2 minutes until you feel you can no longer continue to exercise. This is a maximal test and as such you will be encouraged to push yourself as hard as you can. During the test you will be asked to wear a mask which will cover your mouth and nose which will allow us to measure the amount of oxygen you consume when you exercise.

Visit two: Experimental day one (2.5 - 3 hours)

Preparation and baseline measures

You will be asked to return to the lab for a second time 48 hours - one week after the VO_2 peak test at between 08.00 and 09.30 am in the morning following an eight hour overnight fast. In the 24 hours prior to this session you will be asked to refrain from any strenuous exercise training or alcohol consumption.

Your height, weight, heart rate, blood pressure and blood oxygen saturation will all be measured. You will then have a venous blood sample taken from your forearm by a trained phlebotomist. A finger prick capillary sample will also be taken.

Altitude exposure

You will then be asked to rest for 30 minutes in the altitude chamber. This will either be set to a simulated altitude of 7000ft (16% oxygen in the air) or sea level (21% of oxygen in the air). You will not be told which condition you are in as this may affect your exercise performance. Your heart rate and oxygen saturation will be re-measured and another blood sample will be collected from forearm as well as a second finger prick sample

Exercise

You will be asked to run for one hour at 75% of your maximal capacity. The running speed will be calculated from your VO_2 peak test and adjusted to ensure that you maintain at the correct work rate. Every 15 minutes the run will be paused for up to two minutes for collection of a finger prick sample and to measure your oxygen saturation.

Post exercise

Post exercise blood samples, and biopsies if applicable, will be taken immediately after you finish running.
You are then finished with the exercise part of the session for this day however you will be required to provide one more blood sample (and muscle sample if applicable) three hours after the cessation of exercise. You will not be able to eat until after the final blood sample. For this period you may leave and come back, or if you would prefer there will be a room made available for you with a computer and internet access in which you can do work, surf the net or watch online TV.

Visit three: VO_2 peak test (30 - 45 minutes)

You will be asked to return to the lab for a third time to do a second VO_2 peak test. This will follow the same outline as visit one, with the only difference being that the oxygen content of the air will be different. If during your first test you were exposed to a simulated altitude of 7000ft (16% oxygen in the air) then for the second test you will be exposed to a sea level environment (21% of oxygen in the air) and vice versa. You will not be told which environmental condition you are in.

Visit four: Experimental day two (2.5 - 3 hours)
You will be asked to return to the lab for fourth and final time between 48 hours and one week after the second VO_2 peak test. All procedures will be the same as during visit two, with the exception that the environmental condition will be different. Of note, the exercise intensity that you work at will also be different as will be calculated from the second VO_2 peak test.

Are there any risks or disadvantages that may occur as a result of taking part?
The exercise intervention is low risk as is similar to the exercise training you may do as part of a normal training regime. The reduced oxygen availability will likely reduce the exercise capacity and may result in a participant feeling unwell however, should this happen it can be rectified immediately through removal from the simulated altitude and supine rest.

There is a very low risk of a cardiovascular event occurring during the maximal exercise test, in the highly unlikely event of this occurring the investigator present is trained in first aid and will have access to a defibrillator.

The individuals who are in the biopsy group may have some leg pain the following day and will be strongly advised not partake in any exercise for 24 hours, regardless of pain or not. Individuals who are in the biopsy group will be provided with an additional information sheet regarding the biopsy procedure.

Are there any possible benefits that may occur as a consequence of taking part?
As a participant you will complete a VO_2 peak test which is the gold standard method to measure aerobic fitness used be elite and professional sportsmen and women worldwide. On completion of the study you will be given information on your fitness including your VO_2 peak score which will allow you to see your current fitness level and may be used to tailor your individual training intensities.

What if something goes wrong?
This study has been approved by the University of Ulster ethics committee and accordingly measures are in place to ensure there is no or minimal risk of anything going wrong during the course of this study. However, in the unlikely event that you feel there are any problems or aspects of the study that you are not happy with please discuss these with the research team who are working with you or the chief investigator Dr Davison (contact details below).

Will my taking part be kept confidential?
Yes, all data will be kept in confidence and stored using a coding system rather than your name or initials and any identifying information will be removed prior to publication in accordance with the data protection legislation. However, in the unlikely event that during the course of the investigation any underlying health concerns are disclosed you will be encouraged to share this information with your GP.

Storage of tissue sample and the Human Tissue Act
All samples will be stored recorded and destroyed in accordance with HTA regulations. Samples will only be used in analysis for which you provide consent. If you choose to provide study specific consent only the analysis proposed and outlined above will be conducted, after which they will be destroyed. If you choose to provide enduring consent your samples may be used for additional analysis subject to further ethical approval. Further information regarding the human tissue act can be made available on request.

What will happen to the results of the study?
The results of this study will be used in the thesis for the attainment of a PhD. They will also be submitted for publication in a peer reviewed journal.

Who has reviewed this study?
This study has been reviewed by the University of Ulster Research Ethics Committee. If you would like further information on this process either in general or specifically with regards to this study, please contact Nick Curry (contact details below).

Thank you for taking the time to consider this invitation to partake in this research project. If you are interested in taking part please contact Despoina Tryfidou to arrange a time and date to attend the lab.
Contact details

Name	Role	Contact details
Despoina Tryfidou	**Principle researcher, contact with any questions regarding research participation and if you are interested in taking part in this investigation**	**Tryfidou-D@ulster.ac.uk** **Tel: 028 366 66987** **15C09, Ulster Sports Academy, University of Ulster, Jordanstown, BT37 0QB**
Prof Gareth Davison	Chief investigator	Davison.G@ulster.ac.uk Tel: +44 28 90366664 15C09, Ulster Sports Academy, University of Ulster, Jordanstown, BT37 0QB
Nick Curry	Research and governance senior administrative office University of Ulster.	n.curry@ulster.ac.uk Tel: +44 28 90366629 Room 01H12 Research Office University of Ulster Jordanstown campus Shore Road, Newtownabbey, Co. Antrim, BT37 0QB

Appendix D

Informed Consent

Sport and Exercise Sciences Research Institute

Faculty of Life and Health Science

Consent Form

Please Initial

- I confirm that I have been given and have read and understood the information sheet for the above study and have asked and received answers to any questions raised [ ]
- I understand the nature and risks associated with blood and tissue extractions. I confirm and consent for blood and tissue collection and storage of the stated amounts. [ ]
- I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason and without my rights being affected in any way [ ]
- I understand that the researchers will hold all information and data collected securely and in confidence and that all efforts will be made to ensure that I cannot be identified as a participant in the study (except as might be required by law) and I give permission for the researchers to hold relevant personal data [ ]
- I agree to take part in the above study [ ]
- The potential benefits of keeping my blood or other tissues for future research studies have been explained to me and **(please read carefully and choose only *one*):**

  a. I consent to their indefinite storage and use in any future study pending further ethical approval, **or** [ ]

  b. I consent to their indefinite storage and use in any future study that does not involve the use of my genetic material; **or** [ ]

  c. I do not wish my blood or tissues to be used for any purpose other than this study [ ]

I (name)

.....

of (address)

.....

hereby consent to take part in the above investigation, the nature and purpose of which have been explained to me. Any questions I wished to ask have been answered to my satisfaction. I understand that I may withdraw from the investigation at any stage without being required to give a reason for doing so.

Signed (Volunteer)

..... Date .....

(Investigator)

..... Date .....

Appendix E

Health History Questionnaire

HEALTH HISTORY QUESTIONNAIRE

Any information contained herein will be treated as confidential

Please answer all questions. Circle appropriate answer

Name:_____
Date of Birth:_____/_____/_____

Medical History

Personal History

1.

Have you ever fainted or passed out when exercising?	Yes	No
Do you ever have chest tightness?	Yes	No
Does running ever cause chest tightness?	Yes	No
Have you ever had chest tightness, cough or wheezing that made it difficult for you to perform in sports?	Yes	No
Do you have trouble breathing or do you cough during or after activity?	Yes	No
Have you ever been dizzy during or after exercise?	Yes	No
Have you ever had chest pain during or after exercise?	Yes	No
Do you have or have you ever had racing of you heart or skipped heartbeats?	Yes	No
Have you ever been told you have a heart murmur?	Yes	No
Do you get tired more quickly than your friends do during exercise?	Yes	No
Have you ever been told you have a heart arrhythmia?	Yes	No
Do you have any other history of heart problems?	Yes	No
If you have answered YES to any of the above please give details:		

2.

Have you ever had a seizure?	Yes	No
Have you ever been told:-		
You have epilepsy?	Yes	No
To give up sports because of health problems?	Yes	No
You have high blood pressure?	Yes	No
You have high cholesterol?	Yes	No

You have had rheumatic fever?	Yes	No
You have lung disease?	Yes	No
You have diabetes?	Yes	No
You have thyroid disease?	Yes	No
Have you ever been treated/hospitalised for asthma?	Yes	No
Do you have any allergies?	Yes	No
Have you had a severe viral infection (e.g. myocarditis or mononucleosis) within the last month?	Yes	No
Are you taking any medication at the present time?	Yes	No
Have you routinely taken any medication in the past two years?	Yes	No
If you have answered YES to any of the above please give details:		

3.

Have you had to consult with your doctor within the last six months?	Yes	No
If YES, please give details of reasons, which may affect your participation in the test(s)		

4.

Do you currently have any form of muscle or joint injury?	Yes	No
If YES, please give details:		

5.

Have you had any reason to suspend your normal activity in the past two weeks?	Yes	No
If YES, please give details:		

6.

Family History Has anyone in your family < 50 years of age:		
Died suddenly and unexpectedly?	Yes	No
Being treated for recurrent fainting?	Yes	No
Had unexplained seizure problems?	Yes	No
Had unexplained drowning while swimming?	Yes	No
Had unexplained car accident?	Yes	No
Had heart transplantation?	Yes	No
Had pacemaker or defibrillator implanted?	Yes	No
Had heart surgery?	Yes	No
Experienced sudden infant death (cot death)?	Yes	No

Told they have Marfan Syndrome?	Yes	No
If you have answered YES to any of the above please give details:		

7.

Is there anything to your knowledge that may prevent you from successfully completing the task(s) that have been explained to you?	Yes	No
If YES, please give details:		

Signature of Participant_____Date_____/_____/_____

Signature of Test Supervisor_____Date_____/_____/_____

Please supply the name, address and telephone number for an emergency contact

Appendix F

Borg Scale

6	No exertion
7	
8	
9	
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard (heavy)
16	
17	Very hard
18	
19	
20	Maximal exertion

Appendix G

Supplementary Material

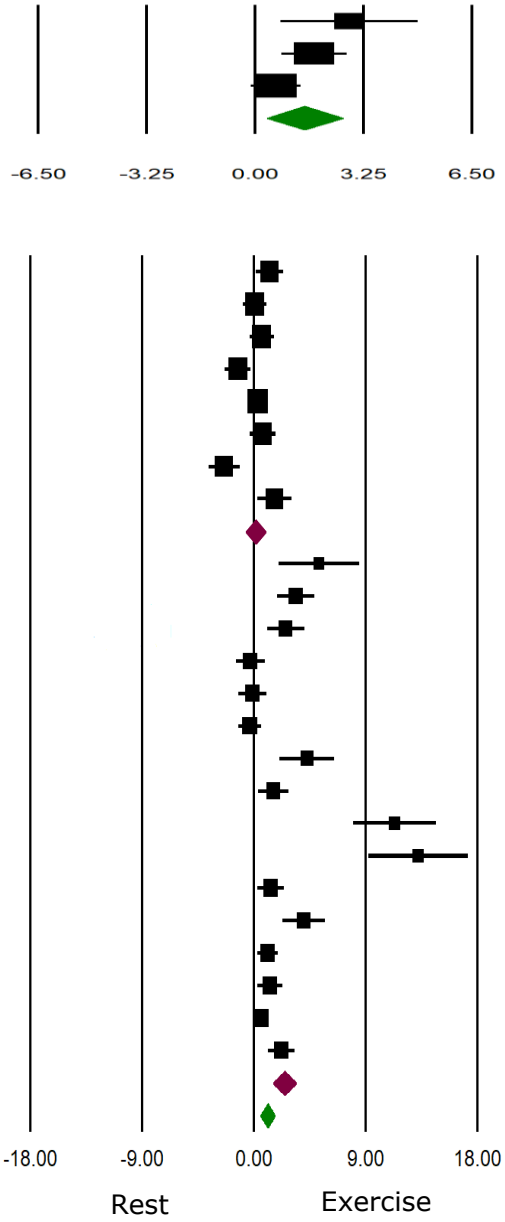
Supplementary Table S1. Studies excluded with reasons.

Study	Reasons for exclusion
Díaz-Castro *et al*. (2012)	The study used urine sampling to report DNA damage.
Mota *et al*. (2010)	No exercise protocol was implemented; only measures of aerobic fitness through $VO_{2\text{-max}}$ test.
Mergener *et al*. (2009)	No exercise protocol was implemented; only physical activity levels reported.
Atli *et al*. (2013)	Exercise protocol was a 3-day football tournament; neither specification of duration nor intensity.
Shockett *et al*. (2016)	Study used cell-free mitochondrial DNA.
Cash *et al*. (2014)	No exercise protocol was implemented; only physical activity levels reported.
Leonardo-Mendonça *et al*. (2014)	Exercise protocol included multiple training days.
Kim *et al*. (2010)	Exercise protocol was a 9-week training program.
Tomasello *et al*. (2012)	No exercise protocol was implemented.
Fogarty *et al*. (2013)	Exercise was 100 isolated knee extension contractions.
Sarmiento *et al*. (2016)	Exercise protocol consisted of 10 resistance type body-building strenuous exercises.
Cuevas *et al*. (2005)	Exercise was anaerobic.
Mrakic-Sposta *et al*. (2015)	Capillary blood was used.
Bloomer *et al*. (2005)	Exercise protocol consisted of both aerobic and anaerobic exercise.
Gray *et al*. (2014)	Exercise was 200 repetitions of eccentric knee contractions.

Supplementary Table S1. Continued

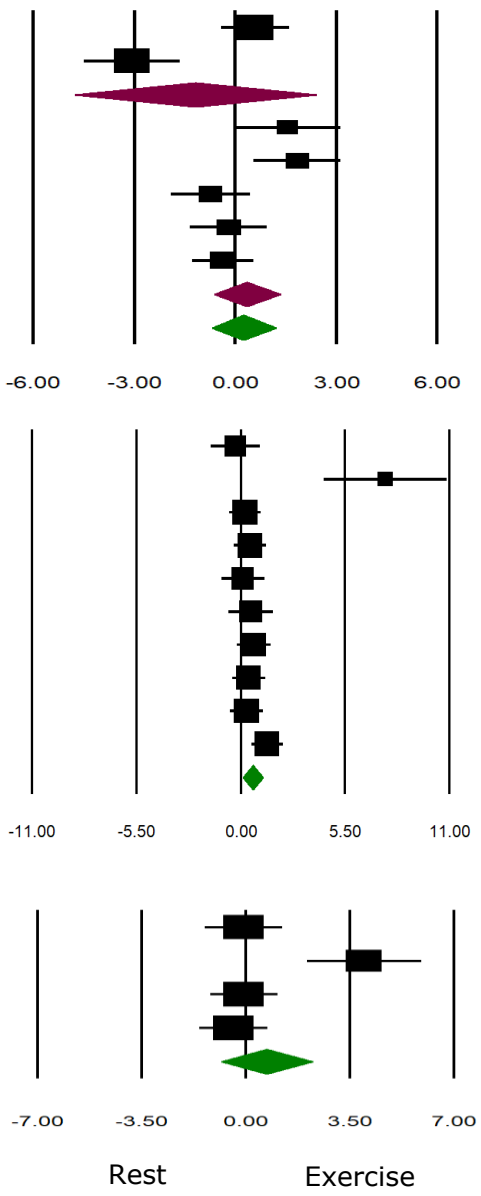
Study	Reasons for exclusion
Shockett *et al*. (2016)	Plasma cell-free mitochondrial DNA was used.
Atamaniuk *et al*. (2004)	Plasma cell-free DNA concentrations were used.
Arazi *et al*. (2015)	Exercise protocol consisted of traditional vs. cluster resistance exercise loading patterns.
Ra *et al*. (2013)	Exercise was eccentric elbow flexor exercises.
Palazzetti *et al*. (2003)	Exercise involved 4 weeks of overload training.
Giacomo *et al*. (2009)	No exercise protocol was implemented.
Demirbağ *et al*. (2005)	Participants were referred for evaluation of suspected coronary artery disease.
Neubauer *et al*. (2010)	Reported the same population as in another study included in the review.

	Study	Method	n	SMD (Hedges' g)	P-Value	RW(%)
a	Hartmann et al. (1994)		3	2.817	0.007	19.23
	Zhang et al. (2004)		11	1.772	0.000	37.97
	Tanimura et al. (2008)		14	0.621	0.099	42.80
	Overall	Comet	28	1.480	0.010	100
	Heterogeneity: Chi^2 = 6.13, df = 2 (P = 0.047); I^2 = 67.37%					
	Test for overall effect: Z score = 2.58 (P = 0.01)					
b	Pittaluga et al. (2013)		7	1.279	0.021	12.16
	Sacheck et al. (2003), young		8	0.090	0.849	13.03
	Sacheck et al. (2003), old		8	0.649	0.182	12.89
	Itoh et al. (2006)		8	-1.290	0.014	12.49
	Saritas et al. (2011)		22	0.347	0.245	14.69
	Sato et al. (2003), active		7	0.707	0.172	12.56
	Sato et al. (2003), sedentary		8	-2.372	0.000	11.35
	Shi et al. (2007)		5	1.673	0.014	10.82
	Subtotal	8-OHdG	73	0.141	0.720	100
	Hartmann et al. (1994)		3	5.250	0.001	3.94
	Hartmann et al. (1995)		8	3.383	0.000	6.46
	Hartmann et al. (1998)		6	2.583	0.001	6.50
	Mastaloudis et al. (2004), m		5	-0.261	0.650	6.99
	Mastaloudis et al. (2004), f		5	-0.101	0.860	7.00
	Møller et al. (2001)		9	-0.305	0.499	7.29
	Niess et al. (1996), u		5	4.285	0.000	5.39
	Niess et al. (1996), t		6	1.586	0.011	6.86
	Sardas et al. (2012), rowers		12	11.335	0.000	3.86
	Sardas et al. (2012), PE s		11	13.253	0.000	3.12
	Tanimura et al. (2010), u		8	1.380	0.009	7.10
	Tanimura et al. (2010), t		8	4.019	0.000	6.19
	Tsai et al. (2001)		14	1.127	0.004	7.40
	Turner et al. (2011)		9	1.329	0.008	7.17
	Wagner et al. (2010)		28	0.606	0.025	7.62
	Zhang et al. (2004)		11	2.232	0.000	7.10
	Subtotal	Comet	148	2.468	0.000	100
	Overall		221	1.113	0.000	
	Heterogeneity: Chi^2 = 14.58, df = 23 (P = 0.000); I^2 = 88.34%					
	Test for overall effect: Z score = 3.71 (P = 0.000)					



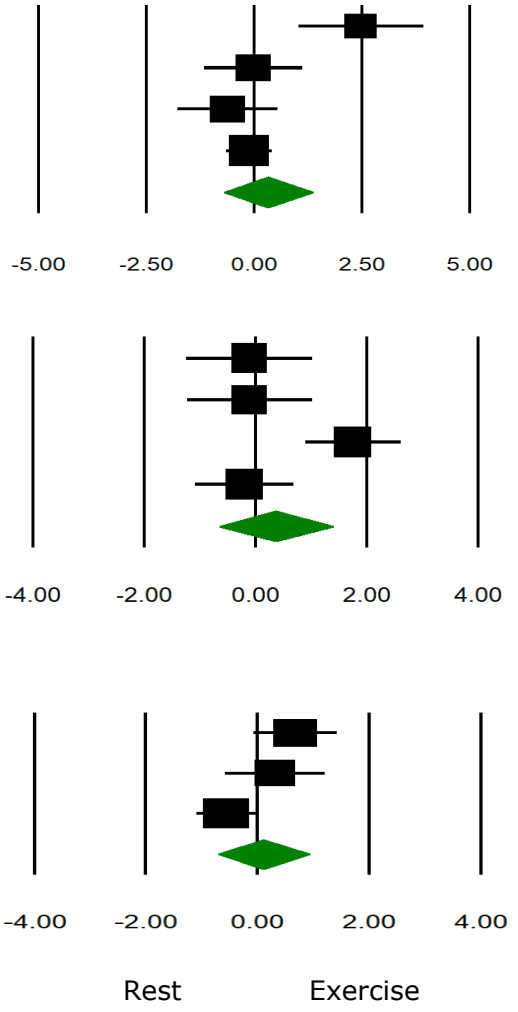
Supplementary Figure S2. Relative weight (RW) standardised mean difference (SMD) and 95% CI (Hedges' g adjusted) of DNA damage compared between rest and after an exercise bout at **(a)** time-point 4 (4-6h) and **(b)** time-point 5 (1d). Values for individual trials and pooled data (Random Model) are shown and grouped by method of quantification. Abbreviations: m, males; f, females; u, untrained; t, trained; PE s, physical education students.

	Study	Method	n	SMD (Hedges' g)	P-Value	RW(%)
a	Sato et al. (2003), active		7	0.585	0.254	50.96
	Sato et al. (2003), sedentary		8	-3.071	0.000	49.04
	Subtotal	8OHdG	15	-1.208	0.509	100
	Hartmann et al. (1994)		3	1.555	0.050	16.58
	Hartmann et al. (1998)		6	1.848	0.005	19.18
	Mastaloudis et al. (2004), m		5	-0.738	0.214	20.33
	Mastaloudis et al. (2004), f		5	-0.190	0.741	20.76
	Møller et al. (2001)		9	-0.369	0.416	23.15
	Subtotal	Comet	28	0.338	0.497	100
	Overall		43	0.231	0.630	
	Heterogeneity: Chi^2 = 0.66, df = 6 (P = 0.41); I^2 = 82.17%					
	Test for overall effect: Z score = 0.48 (P = 0.63)					
b	Hartmann et al. (1994)		3	-0.284	0.666	8.39
	Hartmann et al. (1998)		6	7.625	0.000	2.36
	Kim et al. (2018), olympic c		11	0.221	0.591	11.92
	Kim et al. (2018), O2 c		11	0.485	0.245	11.84
	Mastaloudis et al. (2004), m		5	0.112	0.845	9.53
	Mastaloudis et al. (2004), f		5	0.531	0.363	9.37
	Ryu et al. (2016), 10km		10	0.684	0.122	11.45
	Ryu et al. (2016), 21km		10	0.406	0.349	11.59
	Ryu et al. (2016), 42km		10	0.307	0.476	11.62
	Tsai et al. (2001)		14	1.384	0.001	11.93
	Overall	Comet	85	0.627	0.022	100
	Heterogeneity: Chi^2 = 25.98, df = 9 (P = 0.002); I^2 = 65.36%					
	Test for overall effect: Z score = 2.29 (P = 0.022)					
c	Hartmann et al. (1994)		3	-0.071	0.913	25.59
	Hartmann et al. (1998)		6	3.970	0.000	21.11
	Mastaloudis et al. (2004), m		5	-0.065	0.909	26.70
	Mastaloudis et al. (2004), f		5	-0.419	0.469	26.60
	Overall	Comet	19	0.691	0.380	100
	Heterogeneity: Chi^2 = 16.58, df = 3 (P = 0.001); I^2 = 81.91%					
	Test for overall effect: Z score = 0.88 (P = 0.38)					



Supplementary Figure S3. Relative weight (RW) standardised mean difference (SMD) and 95% CI (Hedges' g adjusted) of DNA damage compared between rest and after an exercise bout at **(a)** time-point 6 (2d), **(b)** time-point 7 (3d) and **(c)** time-point 8 (4d). Values for individual trials and pooled data (Random Model) are shown and grouped by method of quantification. Abbreviations: c, course; m, males; f, females.

	Study	Method	n	SMD (Hedges' g)	P-Value	RW(%)	Random Effects Model (95% CI)
a	Hartmann et al. (1998)		6	2.468	0.001	20.44	
	Mastaloudis et al. (2004), m		5	-0.025	0.965	24.27	
	Mastaloudis et al. (2004), f		5	-0.616	0.294	23.87	
	Wagner et al. (2010)		28	-0.117	0.657	31.42	
	Overall	Comet	44	0.315	0.548	100	
	Heterogeneity: Chi^2 = 12.58, df = 3 (P = 0.006); I^2 = 76.15%						
	Test for overall effect: Z score = 0.6 (P = 0.548)						
b	Mastaloudis et al. (2004), m		5	-0.116	0.839	23.49	
	Mastaloudis et al. (2004), f		5	-0.108	0.850	23.50	
	Tsai et al. (2001)		14	1.745	0.000	26.68	
	Turner et al. (2011)		9	-0.205	0.649	26.33	
	Overall	Comet	33	0.359	0.491	100	
	Heterogeneity: Chi^2 = 12.92, df = 3 (P = 0.005); I^2 = 76.78%						
	Test for overall effect: Z score = 0.69 (P = 0.491)						
c	Tsai et al. (2001)		14	0.679	0.073	32.78	
	Turner et al. (2011)		9	0.314	0.487	29.34	
	Wagner et al. (2010)		28	-0.555	0.039	37.88	
	Overall	Comet	51	0.105	0.801	100	
	Heterogeneity: Chi^2 = 7.9, df = 2 (P = 0.02); I^2 = 74.67%						
	Test for overall effect: Z score = 0.25 (P = 0.801)						

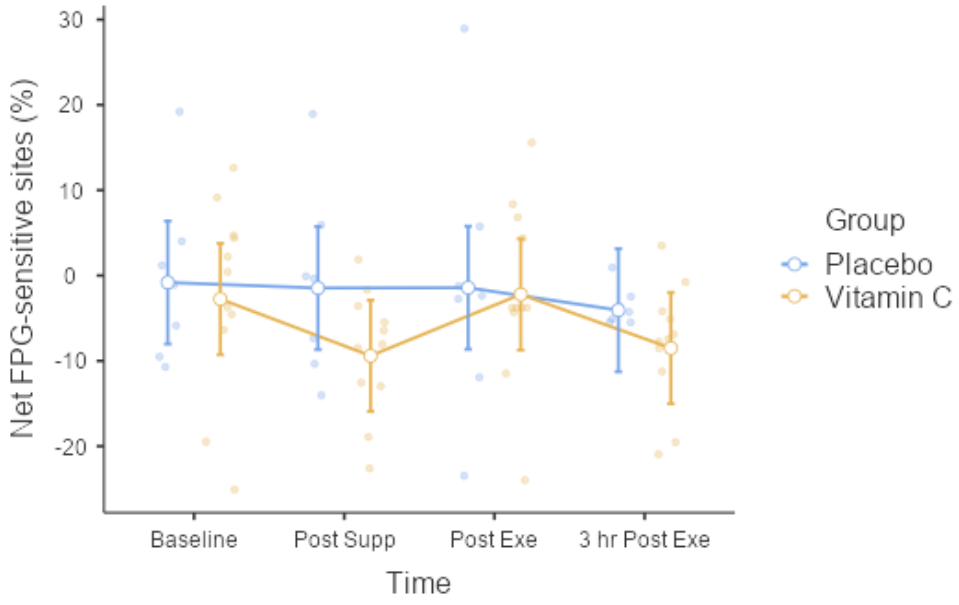


Supplementary Figure S4. Relative weight (RW) standardised mean difference (SMD) and 95% CI (Hedges' g adjusted) of DNA damage compared between rest and after an exercise bout at **(a)** time-point 9 (5d), **(b)** time-point 10 (6d-7d) and **(c)** time-point 11 (14d-28d). Values for individual trials and pooled data (Random Model) are shown and grouped by method of quantification. Abbreviations: m, males; f, females.

The use of the FPG enzyme in combination with the comet assay allows for detection of oxidative DNA base damage as it detects oxidized purines, specifically 8-oxoguanine, one of the most common DNA lesions. The net FPG-sensitive sites are calculated by subtracting the DNA damage in the nucleoids incubated with the buffer from the damage in the presence of the FPG (Azqueta & Collins, 2014) with the outlined calculation below (damage expressed as % in terms of the extent of migration of DNA into the comet tail):

NET FPG-sensitive sites = % tail DNA damage FPG - % tail DNA damage BUFFER

Presented in **Supplementary Figure S5** are the results from chapter 5.



Supplementary Figure S5. NET FPG-sensitive sites (% tail intensity) at baseline, post-supplementation, post-exercise and 3 hours post-exercise across placebo (n = 8) and AA groups (n = 10). Data expressed as EMM (95% CI).

The data appears non-accurate and thus unreliable as the expected values should not consist of minus values. The concentration of FPG when performing the assay is a critical step, as it should be enough to detect all the lesions present in the set incubation time while avoiding interference from nonspecific nucleases (Azqueta & Collins, 2014). It is suspected that

this step caused inaccurate results due to lab and human error. Initially, it was not possible to identify the initial concentration of FPG aliquots correctly and confidently (stored at -80 °C) prior to starting the experiment which resulted to wrong dilutions and concentrations. Thus, FPG aliquots were most likely diluted to the wrong concentration prior to the incubation step of the assay which produced confusing minus values throughout most samples.

7.2.3.5 Microarray Analysis

The pre-existing data set was analysed with microarray technology. Briefly, cRNA was extracted and placed onto a DNA microarray (whole oligonucleotide genome, Agilent, UK) for 17 hours to facilitate binding with its synthetic DNA counterpart. This process results in a fluorescent signal or tag, which is detected by a microarray scanner (DNA microarray, Agilent, UK). The strength of the colour signal on each spot indicates the abundance of RNA in the sample. The raw microarray image files obtained from the scan were processed using feature extraction software (Feature extraction software, 10.5, Agilent, UK) to detect and quantify spots, as well as to flag any quality issues. The resulting data was imported into GeneSpring (GeneSpring GX, Agilent, UK) for analysis, which includes normalization, quality control checks against control metrics, filtering (by flags; present or marginal), grouping (by experimental condition), and statistical analysis (paired T-test with Benjamini & Hochberg False Discovery Rate corrections for multiple comparisons). Genes that were modified by more than 1.5-fold are reported, in accordance with the default settings provided by GeneSpring (McGovern, 2015).