THE UNIVERSITY OF HULL

The effect of high – intensity intermittent exercise on biomarkers of oxidative stress.

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Abstract

There are evidence that high-intensity acute exercise can promote oxidative stress. High-intensity intermittent exercise (HIIE) is a type of structured physical training characterised by repeated bouts of high-intensity exercise interspersed by recovery periods. As the impact of intermittency during acute HIIE has not been extensively studied, it is possible that the repeated intensive bouts within HIIE could induce oxidative stress levels. Plasma biomarkers, including lipid hydroperoxides and markers of DNA damage, have been increasingly applied within acute exercise physiology research to measure oxidative stress. This thesis presents the experimental outcomes of research into the effect of different forms of HIIE on established and novel biomarkers of oxidative stress. For the 1st study (chapter 3) a liquid chromatography-mass spectrometry (LC-MS) method was developed and optimised to measure DNA oxidation in plasma samples. Implementing a range of progressive analytical techniques, the method developed had a sensitivity to detect 8-hydroxy-2'-deoxyguanosine (8-Oxo-dG) in human plasma samples in the range of 5 – 500 nM. The implementation of this LC-MS method along with other oxidative stress biomarkers was thereafter applied to a randomised investigation of different high-intensity intermittent exercise protocols. The 2nd (chapter 4) and 3rd (chapter 5) studies considered the effect of different forms of intermittent exercise undertaken by 9 healthy, regularly active male participants (aged 21.0 ± 3.0 years). Each intermittent exercise session was performed for a total duration of 45 minutes. Each 45 minute exercise protocol consisted of 4 minute stages of high-intensity intermittent running at a mean 75% v$\dot{V}O_{2max}$, followed by 1 minute of passive recovery (halt of running). The experimental protocol

was specifically designed to match for average speed, duration and distance but varied in either the intermittency (chapter 4) or the acceleration/deceleration (chapter 5). The effects of different forms of intermittent exercise (high, moderate, low) or different acceleration/deceleration components (high, moderate, low) in relation to oxidative stress biomarkers were determined. Results showed the absence of significant increases in all the biomarkers examined. However, significant variation in individual oxidative stress responses was observed. Within a final study, the 8-Oxo-dG method developed was compared to a widely used ELISA method, as there are indications in the literature that ELISA may overestimate 8-Oxo-dG. Determination of 8-Oxo-dG was undertaken on blood plasma samples from 30 chronic heart failure patients (males = 23, females = 7) recruited from the Academic Cardiology department at Castle Hill hospital, Hull, UK, as previous studies have characterised the heart failure syndrome to be associated with higher levels of oxidative stress. Results showed that the LC-MS method developed found no detectable levels of 8-Oxo-dG in plasma samples whereas ELISA showed quantifiable amounts of 8-Oxo-dG. Thus, this thesis presented that the impact of intermittency or acceleration/deceleration following acute HIIE does not induce significant oxidative stress as determined by plasma and serum biomarkers, including plasma 8-Oxo-dG.

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I dedicate this PhD thesis to my parents Γιώργο and Ρούλα.

List of abbreviations

%	Percentage
8-Oxo-dG	8-hydroxy-2'-deoxyguanosine
beats.min^{-1}	Beats per minute
BP	Blood pressure
cm	Centimetre
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FOX-1 assay	Ferrous oxidation of xylenol orange assay
GSH	Glutathione
HIIE	High-intensity intermittent exercise
HPLC	High performance liquid chromatography
HR	Heart rate
HR_{max}	Maximum heart rate
kg	Kilograms
km	Kilometres
km.hr^{-1}	Kilometres per hour
LC-MS	Liquid-chromatography mass spectrometry
M^+	Molecular ion
m/z	Mass to charge ratio
ml	Millilitre
mmHg	Millimetre of mercury
MΩ	Megaohm
ng	Nanograms
nM	Nanomolar
nm	Nanometres
NMT	Non-motorised treadmill
ºC	Degrees Celsius
rcf	Relative centrifugal force
ROS	Reactive oxygen species
RPE	Rate of perceived exertion
SOD assay	Superoxide dismutase assay
v / v	Volume/volume
$\dot{V}O_{2max}$	Maximal oxygen consumption
v$\dot{V}O_{2max}$	Velocity at maximal oxygen consumption
VWD	Variable wavelength detector
yrs	Years
µg	Microgram
µl	Microliter
µM	Micromolar

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Chapter 1

Introduction

1.1. Introduction to the area of interest

During the past decades the interest in exercise induced oxidative stress has been the focus of intense research. It is now recognised that free radicals can serve a dual role as they can act as both toxic / poisonous species but also as beneficial ones. This occurs as living organisms can adapt in their presence and utilise them in cellular functions such as cell signalling molecules (Valko et al., 2006). However, when an imbalance between pro-oxidants (free radicals) and antioxidants exists, favouring the former, a state of oxidative stress occurs that causes a disruption in redox signalling and control, as well as molecular damage (Sies and Jones, 2007). The impairment caused by free radicals includes damage in the lipids, the proteins and the DNA (Birben et al., 2012) which results in lipid, protein and DNA oxidation (Finaud et al., 2006).

In the literature the concept of the damaging effects of exercise-induced oxidative stress during acute, intense or prolonged exercise protocols has been investigated over the years (Powers and Jackson, 2008, Gomez-Cabrera et al., 2009, Powers et al., 2007, Reid et al., 1994). Even though there is a wide body of literature investigating the role of exercise induced oxidative stress, extensive research has been focused on the negative effects of exercise-induced oxidative stress (Steinbacher and Eckl, 2015). However, is now accepted that adaptations also occur as a result of chronic exercise which promotes antioxidant activity (Ji, 1999). Moreover, low levels of ROS during exercise are shown to be biologically significant in cell signalling process, causing muscle adaptations and remodelling (Hamilton et al., 2003, Gomez-Cabrera et al., 2005, Paulsen et al., 2014, Merry and Ristow, 2016, Ristow et

al., 2009) as well as promoting muscle contractile function (Reid et al., 1993, Andrade et al., 2001, Mollica et al., 2012).

The difficulty of determining oxidative stress levels during exercise can be caused by the diversity of the different exercise components which could possibly affect the overall result as there is no single “universal exercise protocol” to be implemented for such studies, resulting in contradictions in the literature on multiple occasions (Draeger et al., 2014). A widely used exercise protocol applied in active population is high-intensity intermittent exercise (HIIE), (Thompson, 2015). This type of activity involves brief intervals of high-intensity exercise interspersed with periods of low volume exercise or rest (Gibala et al., 2012). The use of intermittent exercise has been shown to be an effective approach for physiological adaptations and an efficient exercise approach that promotes benefits in healthy (Burgomaster et al., 2008) and diseased individuals (Vogiatzis, 2011, Bogdanis, 2012). However, the manipulation of HIIE variables and the diversity of HIIE regimes that exists may directly affect the physiological responses (Tschakert and Hofmann, 2013). In relation to exercise induced oxidative stress, the utilisation of HIIE has mainly been utilised in the literature for training studies thus, the acute responses or the effect of intermittency or the rate of acceleration and deceleration phases during HIIE have not been documented. Whilst research is still limited for this type of acute activity; additional investigation is required in order to be able to provide further evidence of exercise-induced oxidative stress during acute HIIE.

1.2. Experimental aims and objectives

The central aim of this thesis is to investigate the impact of intermittency and acceleration / deceleration following HIIE. To investigate intermittency 3 distinct running protocols of high, moderate and low intermittency will be implemented. To investigate acceleration and deceleration impact, 3 distinct running protocols of high, moderate and low acceleration / deceleration will be investigated. A control session will also be included that will consist of collection of blood samples at rest at the same time points as they would be collected during an exercise protocol. In order to address the above aims, lipid hydroperoxides, superoxide dismutase and total glutathione levels will be assessed as they are commonly used biomarkers of oxidative stress in acute exercise studies. Moreover, a considerable interest within oxidative stress research has focused on the use of 8-Oxo-dG as a potential biomarker to examine DNA oxidation (Valavanidis et al., 2009, Shigenaga et al., 1989). Accordingly, the development of a valid measure of plasma-derived 8-Oxo-dG is an underpinning aim of this thesis.

Thus, the aims of this thesis are to:

1. Investigate the impact of different protocols of intermittent exercise when the protocols are matched for mean speed, distance and duration but vary in the rate of intermittency.
2. To investigate the impact of different forms of intermittent exercise protocols, when the protocols are matched for mean speed, distance,

duration and intermittency but vary in the rate of acceleration/deceleration.

3. To develop, optimise and implement the measurement of 8-hydroxy-2'-deoxyguanosine (8-Oxo-dG) in plasma samples using a liquid chromatography – mass spectrometric (LC-MS) technique.

1.3. Hypothesis

It is hypothesised that:

1. Low intermittency during HIIE will demonstrate significantly higher levels of oxidative stress compared to moderate and high intermittency protocols.

2. High acceleration/deceleration will demonstrate significantly higher levels of oxidative stress compared to moderate and low acceleration/deceleration protocols.

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Chapter 2

Literature review

2.1. Oxidative stress

Free radicals are unstable, highly reactive molecules with unpaired electrons in their biochemical structure (Alessio & Hagerman, 2006). The most common examples of free radicals (table 2.1) in biological systems are reactive oxygen species (ROS) and reactive nitrogen species (RNS), (Fisher-Wellman & Bloomer, 2009). Examples of ROS include superoxide, hydrogen peroxide and hydroxyl radical (Aprioku, 2013). Even though hydrogen peroxide is not a free radical by definition, due to its toxicity it is regarded a ROS, as it is capable of producing very reactive and toxic hydroxyl radicals upon interaction with metals (Aprioku, 2013; Finaud et al., 2006). In order to counterbalance the reactivity of ROS, enzymatic and non-enzymatic antioxidants exist that act as a protective mechanism (Birben et al., 2012).

Table 2.1 Classification of free radicals.

Free Radical	Contraction	Main effects
Reactive Oxygen species	**ROS**	
Superoxide ion	$O_2^{\cdot -}$	Lipid oxidation and peroxidation protein oxidation DNA damage
Ozone	O_3	
Singlet oxygen	$^1O_2$	
Hydroxyl radical	$OH^{\cdot}$	
Hydrogen peroxide	H_2O_2	
Hypochlorous acid	HOCl	
Alcoxyl radical	$RO^{\cdot}$	
Peroxyl radical	$ROO^{\cdot}$	
Hydroperoxyl radical	$ROOH^{\cdot}$	
Reactive nitrogen species	**RNS**	Lipid peroxidation DNA damage Proteins oxidation
Nitric oxide	$NO_2^{\cdot}$	
Peroxynitrite	$ONOO^{\cdot -}$	

Where R = side chain group. Adapted from Finaud et al. (2006).

The role of ROS and RNS is important as they can have a significant function in many physiological processes (Di Meo et al., 2016). Some of the contributions of ROS include; being part of immunological responses (Knight, 2000); acting as cell signalling molecules (Thannickal & Fanburg, 2000) and having a role in redox regulation of gene transcription (Turpaev, 2002). Thus, their purpose should be considered a function essential for normal cellular processes (Vollaard et al., 2005). However, when an imbalance between pro-oxidants (free radicals) and antioxidants exists, favouring the former, a state of oxidative stress occurs causing a disruption in redox signalling and control, as well as molecular damage (Sies & Jones, 2007). The impairment caused by ROS includes damage in the lipids, the proteins and the DNA (Birben et al., 2012) which results in lipid, protein and DNA oxidation (Finaud et al., 2006). There are evidence which indicates that oxidative stress can occur following an acute, intense or prolonged exercise (Powers et al., 2016) as ROS production is increased (Di Meo et al., 2016). However, the mechanisms by which this increase occurs is still not well understood (Cooper et al., 2002; Di Meo et al., 2016; Powers et al., 2016). It is believed that the main mechanisms of increased of ROS formation include the electron leak at the mitochondrial electron transport chain, ischemia / reperfusion, the activation of xanthine oxidase, neutrophils and the inflammatory response, NADPH oxidase complex and autoxidation of catecholamines (Gomes et al., 2012). Even though the mechanism of increased production of ROS during exercise is not yet fully understood (Gomes et al., 2012), it is thought that different mechanisms may act synergistically (Vollaard et al., 2005).

2.2. Historical background

The toxicity of oxygen has been identified since the time of Lavosier (Hensley & Floyd, 2002) but in 1878 Bert (1878) experimentally demonstrated the significance of oxygen as a poisonous element in living organisms. The beginnings of ROS chemistry started accidentally, when Fenton observed a reaction occurring when hydrogen peroxide was mixed with tartaric acid and ferrous salts (Fenton reaction), (Hensley & Floyd, 2002). However, at the time, the existence of electrons, or Bohr's atomic model was not known thus, Fenton could not suggest an explanation for his observation (Hensley & Floyd, 2002). It was in 1900, when a chemist, Moses Gomberg, was the first to synthesise a stable radical (Gomberg, 1900), as at the time scientists thought that the independent existence of free radicals was not possible (Tomioka, 1997). As Gomberg's work did not receive attention, 30 years passed before Haber and Willstatter suggested that hydroxyl radical may exist (Koppenol, 2001). This was followed by Haber and Weiss proposing that this radical may be generated by the reaction of hydrogen peroxide and superoxide (Haber-Weiss reaction), (Koppenol, 2001). As the existence of radicals became accepted, in 1954 Gerschman et al. (1954) suggested that that the damaging effects in cells in the presence of radiation could be due to radical formation. At the time electron paramagnetic resonance was developed (Powers et al., 2016), which was utilised by Commoner and allowed him to observe that free radicals exist in cells (Commoner et al., 1954). This work was followed by Denham Harman (1956) who suggested the free radical theory of aging by proposing that ageing may be caused by the oxygen radicals which are produced *in vivo* as a by-product of physiological metabolism. These findings and theories set the basis

for a wide interest in the field of free radicals and in 1969, the pioneering work of McCord and Fridovich (1969) indicated that an enzyme (which they named superoxide dismutase) catalyses the dismutation of superoxide radical. This was critical, as it demonstrated that the abundant presence of superoxide dismutase may have a protective role against radical damage (McCord & Fridovich, 1969). By 1981 it was evident that free radicals can be responsible not only for the aging process, but also for damage to lipids and proteins, for DNA mutations and they could play a role in almost all pathological conditions, such as cancer development and cardiovascular diseases (Harman, 1981). By the beginning of the 21st century it was recognised that free radicals can act as both toxic and beneficial species, since living organisms can adapt in their presence and utilise them in cellular functions as cell signalling molecules (Valko et al., 2006).

The beneficial effects of exercise are well known since the ancient times (Tipton, 2014; Vina et al., 2012). Herodicus, who is considered as the father of sports medicine, was advocating the use of regular training and physical activity for a healthy lifestyle (Georgoulis et al., 2007) and Hippocrates was prescribing written exercise recommendations (Tipton, 2014). Since then great advancements have been made in our understanding about exercise physiology and it is an indisputable fact that exercise is beneficial for a healthy lifestyle as well as a medical remedy for many pathological conditions (Warburton et al., 2006). Regular exercise has been shown to prevent or delay the development of medical issues such as cardiovascular disease (Myers, 2003), cancer (Brown et al., 2012; Rajarajeswaran & Vishnupriya, 2009),

osteoporosis (Nikander et al., 2010) and diabetes (Colberg et al., 2016; Sigal et al., 2006). While exercise is beneficial, the work of Dillard et al. (1978) was the first one to suggest that following aerobic exercise increased oxidative stress can be observed, but upon vitamin E supplementation the oxidation can be reduced. With the utilisation of electron paramagnetic resonance spectroscopy Davies et al. (1982) was able to detect a 2-3 fold increase in free radicals in the skeletal muscle of rats. Usually the main focus of research was concentrated on the deleterious effects of exercise induced oxidative stress (Vollaard et al., 2005). However, over the past decades, various exercise modalities have been investigated and it is now well known that regular exercise can have beneficial effects but acute bouts of prolonged or intense exercise can promote oxidative damage to DNA, lipids and proteins (Powers & Jackson, 2008; Radak et al., 1999; Steinbacher & Eckl, 2015). Thus, it is now of equal scientific interest to further investigate the positive role that free radicals may exhibit as an important function which can cause exercise adaptations (Gomez-Cabrera et al., 2005) along with the negative effects. Research in the field of exercise-induced oxidative stress has evidently advanced over the years. However, methodological issues and limitations are yet a major barrier thus, many questions remain unanswered and require further investigation in order to develop our understanding in this field (Powers et al., 2016).

2.3. Methods of detecting and measuring oxidative stress

There is a diverse range of oxidative stress biomarkers (direct or indirect) available and a variety of techniques which can be implemented and utilised to

measure oxidative stress in bodily fluids (e.g. blood and urine) and in muscle (Di Meo & Venditti, 2001; Finaud et al., 2006; Ho et al., 2013; Jenkins, 2000; Leeuwenburgh & Heinecke, 2001). For a biomarker to be considered as a reliable tool in the measurement of oxidative stress certain criteria must be met. These include the chemical uniqueness and detectability, the capacity of the biomarker to be sensitive to potential increases / decreases upon oxidative stress, the ability to retain a relatively long half-life and the constraint in the implication with other cellular processes (Halliwell & Gutteridge, 2007; Powers & Jackson, 2008) Selected techniques, considered as the most important, for the measurement of oxidative stress are described below.

2.3.1. Electron paramagnetic resonance (EPR) spectroscopy

Free radicals are very reactive and short lived, thus detection in body fluids can be difficult (He et al., 2014). The only available method for direct detection and measurement of free radicals available to date includes the use of electron paramagnetic resonance (EPR) spectroscopy (Bailey et al., 2007). Initially this technique was only performed in animal studies (Jackson et al., 1985), but as the use of spin traps became available, it developed to a valuable tool in the study of free radicals in biological samples (Buettner, 1987). Ashton et al. (1998) utilised the method in humans and investigated free radical production in blood, before and after exercise. The detection of free radicals with this technique is possible as spin adducts, such as α-phenyl-tert-butylnitrone (PBN), or 5,5-dimethyl-1- pyrroline N-oxide (DMPO), allow free radicals to react by forming a more stable adduct that can be directly measured using ESR spectroscopy (Xu et al., 2012). Nevertheless, there are several limitations in

the application of this technique. These include difficulty in trapping the radical, poor structural information by the ESR spectra, generation of identical radical adduct intermediates and disturbances in the process, due to spin trap, when there is a high concentration of radicals in a sample (Guo et al., 2004; Swartz et al., 2007).

As the use of a direct method can be complicated, indirect methods exist which allow the examination of end products, by observing the reactions of free radicals with lipids, proteins and DNA (Holley & Cheeseman, 1993). Such techniques allow the quantification of free radicals, by examining the "footprints" that their reactions leave behind (Bailey et al., 2003). These indirect approaches can include measurements of oxidation by implementing a range of tests, such as colorimetric, enzymatic, HPLC and LC-MS, which aid to estimate ROS damage as well as ROS and antioxidant concentrations (Poljsak et al., 2013).

2.3.2. Measurement of lipid peroxidation

Cells contain in their membranes a vast amount of lipids (Coskun & Simons, 2011). Lipid peroxidation can be defined as a process where the attack of ROS in lipids causes their deterioration (Vasilaki & McMillan, 2012). A certain class of lipids, the polyunsaturated fatty acids (PUFAs) are prone to lipid peroxidation, by the reactions mediated by free radicals (Gardner, 1989). The reason PUFAs are attractive for lipid peroxidation is due to the hydrogen containing methylene carbon groups in their biochemical structure (Vasilaki & McMillan, 2012) which upon attack produce PUFA radicals (Kanti Das et al.,

2015). The most prominent ROS involved in the process of lipid peroxidation is the very reactive hydroxyl radical (Ayala et al., 2014). The cascade reaction by which lipid peroxidation can be described includes the initiation, propagation and termination (Porter et al., 1995). The first step involves the presence of a free radical which causes the removal of a hydrogen atom from a methylene group, leading to the formation of a carbon centred radical (Ayala et al., 2014; Halliwell & Gutteridge, 2007). During the propagation step, a lipid peroxyl radical is formed by the reaction of oxygen with the carbon radical (Ayala et al., 2014). As this new radical is formed, another hydrogen atom is removed from another lipid in a chain reaction (Ayala et al., 2014). Yet, the peroxyl radical may also remove a hydrogen from an adjacent fatty acid side chain, in which case a lipid hydroperoxide is formed along with a new peroxyl radical (Halliwell & Gutteridge, 2007). The last step involves the termination of these reactions by the donation of hydrogen atoms from antioxidants to the lipid peroxyl radical (Ayala et al., 2014). The damage to lipids due to lipid peroxidation, can be very significant as it can lead to chain reaction which can trigger further damage to proteins and DNA through the reactive intermediates (Hulbert et al., 2007). As lipids break down due to oxidation, primary products and by-products are formed such as conjugated dienes, lipid hydroperoxides malondialdehyde (MDA), F_2-isoprostanes and expired pentane (El-Aal, 2012). These lipid peroxidation products can be used as a valuable for the measurement of oxidative stress in a biological sample (Devasagayam et al., 2003).

As stated above, a frequently used method to measure oxidative damage is by determining lipid peroxidation through the utilisation of PUFAs, as they are

more prone to ROS attack (Mateos & Bravo, 2007). The most common techniques available include the measurement of by-products of lipid peroxidation such as malondialdehyde (MDA) and F_2-isoprostanes (Monaghan et al., 2009). MDA is formed as it is a by-product in the process of oxidising PUFAs, (Esterbauer et al., 1991), whereas F_2-isoprostanes are compounds of non-enzymatic oxidation of arachidonic acid (Milne et al., 2007). MDA has been widely assessed by implementing the thiobarbituric reactive substances (TBARS) assay, which is used as a colorimetric or fluorescent assay to determine the reaction of thiobarbituric acid in the presence of MDA (Moselhy et al., 2013). Even though TBARS are frequently used, there are some drawbacks and issues in their application. These issues are relevant to the specificity, as MDA can be produced from other reactions in the process of the measurement of the TBARS assay, thus providing overestimating values (Liu et al., 1997). Furthermore, other issues include modifications in the MDA-TBA adduct, the instability of MDA in biological compounds (as it tends to react with other compounds such as proteins), rapid degradation, poor reproducibility and low recovery (Khoubnasabjafari et al., 2015). All the above are concerns which should be considered when this technique is applied and appropriate sample handling and controls must be in place in order to make the measurements as accurate as possible. The use of other methods to measure MDA has also been investigated by the use of HPLC which has been found to be more precise and accurate for lipid peroxidation (Moselhy et al., 2013). However, this method is also experiencing potential issues such as increased cost of the column and time consuming processes (Kil et al., 2014).

Another method to quantify serum lipid hydroperoxides, is through the utilisation of ferrous oxidation-xylenol orange (FOX) assay (Devasagayam et al., 2003). This colorimetric method is easy to use and allows the quantification of lipid hydroperoxides, as hydroperoxides oxidise Fe^{2+} to Fe^{3+} forming a complex between xylenol orange and the ferric ions (Halliwell & Gutteridge, 2007; Meisner & Gebicki, 2009). An advantage compared to MDA is that this assay measures products which are higher in the cascade of lipid oxidation thus, the early products formed in the lipid peroxidation cascade (DeLong et al., 2002).

2.3.3. Measurement of DNA oxidation

It has been shown that exercise can induce oxidative stress and DNA damage (Fogarty et al., 2013b). Methods to examine DNA damage include the measurements of DNA oxidation in leucocytes and these mainly involve the use of the single cell gel electrophoresis (comet assay), (Mastaloudis et al., 2004b). The comet assay can examine DNA damage in individual cells by visualising the migration of the DNA strands following electrophoresis with the use of fluorescent microscopy (Olive & Banath, 2006). Even though this method has been shown to be sensitive (Collins, 2004), there are limitations in the use of the technique. Such limitations include the limit of the cells analysed as it may not be efficient if the sample is heterogeneous, the restriction in the ability to obtain results in the presence of necrotic or apoptotic cells in the sample, the absence of information about DNA fragment size, the complicated analysis and also the confusion in a possible change in inherent sensitivity of the assay (Olive & Banath, 2006).

Research has often used 8-hydroxy-2'-deoxyguanosine (8-Oxo-dG) as a marker of DNA damage, as oxidised guanine has been indicated as the most prone and most abundant site of the DNA mutagenic lesions (Loft & Poulsen, 1996; Wilson et al., 2003). An important aspect in the use of 8-Oxo-dG is that it does not experience degradation once in circulation (Cathcart et al., 1984; Cooke et al., 2008; Shigenaga et al., 1989) and as such, it is considered a stable marker to examine DNA damage. Apart from that, it is also considered to be a biomarker of "whole body" DNA damage (Guetens et al., 2002).

As urine is thought to reflect overall oxidative stress changes (Olinski et al., 2006), techniques have been implemented for the determination of 8-Oxo-dG in this biological fluid. These include the use of immunosorbent assay techniques such as the ELISA (Harms-Ringdahl et al., 2012; Orhan et al., 2004), or chromatographic techniques such as high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS), (Allgayer et al., 2008) gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography with electrochemical detection (EC) and HPLC tandem mass spectrometry (Valavanidis et al., 2009). The use of the ELISA can be easy and inexpensive when compared to chromatography methods (Kau et al., 2006) and it can also be used in different medium like serum (Breton et al., 2003), plasma (Tope & Panemangalore, 2007), cell culture (Kantha et al., 1996), and urine (Orhan et al., 2004). On the other hand, ELISA has important disadvantages in its use as

it is not specific and the results do not correlate efficiently with chromatography methods (Cooke et al., 2008).

The use of chromatography methods, which are described as the “gold standard” (Mistry et al., 2011), provide the means for DNA components to be hydrolysed into monomers (such as bases, nucleosides or nucleotides) and separate with the use of chromatography after which, detection of the lesion is accomplished by the implementation of a detector (Ravanat, 2012). Chromatography methods, especially if used in conjunction with mass spectrometry, are robust as their use, can provide accurate, reliable and sensitive identification of complex biological fluids or minor components (Bergtold et al., 1988). Chromatography methods have also been shown to be more accurate in terms of estimation of DNA damage when compared to ELISA, as ELISA has been shown to overestimate DNA damage (Cooke et al., 2008).

2.3.4. Measurement of antioxidants

The measurement of antioxidants is another class of biomarkers available that can be employed to measure oxidative stress as their concentration can change, compared to baseline levels, upon ROS upregulation (Polidori et al., 2001). Such markers include enzymatic antioxidants such as superoxide dismutase or non-enzymatic antioxidants such as glutathione.

The early work of McCord and Fridovich (1969) demonstrated that superoxide dismutase catalysed the reaction of superoxide radical that was generated from

xanthine and xanthine oxidase reactions to from hydrogen peroxide and oxygen. The importance of superoxide dismutase comes from its high effectiveness as an antioxidant as it blunts cascading reactions caused by superoxide radical, thus it is considered a major antioxidant and the first line of defence (Alscher et al., 2002; Buettner, 2011; Suzuki et al., 2000).

Glutathione can be found in 2 forms; the thiol-reduced (GSH) and the disulphide oxidised (GSSG) and its role is to catalyse free radical reactions, such as by reducing hydrogen peroxide to water (Kaplowitz et al., 1985). This is possible with the utilisation of glutathione peroxidase (GPx) - catalysed reactions that allow hydrogen peroxide and lipid peroxide to be reduced as GSH is oxidised to GSSG (Lu, 2013).

Other biomarkers also exist such as ascorbic acid, catalase and α-tocopherol (Blokhina et al., 2003) each of which can be measured using various techniques. Even though these antioxidant biomarkers are widely used, they can have some disadvantages in their use such as dietary influences or possible auto-oxidation due to sample handling (Powers et al., 2011).

2.4. Exercise terminology

According to Caspersen et al. (1985) exercise can be defined as any planned, structured and repetitive bodily movement produced by skeletal muscles that requires energy expenditure and aims in maintaining and improve fitness. The different modes of exercise can be in terms of muscular contraction (eccentric, concentric and isometric), in terms of frequency (chronic exercise versus acute)

or terms of exercise intensity (aerobic versus anaerobic exercise), (Gomes et al., 2012). The definitions can be of great importance, especially when oxidative stress is discussed, as different modalities can provide different effects in the oxidative stress biomarkers examined.

2.5. The exercise paradox: The deleterious and beneficial effect of exercise and the relationship with oxidative stress levels

In the literature the concept of the damaging effects of exercise-induced oxidative stress during acute, intense or prolonged exercise protocols has been investigated over the years (Gomez-Cabrera et al., 2009; Powers & Jackson, 2008; Powers et al., 2007; Reid et al., 1994). Support in this rationale, that single bouts of acute exercise promote oxidative stress, has been postulated as studies have shown that in trained and untrained human individuals (Fisher-Wellman & Bloomer, 2009) as well as in rats (Bejma & Ji, 1999; Leeuwenburgh et al., 1999) oxidative stress increases. Among the evidence that demonstrate the harmful effects of exercise-induced oxidative stress is a study by McArdle et al. (2004) determining that proteins, such as heat shock proteins, which are designed to prevent muscle damage, were diminished following acute exercise.

However, is now accepted that adaptations also occur as a result of chronic exercise which promotes antioxidant activity (Ji, 1999). Moreover, low levels of ROS during exercise are shown to be biologically significant in cell signalling process, causing muscle adaptations and remodelling (Gomez-Cabrera et al., 2005; Hamilton et al., 2003; Merry & Ristow, 2016; Paulsen et al., 2014; Ristow

et al., 2009) as well as promoting muscle contractile function (Andrade et al., 2001; Mollica et al., 2012; Reid et al., 1993).

Consequently, it is possible that there could be a hormetic effect during physical activity. This means that there is an optimum dosage characterised by low ROS exposure during exercise, which can provide beneficial and adaptive responses during training and performance however, if the dosage is higher or lower, these beneficial effects decline (figure 2.1), (Ji et al., 2006; Merry & Ristow, 2016; Radak et al., 2005; Radak et al., 2008). This assumption could be possibly further supported by evidence showing that chronic anaerobic exercise of specific intensity and duration can neutralise the deleterious effects of exercise-induced oxidative stress by providing adaptations in the skeletal muscle and blood as well as by promoting modifications in antioxidant defence mechanisms therefore, allowing reduced levels of exercise-induced oxidative stress (Bloomer & Goldfarb, 2004). Radak et al. (1999) showed the beneficial responses of regular participation in exercise by indicating reduced 8-Oxo-dG accumulation, which could be due to an activation of a repair system that is stimulated by exercise (Inoue et al., 1993) or due to enhanced antioxidant capacity (Pilger et al., 1997). In addition to the above, in the literature it has also been suggested that in order to achieve muscle hypertrophy by chronic exercise, a certain degree of exercise-induced muscle damage is needed for adaptations to be achieved Evans and Cannon (1991). Thus, a certain ROS induced damage in muscle can lead to adaptations which are desired and can cause beneficial outcomes (Scheele et al., 2009).

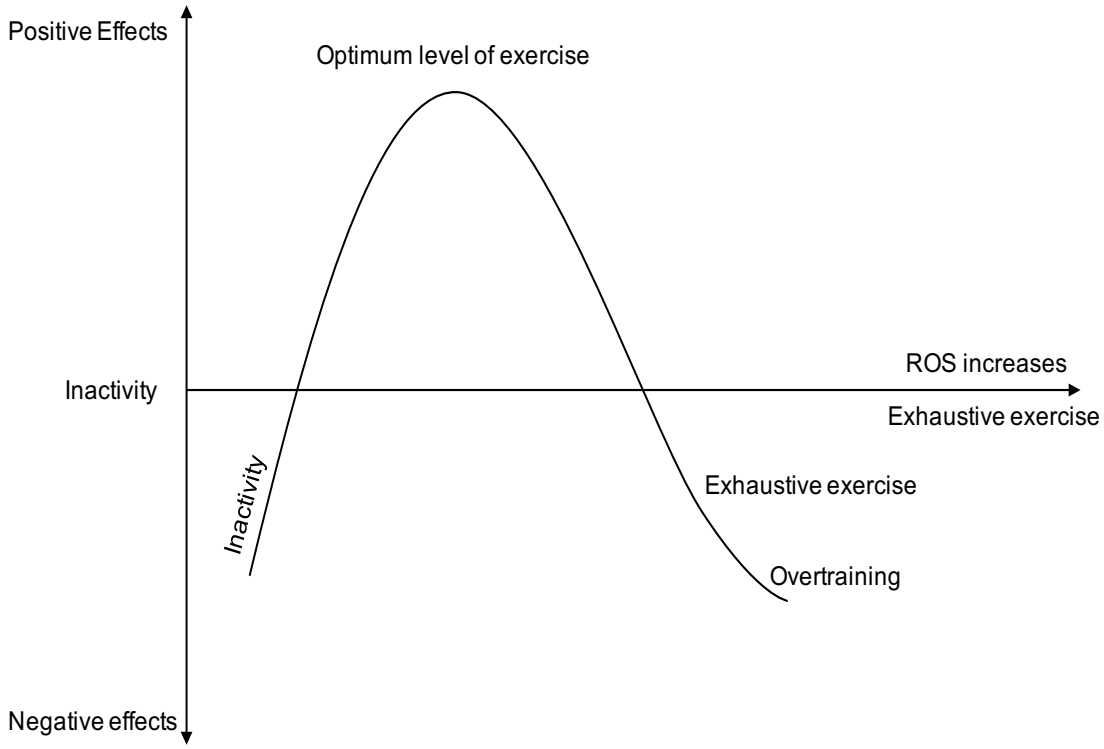

Figure 2.1 Schematic representation of the hormetic effect in exercise. (adapted from Pingitore et al. (2015).

2.6. Oxidative stress in blood and muscle during exercise

Obtaining muscle biopsy samples from human participants, is a complicated and difficult procedure due to the invasive nature that is required to acquire these samples (Berzosa et al., 2011). However, there are limited studies (table 2.2) which have examined oxidative stress in both blood and skeletal muscle during exercise and interestingly few of these have directly compared the same biomarkers between the 2 tissues (Child et al., 1999; Couillard et al., 2003; Fogarty et al., 2013a; Meydani et al., 1993; Quindry et al., 2011). These studies are primarily protocols implemented to induce muscle-damage and increase oxidative stress. Fogarty et al. (2013a) indicated increased levels of 8-oxo-dG in muscle mtDNA and in plasma following exercise. Interestingly, this research also suggested that following α-lipoic acid supplementation, independent mechanisms may exist between the 2 tissues, as suppressed levels of oxidative stress were observed in blood but not in muscle Fogarty et al. (2013a). Quindry et al. (2011) aimed to investigate the relationship between muscle and blood following knee extensions, with results showing a correlation between increases in protein carbonyl levels and type II muscle fibres after low-intensity exercise even though the same was not observed in other fibres types, suggesting that further research is needed to further explore this relationship. In contrast to the above findings, the study of Couillard et al. (2003) failed to show exercise-induced oxidative damage in the muscle of sedentary males, even though plasma creatine kinase levels, which are a biomarker of muscle damage, increased. These results can be conflicting when considering these studies combined. For example the increases observed in protein carbonyls are contradictory between the studies of Fogarty et al. (2013a) with Quindry et al.

(2011) and (Couillard et al., 2003). This could possibly be due to the protocols used in these studies, the choice of participants, sample handling or the choice of the techniques which were utilised for the analysis.

Table 2.2 Overview of human studies investigating exercise-induced oxidative stress in blood and muscle.

Study	Activity	Subjects	Samples	Markers	Effect
Fogarty et al., (2013a)	100 isolated and continuous maximal knee extensions	6 males, apparently healthy	*Blood:* baseline, rest & post exercise *Muscle:* rest & post exercise	8-oxo-dG (plasma) 8-oxo-dG (muscle) Lipid hydroperoxides (serum) Hydrogen peroxide (serum) Protein carbonyls TAC (plasma) TAC (muscle)	↑ after exercise ↑ after exercise ↑ after exercise ↑ after exercise ↑ after exercise ↑ after exercise ↓ after exercise
Quindry et al., (2011)	3x50 eccentric leg contractions with 3 min rest between sets	11 males, UT, recreationally fit	*Blood:* before, post exercise, 24h, 48h post 72h & 96h post exercise *Muscle:* post exercise	lipid hydroperoxides (plasma) Protein carbonyls (plasma) Uric acid (plasma) Creatine kinase (plasma) Antioxidant capacity (plasma) Muscle fibre typing	↔ (slight ↓ post exercise) ↔ post exercise, ↑ 24h and remained ↑ until 72h post exercise ↔ ↔ post exercise, sig. ↑ 24h & remained ↑ until 72h post exercise ↔ correlation between protein carbonyls and type II fibres combined (
Couillard et al. (2003)	knee extensions (30% of max. voluntary capacity) at a pace until exhaustion	10 males, UT, sedentary	*Blood:* baseline, 6h & 48h *Muscle:* baseline & 48h	TBARS (muscle) Protein carbonyls (muscle) Glutathione peroxidase (muscle) Creatine kinase (plasma)	↔ ↔ ↑ after exercise ↑ after exercise
Child et al., (1999)	70 maximal eccentric knee contractions using knee extensors	4 male, 4 females UT, recreationally fit 6 (muscle biopsies)	*Blood:* 5d & 3d before, 3d,4d,5d, 6d,7d,10d &12 d after exercise *Muscle:* before, post exercise 4d & 5d after	Creatine kinase (serum) glucose-6-phosphate dehydrogena β-Glucuronidase (serum) β-Glucuronidase (muscle) Urate (serum) TAC (serum) TAC (muscle) lipid peroxides (plasma) lipid peroxides (muscle)	↑ on days 3,4,5 after exercise. Highest ↑ on day 4 ↑ 7d post exercise ↔ post exercise ↑ with sig. ↑ 7d post exercise ↔ post exercise ↔ post exercise ↑ with sig. ↑ 7d post exercise ↔ post exercise ↔ post exercise
Meydani et al. (1993)	3x15min downhill run with 5 min. rest between bouts intensity at 75% HRrmax	5 males, young, sedentary 6 older males, sedentary	*Blood:* before, post exercise, 3h, 6h, 1d,2d 5d,12 d post exercise *Muscle:* before, post exercise 5d post exercise	lipid peroxides (urine) uric acid (plasma) lipid conjugated dienes (muscle) α-tocopherol (muscle) γ-tocopherol (muscle)	↑ in both groups post exercise, sig. ↑ 12d post exercise ↔ post exercise ↔ post exercise ↔ post exercise ↔ post exercise

Where UT = untrained, h = hours, TBARS = thiobarbituric acid reactive substances, TAC = total antioxidant capacity, d = days, min = minutes, sig = significant.

2.7. Exercise modalities and oxidative stress

Several studies have investigated oxidative stress responses following exercise (Bloomer et al., 2005; Diaz et al., 2011; Friedenreich et al., 2016; Gougoura et al., 2007; Groussard et al., 2003; Kabasakalis et al., 2011; Lovlin et al., 1987; McArdle et al., 2001; Morales-Alamo & Calbet, 2014; Niess et al., 1996; Park & Kwak, 2016; Seifi-Skishahr et al., 2016; Vezzoli et al., 2014). However, between these studies there is a considerable variation in the exercise protocols utilised. The variability is due to the implementation of different intensities, durations, frequencies, training levels, age and mobilisation of different energy systems such as aerobic and anaerobic (Bloomer et al., 2005; Diaz et al., 2011; Friedenreich et al., 2016; Gougoura et al., 2007; Groussard et al., 2003; Kabasakalis et al., 2011; Lovlin et al., 1987; McArdle et al., 2001; Morales-Alamo & Calbet, 2014; Niess et al., 1996; Park & Kwak, 2016; Seifi-Skishahr et al., 2016; Vezzoli et al., 2014). The difficulty of determining oxidative stress during exercise can be caused by the diversity of the different exercise components which could possibly affect the overall result as there is no single “universal exercise protocol” to be implemented for such studies, resulting in contradictions in the literature on multiple occasions (Draeger et al., 2014). This, to some extent, could possibly impede our knowledge for a more definitive answer to the fundamental questions about exercise-induced oxidative stress but on the other hand, exercise is a very dynamic activity that is characterised by this wide variability as it can be found in many forms. Exercise is performed by a large percentage of the population, of all ages thus, research cannot be contained in the investigation of a constrained exercise

protocol applied to all investigations, neither should research focus only on acute exercise as it is expected to induce oxidative stress as this form of exercise is not applicable to everyone.

2.7.1. Exercise-induced stress during aerobic exercise

Studies have shown evidence of lipid peroxidation following acute maximum aerobic exercise using a combination of direct and indirect methods (Ashton et al., 1998; Ashton et al., 1999; Fogarty et al., 2011) or only indirect methods (Jammes et al., 2005; Leaf et al., 1997; Moflehi et al., 2012; Vider et al., 2001). However, there are also studies and evidence that show no effect on lipid peroxidation (Alessio et al., 2000; Hartmann et al., 1995; Jammes et al., 2004). This controversy in the results could be due to many factors such as the volume, intensity and duration of exercise (Fisher-Wellman & Bloomer, 2009) which can lead to an upregulation of the lipid peroxidation biomarkers.

Furthermore, there is evidence that long duration endurance events can promote oxidative stress. The investigation of Machefer et al. (2004) showed that following a 7 day race, well trained, long-distance runners had significant increases in the levels of lipid peroxides 72 hours after the race. As Miyazaki et al., (2001) suggested, even after following 12 weeks of strenuous high-intensity endurance training the antioxidant activities can be elevated and reductions in lipid peroxidation can be observed but the changes in lipid peroxidation compared to rest can still be significant. Other authors have also shown that distance running events such as marathon (Hessel et al., 2000; Liu et al., 1999) half-marathon (Child et al., 2000; Child et al., 1998; Marzatico et al., 1997), 50

km run (Mastaloudis et al., 2001; Mastaloudis et al., 2004a) and 80 km run (Kanter et al., 1988; Nieman et al., 2002) induce oxidative stress as they observed increases in biomarkers of DNA damage, lipid peroxidation and in uric acid. Even in these types of activities though, definite conclusions still cannot be made, as even in these, very stressful conditions such as a marathon event, contradictory evidence have been shown (e.g. the study of Vasankari et al. 1997). The study of Vasankari et al. (1997) suggested the absence of oxidative stress following an acute 31 km and marathon running. The investigators (Vasankari et al., 1997) proposed that the absence of oxidative stress could be due to the participants consumption of food and drinks during the event. Even though it is possible that results could be affected by the consumption of food and drinks (the degree of which is unknown), this study is an interesting finding as research has shown that it is still unclear if antioxidant supplementation can reduce oxidative stress levels (Bentley et al., 2015).

While the levels of oxidative stress are suggested to increase following very intense acute exercise (Fisher-Wellman & Bloomer, 2009), there are some evidence that there are no changes. For example Hartmann et al. (1995) showed evidence that lipid peroxidation using the MDA biomarker was not present following exhaustive exercise. Possibly the sampling time (only a 24 hour post-event time point) was not adequate, as the lipid peroxidation might occur at a different time point (e.g. immediately after or 1 hour post exercise). Interestingly, the maximum acute protocol to exhaustion implemented by Alessio et al. (2000), even though it showed no significant changes in lipid peroxidation after analysis using MDA, an increase of 36% was observed in

lipid hydoperoxides. As the authors suggest the findings could be influenced by the low statistical power as well as due to $\dot{V}O_2$ not being the only mediating factor for the generation of MDA or lipid hydoperoxides Alessio et al. (2000).

An additional issue for consideration is the intensity of acute exercise. McClean et al. (2015) investigated this hypothesis by implementing a continuous running exercise at 3 different exercise intensities, comprising; a mild protocol of 30 minute running at 55% $\dot{V}O_{2max}$, a moderate protocol of 20 minute running at 75% $\dot{V}O_{2max}$ and a maximum running protocol (100% $\dot{V}O_{2max}$). The results of this study suggested that the effects of intensity during acute running evoke different oxidative stress responses, thus more research in this field is required (McClean et al. 2015). More specifically, in terms of continuous moderate intensity aerobic exercise (between 60 – 70% $\dot{V}O_{2max}$), the literature shows contradictions as some authors have shown increased lipid peroxidation (Alessio et al., 1997; Kanter et al., 1993) whereas others (McClean et al., 2011; Morillas-Ruiz et al., 2005; Quindry et al., 2003) have shown no change or no significant change. The results of these studies can be further debated in terms of the overall conclusions provided from these investigations. As an example Morillas-Ruiz et al. (2005) showed that in 30 trained male athletes, following a 90 minute exercise at 70% $\dot{V}O_{2max}$, no significant lipid peroxidation was observed although DNA damage occurred along with protein oxidation. According to the investigators the exercise stimulation has to be sufficient to induce changes in lipid peroxidation markers as the pathways of exercise-induced oxidative stressed can be altered based on the metabolic demands (Morillas-Ruiz et al. 2005). As seen above (e.g. Hartmann et al. 1995), some

studies may not report changes in lipid peroxidation but still report oxidative damage in another biomarker. As both Morillas-Ruiz et al. (2005) and Hartmann et al. (1995) showed, following exercise, lipid peroxidation was not observed even though there were evidence of DNA damage. This might suggest that due to the specificity of the biomarker of choice a different pathway, where oxidative damage may occur may be overlooked thus, the application of several oxidative stress biomarkers is suggested (Powers & Jackson, 2008). However, even though results for moderate exercise are inconsistent, McClean et al. (2011) suggested that increased antioxidant defences during moderate exercise could be evidence for health benefits following this type of activity.

In relation to DNA damage, Fogarty et al. (2011) showed evidence of lipid peroxidation and DNA damage following acute, high-intensity incremental exercise (40 – 100% $\dot{V}O_{2max}$). The results of oxidative DNA damage induced by aerobic exercise are likewise supported by other studies. Okamura et al. (1997) showed that following 8 days of training, 10 well trained distance runners experienced significant increases in DNA damage during the 24 hour measures obtained and values returned to normal 24 hours after the completion of the training. These findings indicate that an accumulation effect of exercise-induced DNA damage occurs, but only lasts as long as the exercise training continues (Okamura et al. 1997). Furthermore, Orhan et al. (2004) recruited 18 moderately trained males and following 1 hour of moderate intensity exercise at 70% $\dot{V}O_{2max}$ they showed evidence of DNA damage (indicated by urinary 8-Oxo-dG) which lasted until the next morning (urine samples were collected at 12 hour intervals). The authors (Orhan et al. 2004) suggested that the observed

increase to DNA damage could be due to exercise induced adaptations, as it was previously shown that DNA repair enzymes are increased following long distance running (Radák et al., 2003). Radák et al. (2000) recruited 5 supra-marathon runners and following a 4 day race (328 km) DNA damage (indicated by urinary 8-Oxo-dG) significantly increased during the first day of the race; possibly due to the increased stress of this type of exhaustive activity. The DNA damage started to decline after day 3, possibly due to adaptations occurring such as by the employment of increased antioxidant defences (Radák et al. 2000). The rationale about the possible beneficial role of increased antioxidant defences during exercise can find further support in the study by Gomez-Cabrera et al. (2006). The authors (Gomez-Cabrera et al. 2006) showed that following completion of a marathon, the generation of ROS acted as signalling molecules, initiating the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and that was succeeded with increases in antioxidant levels that aimed to provide cellular adaptations. This beneficial effect though, as the authors demonstrated, was lost upon inhibition of this pathway, causing a prevention of these valuable adaptations Gomez-Cabrera et al. (2006). Aiming to investigate the relationship of intensive aerobic exercise Sumida et al. (1997) utilised 3 different exercise experiments, after recruitment of long distance runners and untrained participants. The 3 protocols consisted of incremental exercise on a treadmill, incremental exercise on a bicycle and 20 km distance running (Sumida et al. 1997). The authors (Sumida et al. 1997) showed that there were no differences to DNA damage following exercise in all the protocols tested. However, they suggested that even though after a single bout of intense exercise DNA damage could not be observed, there might be

induced DNA damage following training, as their previous research had shown (Okamura et al., 1997).

As the use of intermittent exercise has been shown to be an effective approach for physiological adaptations and an efficient exercise approach that promotes benefits in healthy (Burgomaster et al., 2008) and diseased individuals (Bogdanis, 2012; Vogiatzis, 2011), research has been conducted in this exercise modality in order to examine oxidative stress. However, to the authors knowledge, the effect of acute intermittent exercise on oxidative stress has only been investigated by 2 authors (Kingsley et al., 2005; Thompson et al., 2001) both of which used an intermittent protocol as part of interventions to examine dietary supplementation. These studies also used different intermittent exercise protocols for their investigations. Kingsley et al. (2005) recruited 16 regularly active males and implemented a protocol that consisted of 2 bouts of running at various speeds of a total duration of 45 minutes, followed by a 10 minute recovery. The authors also included a multistage fitness test until volitional exhaustion immediately after the exercise (Kingsley et al. 2005). Thompson et al. (2001) recruited 9 physically active males and employed a 90 minute protocol that included shuttle-running over a 20 m distance. Both Kingsley et al. (2005) and Thompson et al. (2001) observed oxidative stress, with Kingsley et al. (2005) indicating significant increases in lipid hydroperoxides (FOX assay) post exercise and Thompson et al. (2001) indicating significant increases in lipid peroxidation (MDA) post exercise. In contrast to the acute effects of intermittent exercise, training studies have shown adaptations occurring in oxidative stress levels following the completion of training protocols. The 8

week training study of Vezzoli et al. (2014) showed no exercise-induced oxidative stress following high-intensity intermittent training, indicating a lipid peroxidation decrease and a decrease of 25% to DNA damage (as determined with the use of the 8-Oxo-dG marker in urine). As research is still limited for this type of activity more research is needed in order to be able to provide conclusive evidence of exercise-induced oxidative stress.

Overall, as demonstrated above, even though there are evidence that moderate / vigorous intensity continuous and intermittent exercise can induce oxidative stress levels, more research is needed as exercise protocols can be vastly different due to many factors such as intensity, duration, sampling time and physical activity status of the participants. Furthermore, as oxidative stress levels may vary between individual biomarkers, it is possible that these are further evidence which support the hypothesis that different mechanisms may work synergistically (Vollaard et al., 2005). Thus, firm conclusions cannot be made and further research is required to advance and develop our understanding in exercise-induced oxidative stress.

2.7.2. Exercise-induced oxidative stress by eccentric exercise

Eccentric exercise has been shown to induce delayed muscle soreness due to the unaccustomed nature of the activity and promote micro-injuries (Cheung et al., 2003). Delayed muscle soreness has been implicated in increases in oxidative stress (Aoi et al., 2004), and downhill running has been investigated by several authors (Close et al., 2004; Maughan et al., 1989; Park & Lee, 2015; Sacheck et al., 2000; Sacheck et al., 2003), with results indicating an increase

in oxidative stress markers following this type of activity. Thus, interest has evolved in eccentric exercise and oxidative stress studies.

A recent study by Park and Lee (2015) indicated that participants who followed an acute bout of 40 minutes downhill running at moderate intensity (approximately at 70% of $\dot{V}O_{2max}$) elicited an apoptotic response in leucocytes, 24 and 48 hours after exercise suggesting that due to the unaccustomed nature of this exercise, muscle damage is significantly induced and then attenuated if another downhill run follows. However the suggestion of Park and Lee (2015), with regards to the DNA damage, is not supported in the findings of Sacheck et al. (2003). Sacheck et al. (2003) recruited young and old individuals and implemented an acute bout of downhill running with 3 successive 15 minute intervals interspersed with a 5 minute rest at 75% of $\dot{V}O_{2max}$. An overall increase in oxidative stress was found in this study, with evidence of lipid peroxidation (indicated by MDA) to be present immediately post exercise. The results also showed no DNA damage (indicated by 8-Oxo-dG) to be present 24 hours following exercise, suggesting that changes in that biomarker could occur at a later time point (Sacheck et al. 2003). The investigation of Close et al. (2004) attempted to compare eccentric exercise using downhill running and running on a flat surface. The results showed that 30 minutes of 15% downhill running at 65% $\dot{V}O_{2max}$ were enough to induce lipid peroxidation (indicated by MDA) when compared with 30 minutes of running at 65% $\dot{V}O_{2max}$ on a flat surface. Another interesting finding of this study was that downhill running was observed to cause increases in ROS 72 hours post exercise at which point delayed muscle soreness starts to diminish (Close et al. 2004). As the authors suggest,

ROS may act as facilitators for delayed muscle soreness thus the delayed response of increased ROS could be part of the recovery process (Close et al. 2004).

Other authors have also investigated eccentric exercise in relation to oxidative stress utilising resistance exercise protocols, such as eccentric elbow flexion exercise (Childs et al., 2001; Goldfarb et al., 2005; Lee et al., 2002) and lengthening knee flexion exercise (Nikolaidis et al., 2007; Quindry et al., 2011). In relation to this type of exercise as Nikolaidis et al. (2007) suggested, more research is required with more blood samplings to examine late recovery stages in order to be able to better understand the effects of this type of activity.

2.8. Conclusion

In light of the evidence presented above, contradictory findings exist in the literature on the effects of acute exercise on oxidative stress measurements. In addition, there is a lack of substantial research on the effects of acute HIIE on oxidative stress biomarkers. Following, the acute responses, especially when combined with the effect of intermittency or the rate of acceleration and deceleration phases during HIIE have not been documented. Whilst research is still limited for this type of acute activity; additional investigation is required in order to be able to provide further evidence of exercise-induced oxidative stress during acute HIIE.

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Chapter 3

General methods

3.1. General rationale for the experimental design

After acquiring approval from the departmental ethics committee of University of Hull (approval number 1314061) and following the declaration of Helsinki (World Medical, 2013), participants were recruited for the studies through various advertising techniques, such as email communication and flyers.

3.2. Anthropometric measurements

Following informed consent and completion of a medical health history questionnaire, measures of height were performed using a stadiometer (Seca 217, Cardiokinetics, Salford, UK). Ensuring shoes were removed, the participants were instructed to stand straight with the feet together, flat and the heels against the rod facing forwards. The head plate was then lowered to the point where there was a contact with the head. Stature was subsequently recorded to the nearest cm. Body mass measures were performed using a mechanical column balance scale (Seca 700, Cardiokinetics, Salford, UK). Ensuring any extra weight was not carried, the participants were instructed to step on the scale. The weight indicators were then adjusted until the scale was balanced and body mass was measured to the nearest 0.1 kg. Body fat was measured using a body fat monitor (BF306, Omron, Milton Keynes, UK). Measures were performed after calibration and by following the instructions manual. The participants were instructed to stand with feet slightly apart, keep the arms straight and hold the grip of the electrodes by wrapping the fingers around the handle. An immediate reading of the % of body mass was provided on the screen of the monitor and recorded. Resting heart rate was measured

using a heart rate monitor with a portable short angle telemetry device (Polar S801i, Polar electro, Finland). A belt containing 2 electrodes and supplied with a transmitting device was attached to the participant's sternum. The heart rate (beats.min^{-1}) was transmitted via waveforms to a wrist watch that allowed recording and downloading of the heart rate throughout. Measures of blood pressure were performed using an upper arm blood pressure monitor (M6, Omron, Milton Keynes, UK). The participant was instructed to be seated comfortably in an upright position with the back supported at the chair and the feet flat on the floor. The left arm, from which the measurement was taken, was supported on a table and the arm cuff was placed above the elbow. Measurements were then obtained automatically to the nearest systolic and diastolic millimetres of mercury (mmHg).

3.3. Maximal oxygen uptake ($\dot{V}O_{2max}$) testing protocol

Throughout the present thesis participants were required to complete a graded exercise test to volitional exhaustion prior to commencing to any exercise interventions. The $\dot{V}O_{2max}$ was subsequently used to prescribe a specific "dose" of exercise in order to investigate the influence of intermittency and acceleration/deceleration on oxidative stress.

An Oxycon Pro (Cryoservice Ltd, Worchester, UK) was calibrated before each test according to the manufacturer's instructions along with the use of certified calibration gases consisting of 16.4% oxygen (O_2) and 4.5% carbon dioxide (CO_2). A 3 litre syringe (Cosmed srl, Middlesex, UK) was used for the turbine flow meter, to allow the determination of minute ventilation ($\dot{V}_E$). Recording of

cardiorespiratory variables was implemented throughout the procedure. Heart rate was recorded using a heart rate monitor and an Oxycon Pro device was used to obtain respiratory data (MasterScreen™ CPX metabolic cart, BD, Basingstoke, UK).

The $\dot{V}O_{2max}$ determination protocol consisted of motorised treadmill running (Cosmos, H/P Cosmos, quasar, lt) at a set 1% gradient and with an initial speed of 8 km / h which increased by 1 km / h every minute until volitional exhaustion.

Verification criteria for attaining $\dot{V}O_{2max}$ were the following, (2 or more of the criteria had to be achieved in order to meet the requirements); A plateau in $\dot{V}O_2$ ($\dot{V}O_{2max}$ should not change more than 2% despite an increasing workload); a respiratory exchange ratio (RER) greater than 1.15; a maximum heart rate between $\pm$ 10 beats.min^{-1} from the estimated HR_{max} and a RPE of more than 17 (6 – 20 scale, where 6 represents very, very light activity and 20 represents maximum effort activity).

3.4. Collection and processing of blood samples

For the collection of blood samples, each participant had to assume a supine position. After the area of the arm was cleaned using a sterilised swab, containing alcohol, a tourniquet was positioned at the distal end of the bicep brachii. The tourniquet was positioned in the arm for the minimum amount of time required. Venous blood samples (30 ml) were then obtained by a trained phlebotomist, using the Vacutainer® venepuncture technique. Blood collected for plasma was drawn in ethylenediaminetetraacetic acid (EDTA) tubes

(Vacuette®, 6ml K^3EDTA, Ref: 456036, Greiner bio-one, Stonehouse, UK) and for serum in serum separation tubes (SST), (Vacuette®, 5ml Z serum Sep Clot activator, ref: 456071, Greiner bio-one, Stonehouse, UK).

Whole blood collected in EDTA tubes was inverted 9 times and immediately centrifuged at 2500 rcf for 15 minutes at 4°C (Labofuge 400R, Heraeus). A total of 1 ml of plasma (top layer) was aspirated, aliquoted and was immediately stored at -80°C until further analysis. Whole blood collected in SST tubes was inverted 5 times and was allowed to clot for 30 minutes at room temperature. Following, SST tubes were centrifuged at 1800 rcf for 15 minutes at 4°C (Labofuge 400R, Heraeus). A total volume of 1 ml of serum was aspirated and aliquoted and immediately stored at -80°C until further analysis.

3.5. Quantification of DNA oxidation

A combination of liquid chromatography – mass spectrometry (LC-MS) and enzyme-linked immunosorbent assay (ELISA) techniques were used to determine the effect of exercise on the expression of deoxyribonucleic acid (DNA) damage in plasma. Specific details of the LC-MS method can be found in chapter 3, while ELISA methodology is fully described in chapter 6.

3.6. Additional biochemical analysis

The following sections detail the biochemical analysis employed throughout this thesis in relation to human blood samples.

3.7. Measurement of lipid hydroperoxides using ferrous oxidation-xylenol orange (FOX-1) assay.

Serum lipid hydroperoxides (LOOHs) were measured using the ferrous oxidation of xylenol orange (FOX-1) assay. Serum samples were thawed and centrifuged at 3000 rcf for 5 minutes at room temperature (Labofuge 400R, Function line, Heraeus). Standards at concentrations ranging from 0 – 5 μM were prepared using hydrogen peroxide (Alfa Aesar, L14000, Lot: 10180127) mixed with distilled water (18.2 MΩ). FOX-1 reagent was prepared in a total volume of 100 ml of 25 mM sulphuric acid (Amresco, 0499, Lot: 1125C446), supplemented with 250 μM amonium iron (II) sulphate (Acros Organics, 423721000; Lot: A03655885), 100 μM sorbitol (Alfa Aesar, 36404, Lot: T30A064) and 100 μM of xylenol orange (21120010, Sigma-Aldrich, Dorset, UK). Using a clear bottom 96-well plate format (Corning, Costar, CLS3599, Sigma) 180 μl of FOX-1 reagent was added to 20 μl of serum samples or standards in duplicates at a final volume of 200 μl per well. The reaction mix was incubated at room temperature in the dark for 30 minutes. Absorbance was then measured at 560 nm using a plate reader (Tecan, Infinite M200 pro, software: Magellan).

Some serum samples were found to be over the standard curve concentrations, thus a range of dilutions was chosen and these samples were re-tested to find the optimum dilution that would be in the middle range of the standard curve.

For the analysis, a standard curve was constructed using Microsoft office Excel® (version 15.26) after calculating the blank corrected values of each duplicate well of standards against the known concentrations of the hydrogen peroxide. The y-intercept was set at 0 and Pearson coefficient of determination (R^2) value was examined to indicate the proportion of variance in the samples after they were corrected against the blank. The assay was considered to be successful when R^2 was ≥ 0.98. The average values of each duplicate well of sample were calculated and the concentration of each sample was determined in µM by dividing the average value of each well against the slope. The final concentration was then calculated by multiplying the concentration previously found in µM with the dilution factor to determine the final concentration.

3.8. Measurement of Superoxide Dismutase (SOD).

Superoxide dismutase (SOD) concentrations were determined using a commercially available kit purchased from Cambridge Bioscience (catalogue number: 706002, Cambridge, UK) according to the manufacturer's guidelines. Standards were prepared by diluting 20 µl of SOD standards provided in the kit with 1.98 ml of sample buffer. The diluted solution was used to prepare the standards at concentrations ranging from 0 - 0.050 U / ml. Serum samples were then thawed and diluted in 1X sample buffer at a ratio of 1:5 and 10 µl of sample was added in each well of a clear bottom 96-well plate. Radical detector was then prepared by mixing 50 µl of radical detector with 19.95 ml of diluted assay buffer and 200 µl of the diluted radical detector was added in each well. The reactions were initiated by adding 20 µl of diluted xanthine oxidase (50 µl of xanthine oxidase in 1.95 ml 1X sample buffer). The 96-well plate was left to

incubate for 30 minutes at room temperature and absorbance was measured at 450 nm using a plate reader (Tecan, Infinite M200 pro, software: Magellan).

For the analysis, a linearised rate standard (LR) curve was constructed using Microsoft office Excel® (version 15.26). The assay was considered to be successful when R^2 was $\geq$ 0.98. The average absorbance of each standard and sample was calculated and was blank corrected. Blank corrected average values were divided by itself and divided to yield the LR. The average values of each sample were also determined by dividing the blank corrected average value with the average blank to determine the linear rate. Following, the LR of each average sample was subtracted by the y-intercept (LR – y-intercept) and the result was further divided by the slope [(LR – y-intercept)] / slope. Sample volume correction followed and the final concentration of SOD (U / ml) was calculated by multiplying the result of the LR – y-intercept)] / slope with the sample volume correction. Finally, to correct for dilution, each average well sample was multiplied by the dilution factor.

Some serum samples were found to be over the standard curve concentrations, thus a range of dilutions was chosen and these samples were re-tested to find the optimum dilution that would be in the middle range of the standard curve.

3.9. Measurement of total glutathione (GSH).

Total glutathione (GSH) (oxidized and reduced) was determined according to the manufacturer's guidelines, using a commercially available kit (catalogue number: 703002, Cambridge Bioscience Ltd, Cambridge, UK). A solution of

metaphosphoric acid was prepared for the deproteination of serum samples by mixing 5 g of metaphosphoric acid (Sigma-Aldrich, 239275) in 50 ml of distilled water (18.2 MΩ). In a total final volume of 400 µl, 200 µl of serum sample were mixed with 200 µl of metaphosphoric acid and left to incubate for 5 minutes at room temperature. Samples were then centrifuged at 3000 rcf for 5 minutes at room temperature. The supernatant was collected and stored at -20°C. Samples were then concentrated by lyophilisation (Edwards Modulyo Freeze Dryer) for 24 hours. The residues were reconstituted using 1x MES buffer to 1 / 3 of its original recovered volume that was retrieved after the post-acid precipitation step. The pH was tested in each sample using Whatman® universal indicator papers (pH 1-11). Sample pH was adjusted to pH 7 by the addition of TEAM reagent (50 µl per ml of the original recovered volume), that was previously prepared using 531 µl of triethanolamine (Sigma-Aldrich, T58300) and 469 µl of distilled water (18.2 MΩ). Standards were also prepared using glutathione disulphide (GSSG) mixed with MES buffer at concentrations ranging from 0 - 8.0 µM GSSG. Fifty µl of sample or standard was added in duplicates to a clear bottom 96-well plate and assay cocktail was immediately prepared by mixing 11.25 ml of 1x MES buffer, 0.45 ml reconstituted cofactor mixture, 2.1 ml enzyme mixture and 2.3 ml of distilled water (18.2 MΩ). A total of 150 µl of assay cocktail mixture was immediately added in each well. After a 25 minute incubation at room temperature, using the end point method, absorbance was measured at 410 nm using a plate reader (Tecan, Infinite M200 pro, software, Magellan).

For the analysis, a standard curve was constructed using Microsoft office Excel® (version 15.26). The y-intercept was set at 0 and the assay was considered to be successful when R^2 was $\geq$ 0.98. The average absorbance value was determined by subtracting the blank average value from itself and calculating the rest of the standards and samples based on the blank corrected value. To calculate the concentration of the corrected absorbance values for each sample, blank corrected value was divided with the slope and multiplied with 2 (to account for the addition of metaphosphoric acid reagent) and was then multiplied by sample dilution to determine the final concentration in µM.

Some serum samples were found to be over the standard curve concentrations, thus a range of dilutions were chosen and these samples were re-tested to find the optimum dilution that would be in the middle range of the standard curve.

3.10. Statistical Analysis

A variety of statistical techniques were adapted throughout this series of experiments and were altered dependent on the individual methodological design. Full details of the statistics employed can be found in each of the subsequent experimental investigations.

3.11. Chapter references

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Chapter 4

Methodological development and optimisation of 8-hydroxy-2'-deoxyguanosine in plasma samples using liquid chromatography – mass spectrometry.

4.1. Introduction

Of the 4 DNA nucleobases (adenine, thymine, cytosine and guanine), guanine is the most prominent to oxidation due to its low ionisation potential (Kroese and Scheffer, 2014). A common ROS attack involves the reaction of the hydroxyl radical with the guanine base (at the C-8 position), causing the formation of 8-hydroxy-2'-deoxyguanosine (8-Oxo-dG), (Valavanidis et al., 2009, Dizdaroglu, 1991). It has been shown that the accumulation of 8-Oxo-dG can result in gene mutation and / or gene malfunction (Lan et al., 2003) as it can induce C to T transversions (Marnett, 2000). Since 8-Oxo-dG is a stable product of oxidation, it can serve as an indicator of DNA damage (de Souza-Pinto et al., 2001). Thus, a great interest has been focused on 8-Oxo-dG as a potential biomarker to examine DNA oxidation (Valavanidis et al., 2009, Shigenaga et al., 1989).

The most common biological fluid to detect 8-Oxo-dG has been in urine, as it is an easy and non-invasive method of sample collection. However, other fluids such as blood and saliva have been used (Lam et al., 2012). Even though it is still debatable which bodily fluid would be more applicable for the assessment of 8-Oxo-dG, Hu et al. (2015) suggested that apart from urine, blood is also a an appropriate bodily fluid to be used to examine 8-Oxo-dG concentrations. Indeed, the use of blood, can possibly be advantageous as changes at specific time intervals can be investigated, whereas in urine repeated collections at specific time-points can be more difficult to obtain (Lagadu et al., 2010).

A range of techniques have been utilised to examine DNA damage in blood and urine, including enzyme-linked immunosorbent assays (ELISA) and analytical techniques such liquid-chromatography mass spectrometry (LC-MS) (Valavanidis et al., 2009). Each of these methods has advantages and disadvantages. As an example, ELISA is easy and simple to perform (Wu et al., 2004) but, ELISA selectivity is solely reliant upon the specificity of the detection antibody (Lequin, 2005). As this detection occurs in a complex sample and there is no form of sample fractionation or separation, there is a real risk of non-specific interactions with related chemicals to the intended antibody target. LC-MS on the other hand, is a highly sensitive analytical technique used in biochemistry (Pitt, 2009). LC-MS can be used to chromatographically separate a sample based on its physiochemical properties and also simultaneously measure mass to charge ratio *(m/z)*. The addition in the detection of a substance of the *m/z* ratio can be of importance, as *m/z* can be fairly characteristic of a substance (Pitt, 2009, Gross, 2011). Therefore, LC-MS may provide a more powerful and robust approach to the analysis and overall quantification of 8-Oxo-dG than ELISA. Indeed, DNA damage studied in urine and mononuclear cell samples by ELISA shows a discrepancy in published results when analytical methods such as LC-MS have been used, formulating the conclusion that ELISA may overestimate the levels of 8-Oxo-dG (Song et al., 2009, Yoshida et al., 2002, Shimoi et al., 2002, Chiou et al., 2003). Furthermore, Shimoi et al. (2002) also demonstrated an overestimation of ELISA concentrations by showing that in urine, between ELISA and high performance liquid chromatography (HPLC), a 2-fold higher concentration was

observed in ELISA and 10% of the samples had a 4-fold increase. These discrepancies could be due to the factors stated above.

The measurement of 8-Oxo-dG using LC-MS in plasma samples, to our knowledge, has been investigated by Cooke's laboratory (Lam et al., 2012) and more recently by Villano et al. (2015). Villano et al. (2015) found that in triathletes there were no significant changes following 2 weeks of training, with 8-Oxo-dG concentrations being approximately less than 0.01 nM at baseline and less than 0.02 nM following 2 weeks of training. Also, Lam et al. (2012) reported basal levels of 8-Oxo-dG that are lower (0.14 nM) than those compared with an ELISA (e.g. 118.75 nM, Fogarty et al., 2013).

As the evidence shown above indicate, the use of LC-MS in a complex sample, such as plasma, could be a more appropriate method to determine the presence of 8-Oxo-dG compared to an ELISA. Thus, the aim of this chapter is to develop and optimise an LC-MS method for the detection of 8-Oxo-dG in human plasma samples.

4.2. Experiment 1

Aim: To optimise chromatography for 8-Oxo-dG.

4.2.1. Methods and results

Since HPLC is mainly used as a separation technique, the initial chromatography was conducted using this method, as the objective was to

determine if the separation and identification of 8-Oxo-dG was possible. For this experiment, initially a variety of HPLC conditions were attempted (1260 Infinity, Agilent Technologies, Cheshire, UK), in order to develop an optimised chromatography. A C_{18} column was chosen and an isocratic method was implemented, using methanol and water as running buffers and measuring at a wavelength of 280 nm with a variable wavelength detector (VWD), (Lam et al., 2012, Bolin et al., 2004). During these stages, aiming to achieve optimal conditions, 8-Oxo-dG stocks (H5653 Sigma-Aldrich, Poole, UK) of 1μM in methanol were prepared but this lead to an observed peak broadening (figure 4.1) in the chromatography. This was resolved by diluting 8-Oxo-dG standard in distilled water (18.2 MΩ), (figure 4.2). Implementing a combination of different concentrations of methanol and formic acid in the running buffers and by testing a variety of isocratic methods (table 4.1), the chromatography of 8-Oxo-dG was optimised. The optimised peak shape for 8-Oxo-dG was achieved using an isocratic method of 15% v / v of methanol containing 0.1% v / v formic acid, with 85% v / v of water containing 0.1% v /v formic acid.

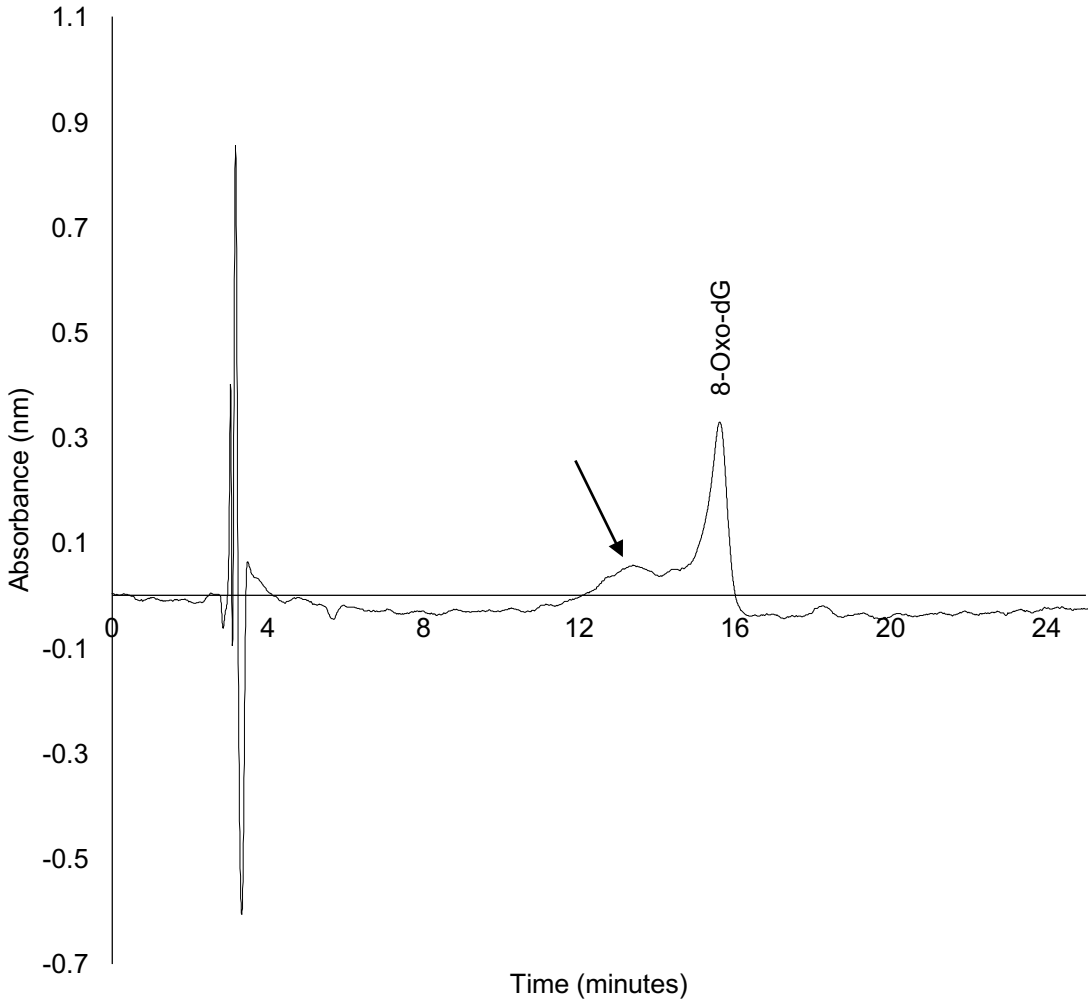

Figure 4.1 Representative figure of peak broadening (indicated by the arrow), observed during 8-Oxo-dG optimisation using HPLC. Following tests, it was concluded that the broadening was observed to due to the stock preparation of 8-Oxo-dG in methanol (VWD = 280nm).

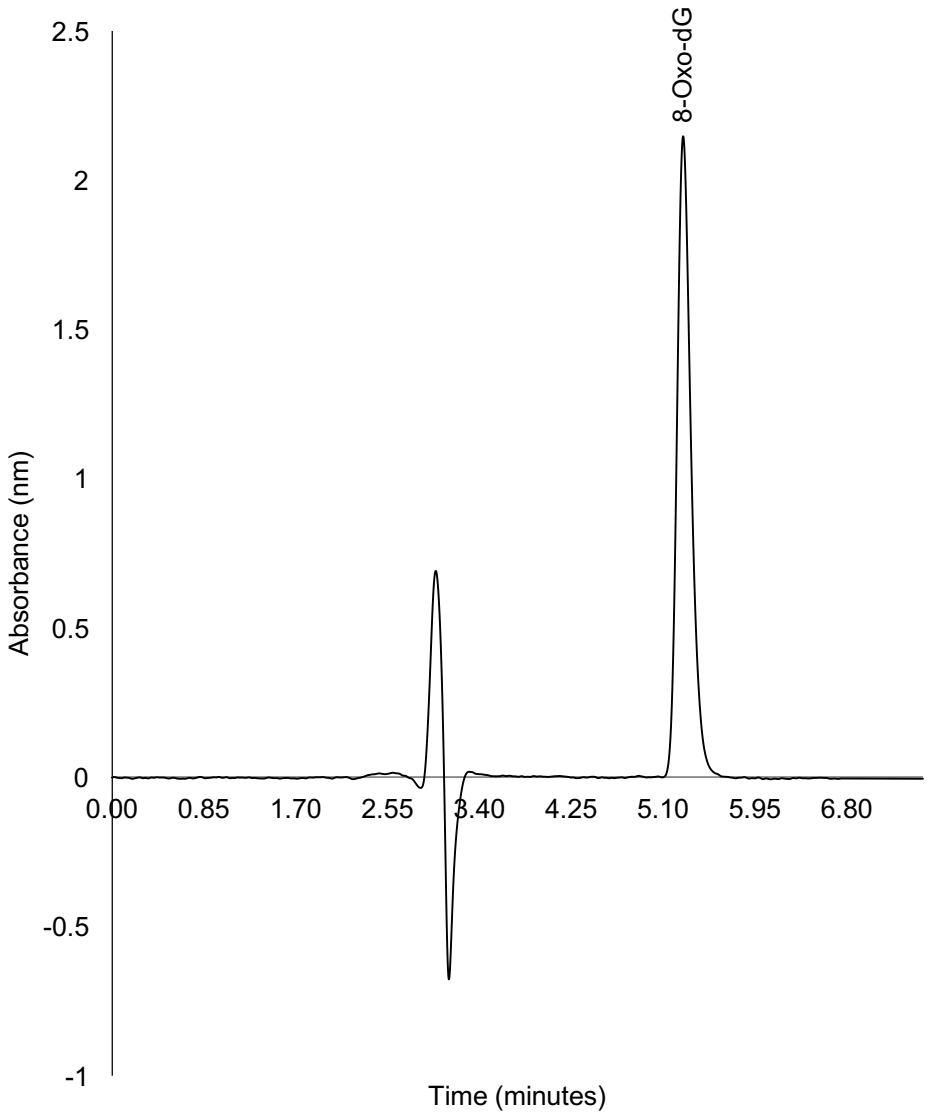

Figure 4.2 Representative figure of optimised chromatography using HPLC for the quantification of 8-Oxo-dG using 1 μM of 8-Oxo-dG standard. In the optimised method an isocratic protocol was used with running solvents in the mobile phase consisting of 15% methanol supplemented with 0.1% formic acid and 85% water supplemented with 0.1% formic acid (VWD = 280 nm).

Table 4.1 Representation of the different isocratic conditions tested for 8-Oxo-dG method optimisation with the use of HPLC.

	Methanol (%)	Water (%)	Methanol concentration of formic acid in buffer (%)	Water concentration of formic acid in buffer (%)
Condition 1*	15	85	0.1	0.1
Condition 2	5	95	0.1	0.1
Condition 3	5	95	0	0
Condition 4	15	85	0.5	0.5
Condition 5	5	95	0.5	0.5
Condition 6	25	75	0.1	0.1
Condition 7	10	90	0.1	0.1

*Where * indicates the optimised method chosen for HPLC method.*

4.2.2. Discussion

In this initial approach to detect 8-Oxo-dG an HPLC technique was utilised as it is a simple separation technique for the initial optimisation. A viable HPLC chromatography method has been demonstrated for the detection of 8-Oxo-dG. Observed peak broadening using isocratic conditions was corrected when 8-Oxo-dG standard was diluted in distilled water (18.2 MΩ). After testing different conditions, the optimised final isocratic method included the use of 15% v / v of methanol containing 0.1% v / v formic acid, with 85% v / v of water containing 0.1% v /v formic acid. As discussed in the introduction, LC-MS can be used to chromatographically separate a sample based on its physiochemical properties and also simultaneously measure *m/z* ratio*,* which allows a more powerful approach to the analysis and the overall quantification of 8-Oxo-dG compared to HPLC. Thus, the method developed in the HPLC will be carried to LC-MS for further development and optimisation.

4.3. Experiment 2

Aim: To apply the HPLC method developed to the LC-MS system.

4.3.1. Methods and results

The parameters of optimisation involved single ion monitoring in both negative (figure 4.3) and positive mode (figure 4.4). A *m/z* of 282.2 in negative mode was chosen, since it provided a taller and symmetrical peak. Voltages (DC, DL and RF) were also examined and it was concluded that the default settings were

appropriate (figures showing the results following the analysis of the voltages can be found in appendix 2).

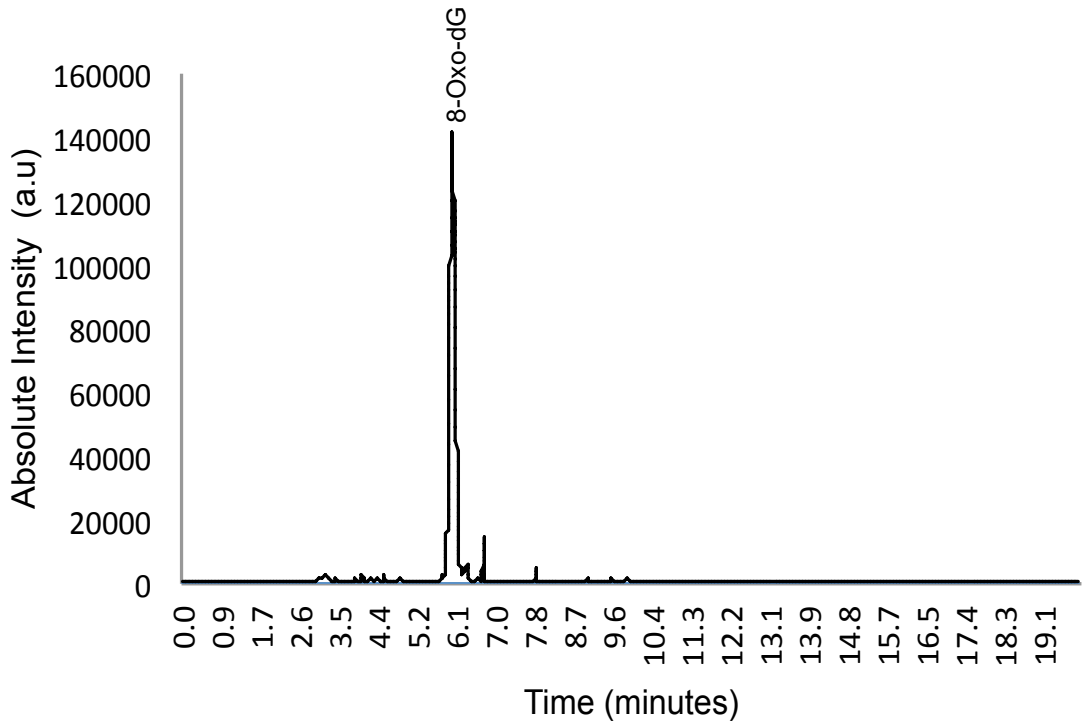

Figure 4.3 Representative figure of ion scan for the examination of 8-Oxo-dG in negative mode for an *m/z* 282.2 using LC-MS.

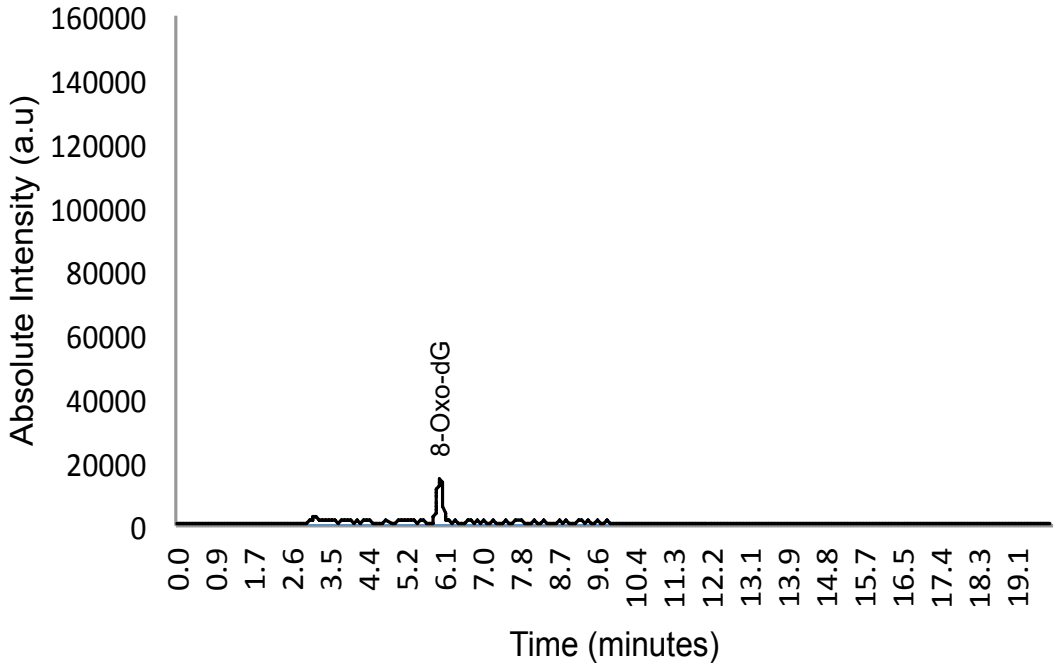

Figure 4.4 Representative figure of ion scan for the examination of 8-Oxo-dG in positive mode for an *m/z* 284.2 using LC-MS.

4.3.2. Discussion

The HPLC method was subsequently applied in LC-MS to detect 8-Oxo-dG. A *m/z* of 282.2 in negative mode was chosen for the detection of 8-Oxo-dG, as ionisation was enhanced resulting in a stronger signal and improved sensitivity, which provided the best possible result in the quality and symmetry of the peak. In order to proceed with the method development, an internal standard that is stable has similar properties to 8-Oxo-dG but does not interfere must be applied to account for reproducibility and reduction of matrix and ionisation effects (Bergeron et al., 2009).

4.4. Experiment 3

Aim: To optimise 2,6-diaminopurine to be used as an internal standard.

4.4.1. Methods and results

2,6-diaminopurine has been shown to have a similar structure to 8-Oxo-dG but not found in samples and has been previously used in the measurement of DNA damage (Ravanat et al., 1995, Cooke et al., 1998). Thus, it was chosen as an internal standard (247847, Sigma-Aldrich, Poole, UK) and was subsequently tested and optimised. An issue was observed when using the 8-Oxo-dG isocratic method, as 2,6-diaminopurine eluted in the solvent front. To correct this, a gradient method was set as follows (figure 4.5). Methanol (with 0.1% v / v formic acid) increased over the first 5 minutes from 5% to 15% at which point then concentration was retained at 15% until minute 10. From minute 11 until minute 15.50, 95% methanol (with 0.1% formic acid) was included as a wash-

out step and then from minute 16 until minute 30 an equilibration period followed with 5% of methanol (with 0.1% formic acid). This outcome, of implementing a gradient protocol, resulted in 2,6-diaminopurine peak showing after the solvent front (retention time = 3.7 minutes) and 8-Oxo-dG occurring latter on during the 15% concentration (retention time = 9.8 minutes). For the voltages and the examination of positive and negative mode, the default settings were chosen to be used. After detection of 2,6-diaminopurine in positive (figure 4.6) and negative mode (figure 4.7), it was determined (based on the same principles of optimisation as previously stated in experiment 2) that the positive mode was the most appropriate to be used for the analysis. On positive mode, single ion monitoring, 2,6-diaminopurine was detected for *m/z* of 151. A stock of 7 mM of 2,6-diaminopurine in DMSO was prepared every 5 days, since results of subsequent daily tests showed that 2,6-diaminopurine stock was stable for a maximum of 7 days. A range of 2,6-diaminopurine dilutions were examined in LC-MS and it was determined that the optimal concentration to be used was 100 µM diluted in distilled water (18.2 MΩ).

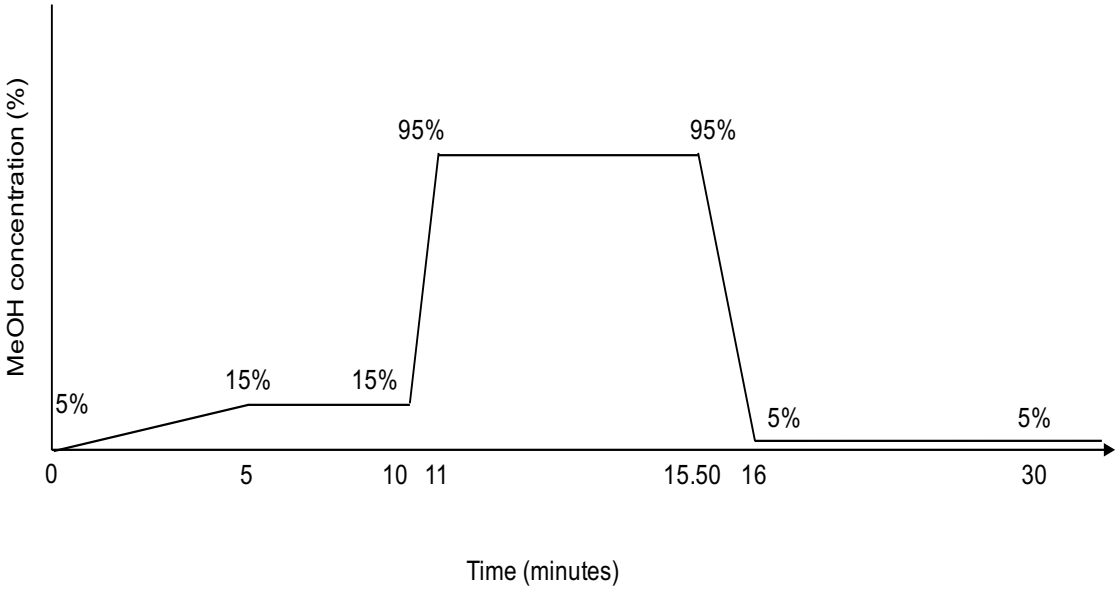

Figure 4.5 Optimised gradient protocol for LC-MS analysis of 8-Oxo-dG. Percentages shown represent the mobile phase of methanol (MeOH) concentration (0.1% formic acid v / v in methanol). Beginning at time point zero, a solution of 5% methanol with distilled water (18.2 MΩ) was used that was gradually increased to 15% of methanol over a period of 5 minutes and remained at this concentration until the 10th minute. From minute 10 until minute 11 methanol concentration increased to 95% and remained at this concentration until minute 15.50. Methanol then decreased to 5% at minute 16, and remained at this concentration until minute 30.

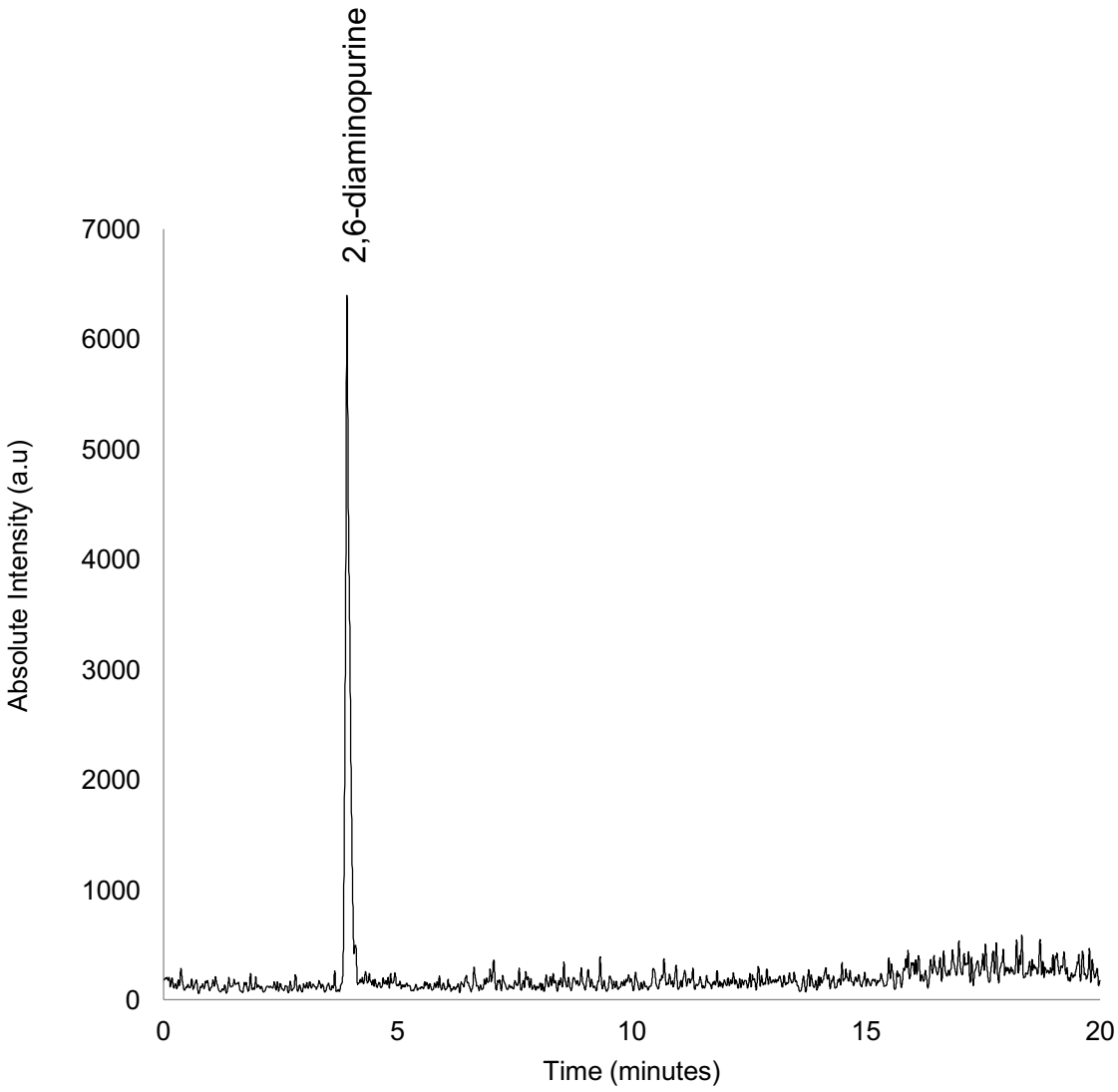

Figure 4.6 Positive ion scan of 2,6-diaminopurine (internal standard) with *m/z* = 151 selected. This m/z corresponds to that predicted for 2,6-diaminopurine (M^+ [1]).

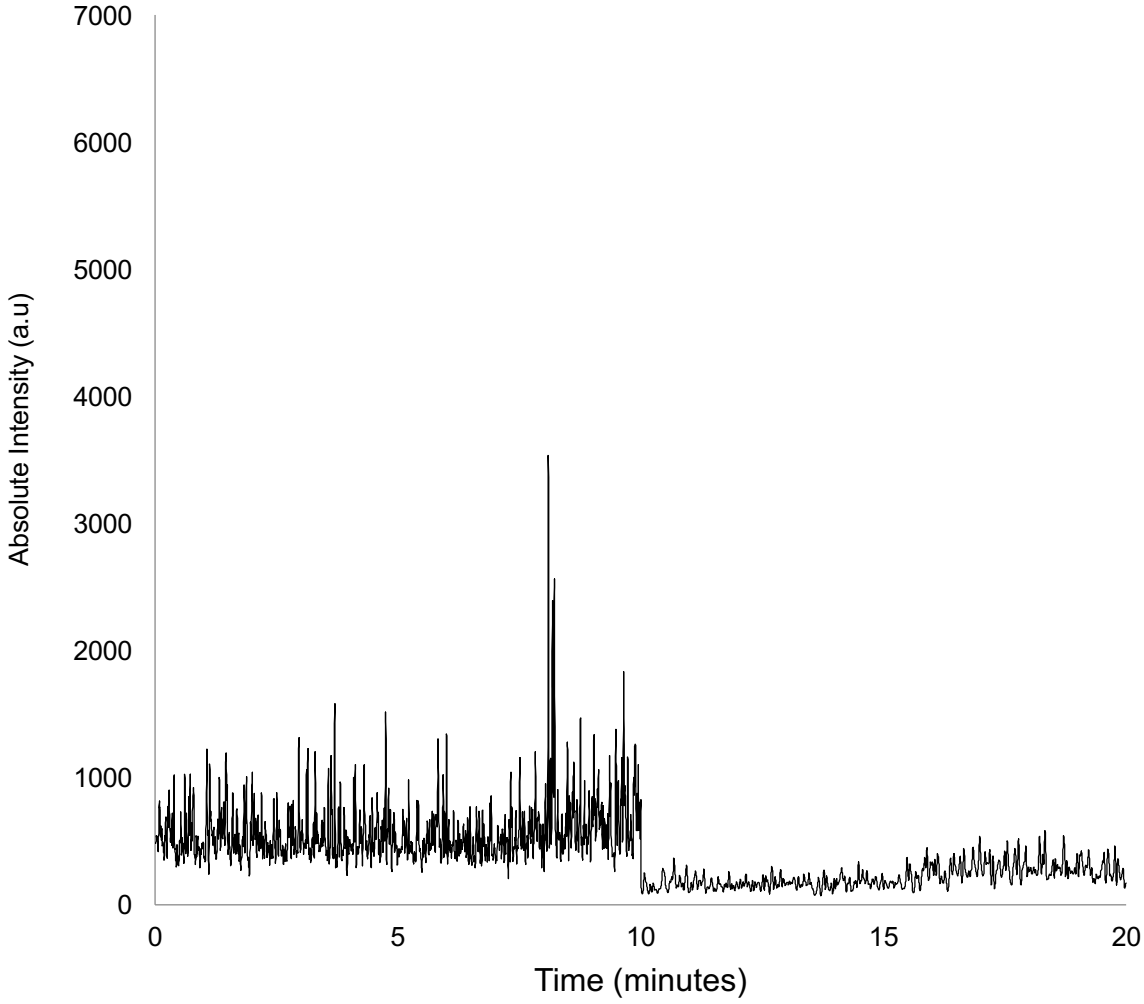

Figure 4.7 Negative ion scan of 2,6-diaminopurine (internal standard). This *m/z* corresponds to that predicted for 2,6-diaminopurine (M^{-1}).

4.4.2. Discussion

A viable method that includes an internal standard, 2,6-diaminopurine, has been demonstrated by implementing a gradient method. The choice of applying a gradient instead of an isocratic method allowed to correct 2,6-diaminopurine eluting in the solvent front. A *m/z* of 151 on positive mode was chosen for the detection of 2,6-diaminopurine, as it provided a robust signal of the peak, which was determined using the same principles as described in experiment 2. Following tests using different dilutions, the optimal concentration chosen to be used was 100 µM diluted in distilled water (18.2 MΩ). To proceed, a recovery of 8-Oxo-dG in plasma samples must be examined to determine the best deproteination method to apply in the method.

4.5. Experiment 4

Aim: To recover 8-Oxo-dG in plasma samples.

4.5.1. Methods and results

In order to examine the recovery of 8-Oxo-dG in plasma samples, different deproteination methods were tested by employing common deproteination mixtures. Using 100 µl of plasma sample, 1 ml of methanol (using concentrations of 25%, 50% 75% and 100%) or Dimethylformamide (DMF) or acetone was added to each sample. Following an overnight incubation at -20ºC samples were centrifuged at 16100 rcf for 10 minutes at 4ºC and then injected in LC-MS. Results showed that DMF did not show a peak during LC-MS analysis (figure 4.8) and acetone could not be used as it did not deproteinate

the samples efficiently in order to be used down the column. On the contrary 100% methanol was shown to be the best deproteination technique and thus was chosen to be used in the protocol (figure 4.9).

To examine if the drying down of the samples would not oxidise the samples, plasma samples were tested dried versus non-dried and incubated (37 ºC) which is a temperature also used in digestion, versus non incubated (immediately frozen at -20ºC). Using 100 µl of plasma at 500 nM of 8-Oxo-dG and by following the methanol deproteination technique described above, samples were left to either dry for 4 hours using a centrifugal evaporator or they were tested without being dried. Dried samples were corrected for concentration by the addition of the same volume of solvent (1 ml) as in the non-dried samples to ensure there was no concentration effect by the drying down method. Results using LC-MS analysis showed that dried samples did not cause an increase in signal which suggested that there was no loss, damage or increase in oxidation with the use of drying down process (figure 4.10).

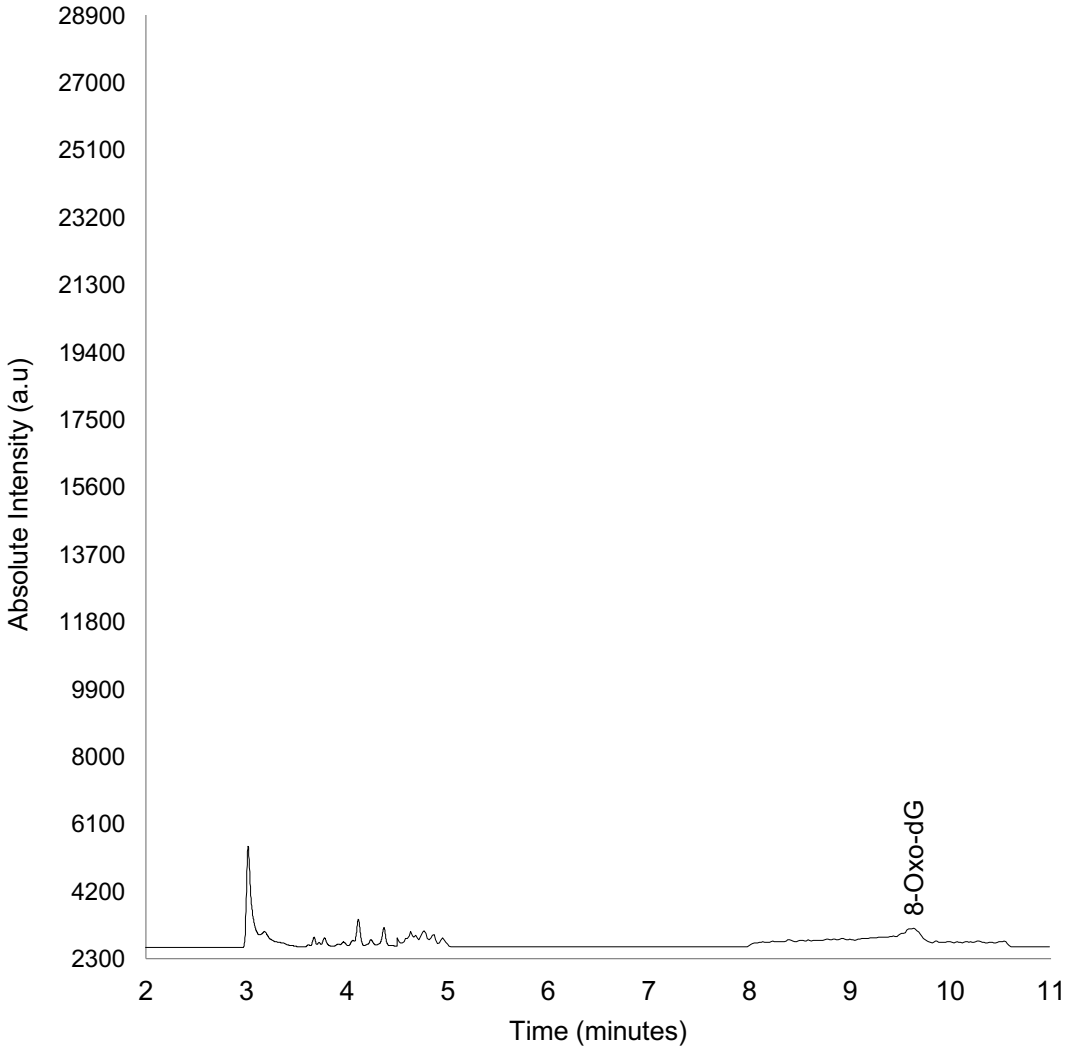

Figure 4.8 Representative figure showing plasma samples (100 nM) after DMF deproteination.

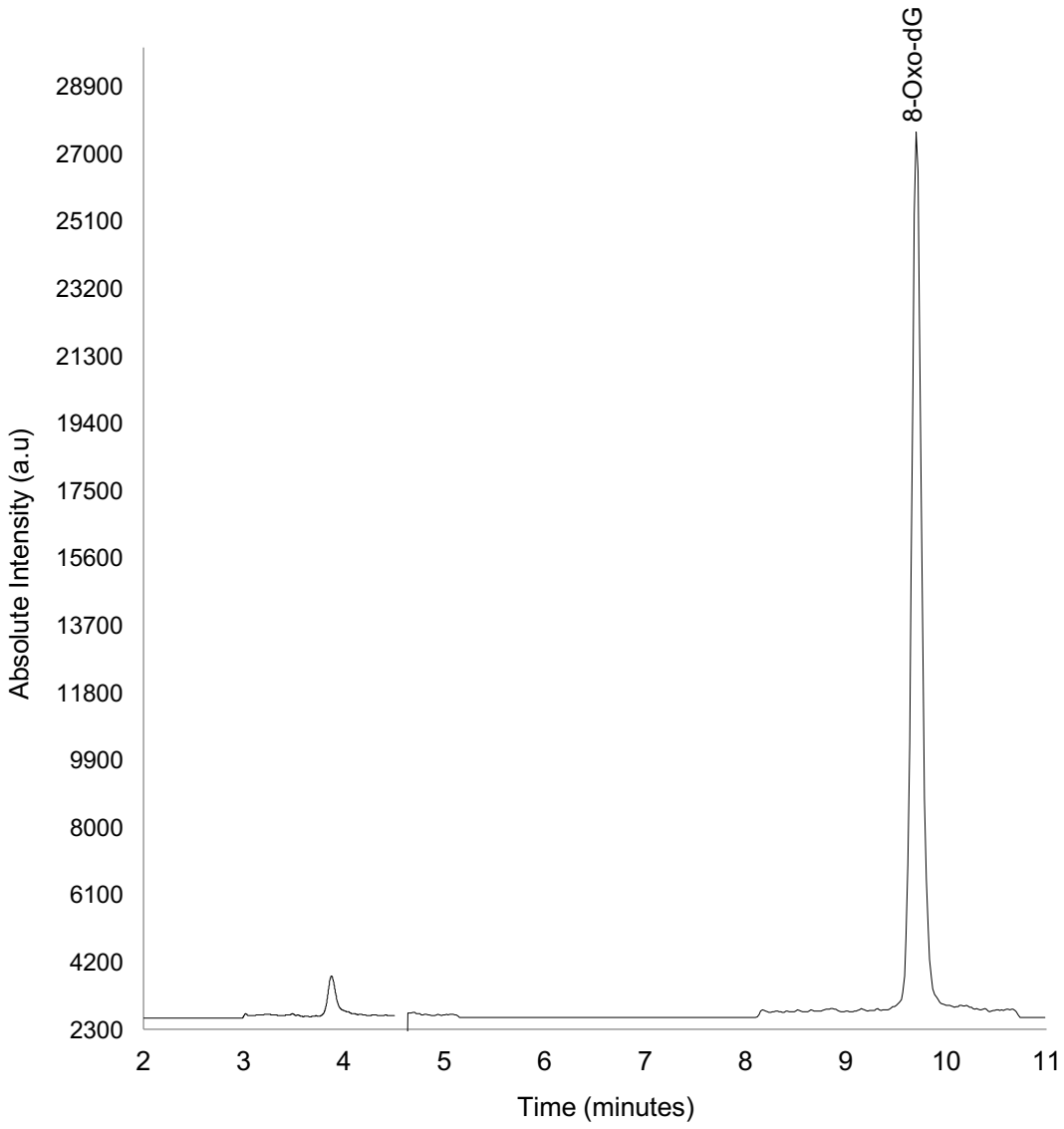

Figure 4.9 Representative figure showing plasma samples (100 nM) after deproteination using 100% methanol.

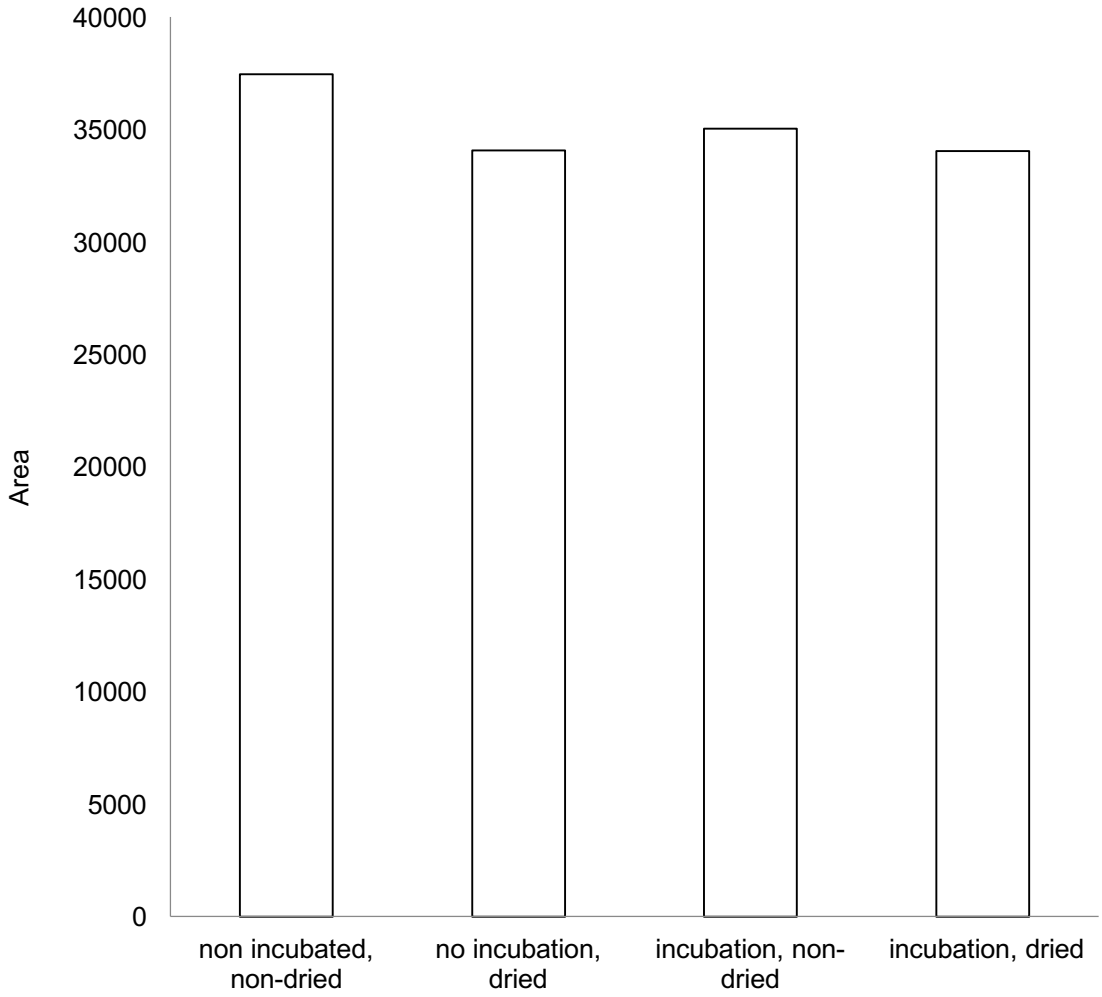

Figure 4.10 Comparison of either concentrated (dried) or non-concentrated and incubated or non-incubated 100 μM 8-Oxo-dG standards for the recovery of 8-Oxo-dG in plasma samples (n = 1 experiments).

4.5.2. Discussion

The use of 100% methanol was chosen as the best method to achieve deproteination of plasma samples following an overnight incubation at -20ºC. To ensure samples were concentrated to allow improved detection, the use of a centrifugal evaporator was implemented. Following tests to ensure that the use of the centrifugal evaporator did not damage or oxidise samples after a drying down period of 4 hours, the method was further developed. As a result, the use of 100% methanol was implemented in the protocol and a drying down period of the samples for 4 hours. To proceed, a linearity in the standard curve must be examined to determine the dynamic range of the assay in plasma samples.

4.6. Experiment 5

Aim: To examine linearity in standard curves

4.6.1. Methods and results

A range of standard curves were examined, looking at the linearity of the standard curve. Standard curves were prepared in water and plasma. Water was chosen as this is a typical buffer used in ELISA analysis of 8-Oxo-dG. However, as plasma is a complicated fluid due to interactions with other components (matrix effect), (Chiu et al., 2010) it was decided that the 2 methods should be investigated and compared. A range of 8-Oxo-dG concentrations (0 nM – 500 nM) was also chosen as these represent is a typical range of concentrations used in ELISA. Results showed that when the standard curve was prepared in water the recovery was greater compared to plasma

(figure 4.11). Thus, it was determined that the standard curve should be made in plasma in order to provide an accurate quantification of the 8-Oxo-dG concentrations.

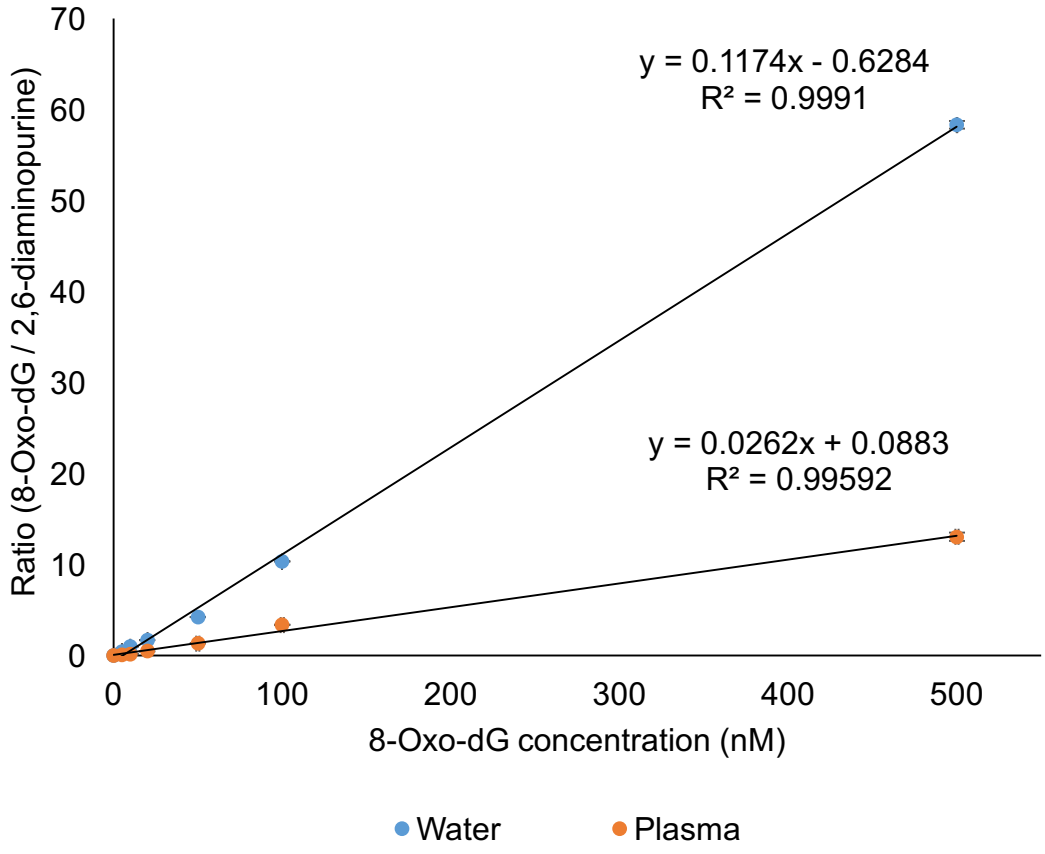

Figure 4.11 Representative standard curve (0 – 500 nM) to examine linearity and recovery rate of plasma and water samples. Results showed that the recovery rate in plasma was lower compared to water thus, it was decided that the standard curve should be made in plasma (n = 2 independent experiments).

4.6.2. Discussion

Standard curve in plasma was chosen as the more appropriate method to be used since results showed that 8-Oxo-dG signal was lower in plasma compared to water which could be due to the matrix effect. Consequently, the use of plasma could provide more reliable quantification of 8-Oxo-dG. From the results of this experiment, an enzymatic digestion should be applied next to determine the optimum approach to release the free nucleotides. It is considered that the ELISA may also detect 8-Oxo-dG in free solution and small DNA and RNA molecules such as those found in plasma (Lo, 2001). This can be important as digestion can break DNA molecules and release free nucleotides thus, provide more representative quantification of 8-Oxo-dG concentrations when compared to ELISA methods. As this method in addition to Lam et al. (2012) and Villano et al. (2015) measured free 8-Oxo-dG concentrations without implementing a digestion, it is possible that the results could underestimate 8-Oxo-dG. Thus, with the addition of a digestion step it could be possible to break DNA molecules and release free nucleotides which may result in more representative quantification of 8-Oxo-dG concentrations.

4.7. Experiment 6

Aim: To use an enzymatic digestion in the method to release free nucleotides and optimise the digestion.

4.7.1. Methods and results

As digestion can increase the detection of 8-Oxo-dG by breaking DNA molecules and release free nucleotides, it was decided that there is a need to compare a positive standard ELISA kit control (EpiQuick 8-OHdG DNA Damage, P-6003-96, Epigentek, Farmingdale, NY) with the 8-Oxo-dG used in LC-MS method developed. This addition was implemented as ELISA sometimes use digestion of the DNA thus, an increased signal of the ELISA standard in the LC-MS method would imply that digestion can increase the signal by breaking DNA and releasing free nucleotides. Consequently, an ELISA kit standard was used containing an 8-Oxo-dG oligonucleotide, to examine if digestion could play an important role in the resulting quantification of 8-Oxo-dG. Using a published method (Ravanat et al., 1998) with selected modifications, water samples supplemented with 10 ng of ELISA positive control were tested in LC-MS, to determine whether a 2 hour digestion could be implemented to detect the resulting nucleotides. The method involved a 2 hour digestion using DNase I (10104159001, Sigma-Aldrich, Dorset, UK) and a further 1 hour de-phosphorylation using alkaline phosphatase (A3907.0100 Sigma-Aldrich, Dorset, UK). Results showed that detectable peaks could only be observed following digestion (figure 4.12) whereas no detectable peaks could be observed when a digestion step was not included in the method (figure 4.13).

To examine if detection of 8-Oxo-dG is improved when incubation time of digestion increases, water samples supplemented with 10 ng of ELISA standard were also incubated for 4 hours of digest with 2 hours to de-

phosphorylate. This also included samples of ELISA positive control that were incubated for 4 hours without the addition of digestive enzymes. Results showed quantifiable peaks following 4 hours of digestion (figure 4.14) whereas samples without digestion showed no quantifiable peaks (figure 4.15). After comparison between 2 and 4 hours, the method that included 2 hours of digestion was chosen, as during the 4 hours there was a decrease in signal which could imply that there is a spontaneous breakdown of DNA.

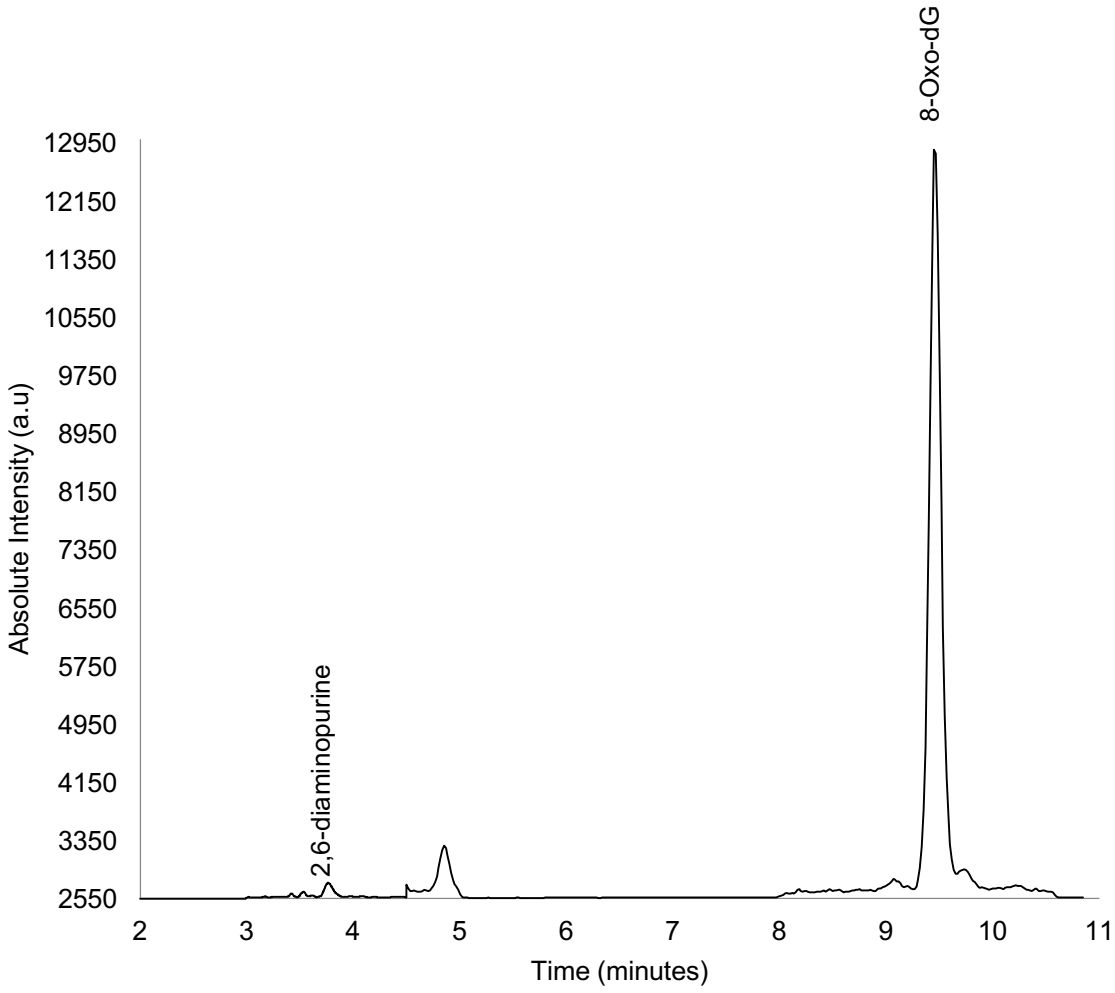

Figure 4.12 Representative chromatography of ELISA positive control (10 ng) following 2 hours of digestion in water samples supplemented with 500 nM 8-Oxo-dG. Digestion provided a quantifiable peak for 8-Oxo-dG of *m/z* = 282.2

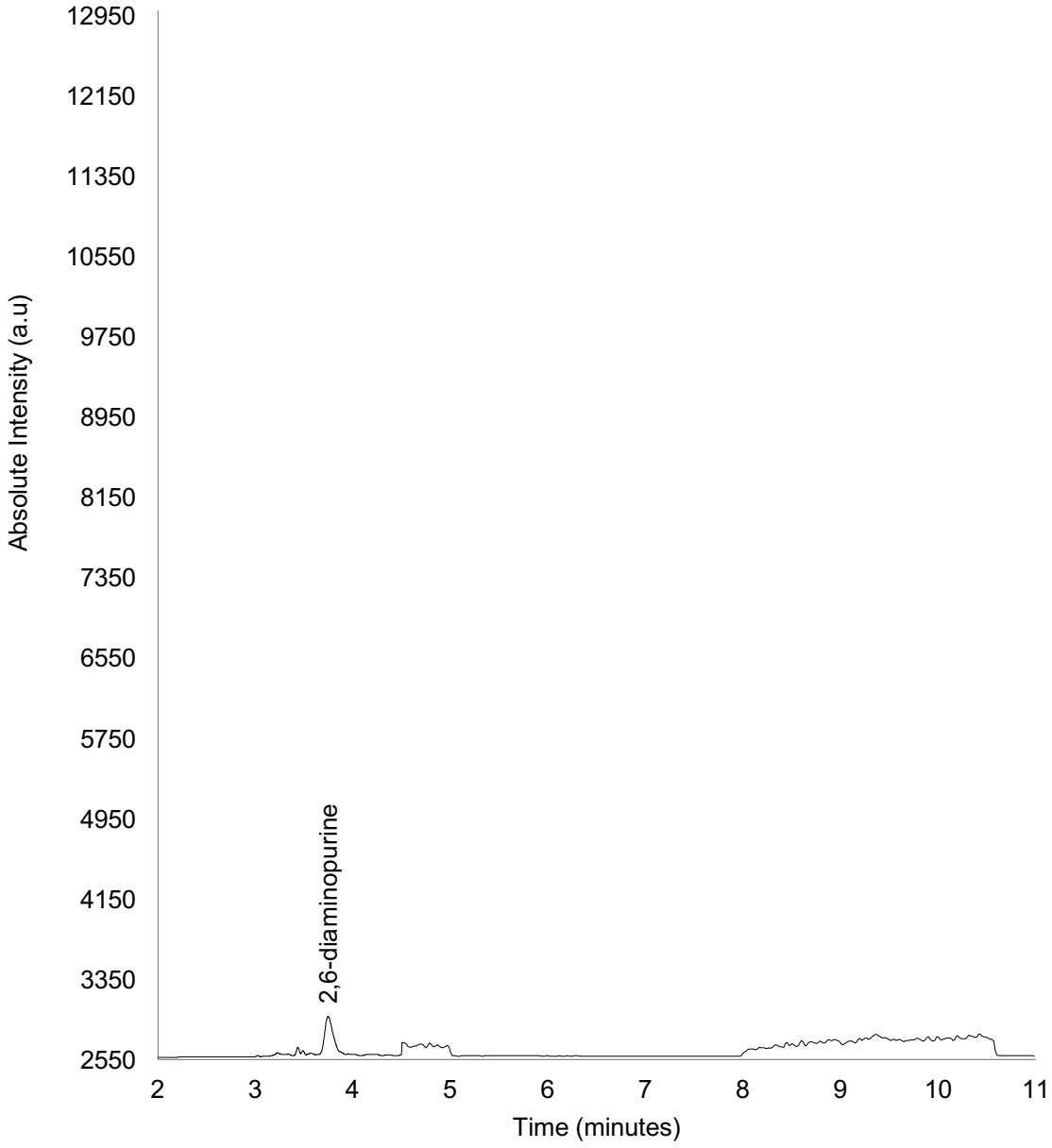

Figure 4.13 Representative chromatography of ELISA positive control (10 ng) of 2 hour incubation without the addition of digestive enzymes for water samples. Results did not provide a quantifiable peak for 8-Oxo-dG of *m/z* = 282.2.

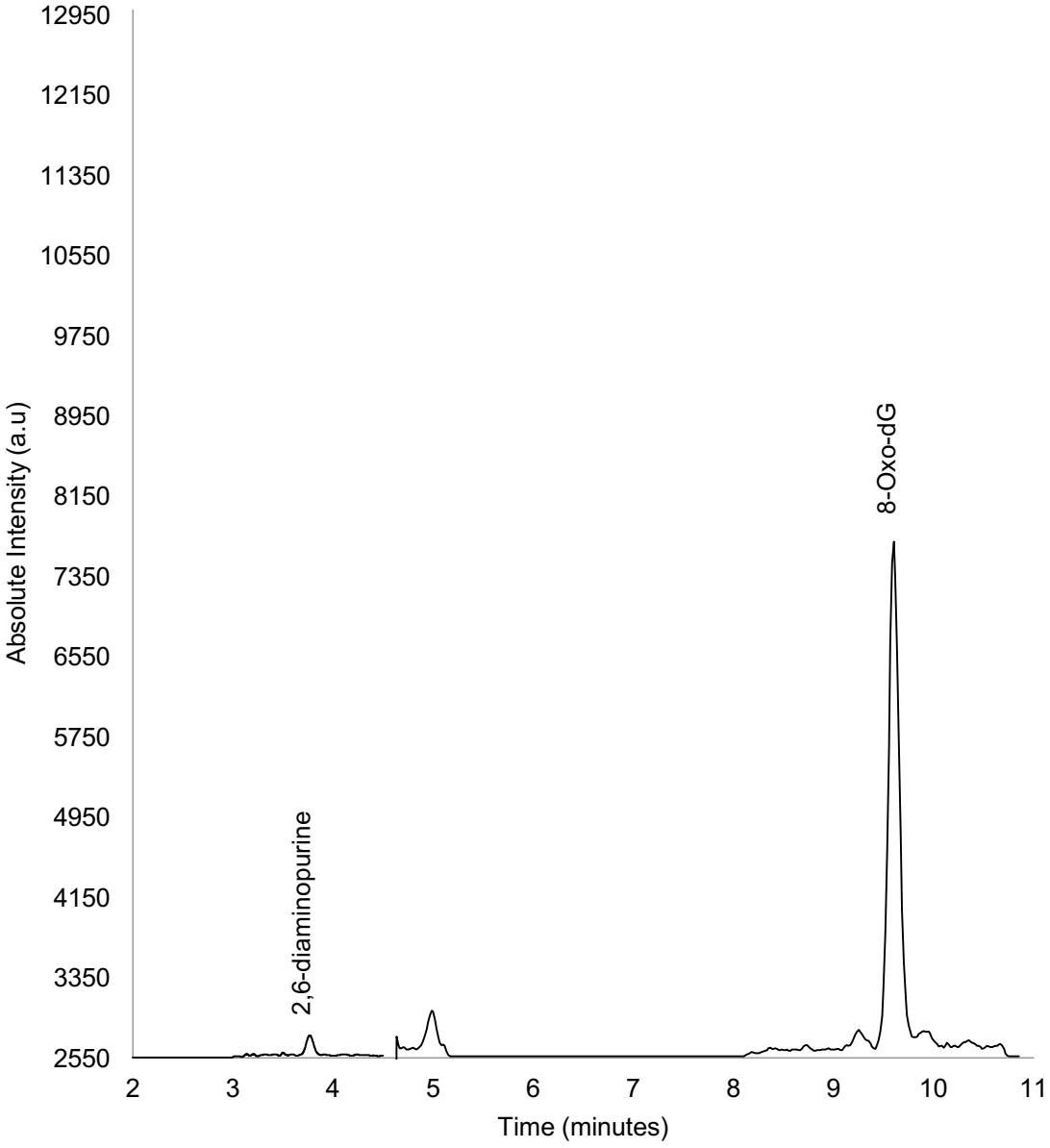

Figure 4.14 Representative chromatography of ELISA positive control (10 ng) following 4 hours of digestion in plasma samples supplemented with 500 nM 8-Oxo-dG. Digestion provided a quantifiable peak for 8-Oxo-dG of *m/z* = 282.2.

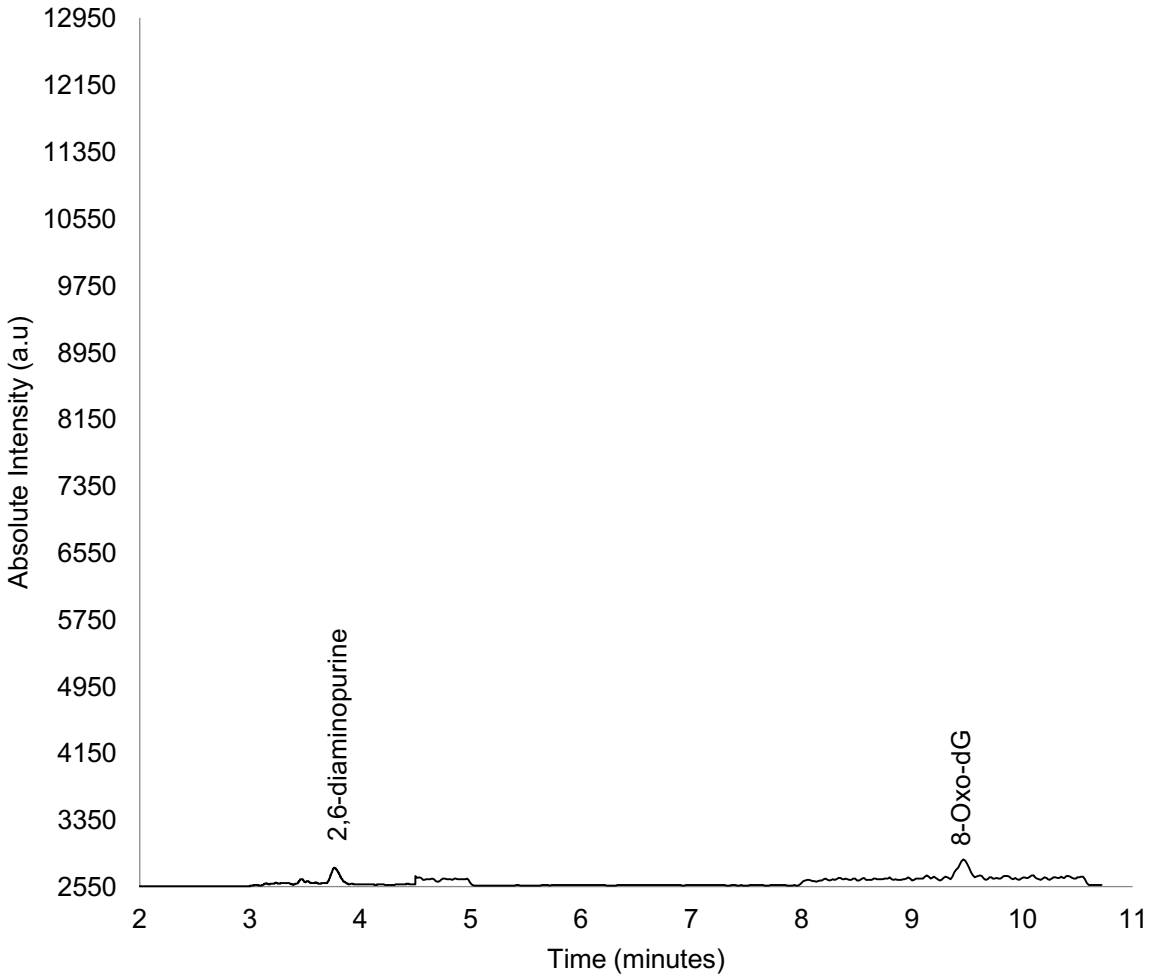

Figure 4.15 Representative chromatography of ELISA positive control (10 ng) for 4 hours incubation without the addition of digestive enzymes for 4 hours of plasma samples. Results showed a loss in signal of 8-Oxo-dG of *m/z* = 282.2.

4.7.2. Discussion

This was the final step in method development and optimisation. Enzymatic digestion was accomplished and provided a quantifiable peak for 8-Oxo-dG. This was achieved by implementing a 2 hour digestion using DNase I and a further 1 hour de-phosphorylation using alkaline phosphatase. Results also showed that following an increase in the time of digestion 8-Oxo-dG peaks did not enhance. As a result, a 2 hour digestion followed by 1 hour of de-phosphorylation was chosen as a more appropriate method to proceed.

4.8. Final LC-MS method for the detection of 8-Oxo-dG in plasma samples

A LC-MS detection method for the quantification of 8-Oxo-dG was developed and optimised as part of this thesis. Samples were enzymatically digested to individual de-phosphorylated nucleotides using a previously published method (Ravanat et al., 1998) with the modifications stated below.

After selecting plasma samples for each analysis in random order, a 2 hour digestion at 37°C was achieved by supplementing 100 µl of plasma sample with 20 U of DNase I (10104159001, Sigma-Aldrich, Dorset, UK), and 100 µl of Hanks' Balanced Salt Solution (HBSS), (H8264, Sigma-Aldrich, Dorset, UK). Samples were then dephosphorylated for 1 hour at 37°C with the addition of 24 µl of Tris-EDTA buffer solution (93283, Sigma-Aldrich, Dorset, UK) and 2 U of grade I alkaline phosphatase (from calf intestine), (A3907.0100 Sigma-Aldrich, Dorset, UK). Samples were then incubated overnight at -20°C after the addition

of 500 µl of chilled HPLC grade methanol (M / 4056 / 17, Fisher Scientific, Loughborough, UK). Samples were centrifuged at 16100 rcf, for 10 minutes at 4°C (17R micro star, VWR, Leicestershire, UK). After supernatant was recovered, it was evaporated to dryness for 4 hours at room temperature, using a centrifugal evaporator (GeneVac, MiVac, Suffolk, UK). Residues were re-suspended into 50 µl of distilled water (18.2 MΩ) supplemented with 100 µM of 2,6-diaminopurine (247847 Sigma-Aldrich, Dorset, UK) and centrifuged at 16100 rcf, for 10 minutes at 21°C (17R micro star, VWR, Leicestershire, UK). The supernatant was then recovered and aliquots were placed into HPLC fixed insert amber vials (Thermo Scientific, Chromacol, USA) in order to be analysed using LC-MS. The analysis to quantify 8-Oxo-dG involved the implementation of a gradient protocol running for a total duration of 30 minutes per sample (figure 4.5), using the parameters found in table 4.2 and the LC-MS units found in table 4.3. From a stock solution of 8-Oxo-dG (H5653, Sigma, Dorset, UK) that was previously prepared at a concentration of 3.53 mM in dimethyl sulfoxide (DMSO), (0231, VWR, Leicestershire, UK) standards were made by serial dilution, at concentrations ranging from 0 - 500 nM. Standards were prepared in resting plasma following the same method as for the samples without the addition of digestive enzymes. Data were then obtained, integrated and analysed using LC-MS solution data analysis software from Shimadzu (LabSolutions, version: 5.42 SP4, Shimadzu Corporation).

Table 4.2 Optimised LC-MS method parameters.

8-Oxo-dG	H5653 (Sigma-Aldrich, Poole, UK), m/z = 282.2 negative mode, Retention time = 10.1 minutes
2,6 diaminopurine (Internal Standard)	247847 (Sigma-Aldrich, Poole, UK), m/z = 151 positive mode Retention time = 3.8 minutes
Column	C_{18} (Agilent, Eclipse XDB-C18, 4.6 × 150 mm, 5 µm, Lot number: B12181
Column Temperature	40°C
Solvents	0.1% formic acid v / v in methanol 0.1 % formic v / v in distilled water (18.2 MΩ)
Flow rate	0.500 ml / min
Injection volume	20 µl
Total run time	30 minutes
DL Volt	Default
Qarray DC	Default
Qarray RF	Default (tune file)
Interface Volt	Tune file
Event time	0.1 seconds
Microscan	0 U
Interface	Tune file settings
Drying gas flow	18 l/min

Table 4.3 Details of the LC-MS units used for the analysis of 8-Oxo-dG.

Module	Model
LC-MS	LCMS - 2020
Valve Unit	FCV – $20AH_2$
Column Oven	CTO-$10AS_{VP}$
Liquid Chromatograph	LC-20AD
Degasser	DGU-$20A_5$
Nitrogen Generator	NM32LA (Peak scientific Ltd, Inchinnan, UK)

All the units described in this table are Shimadzu, (Shimadzu Corporation, Milton Keynes, UK) unless stated otherwise.

4.9. Method and optimisation discussion

A method to detect 8-Oxo-dG by LC-MS in human plasma and isolated DNA samples has been optimised. This method builds upon previous ones (Lam et al., 2012, Villano et al., 2015) with a couple of modifications to the sample handling that may address some limitations of these methods as follows. Firstly, the inclusion of a DNase I digestion step for plasma samples. Neither Lam et al. (2012) nor Villano et al. (2015) included in their methods a digestion and this may explain why the results of their LC-MS quantification of 8-Oxo-dG in plasma is substantially lower to that reported in ELISA experiments. A second difference is that the method developed in this chapter has used a much simpler sample handling procedure (liquid-liquid extraction rather than solid phase extraction). A third difference is that Villano et al. (2015) used a more sensitive detector compared to this method. The optimised method does not have the same level of sensitivity as that reported by Lam et al. (2012). However, a limit of quantification of 5 nM is reported. Nevertheless, this method allows detection to a level comparable to that reported for ELISA (Fogarty et al., 2013, Karpouzi et al., 2016), and based on these ELISA results it is anticipated to be able to detect and quantify 8-Oxo-dG in plasma and DNA samples. Further similarities were also seen between data from this method and that reported by Lam et al. (2012), including the requirement to use plasma for standard curves (due to the reduced recovery of 8-Oxo-dG compared with water as the calculated concentrations).

Chapter 5 of this thesis will apply this method developed as part of the aim to investigate the impact of different protocols of intermittent exercise when the

protocols are matched for mean speed, distance and duration but vary in the rate of intermittency.

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Chapter 5

The impact of exercise intermittency on oxidative stress levels

5.1. Introduction

According to a recent survey by the American College of Sports Medicine, high-intensity intermittent exercise (HIIE) is ranked as one of the most popular fitness routines, holding the top place during 2015 and currently being ranked as the third most popular form of exercise activity (Thompson, 2015). HIIE is characterised by a set of repeated high-intensity exercise activities, interspersed by recovery periods both of which can vary in either intensity or duration (Driver, 2012). One of the main reasons for the preference of HIIE over other training routines (e.g. endurance training) is due to the beneficial effects of training adaptations (e.g. skeletal muscle remodelling, increased muscle oxidative capacity) in a time-efficient manner (Gibala, 2007, Gibala et al., 2006, Burgomaster et al., 2007). Furthermore, the benefits of HIIE over continuous exercise routines have also been documented in diseased populations (Elliott et al., 2015). Results indicate an advantage of HIIE, in terms of cardiovascular function and aerobic fitness as well as in efficiency, quality of life and exercise adherence (Gayda et al., 2016, Rognmo et al., 2004).

There are indications that acute or high-intensity exercise can induce oxidative stress (Fisher-Wellman and Bloomer, 2009, Powers and Jackson, 2008, Gomez-Cabrera et al., 2009, Sureda et al., 2009). Similarly, as acute HIIE is a form of exercise activity that contains such features, it is possible that oxidative stress is also increased. However, most of the studies investigating HIIE, focus on the training effects and not the on the acute responses (Fisher et al., 2011, Bogdanis et al., 2013, Vezzoli et al., 2014). Upon closer observation of these studies, with regards to the baseline measurements, it can be suggested that

acutely, HIIE promotes oxidative stress and any attenuations that might occur are shown following a training period. Indeed, Bogdanis et al. (2013) showed that an acute session of high-intensity intermittent sprints can increase oxidative stress for up to 24 hours. It has been suggested that more intermittent sessions can be equally or more beneficial in terms of overall health, than continuous exercise, when these are matched for total energy expenditure (Lee et al., 2000) or duration (Schmidt et al., 2001, Gill et al., 1998). Still, the effect of how intermittency impacts on oxidative stress levels, when HIIE is of similar speed, duration and distance is unknown.

Therefore, the purpose of this study was to investigate the impact of exercise intermittency on oxidative stress biomarkers when exercise is matched for average speed, duration and distance, while intermittency is varied.

5.2. Methods

5.2.1. Non-motorised treadmill

As the changes in running velocity can be better reflected and monitored with the use of a non-motorised treadmill (Lakomy, 1987), the choice in the use of this type of treadmill has certain benefits. The first advantage is that the choice of this type of treadmill has a novel aspect in the approach to examine the impact of intermittency and oxidative stress. Secondly, the use of a non-motorised treadmill also better reflects team-sport activities such as football and rugby, as it has been shown to imitate better over ground locomotion, since the participant has to actively propel the belt for locomotion to occur (Franks et al.,

2012). From the above, it is possible that the choice in the use of this type of treadmill can reflect more accurately activities that include aspects of running in team sports, as the rapid velocity changes can be examined in a controlled environment and the changes in performance can be measured (Tofari et al., 2015). Consequently, the use of a non-motorised treadmill to study oxidative stress could serve a dual purpose. It may better reflect the mechanics of over ground running, as individuals are responsible for driving forward the belt and at the same time, without the interference of a motor, a greater margin for manipulations in the intermittency can be made.

5.2.2. Participant characteristics

Regularly active, male, non-smokers aged 21 ± 3 years who were not taking any medication or supplements were recruited for the study (n = 9). The physiological characteristics of the participants recruited are shown in table 5.1. The inclusion / exclusion criteria were the following: participants were regularly active males, between the age of 18 - 30 years old, they were apparently healthy and physically active (i.e. participated in exercise sessions at least 3 times per week), they should also not consume any medications or supplements and they should be non-smokers. Anthropometric measurements were performed as described in chapter 3.

Table 5.1 Participant characteristics for the study (n = 9).

Participant characteristics	Value
Age (yrs)	21.0 ± 3.0
Height (cm)	180.0 ± 4.0
Body mass (kg)	79.4 ± 7.9
Body fat (%)	12.0 ± 3.0
systolic BP (mmHg)	133.0 ± 10.0
diastolic BP (mmHg)	71.0 ± 6.0
Resting HR (beats·min^{-1})	58.0 ± 13.0
predicted HR (beats·min^{-1})	192.0 ± 2.0
Peak HR (beats·min-1)	183.0 ± 9.0
v$\dot{V}O_{2max}$ (km·hr^{-1})	14.0 ± 6.0
$\dot{V}O_{2max}$ (mL·kg^{-1}·min^{-1})	52.0 ± 6.0

All data are presented as mean ± standard deviation. Note: yrs = years, cm = centimetres, kg = kilograms, % = percentage, mmHg = millimetres of mercury, beats.min^{-1} = beats per minute, v$\dot{V}O_{2max}$ = velocity at maximal oxygen consumption, $\dot{V}O_{2max}$ = maximal oxygen consumption km.hr^{-1} = kilometres per hour, mL.kg.$^{-1}$min^{-1}= millilitres per kilogram per minute.

5.2.3. Familiarisation protocols.

Participants were familiarised to all experimental equipment and procedures prior to engaging with exercise intervention protocols. During familiarisation to the NMT participants walked (4 $km \cdot h^{-1} \cdot min^{-1}$ for 30 seconds interspersed with 30 seconds rest), jogged (between 6 – 8 $km \cdot h^{-1} \cdot min^{-1}$ for 30 seconds interspersed with 30 seconds rest) or ran (between 8 – 10 $km.h^{-1} \cdot min^{-1}$ for 30 seconds interspersed with 30 seconds rest) for 15 minutes (4 minutes exercise, 1 minute rest between each mode). During the 1 minute rest period, participants dismounted from the NMT and walked around the laboratory. These sessions were repeated until participants were fully familiarised. Familiarisation was considered complete when the participant was able to achieve and maintain the desired speed within a 2 second period.

5.2.4. HIIE protocols

The exercise interventions were performed on a non-motorised treadmill (NMT), (Woodway Force 3 NMT, Woodway Ltd). Exercise protocols were matched for average speed, duration and distance, but varied in intermittency. Due to the study design, and also to discount any possible training adaptations, each participant had to complete 1 exercise intervention per week in a random order. Randomisation was achieved with the use of Research Randomiser online Software (https://www.randomizer.org). The following interventions were formulated in conjunction with Evans, W. (unpublished data).

Highly intermittent protocol: This exercise served as the most intermittent, as very brief runs were followed by very brief periods of active recovery. Each participant had to run for 5 seconds at 2 different individualised running speeds, aiming to reach target speed within 2 seconds. The first 5 second run represented 95% v$\dot{V}O_{2max}$ and this was followed by another 5 second run representing 55% v$\dot{V}O_{2max}$. After these 2 bouts of 5 seconds, a standardised across the participants active recovery period of walking at 4 $km \cdot h^{-1}$ proceeded. A total of 9 stages of 4 minute were performed, with 1 minute of rest between each stage. A total mean v$\dot{V}O_{2max}$ 75% v$\dot{V}O_{2max}$ was achieved over the 45 minute period was of. In this protocol, a total of 48 changes in speed occurred during each 4 minute stage (figure 5.1).

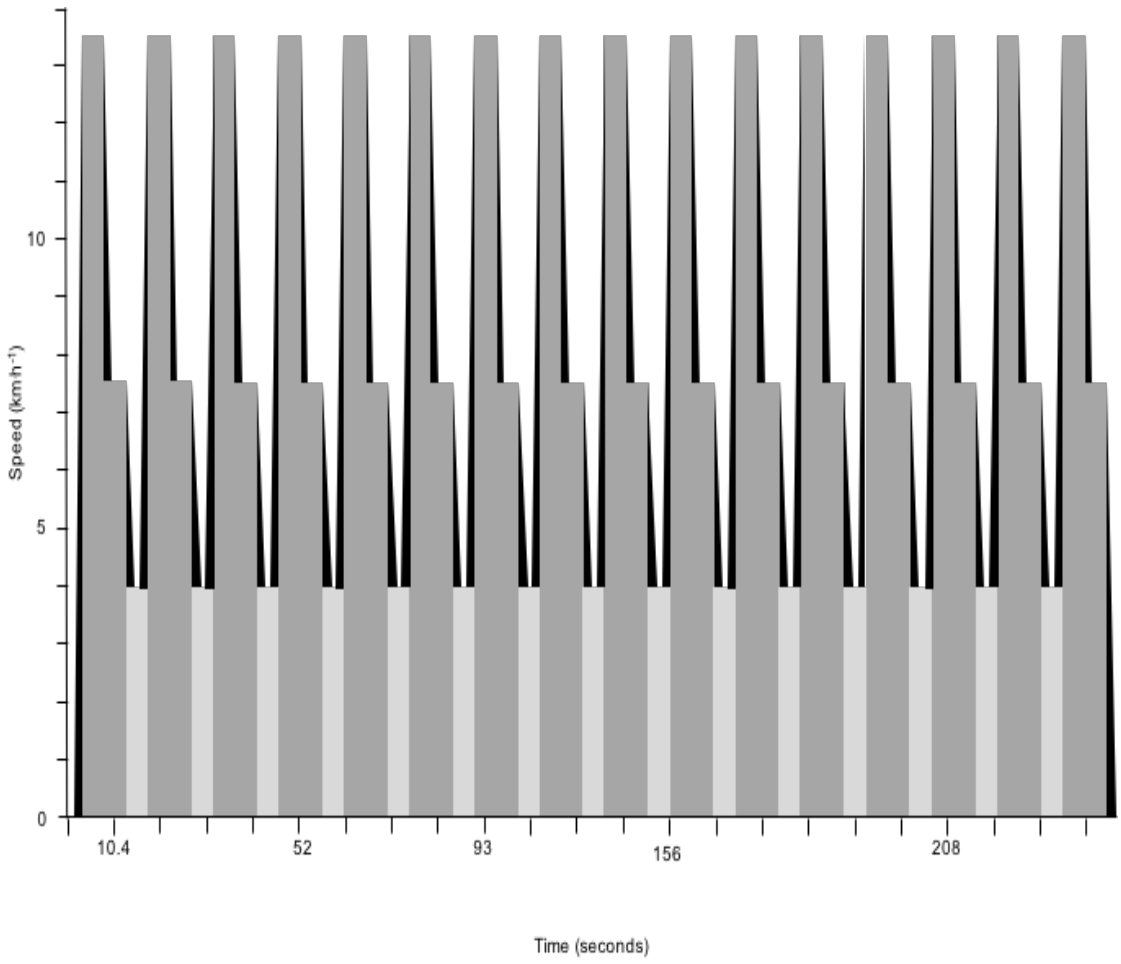

Figure 5.1 Representative diagram of the highly intermittent, 4 minute exercise bout. Each dark line represents a 2 seconds acceleration or deceleration phase. After 2 seconds of acceleration each participant had to run at 2 different individualised speeds for 5 seconds between 95% and 55% v$\dot{V}O_{2max}$ (grey bars). This was followed by an active recovery (walking) at 4 km·h^{-1} (light grey bars).

Moderately intermittent protocol: The same principles as the highly intermittent protocol were followed, but this intervention was less intermittent, as the changes in speed were 1 / 4 of that in the highly intermittent. Each participant had to run for 20 seconds at 2 different individualised running speeds aiming to reach target speed within 2 seconds. The first 20 second run was set to represent 95% v$\dot{V}O_{2max}$ and this was followed by another 20 second run at 55% v$\dot{V}O_{2max}$. After each of these sets of 20 second bouts, a standardised across the participants active recovery period of walking at 4 $km \cdot h^{-1}$ proceeded. A total of 9 stages of 4 minute bouts were performed, with 1 minute of rest between each stage. The total mean workload achieved over the 45 minute period was of 75% v$\dot{V}O_{2max}$. In this protocol, a total of 12 changes in speed occurred during each 4 minute stage (figure 5.2).

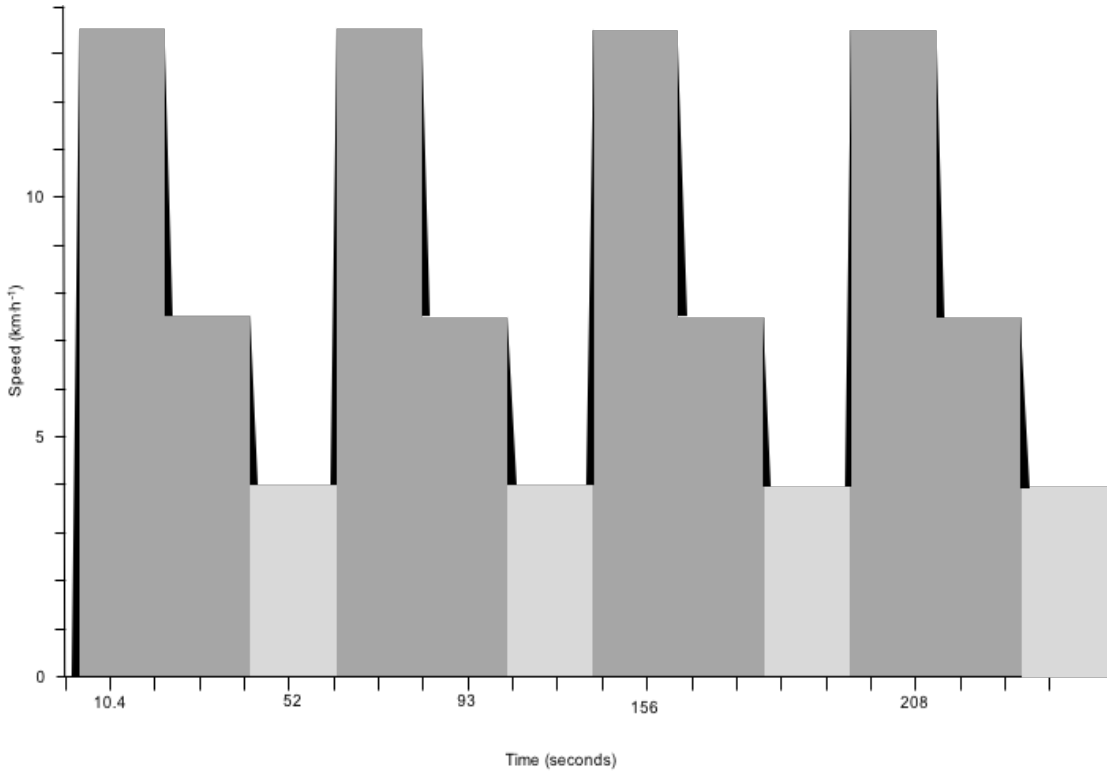

Figure 5.2 Representative diagram of the moderately intermittent, 4 minute exercise bout. Each dark line represents a 2 seconds acceleration or deceleration phase. After 2 seconds of acceleration each participant had to run at 2 different individualised speeds for 20 seconds between 95% and 55% v$\dot{V}O_{2max}$ (grey bars). This was followed by an active recovery (walking) at 4 km·h^{-1} (light grey bars).

Low intermittent protocol: This intervention served as the least intermittent (with 1 / 4 of the changes in speed compared to moderately intermittent), as continuous running on a NMT for a longer period of time would not be easily achieved (De Witt et al., 2009). Each participant had to run for 80 seconds at an individualised running speed representing a total workload of 75% v$\dot{V}O_{2max}$. The aim was to reach target speed within 2 seconds. Following each 80 second run, a standardised across the participants active recovery period proceeded (walking at 4 $km \cdot h^{-1}$). A total of 9 stages of 4 minute bouts were performed, with 1 minute of rest between each stage. The total mean workload achieved over the 45 minute period was of 75% v$\dot{V}O_{2max}$. In this protocol, a total of 3 changes in speed occurred during each 4 minute stage (figure 5.3).

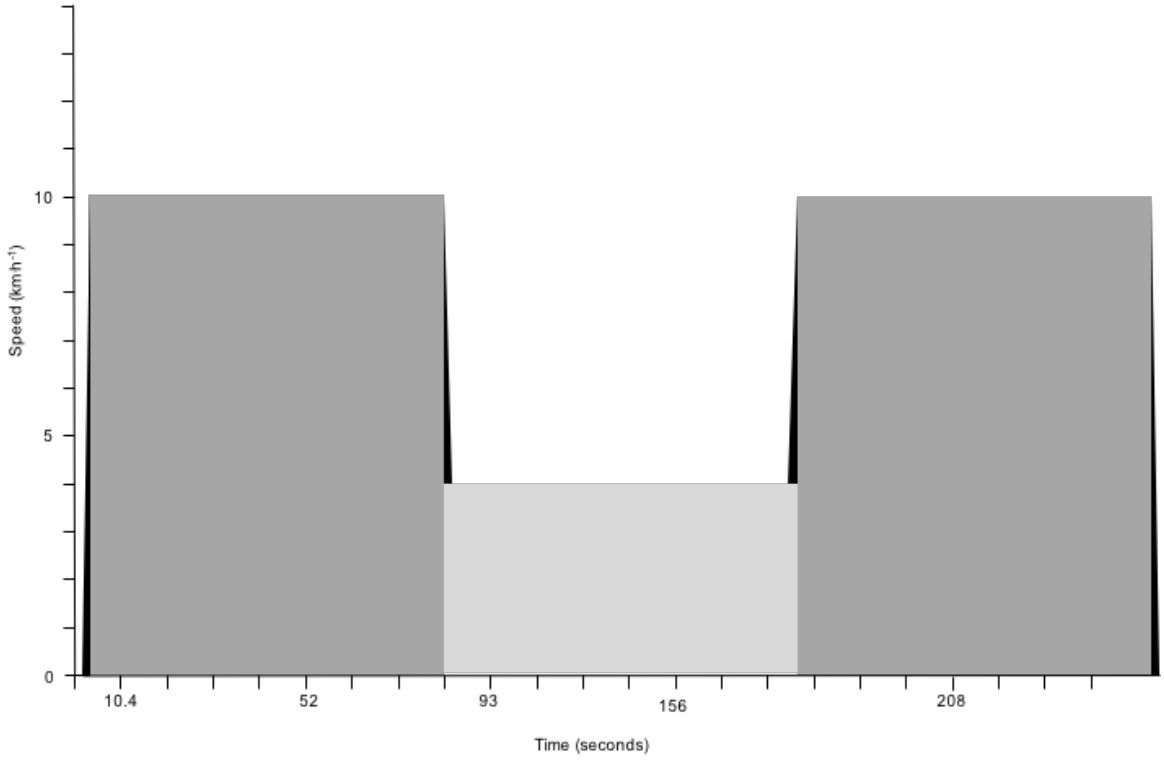

Figure 5.3 Representative figure of a low intermittent 4 minute exercise bout. Each dark line represents a 2 second acceleration or deceleration phase. After 2 seconds of acceleration each participant had to run for 80 seconds at 75% v$\dot{V}O_{2max}$ (dark grey bars) followed by 80 seconds of walking at 4 km·h^{-1}(light grey bar).

5.2.5. General exercise protocol design

In order to account for the diurnal variations, a time frame commencing at 06:30 - 08:30 was provided. During this time frame, each participant had to choose a specific time period during which, they would attend the laboratory at the exact same time (± 15 minutes) each week for the remaining duration of the study. In addition, participants were also required to abide to the following: refrain from any form of exercise or physical activity for 48 hours; excluding light walking, fast for 12 hours prior to each laboratory visit, refrain from the use of alcohol for 24 hours, drink water as required and refrain from the use of supplements or medication.

During all exercise sessions, a rest period of 10 minutes after the arrival at the laboratory was provided. This time also served to communicate any potential changes in the physical activity status, health or lifestyle of the participant, who was also required to sign the relevant informed consent declaration form and pre-exercise medical questionnaire (appendix 2). After this, heart rate was measured and a venous blood sample was collected that served as the pre-exercise (baseline) sample. A 5 minute warm-up session proceeded, comprising of an intermittent running on the NMT of altering speeds every 15 seconds between 55% of $v\dot{V}O_{2max}$ and a walking speed of 4 $km \cdot h^{-1}$.

Each exercise session was performed for a total duration of 45 minutes, without accounting the warm-up time. Each 45 minute exercise protocol consisted of 4 minute stages of high-intensity intermittent running at a mean 75% of $v\dot{V}O_{2max}$, followed by 1 minute of passive recovery (halting of running). Once exercise

was completed, venous blood samples were collected at: 1 hour, 2 hours and 24 hours post exercise (figure 5.4). For recording rate of perceived exertion (RPE), a Borg's Scale (Borg, 1982), (scale from 6 - 20, where 6 represents very, very light activity and 20 represents a maximum effort activity) was used in the final 15 seconds of each 4 minute bout.

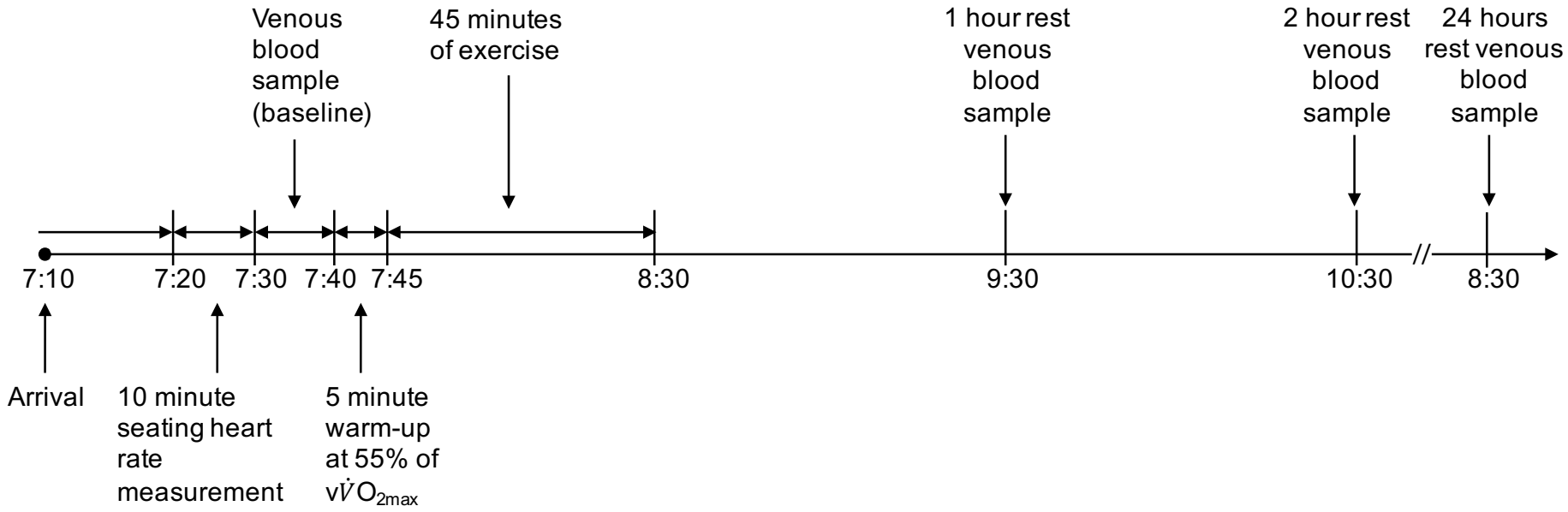

Figure 5.4 Schematic overview of a typical experimental protocol.

5.2.6. Collection of blood samples for the analysis of oxidative stress biomarkers

Blood samples were collected for serum and plasma, as described in chapter 3, at baseline, 1 hour, 2 hours and 24 hours post exercise. Analysis of lipid hydroperoxides, total glutathione and superoxide dismutase was conducted as described in chapter 2 and analysis of 8-hydroxy-2'-deoxyguanosine (8-Oxo-dG) was performed as described in chapter 4.

5.2.7. Analysis of normality of the data

Using SPSS (version 24, IBM Corp., NY.USA), the data were assessed for normal distribution using Shaphiro Wilk test ($p < 0.05$). As the requirements of normal distribution criteria were not met, statistical analysis was conducted using Kruskal-Wallis test ($p < 0.05$) using SPSS (version 24).

5.3. Results

5.3.1. 8-Oxo-dG

8-Oxo-dG concentrations were not determined above the limit of detection of the LC-MS method used (figure 5.5). The limit of detection at 5 nM (figure 5.6), the internal standard (figure 5.7), the digestion controls (figure 5.8) and repeated standard injections all confirmed that the detection method was working and

demonstrated that the technique that was implemented showed appropriate sensitivity as described in chapter 4 of this thesis.

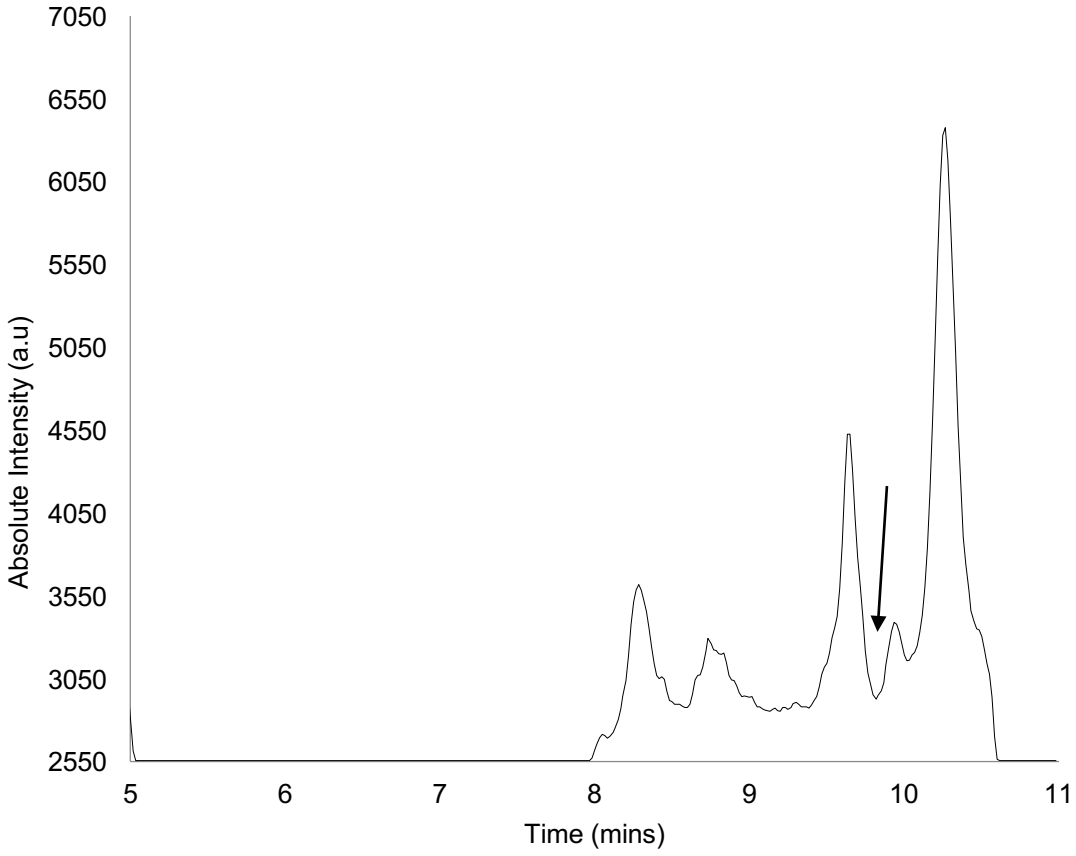

Figure 5.5 Representative chromatography of plasma samples for the presence of 8-Oxo-dG during HIIE. Results show no quantifiable peak at the retention time of interest (indicated by the arrow).

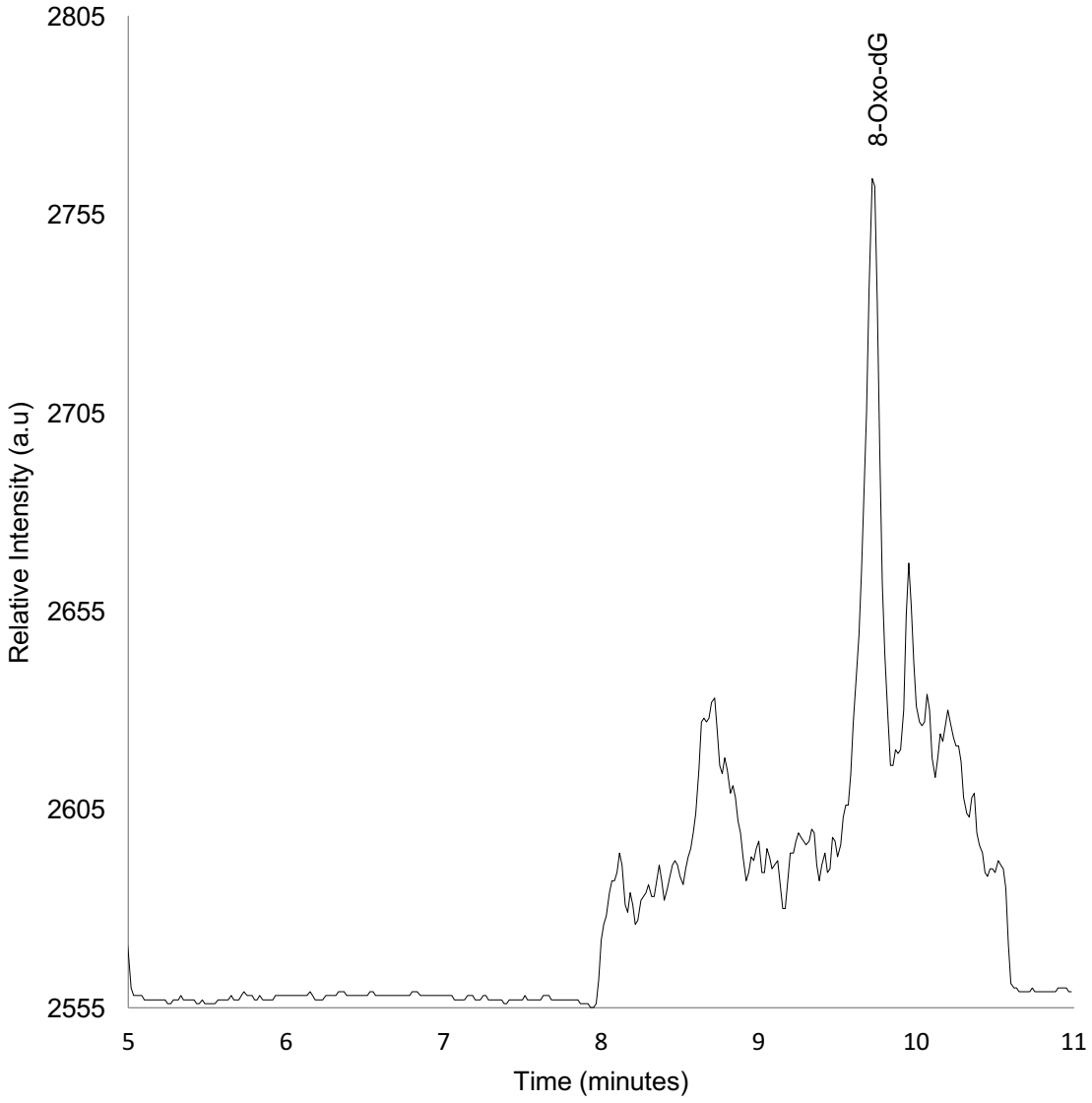

Figure 5.6 Representative chromatography of 5 nM 8-Oxo-dG standard sample.

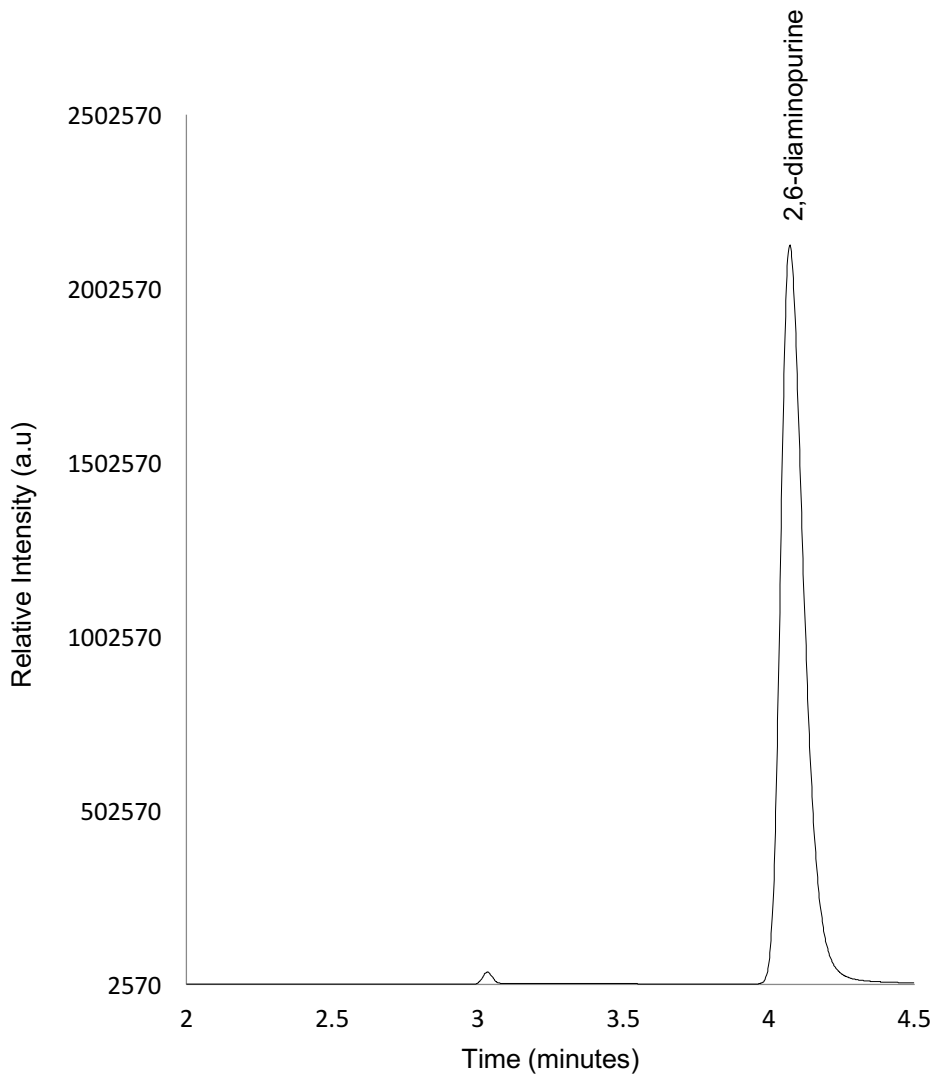

Figure 5.7 Representative chromatography of the internal standard (2,6-diaminopurine).

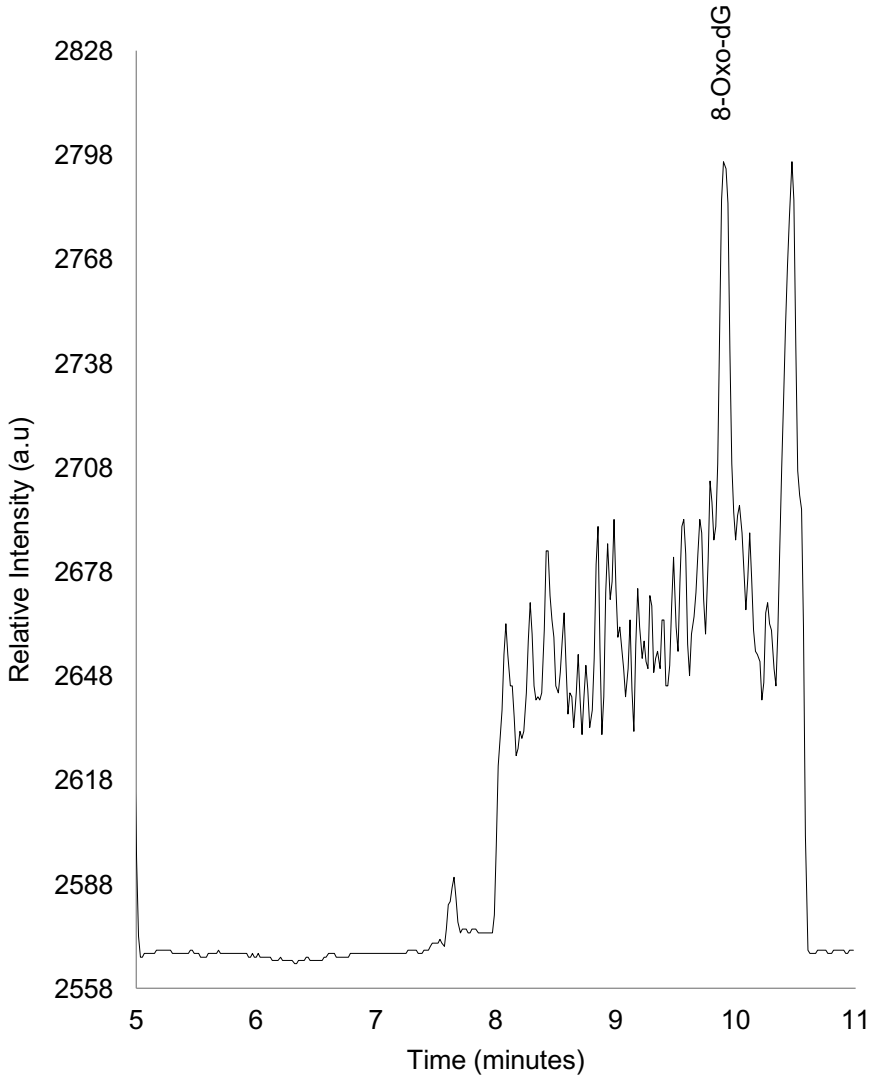

Figure 5.8 Representative chromatography of ELISA standard following digestion.

5.3.2. Lipid hydroperoxides

All exercise protocols showed similar lipid hydroperoxide concentrations in serum to the control conditions (figure 5.9). Statistical analysis showed no significance ($p < 0.05$). Substantial variation was seen between conditions and time points in the range of 1.6 – 24.9 $\mu mol.L^{-1}$.

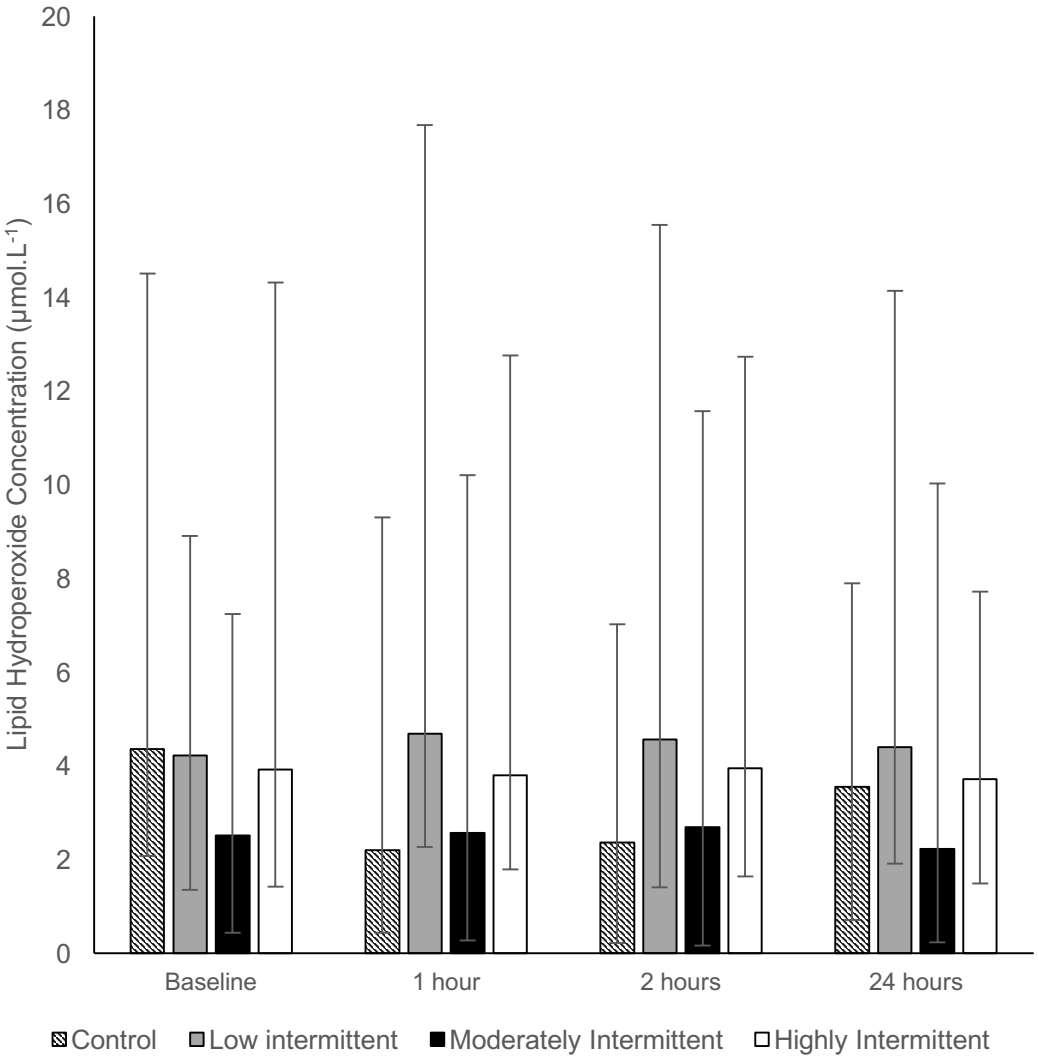

Figure 5.9 Lipid hydroperoxide concentrations for HIIE and a true control at baseline 1 hour, 2 hours and 24 hours. Median values with error bars indicating 75% upper and 25% lower percentiles.

5.3.3. Total glutathione

All exercise protocols showed similar total glutathione concentrations in plasma to the control conditions (figure 5.10) and statistical analysis showed no significance ($p < 0.05$). Considerable variation was observed in all conditions in the range of 0.32 – 5.50 μM.

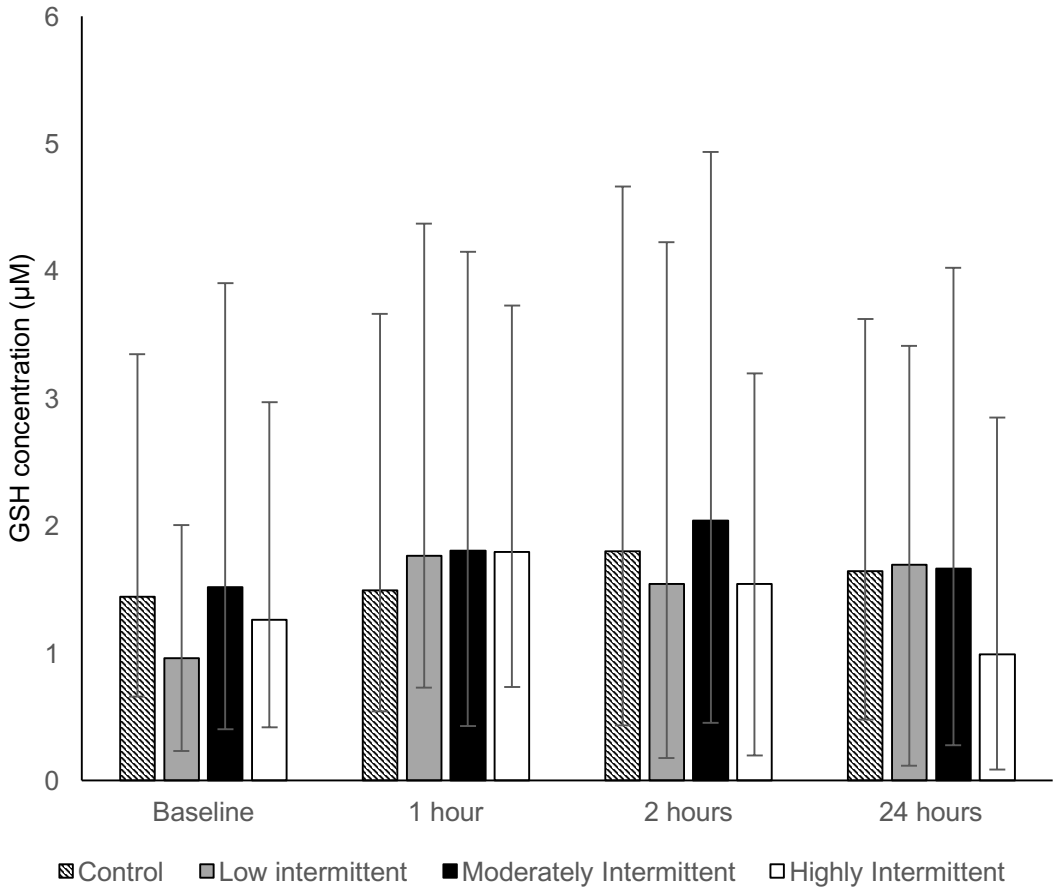

Figure 5.10 Total GSH concentration following 3 different HIIE protocols of varying intermittency and a true control at baseline 1 hour, 2 hours and 24 hours (n = 9). Median values with error bars indicating 75% upper and 25% lower percentiles.

5.3.4. Superoxide dismutase activity

Figure 5.11 demonstrates that all exercise protocols showed similar superoxide dismutase activities in plasma to the control conditions with no statistical significance being observed ($p < 0.05$). Substantial variation was observed in the range of 0.17 – 6.93 U / ml.

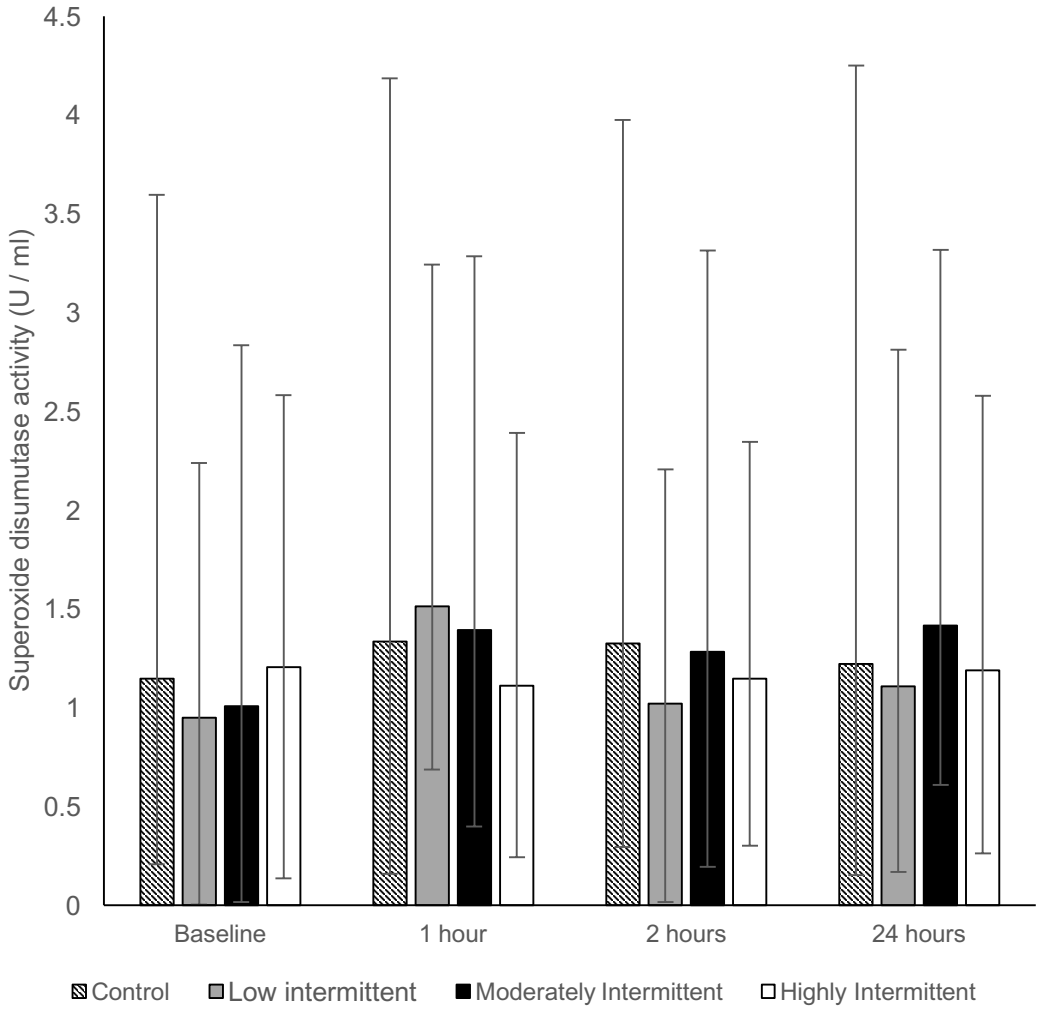

Figure 5.11 Superoxide dismutase activity (U / ml) following 3 different HIIE protocols of varying intermittency and a true control at baseline 1 hour, 2 hours and 24 hours (n = 9). Median values with error bars indicating 75% upper and 25% lower percentiles.

5.4. Discussion

Different forms of HIIE were examined as part of this study that were matched for distance, mean speed and duration, but varied in intermittency. Also a true control session was implemented to examine oxidative stress throughout the experimental time-course. Results showed that the biomarkers tested were not significantly altered in any of the exercise protocols, at any time point. However, Margaritelis et al. (2016) showed the oxidative stress can be vastly different among individuals, with some participants experiencing high levels while other experience low levels of oxidative stress following the same bout of exercise.

An interesting finding of this study is that control measurements showed a variation among and within groups and individual participants. Most oxidative stress experiments only obtain a baseline measure (Bloomer et al., 2006, Bogdanis et al., 2013, Deminice et al., 2010). However, this study showed that baseline measures alone might not be as appropriate, since variations in oxidative stress levels may occur from day to day, hour to hour and from participant to participant. Goldfarb et al. (2014) investigated the daily variations of blood oxidative stress levels at rest in healthy young participants over a four week period and observed that there are great daily fluctuations, suggesting that when investigating oxidative stress biomarkers proper controls to account for these variations must be in place. The variation is also supported by Kanabrocki et al. (2002) who observed that there is a statistically significant difference in temporal circadian rhythms and oxidative stress biomarkers. Thus, the baseline measures should be considered in more

depth in future studies to determine if the oxidative stress observed in exercise investigations is a true and accurate reflection of the exercise input and not due to confounding influences, such as normal diurnal variations.

As most investigations into oxidative stress have used exhaustive protocols, this study aimed to examine oxidative stress under more real case scenarios of exercise and mimic more a daily exercise routine. Thus, the HIIE protocols implemented in this study provided an average v$\dot{V}O_{2max}$ of 75% which has been suggested to be an optimal training intensity for moderately trained athletes (Midgley et al., 2006). Bloomer et al. (2005) used a submaximal intensity similar to this study with results also indicating an absence of oxidative stress in the biomarkers examined (8-Oxo-dG and malondialdehyde) at 1 hour and 24 hours post exercise, suggesting that higher intensity protocols may be required to elicit oxidative stress.

A finding of this study is that 8-Oxo-dG was not detected in any exercise conditions. As this method developed covers a dynamic range similar to that of commercial ELISA, it is possible that ELISA overestimates the concentrations of 8-Oxo-dG thus, further research is required.

5.5. Conclusion

This study has shown that HIIE protocols based on different intermittencies did not significantly change oxidative stress biomarkers. It is possible that the effect of intermittency as a single factor to induce oxidative stress is not an adequate stimulus. The use of 8-Oxo-dG using the LC-MS method developed, to determine DNA damage in plasma samples, showed that concentrations are below the limit of detection. Supporting evidence of controls showed that this method is operational, which may suggest that the use of ELISA overestimates concentrations. Thus, careful consideration should be given in the technique used when investigating this biomarker in acute exercise interventions.

Chapter 6 will investigate the impact of HIIE protocols, when the protocols are matched for mean speed, distance, duration and intermittency but vary in the rate of acceleration/deceleration.

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Chapter 6

The role of acceleration/deceleration during high-intensity intermittent exercise on oxidative stress biomarkers

6.1. Introduction

High-intensity intermittent exercise protocols can have a large amount of variability in terms of intermittency but also in terms of acceleration and declaration. During human movement such as running, muscles undergo a series of stretch-shortening cycles which are comprised of eccentric and concentric contractions (Komi, 2000). In level locomotion the highest forces are demonstrated upon the eccentric phase of the contractions, as the active tension is used in order to decelerate the centre of mass as the foot is in contact with the ground (Armstrong et al., 1983) but also as the soleus and gastrocnemius muscles work eccentrically to lift the heel and propel forward (Eston et al., 1995). The eccentric contractions have been shown to be involved in muscle related injuries, inflammation, fatigue and delayed muscle soreness (DOMS), (Proske and Morgan, 2001). The damage induced in the muscle due to eccentric exercise, is believed to be fibre specific with fast-twitch glycolytic fibres showing greater changes and the Z disk being the most prone to injury (Friden and Lieber, 2001). Mizrahia et al. (2000) showed that muscular fatigue and associated kinematic changes, which could lead to injuries, were caused as a result of higher impact accelerations during running. This is also suggested by Armstrong et al. (1983).

There are also evidence that induced muscle damage can result due to exercise of long duration or high-intensity (Tee et al., 2007). This could be due to the mechanical stress placed upon the muscle in the presence of an eccentric activity (Howatson and van Someren, 2008). As an example, during the stance and late

swing phase of running, the very high forces experienced can reach 8 times the body weight (Sun et al., 2015) and may lead to injuries (McNair, 2006). Moreover, the repeated stretch-shortening cycles of the muscolotendon unit, such as that occurring during running, has been suggested to cause damage in muscle fibres (Butterfield and Herzog, 2005). Thus, it is possible that these forces and the repeated eccentric contractions during high-intensity intermittent running could induce more stress and strain in the muscle that could lead to a state of oxidative stress. Indeed, authors have shown that eccentric exercise can cause increases in oxidative stress levels (Goldfarb et al., 2005, Nikolaidis et al., 2008, Nikolaidis et al., 2007), while the soreness observed has also been shown to be related to an increased ratio of oxidised to reduced glutathione levels (Faulkner et al., 1993). It is also interesting that football, which is highly associated with HIIE as it is characterised by acute sprints lasting a few seconds (Bangsbo, 1994), has also been shown to cause upregulation of free radicals, as phagocytes permeate the muscle (Fatouros et al., 2010).

Thus, it is possible that the impact of acceleration and deceleration during HIIE may have an effect on oxidative stress biomarkers. However, this is currently unknown, as the impact of acceleration and deceleration has been widely overlooked and there are no studies investigating the effect of these factors on oxidative stress changes following HIIE. Therefore, the aim of this chapter is to investigate` oxidative stress responses using different HIIE protocols with a fixed degree of intermittency while manipulating the acceleration and deceleration

phases. The principle of these exercise protocols was to manipulate the acceleration phase (i.e. the time required to reach target speed), while at the same time maintaining the remaining variables (i.e. intermittency, duration, speed and distance) the same for all protocols. It is hypothesised that the more rapid changes in acceleration and deceleration will provide greater exercise-induced oxidative stress.

6.2. Methods

6.2.1. Participant characteristics

Regularly active, male, non-smokers aged 21 ± 3 years who were not taking any medication or supplements were recruited for the study (n = 9). The physiological characteristics of the participants recruited are shown in table 6.1. The inclusion / exclusion criteria were the following: participants were regularly active males, between the age of 18 - 30 years old, they were apparently healthy and physically active (i.e. participated in exercise sessions at least 3 times per week), they should also not consume any medications or supplements and they should be non-smokers. Anthropometric measurements were performed as described on chapter 2.

Table 6.1 Participant characteristics for the study (n = 9).

Participant characteristics	Value
Age (yrs)	21.0 ± 3.0
Height (cm)	180.0 ± 4.0
Body mass (kg)	79.4 ± 7.9
Body fat (%)	12.0 ± 3.0
systolic BP (mmHg)	133.0 ± 10.0
diastolic BP (mmHg)	71.0 ± 6.0
Resting HR (beats·min^{-1})	58.0 ± 13.0
predicted HR (beats·min^{-1})	192.0 ± 2.0
Peak HR (beats·min-1)	183.0 ± 9.0
v$\dot{V}O_{2max}$ (km·hr^{-1})	14.0 ± 6.0
$\dot{V}O_{2max}$ (mL·kg^{-1}·min^{-1})	52.0 ± 6.0

All data are presented as mean ± standard deviation. Note: yrs = years, cm = centimetres, kg = kilograms, % = percentage, mmHg = millimetres of mercury, beats.min^{-1} = beats per minute, v$\dot{V}O_{2max}$ = velocity at maximal oxygen consumption, $\dot{V}O_{2max}$ = maximal oxygen consumption, km.hr^{-1} = kilometres per hour, mL.kg.$^{-1}$min^{-1} = millilitres per kilogram per minute.

6.2.2. HIIE protocols with fixed intermittency and altered acceleration/deceleration phases

The familiarisation protocols and the general exercise protocol design used in this study can be found in chapter 5.

High acceleration protocol: Each participant had to run for 20 seconds at 2 different individualised running speeds aiming to reach target speed within 2 seconds. The first 20 second run was set to represent a 95% v$\dot{V}O_{2max}$ and this was followed by another 20 second run at 55% v$\dot{V}O_{2max}$. After each of these sets of 20 second bouts, a standardised across the participants active recovery period of walking at 4 $km \cdot h^{-1}$ proceeded. A total of 9 stages of 4 minute bouts were performed, with 1 minute of rest between each stage. The total mean workload achieved over the 45 minute period was of 75% v$\dot{V}O_{2max}$ (figure 6.1).

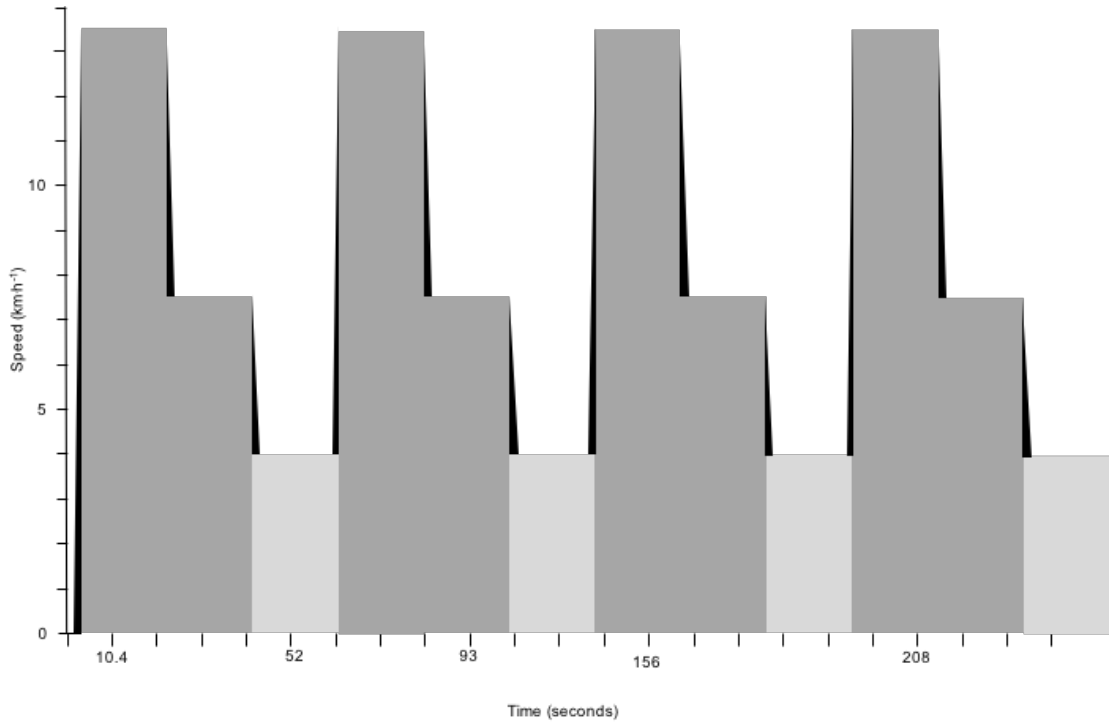

Figure 6.1 Representative diagram of a high acceleration, 4 minute exercise bout. Each dark line represents a 2 seconds acceleration or deceleration phase. After 2 seconds of acceleration each participant had to run at 2 different individualised speeds for 20 seconds between 95% and 55% v$\dot{V}O_{2max}$ (grey bars). This was followed by an active recovery (walking) at 4 km·h^{-1} (light grey bars).

Moderate acceleration protocol: Each participant had to run for 20 seconds at 2 different individualised running speeds aiming to reach target speed within 4 seconds. The first 20 second run was set to represent a 95% v$\dot{V}O_{2max}$ and this was followed by another 20 second run at 55% v$\dot{V}O_{2max}$. After each of these sets of 20 second bouts, a standardised across the participants active recovery period of walking at 4 $km \cdot h^{-1}$ proceeded. A total of 9 stages of 4 minute bouts were performed, with 1 minute of rest between each stage. The total mean workload achieved over the 45 minute period was of 75% v$\dot{V}O_{2max}$. (figure 6.2).

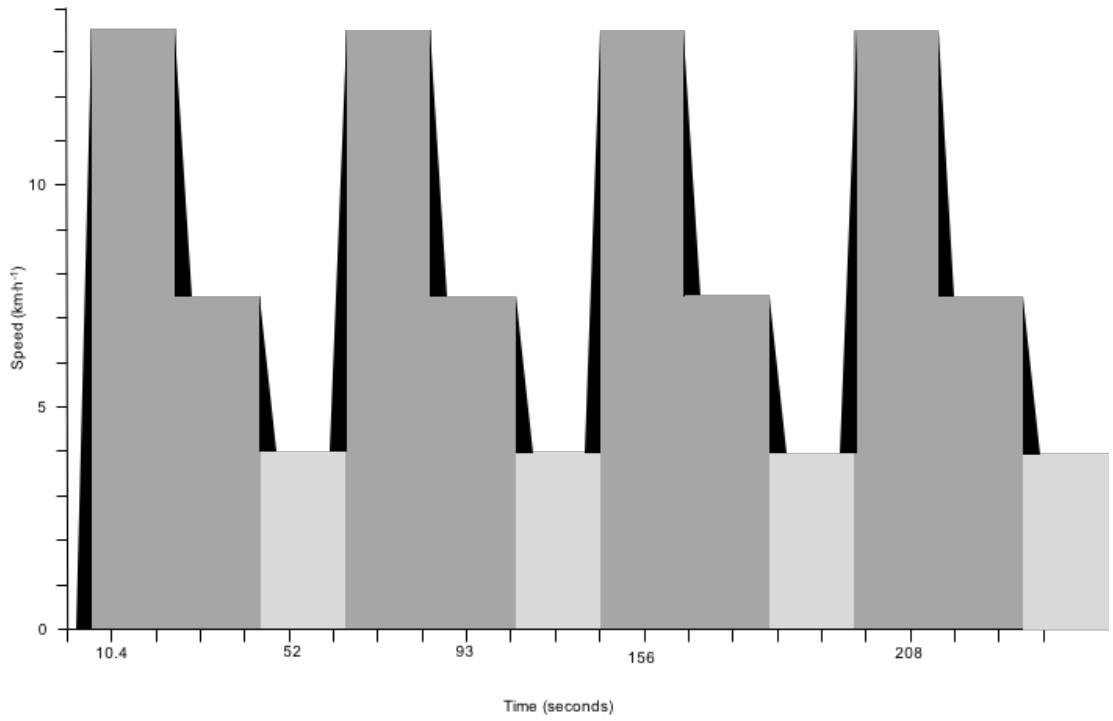

Figure 6.2 Representative diagram of a moderate acceleration, 4 minute exercise bout. Each dark line represents a 4 seconds acceleration or deceleration phase. After 4 seconds of acceleration each participant had to run at 2 different individualised speeds for 20 seconds between 95% and 55% v$\dot{V}O_{2max}$ (grey bars). This was followed by an active recovery (walking) at 4 km·h^{-1} (light grey bars).

Low acceleration protocol: Each participant had to run for 20 seconds at 2 different individualised running speeds aiming to reach target speed within 6 seconds. The first 20 second run was set to represent a 95% v$\dot{V}O_{2max}$ and this was followed by another 20 second run at 55% v$\dot{V}O_{2max}$. After each of these sets of 20 second bouts, a standardised across the participants active recovery period of walking at 4 $km \cdot h^{-1}$ proceeded. A total of 9 stages of 4 minute bouts were performed, with 1 minute of rest between each stage. The total mean workload achieved over the 45 minute period was of 75% v$\dot{V}O_{2max}$ (figure 6.3).

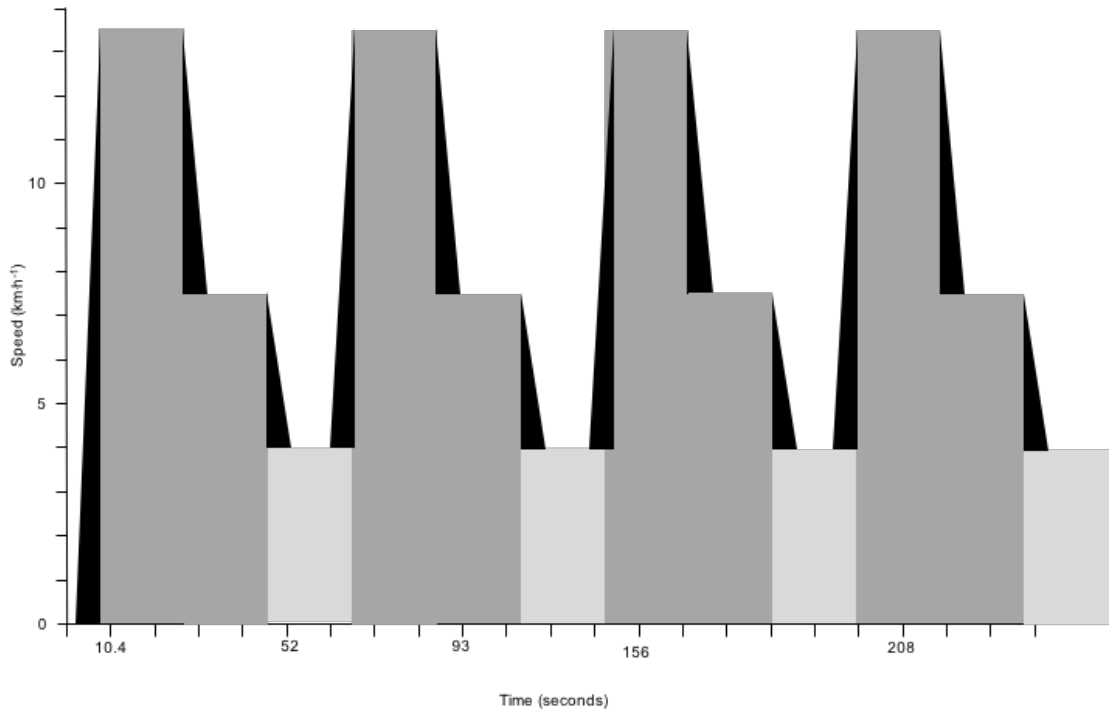

Figure 6.3 Representative diagram of a low acceleration, 4 minute exercise bout. Each dark line represents a 6 seconds acceleration or deceleration phase. After 6 seconds of acceleration each participant had to run at 2 different individualised speeds for 20 seconds between 95% and 55% v$\dot{V}O_{2max}$ (grey bars). This was followed by an active recovery (walking) at 4 km·h^{-1} (light grey bars).

6.2.3. Collection of blood samples for the analysis of oxidative stress biomarkers

Blood samples were collected for serum and plasma as described in chapter 2. Briefly venous blood samples were collected before, 1 hour, 2 hours and 24 hours post exercise for the analysis of lipid hydroperoxides, 8-oxo-2'-deoxyguanosine (8-Oxo-dG), total glutathione and superoxide dismutase activity. Analysis of lipid hydroperoxides, total glutathione and superoxide dismutase was conducted as described on chapter 3 and analysis of 8-Oxo-dG was performed using a combination of assays as described on chapter 4.

6.2.4. Statistical analysis

Using SPSS (version 24), the data were assessed for normal distribution using Shaphiro Wilk test ($p < 0.05$). As the requirements of normal distribution criteria were not met, statistical analysis was conducted using Kruskal-Wallis test ($p < 0.05$) using SPSS (version 24).

6.3. Results

6.3.1. 8-Oxo-dG

For all the conditions tested 8-Oxo-dG concentrations could not be detected (figure 6.4). The limit of detection at 5 nM (figure 6.5), the internal standard (figure 6.6), the digestion controls (figure 6.7) and repeated standard injections all confirmed that the detection method was working and demonstrated that the technique that

was implemented showed appropriate sensitivity as described in chapter 4 of this thesis.

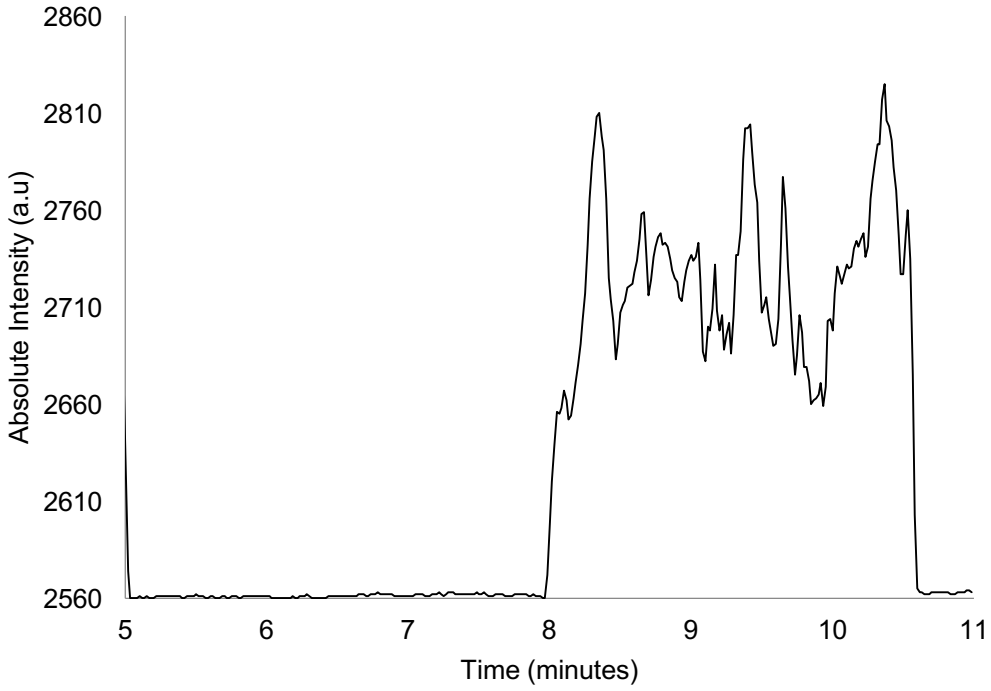

Figure 6.4 Representative chromatography of plasma samples for the presence of 8-Oxo-dG obtained during this study. Results show no quantifiable peak.

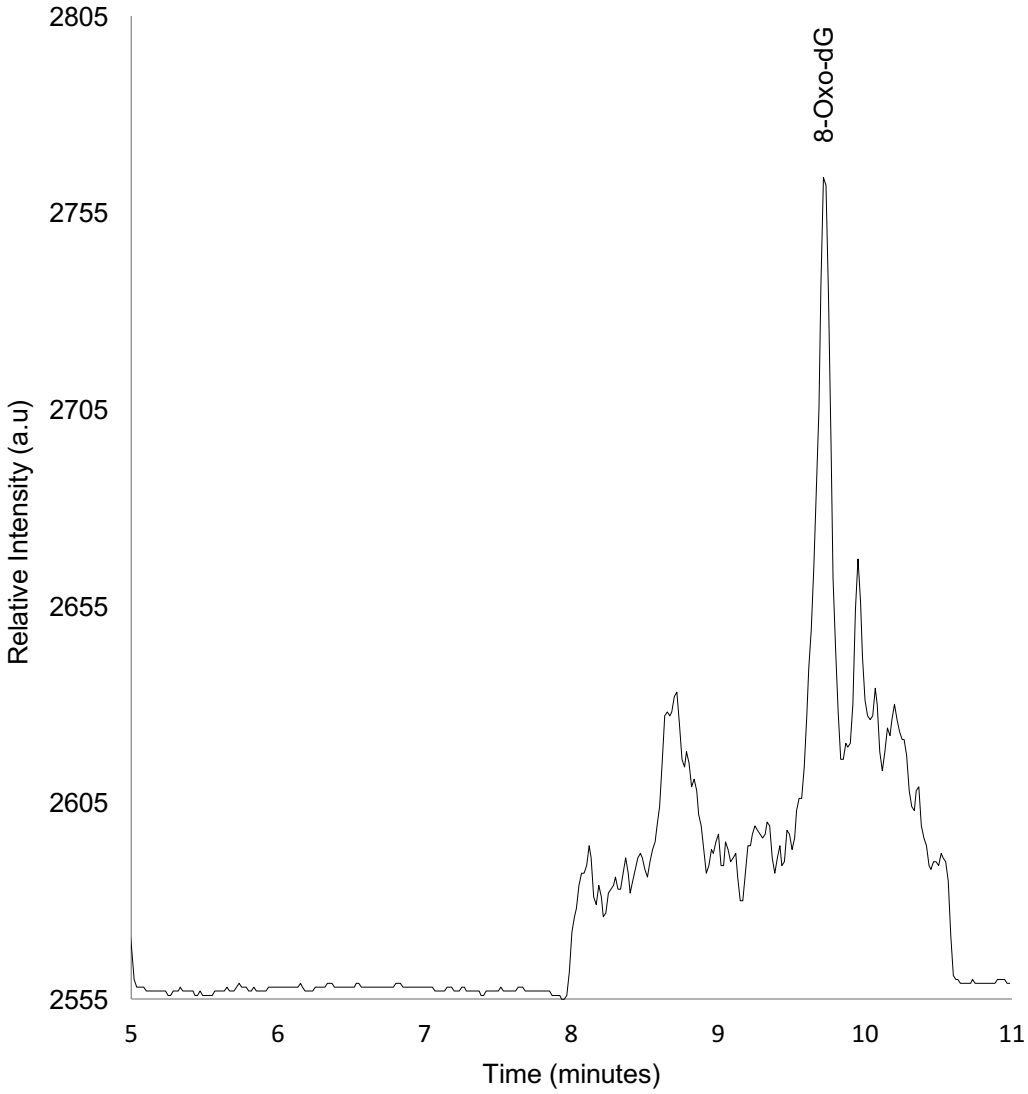

Figure 6.5 Representative chromatography of 5 nM 8-Oxo-dG standard sample.

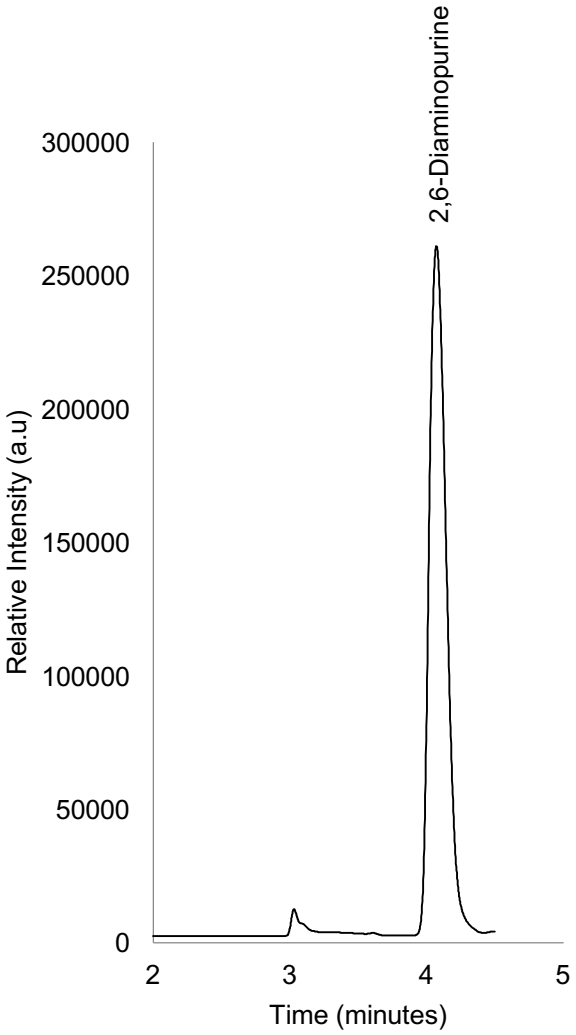

Figure 6.6 Representative chromatography of the internal standard (2,6-Diaminopurine) obtained during this study. Results show a quantifiable peak and appropriate sensitivity for the analysis.

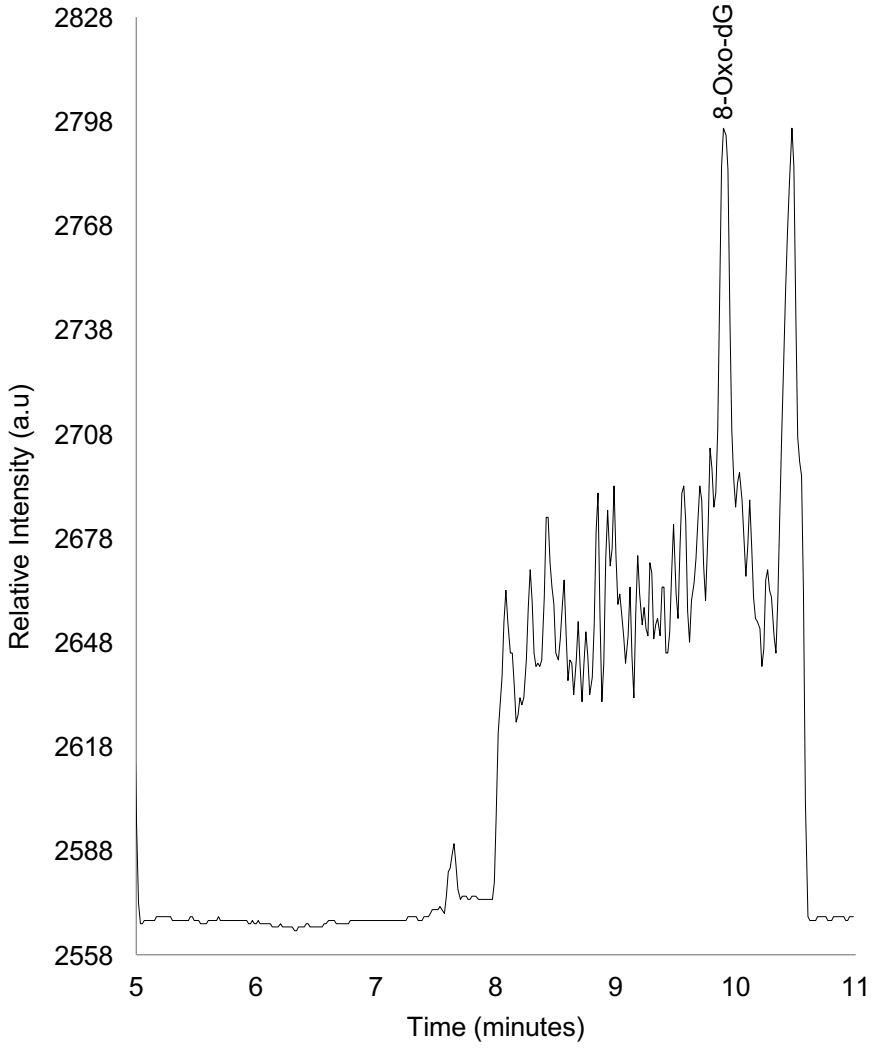

Figure 6.7 Representative chromatography of ELISA standard following digestion.

6.3.2. Lipid hydroperoxides

For all the conditions tested no statistical significance was observed (figure 6.8). All exercise protocols showed similar lipid hydroperoxide concentrations in serum to the control conditions ($p < 0.05$). Substantial variation was observed in all conditions in the range of 1.6 – 24.9 $\mu mol.L^{-1}$.

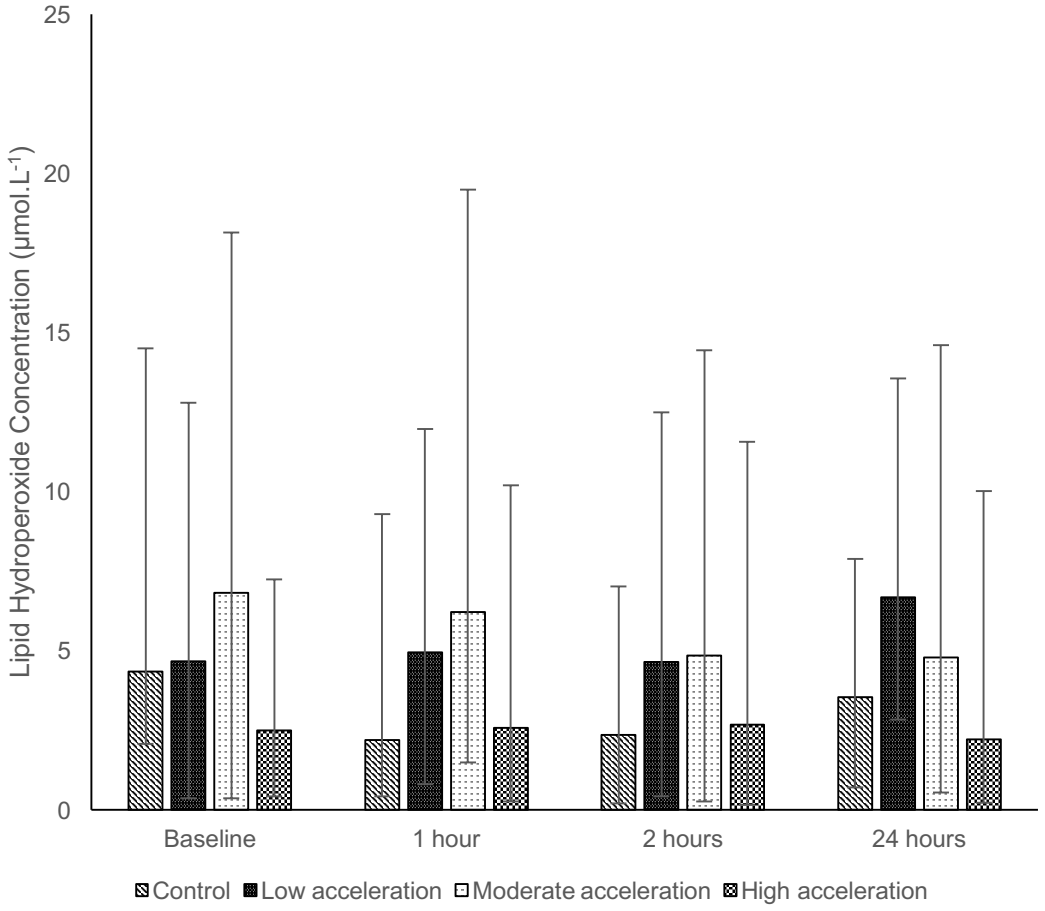

Figure 6.8 Lipid hydroperoxide concentrations ($\mu mol.L^{-1}$) following 3 different HIIE protocols of varying acceleration and a control session at baseline 1 hour, 2 hours and 24 hours (n = 9). Median values with error bars indicating 75% upper and 25% lower percentiles.

6.3.3. Total glutathione

For all the conditions tested no statistical significance was observed (figure 6.9). All exercise protocols showed similar total glutathione concentrations in plasma to the control conditions for $p < 0.05$. The variation in all the conditions was in the range of 0.32 – 5.50 μM.

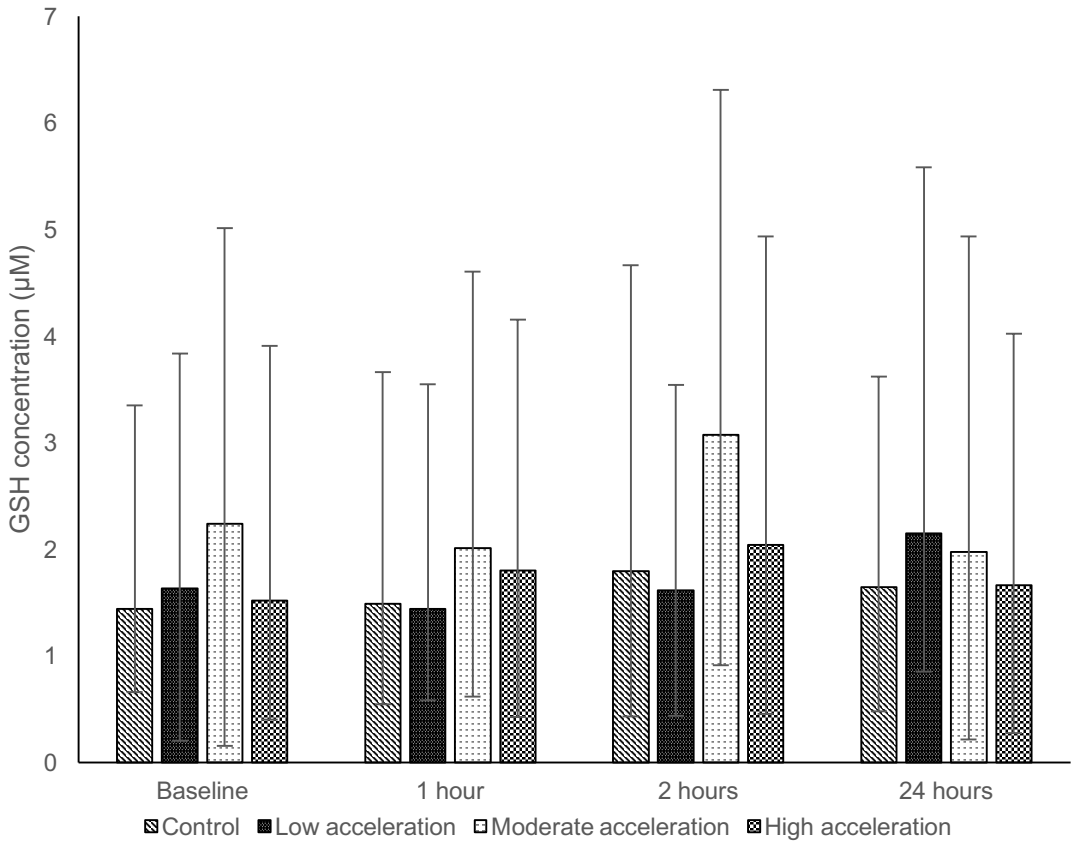

Figure 6.9 Total GSH concentration (µM) following 3 different HIIE protocols of varying intermittency and a control session at baseline 1 hour, 2 hours and 24 hours (n = 9). Median values with error bars indicating 75% upper and 25% lower percentiles.

6.3.4. Superoxide dismutase

For all the conditions tested no statistical significance was observed (figure 6.10). All exercise protocols showed similar superoxide dismutase activities to the control conditions for $p < 0.05$. Considerable variation was observed in all conditions examined. The variation was in the range of 0.43 – 6.93 U / µl.

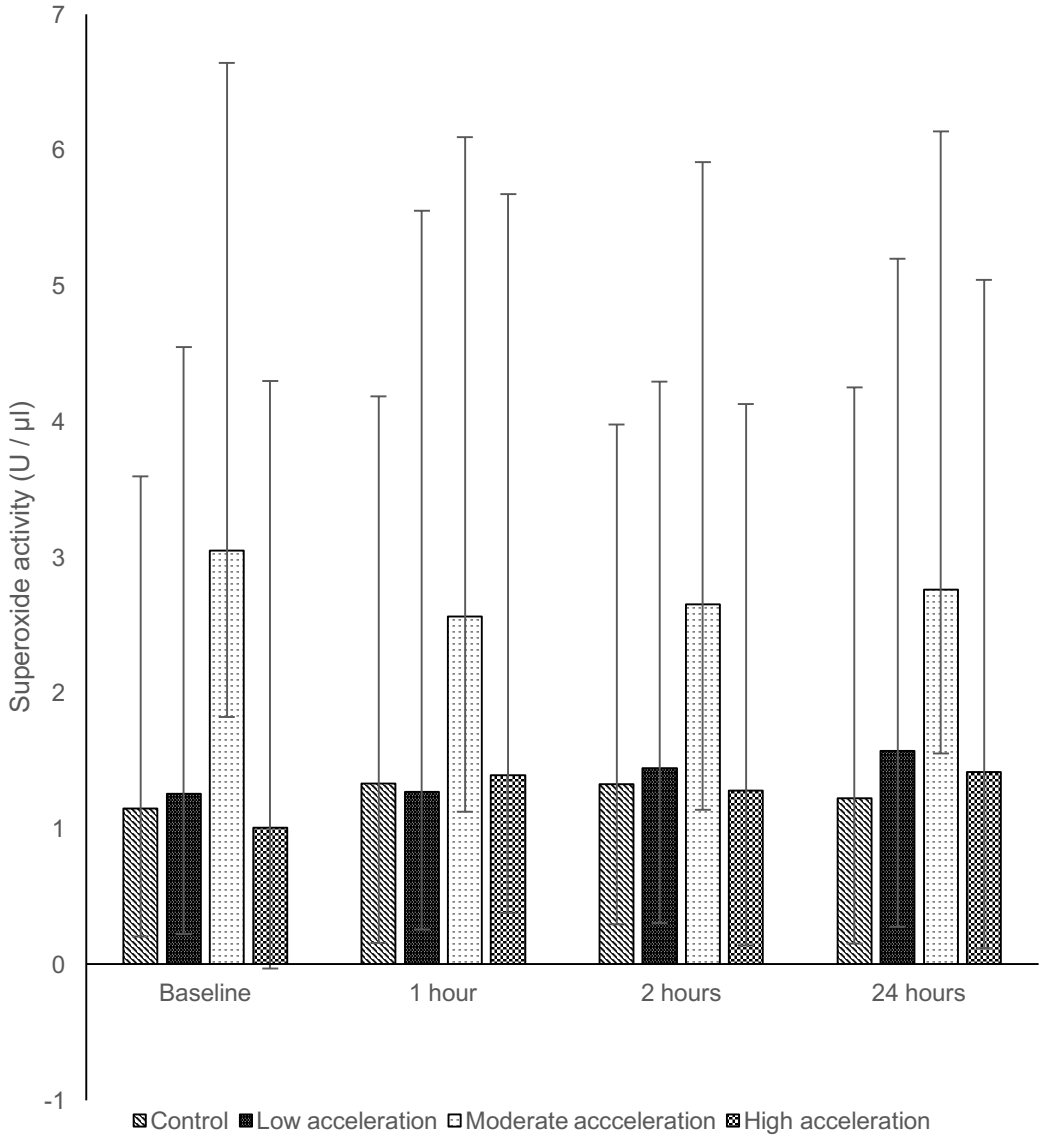

Figure 6.10 Superoxide dismutase activity (U / µl) following 3 different HIIE protocols of varying acceleration and a control session at baseline 1 hour, 2 hours and 24 hours (n = 9). Median values with error bars indicating 75% upper and 25% lower percentiles.

6.4. Discussion

This is the first study to examine the role of acceleration and deceleration in HIIE on oxidative stress parameters. Results of this research indicate that oxidative stress was not significantly changed as a result of the acceleration and deceleration components of the activity. Since no direct comparison to the existing literature can be made, comparisons with eccentric exercise research investigating oxidative stress are presented. Interestingly, Margaritelis et al. (2014) showed that individuals performing acute eccentric exercise, aiming to induce oxidative stress, vary in the mean response. It was observed that some individuals experience negative responses, while other experience no increases in the oxidative stress biomarkers tested (F_2-isoprostanes, protein carbonyls, glutathione), (Margaritelis et al. 2014). Thus, due to the great heterogeneity, basal levels of the biomarkers of oxidative stress are considered important when these parameters are examined (Margaritelis et al., 2014). It is possible that as there were no samples from muscle biopsies, the structural damages caused to the muscle due to the eccentric nature of the exercise to be more prominent in the muscle compared to blood biomarkers of oxidative stress. Previous literature findings support that eccentric exercise does not show an increase in glutathione status (Camus et al., 1994) possibly due to insufficient exercise stimulus, which is also supported in this study as total glutathione levels were not increased .

Bloomer et al. (2006) investigated blood oxidative stress biomarkers and conducted muscle biopsies to determine the responses in squats versus sprints.

The findings of this study showed that there are minimal changes in oxidative stress levels between these exercise conditions when the participants are trained, which could imply that trained individuals possibly have experienced adaptations due to their training status leading to non-significant changes in the oxidative stress levels. Accordingly, research by McAnulty et al. (2005) examining blood biomarkers and muscle biopsies showed that exhaustive resistance exercise in trained men shows no upregulation on oxidative stress as indicated by F_2-isoprostanes, which could be due to different modes of exercise causing different mechanical stress that affects the levels of oxidative stress. Another study made by Quindry et al. (2011) investigated the role of eccentric exercise in participants with homogenous muscle fibre type compositions and found that the fibre type composition may influence oxidative stress levels, as correlations existed between type II muscle fibres and protein carbonyls. Thus, it is possible that individuals with a higher percentage of type II muscle fibres could show higher oxidative stress compared to individuals with type I muscle fibres (Quindry et al., 2011).

As literature shows (Bloomer et al. 2006, Margaritelis et al. 2014, McAnulty et al. 2005) the link between eccentric exercise and individual responses together with the training status of the participants and the physiological stimuli can significantly influence oxidative stress levels. Thus, more research is needed to determine the biomechanical impact of acceleration and deceleration on oxidative stress during HIIE and examine whether the eccentric nature of the running component, accompanied with the increased impact forces, may promote oxidative stress.

6.5. Conclusion

This is the first study to investigate changes in oxidative stress status following HIIE where acceleration and deceleration have been specifically manipulated. Even though no evidence to support oxidative stress responses were found, more research is needed to investigate further the role of acceleration/deceleration on oxidative stress following HIIE using various rates of acceleration and deceleration.

As interesting observation following the investigations presented in both chapters 5 and 6 was that 8-Oxo-dG concentrations were not observed following HIIE using the LC-MS method developed. The absence of 8-Oxo-dG detection in the samples that was previously investigated in these chapters, using the LC-MS method developed, could represent more specifically the absence of DNA damage occurring following exercise than ELISA methods. As increases in 8-Oxo-dG are shown to be associated with heart failure (Kroese and Scheffer, 2014), chapter 7 will utilise a heart failure patient group to compare the same plasma samples of a heart failure group using the 2 methods (ELISA and the LC-MS method developed) aiming to determine if the levels of 8-Oxo-dG are in agreement between the techniques.

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Chapter 7

Quantification of DNA damage in plasma samples from heart failure patients for the comparison of ELISA method and LC-MS

7.1. Introduction

An interesting observation in this thesis was that 8-Oxo-dG concentrations were not observed following HIIE using the LC-MS method developed. As increased oxidative DNA damage has been linked to cardiovascular disease (Cooke et al., 2006a), various techniques and approaches such as ELISA and LC-MS have been applied to quantify the oxidation of DNA (Kroese and Scheffer, 2014). The importance of aiming to measure biomarkers as accurately as possible, such as by quantifying DNA damage, can be of interest as it can provide a risk prediction, which in turn can be used for treatment strategies or patient management (Wang, 2011, Cooke et al., 2009). Even though the techniques implemented to assess DNA damage have used a wide range of biological matrices, including plasma (Tope and Panemangalore, 2007) and urine (Yoshida et al., 2002), discrepancies in the quantification levels have been observed between methods such as LC-MS and ELISA (Rossner et al., 2016). Cooke et al. (2008) showed (figure 7.1) that in urine, even when ELISA techniques are improved in sensitivity, such as by using a fixed protein-bound 8-hydroxy-2'-deoxyguanosine (8-Oxo-dG), (ELISA improved kit), the results are still disproportional to the observed values of 8-Oxo-dG concentrations found with analytical techniques (4 – 10 fold difference). Also Rossner et al. (2016) concluded that the use of ELISA in urine samples is not suitable as it overestimates concentrations of 8-Oxo-dG thus, chromatographic methods should be used to avoid misleading data interpretations.

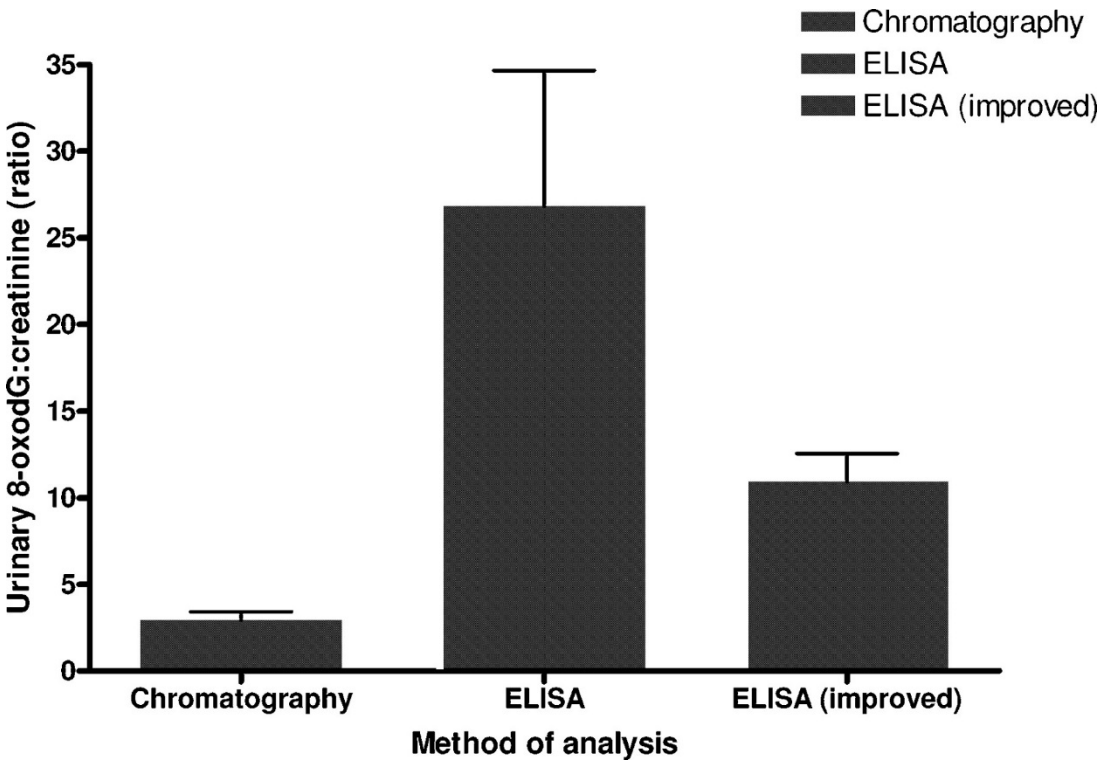

Figure 7.1 Comparison of different techniques used for the analysis of 8-Oxo-dG in urine samples. 8-Oxo-dG in analytical techniques demonstrate a urinary 8-Oxo-dG ratio at least 4-10 fold less compared with ELISA techniques available. Image reprinted from Cooke et al. (2008).

With regards to this thesis, as shown in previous chapters (5 and 6), DNA damage was not observed in healthy male participants following high-intensity intermittent exercise. Due to the age and fitness status of these individuals, it is possible that DNA damage following exercise was below the limit of detection for the 8-Oxo-dG method developed. However, it is also possible that the ELISA overestimation of DNA damage shown in urine, could also be applied to other biological fluids such as plasma. Thus, the absence of 8-Oxo-dG detection in the samples previously investigated (chapters 5 and 6) using the LC-MS method developed could represent more specifically the absence of DNA damage occurring following exercise than ELISA.

Increases in 8-Oxo-dG are shown to be associated with heart failure (Kroese and Scheffer, 2014). More specifically, researchers have shown that 8-Oxo-dG is significantly increased in heart failure patients when ELISA technique is implemented (Nagayoshi et al., 2009, Watanabe et al., 2006, Kono et al., 2006). Reflecting on the effect of heart failure in increased 8-Oxo-dG levels, as well as the possibility of overestimation of 8-Oxo-dG using ELISA assays, it was decided to examine DNA damage in plasma, by comparing the method developed against ELISA using a heart failure clinical group. To investigate this, it was decided to compare the same plasma samples of a heart failure group using the 2 methods (ELISA and the LC-MS method developed) to determine if the levels of 8-Oxo-dG are in agreement between the techniques.

Thus, the aim of this investigation is to quantify DNA damage using LC-MS and ELISA methods implementing an analysis of plasma samples obtained from cardiac heart failure patients.

7.2. Methods

A total of 30 heart failure patients were recruited (males n = 23, females n = 7) from the cardiology department at Castle Hill hospital, Hull, UK for this pilot study. The patients were allocated into 3 groups as follows; the cardiac heart failure (CHF) responders (n = 12) who showed improvements in the clinical marker of chronic heart failure (NT-proBNP), the CHF non-responders group (n = 12) that showed an increase in NT-proBNP over 4 months and the non-heart failure control group (negative diagnosis for CHF), (n = 10). All patients had a fasting venous blood sample obtained using the Vacutainer System (Becton-Dickinson, Oxford, UK) during the first visit to the cardiology department.

7.2.1. ELISA analysis of 8-Oxo-dG

Using a commercially available ELISA kit (Cell Biolabs, OxiSelect™ Oxidative DNA Damage ELISA, Kit, STA-320, Oxford, UK) the 8-Oxo-dG conjugate was diluted to a concentration of 1mg / ml with 1 µg / ml of PBS. Using a 96-well protein binding plate, 100 µl of the diluted conjugate was added to each well and left to incubate overnight at 4°C. Next, the 8-Oxo-dG coating solution was removed and the plate was washed with distilled water (18.2 MΩ) and blotted to remove the excess liquid. Then, 200 µl of assay diluent was added to each well and blocked for 1 hour at

room temperature. Immediately before use, the anti–8-Oxo-dG antibody and secondary antibody were diluted 1:500 and 1:1000, respectively, along with the 8-Oxo-dG standard using the assay diluent and a standard curve ranging from 0 - 20 ng / ml was prepared. Each of the plasma samples was diluted 1:100 in assay diluents, and 50 µl of the final concentration was added in duplicate along with the standards to the wells of the 8-Oxo-dG conjugate-coated plate and incubated for 10 minutes at room temperature in an orbital shaker. After incubation, each well was washed three times with the assay wash buffer before adding 100 µl of the secondary antibody to each well and incubated for 1 hour at room temperature in an orbital shaker. Each well was washed three times and 100 µl of the substrate solution was added to each well and incubated in an orbital shaker at room temperature until a noticeable colour change had taken place. The reaction was terminated by adding 100 µl of the stop solution to each well. Absorbance was read immediately on a plate reader (Dynatech MRX 650 Plate Reader, Haverhill, MA) at a wavelength of 450 nm.

7.2.2. LC-MS analysis of 8-Oxo-dG

The LC-MS method for this study follows the protocol previously described in chapter 4.

7.3. Results

7.3.1. Plasma 8-Oxo-dG levels of heart failure patients using the ELISA technique.

The average 8-Oxo-dG concentration determined using the ELISA was converted from ng / ml to nM for comparison reasons. The average 8-Oxo-dG concentration between the conditions was 5.3 nM (SD ± 0.7 nM), (figure 7.2). Statistical analysis showed no significant relationship to exist between conditions for $p < 0.05$.

7.3.2. Plasma 8-Oxo-dG levels of heart failure patients using the LC-MS technique.

The concentrations of 8-Oxo-dG could not be quantified in LC-MS as they were below the limit of detection (figure 7.3). Controls such as internal standard (figure 7.4), ELISA with digestion (figure 7.5), ELISA without digestion (figure 7.6) and standard 8-Oxo-dG samples (figure 7.7) all confirmed that the detection method was working and demonstrated that the technique implemented showed appropriate sensitivity as described in chapter 4.

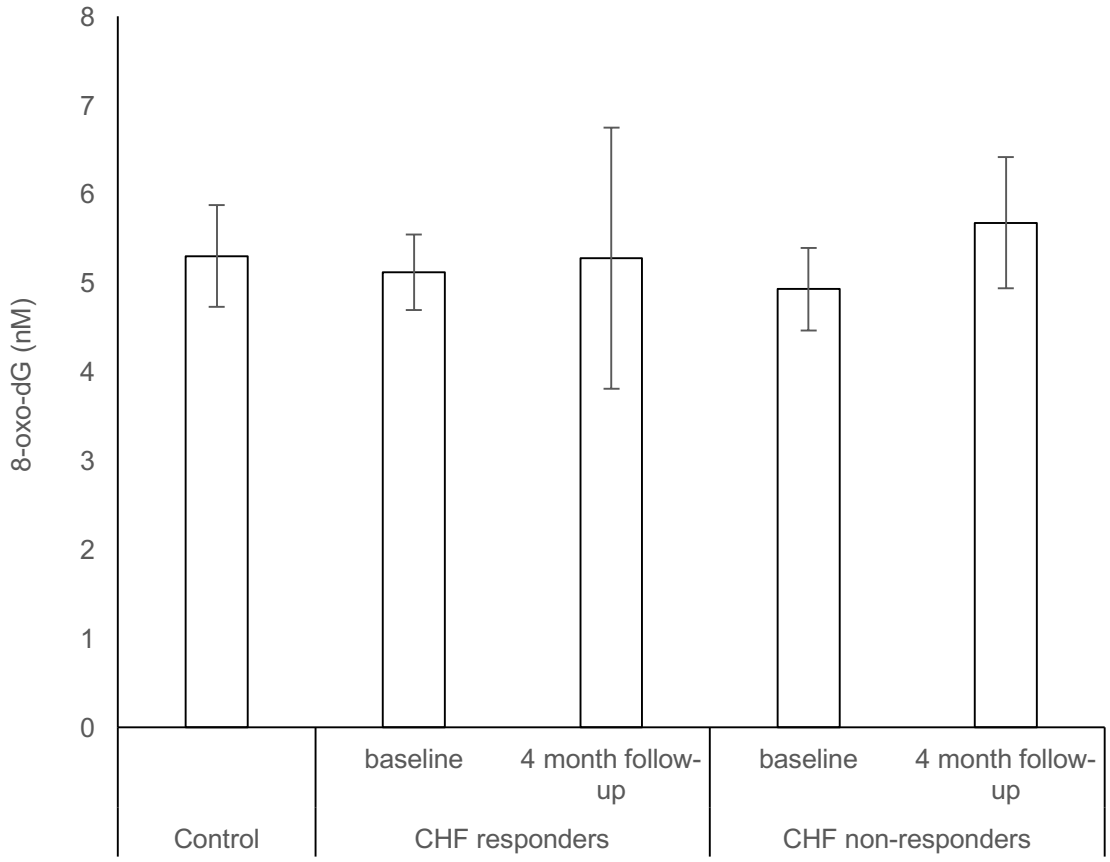

Figure 7.2 DNA damage for the presence of 8-Oxo-dG (nM) as indicated using ELISA. Figure demonstrates the concentration for the control group as well as for CHF responders and non-responders at baseline and after a follow-up period of 4 months. (control n= 10, CHF responders n = 12, CHF non-responders n = 10).

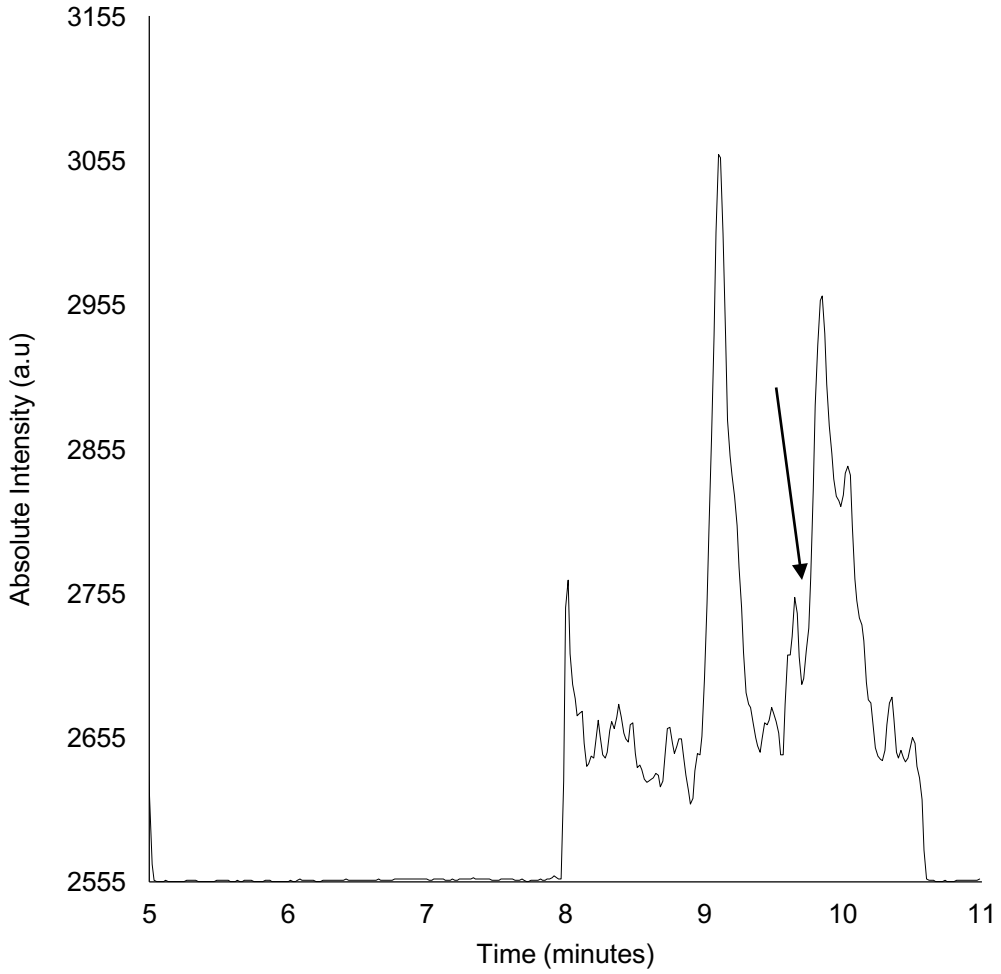

Figure 7.3 Representative chromatography of CHF patients plasma samples for the presence of 8-Oxo-dG. Results show no quantifiable peak at the retention time of interest (indicated by the arrow).

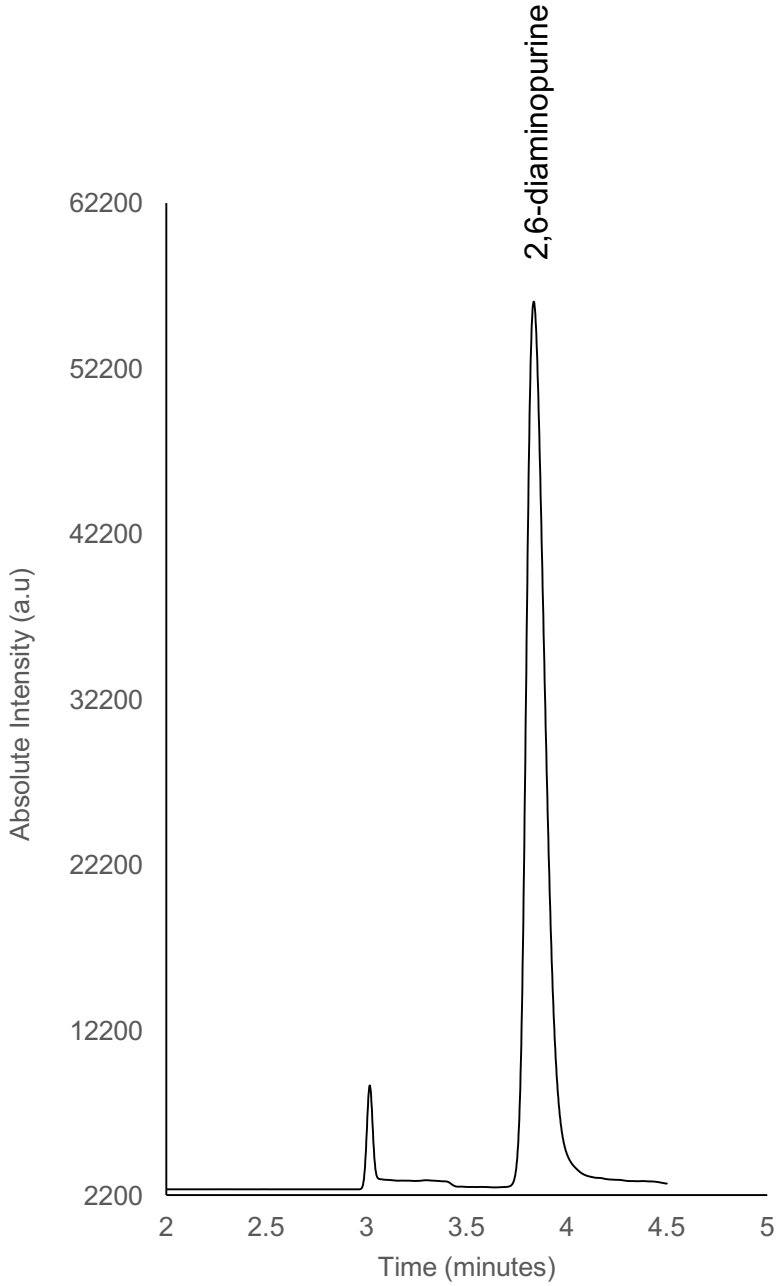

Figure 7.4 Representative chromatography of the internal standard (2,6-Diaminopurine).

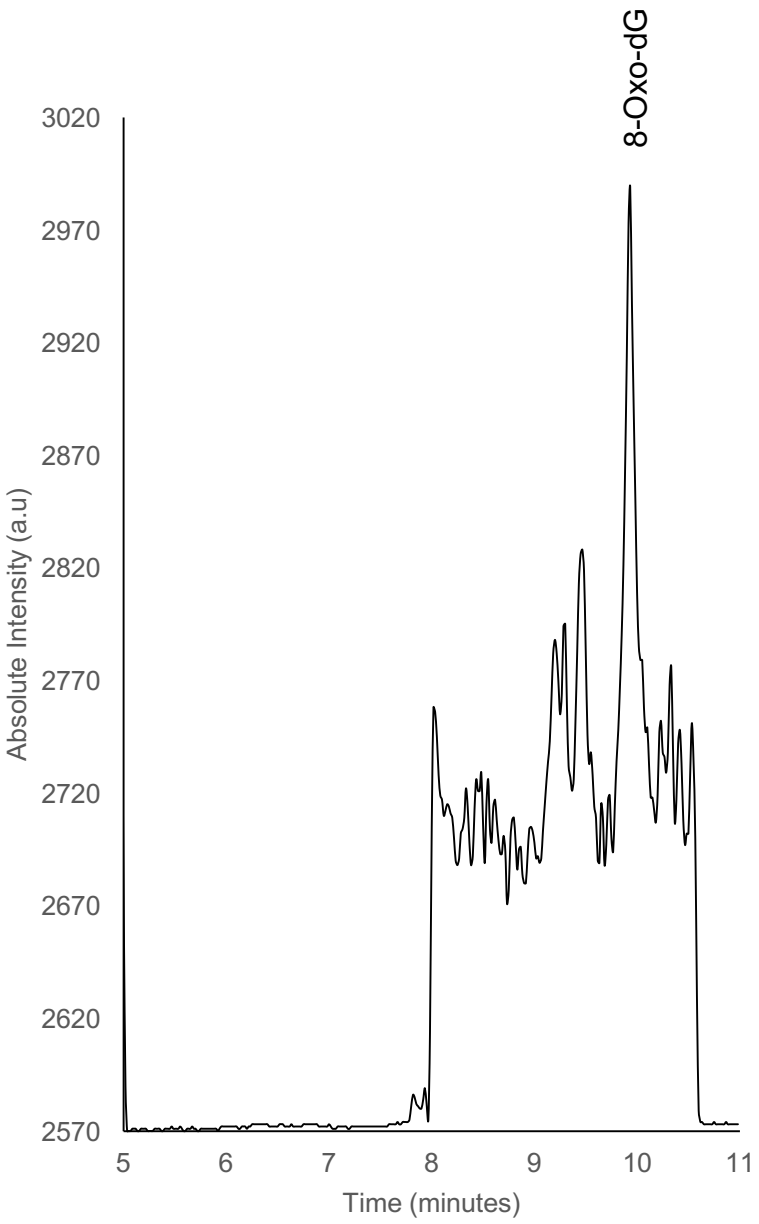

Figure 7.5 Representative chromatography of the ELISA standard with digestion.

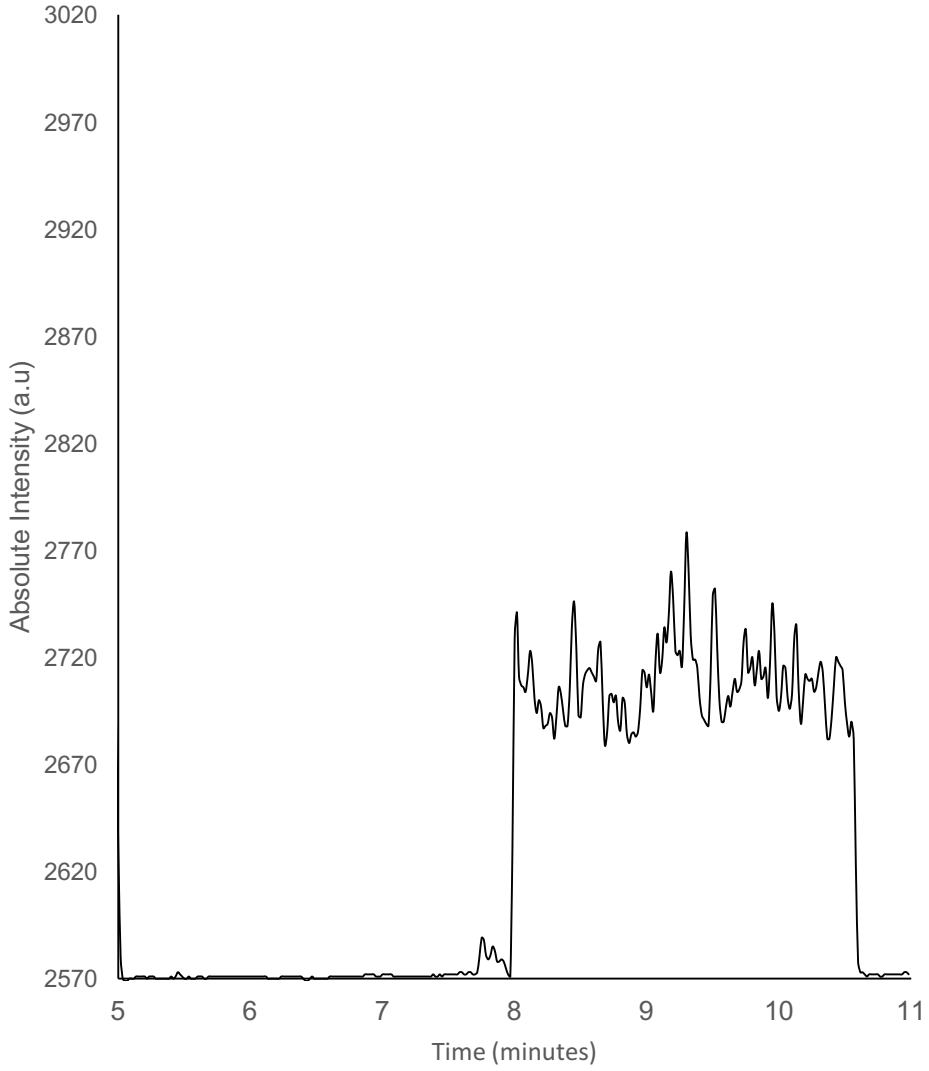

Figure 7.6 Representative chromatography of the ELISA standard without digestion.

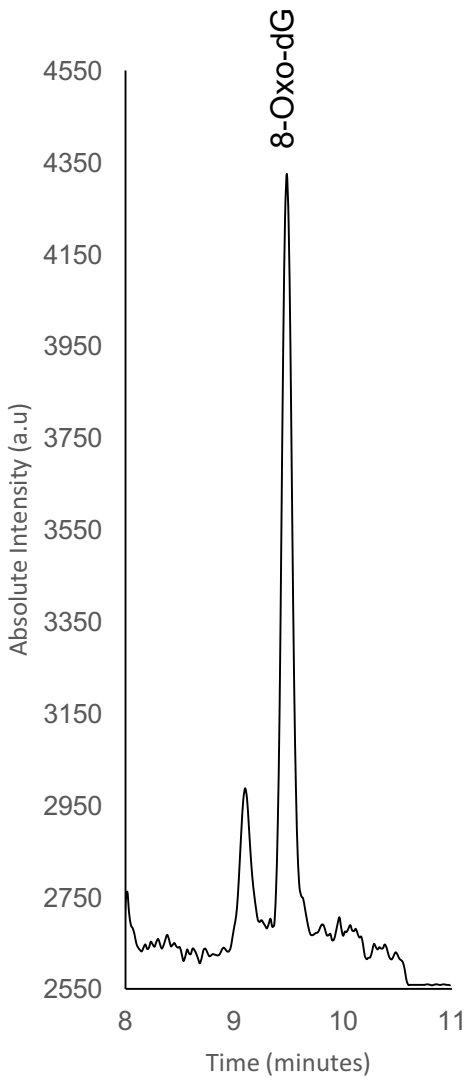

Figure 7.7 Representative chromatography of the 50 nM standard.

7.4. Discussion

This study showed a discrepancy in the ELISA method when compared with the LC-MS method developed. Comparing the 2 methods, results showed 8-Oxo-dG production in ELISA that was not present using LC-MS for a range of detection between 5 nM - 500 nM. Even though the ELISA concentrations observed should be in the low end of the standard curve of the LC-MS method (approximately around 5 nM) the results did not verify this and quantification was not possible. This finding could support the belief that ELISA overestimates 8-Oxo-dG concentrations thus, particular attention and caution should be given when reporting findings.

Research has shown that the use of ELISA may have certain issues (Guven et al., 2014, Terato et al., 2014, Cooke et al., 2006b) which could also influence the results observed in this study. Possible reason for these differences observed between the 2 methods could be due to ELISA's non-specific binding or the cross reactivity with other oxidised bases that could overestimate DNA damage (Pilger and Rudiger, 2006). Also as ELISA standards are in water and not plasma the matrix effect may interfere with the observed values, as plasma is a complicated fluid and possible interactions are possible (Chiu et al., 2010). Additionally, Cooke et al. (2006b) suggested that ELISA may not be specific for oxidised guanine quantification, as compounds that exhibit a high molecular weight such as proteins, may interfere in the analysis. Thus, as plasma samples using the LC-MS method were de-proteinated, it is possible that the quantification using this analytical

method could be more sensitive, which could explain the differences between the methods used. In addition, the use of chromatographic techniques coupled with mass spectrometry are more specific as the detection of a substance (e.g. 8-Oxo-dG) is achieved based on the *m/z* ratio that allows more precise detection. On the other hand, possible issues with the application of the LC-MS technique were limited, as an internal standard was used (figure 7.4) in each run and enzymatic digestion was performed prior to injections as previously described (chapter 4). The use of enzymatic digestion in the ELISA positive control also showed that this LC-MS method had a limit of detection at 0.25 nM (figure 7.5) thus, any presence of 8-Oxo-dG within this range would be detected. It is possible that if this LC-MS method was applied in a more advanced setting, such as with the implementation of a more sensitive detector, the limit of quantification in the standard curve would be lower which could possibly allow the quantification of DNA damage at lower levels below 5nM.

7.5. Conclusion

This study aimed to examine the presence of 8-Oxo-dG in a clinical population as it was expected that the levels of DNA damage would be increased since literature suggests that cardiovascular conditions exacerbate oxidative stress. As it was observed in previous studies (chapter 5 and 6) that DNA damage was not detected using the method developed, an attempt to compare the technique against ELISA, using a clinical setting of heart failure patients was considered. Results showed that there are discrepancies in the concentrations of 8-Oxo-dG observed between

the 2 methods in plasma, suggesting further research to determine more accurately the amounts of DNA damage present in plasma samples.

7.6. Chapter References

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Chapter 8

General discussion

8.1. Summary of findings

The present thesis investigated the impact of high-intensity intermittent exercise (HIIE) exercise on oxidative stress. To this end, biomarkers of oxidative stress were examined and a liquid chromatography – mass spectrometry (LC-MS) method was developed aiming to detect 8-hydroxy-2'- deoxyguanosine (8-Oxo-dG) in plasma samples**.** Distinct HIIE exercise protocols were implemented aiming to manipulate either the intermittency or the acceleration/deceleration and blood samples were analysed before and following 1, 2 and 24 hours post exercise. The main finding of these studies was that there were no significant changes in oxidative stress however, a substantial variation in the biomarkers analysed was observed. More specifically the main findings observed following the investigations in this thesis were the following:

Study 1: Methodological development and optimisation of 8-hydroxy-2'-deoxyguanosine (8-Oxo-dG) in plasma samples using liquid chromatography – mass spectrometry (LC-MS).

As part of this thesis, a method to measure DNA damage and specifically the oxidised version of guanine (8-oxo-dG) using LC-MS was developed and optimised using a simple handling procedure (liquid-liquid extraction). In addition, this method also implemented an enzymatic digestion protocol, which allowed the release of free nucleotides by subsequently allowing a more representative

quantification of 8-Oxo-dG concentrations. The limit of detection using the method developed has a limit of quantification of 5 nM and a limit of detection of 0.25 nM.

Study 2: The impact of exercise intermittency on oxidative stress levels

The primary aim of this study (chapter 5) was to investigate high - intensity intermittent exercise, in order to examine oxidative stress responses, by manipulating the intermittency while exercise duration, distance and average speed was matched. To this end, 3 distinct HIIE protocols were implemented (high, moderate and low intermittency), on a non-motorised treadmill, for 45 minutes at total workload of 75% v$\dot{V}O_{2max}$. A control session was also included which consisted the collection of blood samples at rest at the same time points as they would be collected during an exercise protocol. The results of this study showed that oxidative stress was not significantly increased in any of the protocols for the biomarkers tested and 8-Oxo-dG was below the limit of detection. However, substantial variation was observed between the different HIIE conditions for lipid hydroperoxides (1.6 – 24.9 μmol.L^{-1}), total glutathione (0.32 – 5.50 μM) and superoxide dismutase (0.17 – 6.93 U / ml).

Study 3: The role of acceleration/deceleration during high-intensity intermittent exercise on oxidative stress biomarkers.

The primary aim of this study (chapter 6) was to investigate the role of acceleration and deceleration during HIIE in order to examine oxidative stress responses, by manipulating the acceleration and deceleration while exercise intermittency, duration, distance and average speed was matched. For this investigation, 3 distinct HIIE protocols were implemented (high, moderate and low acceleration), on a non-motorised treadmill, for 45 minutes at total workload of 75% v$\dot{V}O_{2max}$. A control session was also included which consisted the collection of blood samples at rest at the same time points as they would be collected during an exercise protocol. The results of this study showed that oxidative stress was not significantly increased in any of the protocols for the biomarkers tested and 8-Oxo-dG was below the limit of detection. However, substantial variation was observed in lipid hydroperoxides (1.6 – 24.9 μmol.L^{-1}), total glutathione (0.32 – 5.50 μM) and superoxide dismutase (0.43 – 6.93 U / ml).

Study 4: Quantification of DNA damage in plasma samples from heart failure patients for the comparison of ELISA method and LC-MS.

As the previous studies of this thesis showed (study 2 and 3) 8-Oxo-dG concentrations were not observed following HIIE using the LC-MS method developed. The absence of 8-Oxo-dG detection in the samples that was previously

investigated in these chapters using the LC-MS method developed could represent more specifically the absence of DNA damage occurring following exercise than ELISA methods. As increases in 8-Oxo-dG are shown to be associated with heart failure (Kroese and Scheffer, 2014), this study employed a heart failure patient group to compare the same plasma samples of a heart failure group using the 2 methods (ELISA and the LC-MS method developed) aiming to determine if the levels of 8-Oxo-dG are in agreement between the techniques. Following the comparison between the 2 methods, results showed that 8-Oxo-dG production in ELISA was observed in the low end of the standard curve (approximately 5 nM), whereas using the LC-MS method the quantification was not possible in the range of 5 – 500 nM. This finding could support the belief that ELISA overestimates 8-Oxo-dG concentrations thus, particular attention and caution should be given when reporting findings.

8.2. Exercise as a stimulus for oxidative stress

The experimental investigations undertaken in this thesis, examined the impact of high-intensity intermittent exercise (HIIE) on oxidative stress biomarkers by manipulating the intermittency and the acceleration/deceleration of the HIIE protocols (chapter 5 and 6). While no previous study, to our knowledge, has examined the impact of intermittency or acceleration/deceleration during acute HIIE, comparisons can be made with similar exercise modalities, such as acute aerobic exercise.

In relation to acute exercise, the findings of this thesis seem to be conflicting with previous literature findings, as the investigators have shown evidence of increased oxidative stress (Fogarty et al., 2011; Jammes et al., 2004; Kyparos et al., 2007). However, the results are supported by previous findings indicating no significant changes in oxidative stress status (Taghiyar et al., 2013; Vasankari et al., 1997). Potentially, as participants were physically active individuals, the prior adaptations to the exercise stimulus could not induce exercise related oxidative stress (Radak et al., 1999; Seifi-Skishahr et al., 2016).

Moreover, associations have been made in regards to different nutritional status between active and sedentary population, with inactive individuals exhibiting unhealthy diet patterns which are, deficient intake of fruits and vegetables (Lowry et al., 2015). This could explain increases in oxidative stress following exercise in less trained individuals, as physical activity and a balanced diet, rich in fruits and vegetables, could reduce exercise-induced oxidative stress by inducing better antioxidant defences (Yavari et al., 2015). However, as in these studies the participants were physically active, it is possible that in their nutritional diet could serve as protective mechanism.

It has also been suggested that oxidative stress levels are not affected by the absolute metabolic workload (Quindry et al., 2003) but from the exercise duration (Bloomer et al., 2005) and intensity (Bailey et al., 2004; Quindry et al., 2003). In addition to the above, this thesis has shown that when exercise is matched for

external workload (speed, duration and distance) but the intermittency or the acceleration varies, there are no changes in oxidative stress. Thus, it is hypothesised that the increased rate of intermittency or acceleration and deceleration, as a single factor is not a sufficient contributor to induce oxidative stress, but a combination of increased intensity, duration and intermittency or acceleration is required to observe changes in oxidative stress status during HIIE. Findings of a recent study by Kaspar et al. (2016) demonstrated that acute continuous exercise elicits adverse inflammatory responses compared to a similar HIIE session and as the authors suggest, the observed reduction in chemoattractant protein-1 (MPC-1) following HIIE could be due to the effective reduction in oxidative stress. Thus, it is possible that oxidative stress is reduced as a result of decreased inflammation by this type of activity.

8.3. Temporal responses of ROS during exercise

8.3.1. Day to day variation in oxidative stress responses

Goldfarb et al. (2014) reported a wide variability in daily oxidative stress levels between participants at rest, even when the time, the diet and the physical activity status were taken into account. Investigating this observation in the present thesis, there are indications that this hypothesis could be supported. Examining the baseline concentration of lipid hydroperoxides of the participants following each visit in the laboratory (figure 8.1), data showed an mean value of 6.42 $\mu mol.L^{-1}$ in the range of 1.80 – 20.69 $\mu mol.L^{-1}$. As this is a considerable disparity, day to day

variations during the baselines measurements can be rather important, since this shows that there may be an 11-fold difference in the daily fluctuations of the concentration of lipid hydroperoxides.

It was also interesting that upon closer inspection of intra-individual day to day concentrations of lipid hydroperoxides at rest, individuals showed variability in their rest values between visits. As an example, the concentration of lipid hydroperoxides at baseline for P2 varied within the range of 3.09 $\mu mol.L^{-1}$ to 20.69 $\mu mol.L^{-1}$ between the 6 visits. Conversely, other individuals maintained relatively higher concentration of lipid hydroperoxides during all visits in the laboratory. As an example P6 maintained relatively high concentrations in the range of 12.19 $\mu mol.L^{-1}$ – 18.71 $\mu mol.L^{-1}$. This observation, of individual variability, could imply the existence of “outliers”. From the results we can tentatively suggest that individuals exhibit a pronounced day to day variation in blood oxidative biomarkers, which requires careful control (figure 8.2). This variation can possibly be caused by diet, inflammation, or it could be due to normal fluctuations of daily ROS production which may be part of the normal physiological processes. When investigating biological biomarkers, there is a possibility that there could be a cyclical rhythm that could cause daily, monthly or seasonal fluctuations (Fraser & Fogarty, 1989). Considering this, the daily, monthly or seasonal physiological production of ROS levels are not fully understood, as the observations about increased oxidative stress levels in exercise studies are usually indicated against baseline measures (Kliszczewicz et al., 2015; Välimäki et al., 2016; Wadley et al., 2016). Likewise,

individual inherent fluctuations may be present, which may be described as random variation near a homeostatic set point (Fraser & Fogarty, 1989).

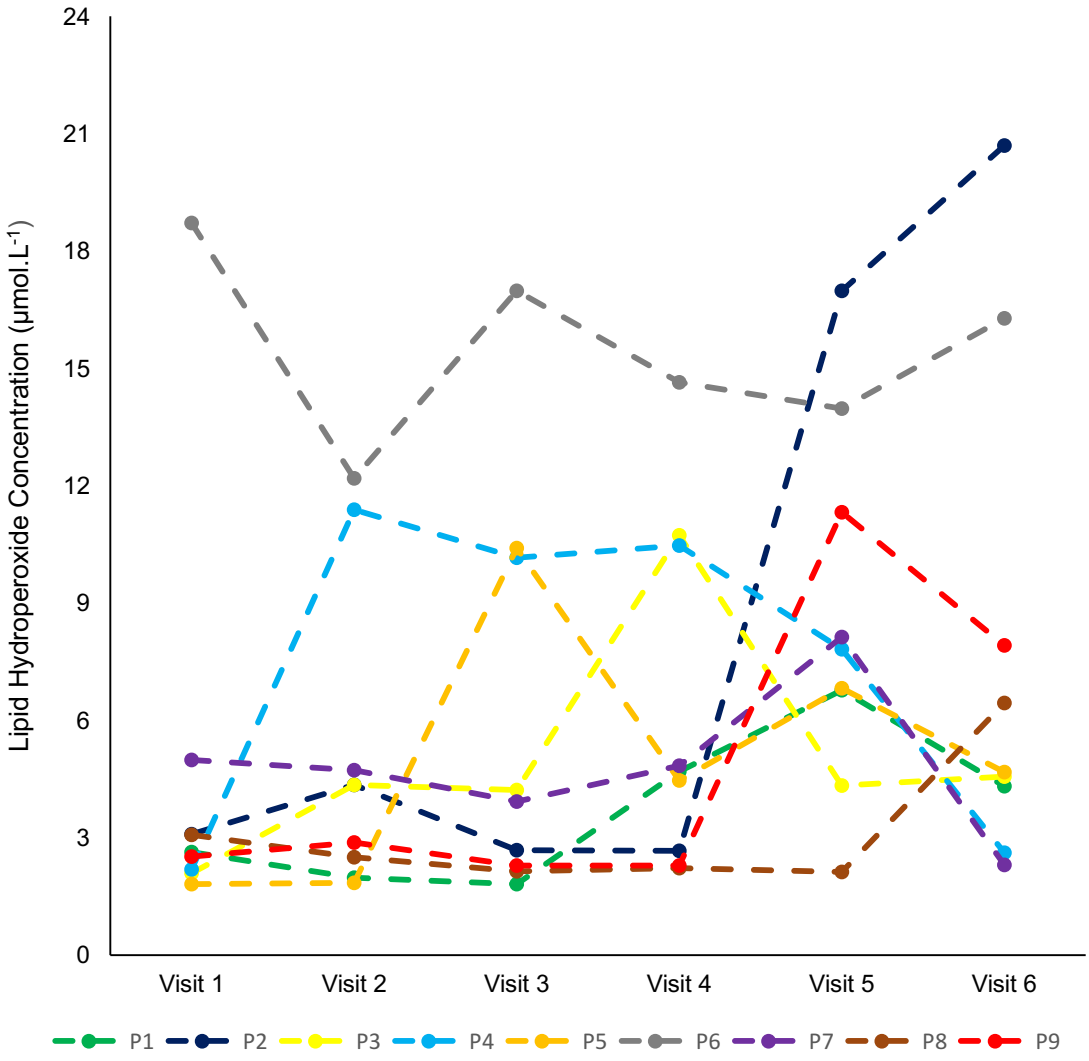


Figure 8.1 Day to day variation for individual participants (n = 9) of baseline lipid hydroperoxides following 6 visits in the laboratory.

8.3.2. Diurnal variation in ROS responses

In accordance to the above section, intra-individual diurnal fluctuations are also of interest. Upon a closer examination of the control session, where exercise was not performed, individuals showed a considerable variation during a 24 hour response (figure 8.2), with some individuals showing increases in lipid hydroperoxides from baseline to 1 hour (P6, figure 8.2), while others showed decreases (P3, figure 8.2) and others experienced minimum changes (P8, figure 8.2). This observation could further supplement the hypothesis that individuals experience different diurnal changes as part of the daily physiological and metabolic processes. As low ROS have been shown play an dual role in humans (Bartosz, 2009), it is possible that the observed intra-individual fluctuations are part of this process. Thus, those differences observed during a 24 hour period could possibly be within the normal range of lipid hydroperoxide production, which could serve as play a role in biological functions such as in signal transduction (Girotti, 1998).

Consequently, it would be sensible to include control sessions where no exercise is performed, with blood samples obtained at the same time points as they would during an exercise session. This method could possibly decrease the artefact of sampling time and individuality in the biomarkers tested, as the samples can be compared against a control session that takes into account these factors. As reference values of oxidative stress biomarkers are currently unknown (Davison et al., 2012) more research is required in an attempt to study and determine the physiological levels of these markers in healthy population and investigate the

possibility of an upper or lower threshold. That way the exercise-induced oxidative stress studies could provide better understanding, as any increases observed would be determined to be due to the exercise stimulus and not due to the artefact of the physiological fluctuations.

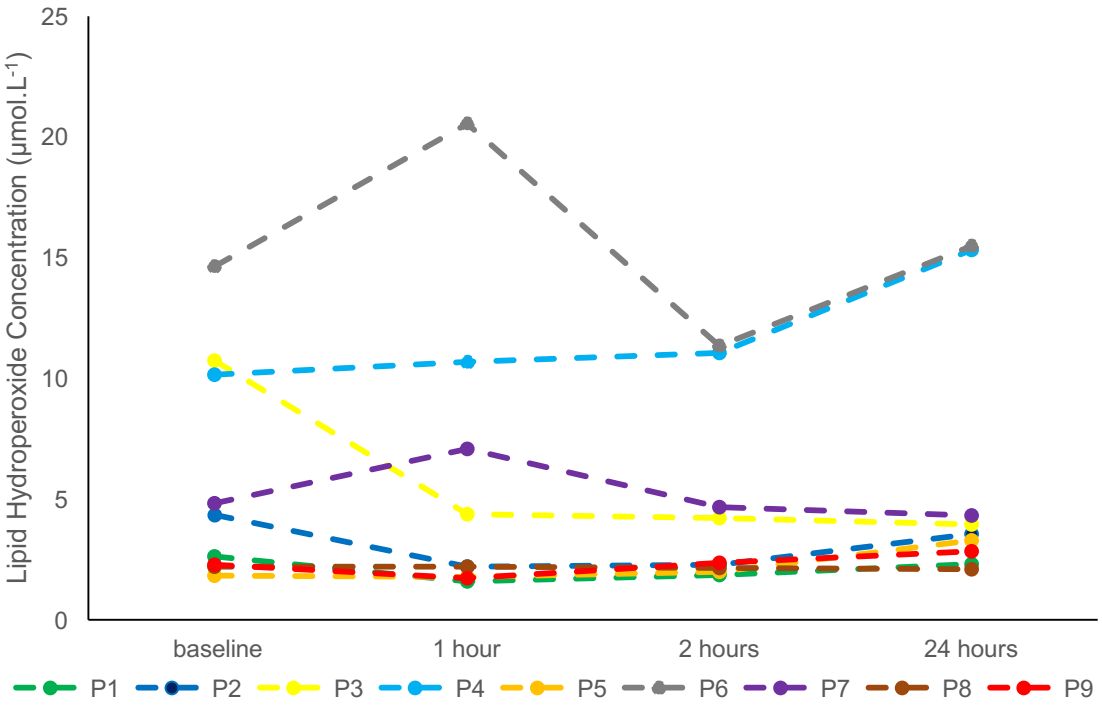


Figure 8.2 Diurnal variations of individual participants (n = 9) during control session. Blood samples were analysed at baseline, 1 hour, 2 hours and 24 hours to examine lipid hydroperoxides. Each dashed line represents an individual participant (P1- P9).

8.3.3. Variation of ROS in population mean

As discussed in sections 8.2.1 and 8.2.2, the day to day variations as well as the diurnal variations can have an effect in the resulting outcome and the assumptions made about exercise-induced oxidative stress. Margaritelis et al. (2016) proposed the “regression to the mean artefact” in exercise-induced oxidative stress studies, which is a concept that could also be linked in this thesis. Margaritelis et al. (2016) proposed that the recruitment of participants could be classified on groups based on baseline measurements, as some individuals may experience extreme (low or high) values. This research also showed that even when this artefact is considered individuals may still experience extreme (low or high) values to the same exercise stimulus.

Examining lipid hydroperoxides from this thesis outcomes, for “regression to the mean artefact”, data showed that individuals varied in responses during control (figure 8.3 A), low intermittency (figure 8.4 A), moderate intermittency (figure 8.5 A) and high intermittency (figure 8.6 A) protocols. While some participants maintained a high lipid hydroperoxide concentration throughout the exercise protocols (e.g. figure 8.3 A – 8.6 A, participant P6), others demonstrated low levels (e.g. figure 8.3 A – 8.6 A, participant P9). Attempting to employ the concept of applying high, medium and low groups based on baseline levels following Margaritelis et al. (2016) was not possible as this thesis had 9 participants in total. However, in an attempt to apply this concept, it was decided to examine lipid hydroperoxide measurements by inspecting the results after excluding participants

from the exercise protocols if extreme values were observed at baseline or control. Results showed that the variation in responses were decreased during control (figure 8.3 B), low intermittency (figure 8.4 B), moderate intermittency (figure 8.5 B) and high intermittency (figure 8.6 B) protocols and after the exclusion of the extremes the upper and lower percentiles also declined (tables 8.1 – table 8.3). The median had a total decrease of 20.78%. 75% upper percentiles had a total decrease of 53.68%, while 25% lower percentiles had a decrease of 6.19%, indicating that the variation between participants was more symmetrical and the sample was more homogeneous. This demonstrates that if groups were included based on baseline or control measurements, the physiological variation and characteristics of individuals tested would possibly provide more accurate representation of the impact of the exercise protocols. However, even after the exclusion of the extremes, some individuals still experienced higher responses to the same exercise stimulus (e.g. figure 8.4 participant P1 and P2, 2 hours, low intermittency protocol). Considering the impact of exercise, it is interesting that both of these individuals experienced the highest levels of oxidative stress in the same protocol and at the same time point. Specifically, in P1 the increase from baseline to 2 hour post exercise is 134% and in P2 the increase is 426%. If these values are of physiological significance remains unknown. However, it would be interesting to examine if this response could be an indication of induced oxidative stress as a result of low intermittency.

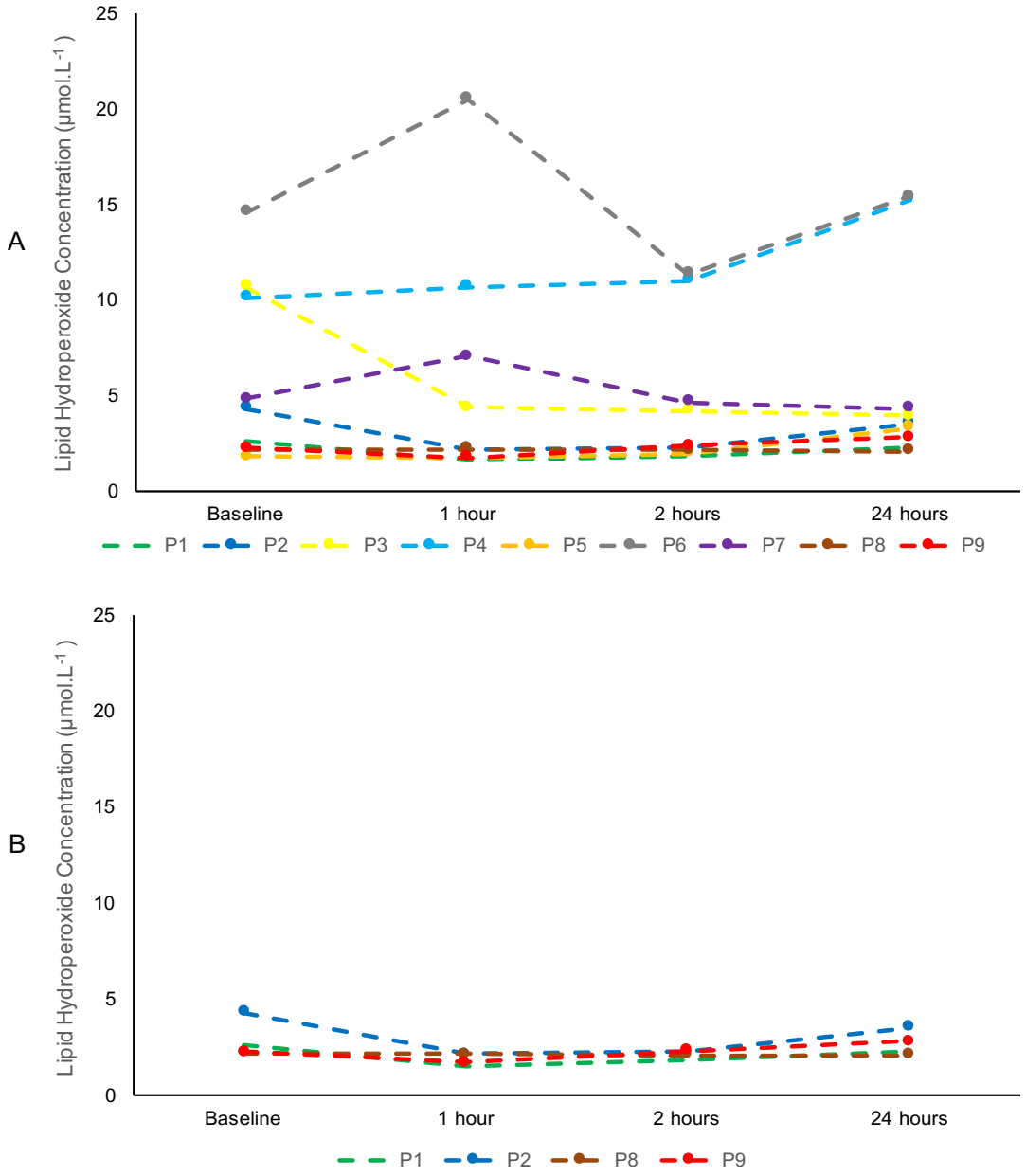
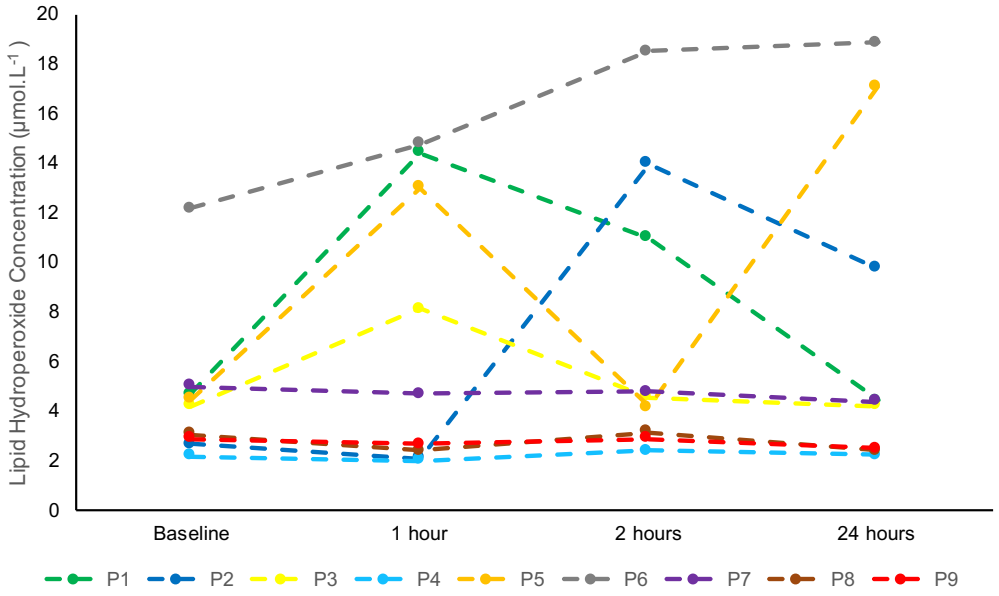


Figure 8.3 Concentration of lipid hydroperoxides (µmol.L^{-1}) for control session for all participants (n = 9), (figure A) and following rejection of participants with extreme values (n = 4), (figure B).

A



B

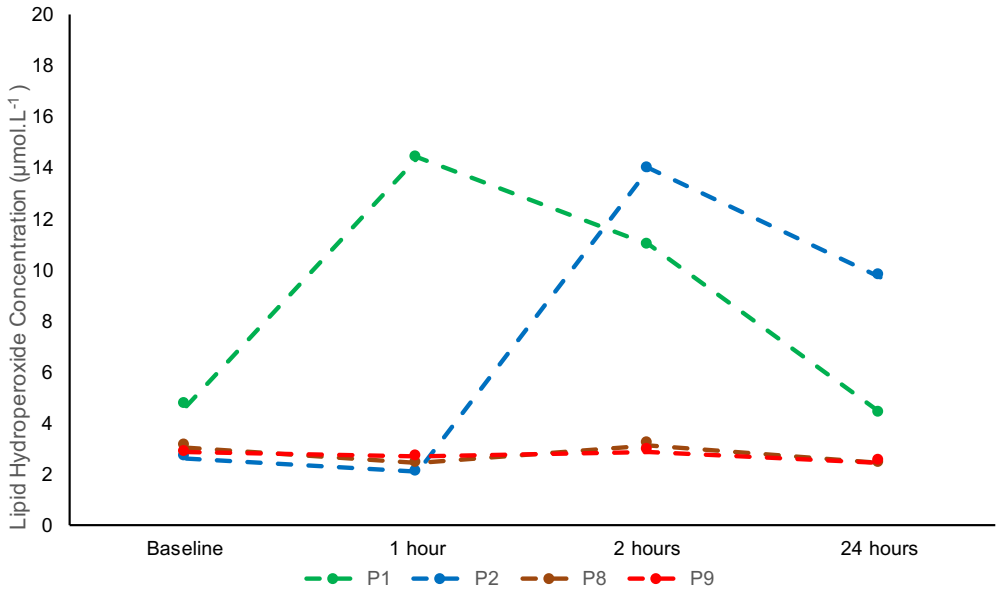


Figure 8.4 Concentration of lipid hydroperoxides (µmol.L^{-1}) for low intermittency protocol for all participants (n = 9), (figure A) and following rejection of participants with extreme values (n = 4), (figure B).

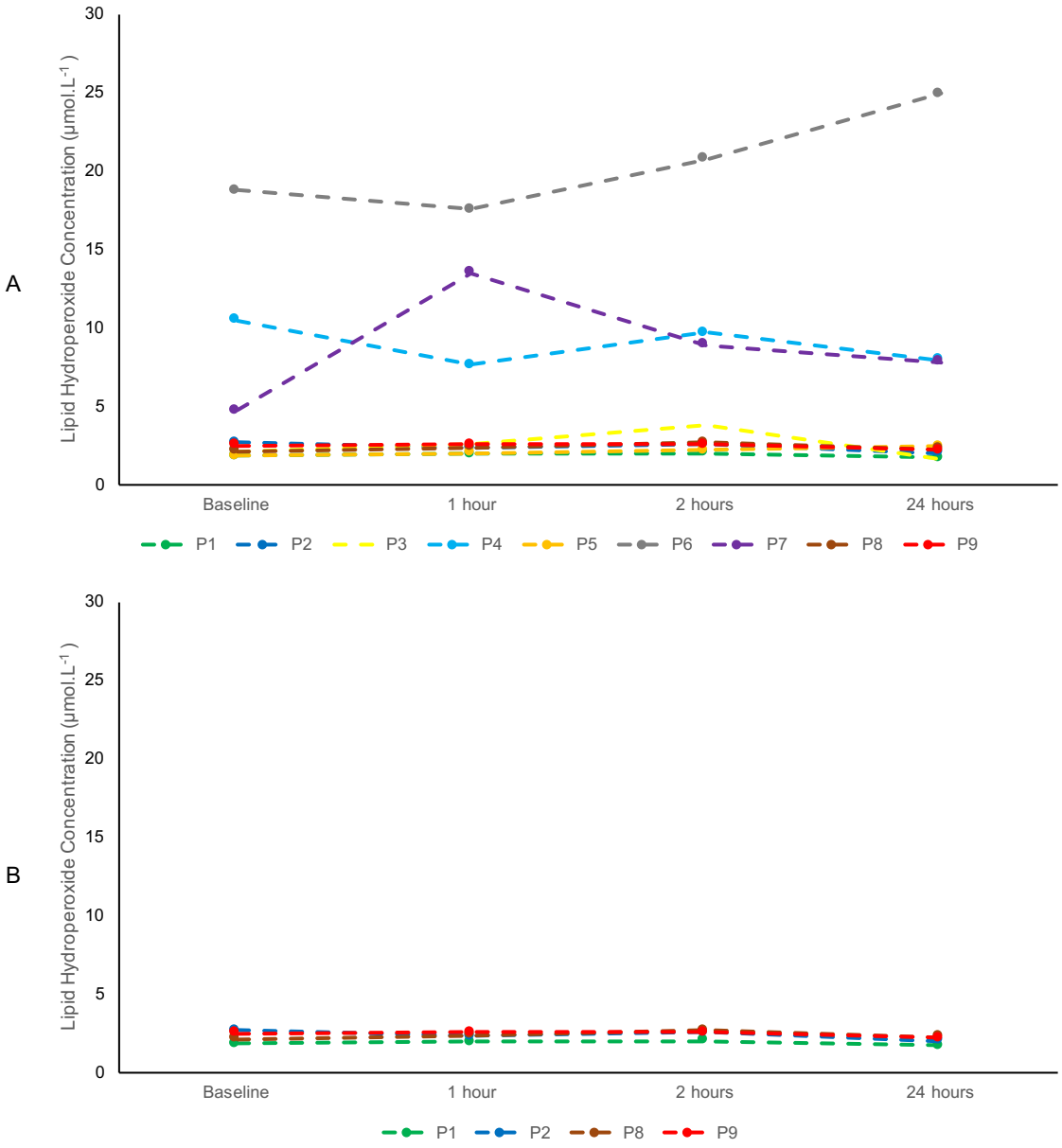


Figure 8.5 Concentration of lipid hydroperoxides (µmol.L^{-1}) for moderate intermittency protocol for all participants (n = 9), (figure A) and following rejection of participants with extreme values (n = 4), (figure B).

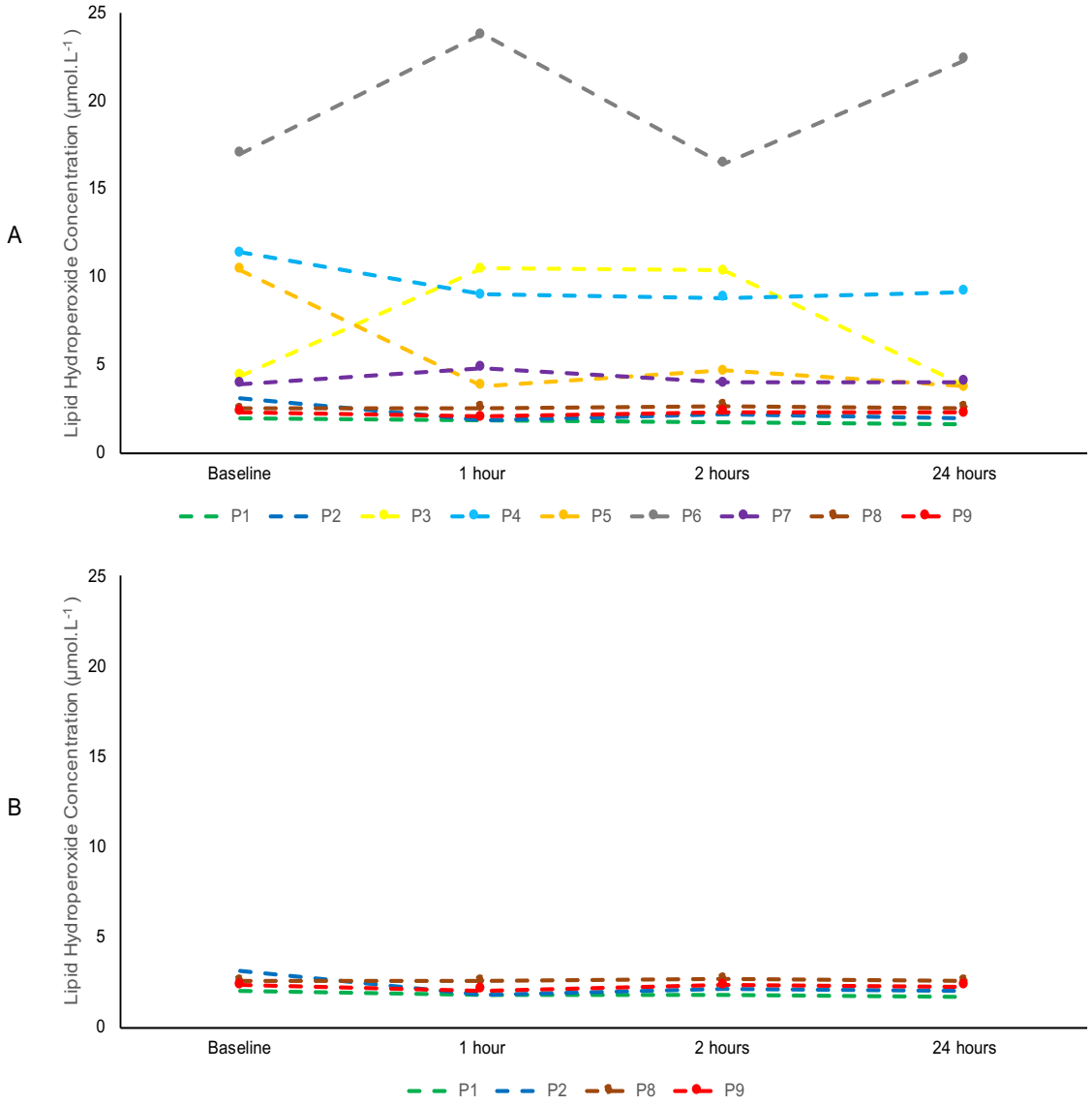


Figure 8.6 Concentration of lipid hydroperoxides (µmol.L^{-1}) for high intermittency protocol for all participants (n = 9), (figure A) and following rejection of participants with extreme values (n = 4), (figure B).

Table 8.1 Percentage difference of median following exclusion of participants with extreme measurements.

Time	Condition	Before	After	% difference
Baseline	Control	4.35	2.45	43.57
	Low intermittency	4.22	2.98	29.50
	Moderate intermittency	2.51	2.32	7.45
	High intermittency	3.92	2.40	38.82
1 hour	Control	2.20	1.97	10.53
	Low intermittency	4.68	2.55	45.57
	Moderate intermittency	2.57	2.34	8.93
	High intermittency	3.79	1.91	49.66
2 hours	Control	2.36	2.22	5.73
	Low intermittency	4.56	7.07	-54.91
	Moderate intermittency	2.69	2.54	5.54
	High intermittency	3.94	2.22	43.60
24 hours	Control	3.55	2.58	27.35
	Low intermittency	4.39	3.45	21.36
	Moderate intermittency	2.23	2.09	6.20
	High intermittency	3.72	2.10	43.51

Positive values shown in percentage difference (% difference) indicate a decrease and negative values indicate an increase.

Table 8.2 Difference in the 75% upper percentile following the exclusion of extreme measurements.

Time	Condition	Before	After	% difference
Baseline	Control	10.16	3.06	69.87
	Low intermittency	4.68	3.48	25.74
	Moderate intermittency	4.73	2.55	46.04
	High intermittency	10.39	2.65	74.51
1 hour	Control	7.09	2.20	69.02
	Low intermittency	13.00	5.61	56.81
	Moderate intermittency	7.62	2.43	68.16
	High intermittency	8.97	2.14	76.11
2 hours	Control	4.67	2.31	50.43
	Low intermittency	10.98	11.73	-6.83
	Moderate intermittency	8.88	2.58	70.90
	High intermittency	8.78	2.39	72.83
24 hours	Control	4.33	3.03	30.16
	Low intermittency	9.74	5.75	40.99
	Moderate intermittency	7.79	2.19	71.86
	High intermittency	4.00	2.31	42.35

Positive values shown in percentage difference (% difference) indicate a decrease and negative values indicate an increase.

Table 8.3 Difference in the 25% lower percentile following the exclusion of extreme measurements.

Time	Condition	Before	After	% difference
Baseline	Control	2.28	2.26	0.68
	Low intermittency	2.88	2.82	1.95
	Moderate intermittency	2.08	2.05	1.35
	High intermittency	2.50	2.21	11.64
1 hour	Control	1.77	1.70	4.07
	Low intermittency	2.41	2.33	3.14
	Moderate intermittency	2.30	2.21	3.96
	High intermittency	2.01	1.80	10.13
2 hours	Control	2.14	2.07	3.29
	Low intermittency	3.16	3.09	2.11
	Moderate intermittency	2.53	2.40	5.13
	High intermittency	2.30	2.05	11.01
24 hours	Control	2.85	2.26	20.66
	Low intermittency	2.49	2.47	0.68
	Moderate intermittency	2.00	1.92	3.89
	High intermittency	2.23	1.88	15.34

Positive values shown in percentage difference (% difference) indicate a decrease.

8.4. The impact of biomechanics on exercise-induced oxidative stress

During eccentric contractions there is higher peak torque, reduced motor unit activation and longer length of the muscle during the contraction which induce the mechanical stress in muscle (Assumpcao Cde et al., 2013). As HIIE involves this type of activity, it is possible that the impact of eccentric contraction can possibly induce muscle damage, which may lead to delayed muscle soreness (DOMS), disruptions in myofibrilar structure, myofibre necrosis and inflammation (Paulsen et al., 2012). However, if participants are accustomed to the exercise protocols implemented because these closely represent their regular training, the impact of muscle damage may be to a lesser degree (Paulsen et al., 2012). This may also be true for this thesis, as participants were regularly trained and their exercise regimes could reflect closely the exercise protocols implemented in these studies (i.e. training that involves repeated sprints with brief rest periods). Also, as the repeated bout effect shows, a single session of eccentric exercise, before the subsequent eccentric exercise bouts, may have a protective role against muscle damage (McHugh, 2003; Nosaka & Clarkson, 1995). Attempting to relate this effect to oxidative stress, it is possible that the intermittency of HIIE could serve in a similar fashion (i.e. faster recovery and attenuated changes in oxidative stress status). Thus, it is possible that the eccentric component of this type of activity was not significant to cause biomechanical stress, which could stimulate increased ROS production. Therefore, further research is required to provide evidence of biomechanical factors related to the eccentric nature of running related oxidative stress.

8.5. Future work

Although this thesis attempted to investigate the impact of HIIE on oxidative stress biomarkers, further research on the effect of intermittency and acceleration during this type of activity is required. Recommendations for future work could include the impact of intermittency or acceleration on biomarkers of oxidative stress in sedentary individuals or females to examine if the training status or the gender can impact oxidative stress following this type of activity. As this thesis had 9 participants, the use of these protocols could be further investigated using a larger population sample to examine if significant changes occur. It is also possible that oxidative stress was not observed in the biomarkers tested in this thesis due to the rest periods, which did not permit oxidation to be measured to a significant level but it is also possible that with the addition of an immediately after exercise sample oxidative stress would be more significant when compared to samples obtained at a later stage.

Another interesting observation of the present thesis is the observation of substantial variation in the biomarkers tested. As variation was also present in in baseline measurements, future studies investigating oxidative stress should take into account the “regression to the mean” artefact and categorise participants based on their baseline measures into groups before further analysis is conducted. As shown in this thesis, the variation in baseline measurements may vary substantially in individuals (day to day variation and diurnal variation) thus, research should aim to further investigate the variability in an attempt to determine

if a possible threshold exists after which oxidative stress occurs that is beyond the normal range.

Finally, the use of 8-hydroxy-2'deoxyguanosine (8-Oxo-dG) LC-MS method that was developed and optimised in this thesis, could be further advanced by implementing a more sensitive detector and by simultaneously measuring all DNA bases to determine possible oxidation which would provide an overall representation of DNA damage.

Redox biology is a very important field of research in order to better understand and appreciate the impact of exercise due to the biological importance. Consequently, further investigation is required in order to enhance our understanding of redox biology and attempt to answer complex question of redox signalling and biological adaptations occurring during exercise as a result of redox processes. Furthermore, as the tools and the techniques to measure oxidative stress still exhibit certain limitations, more research is still needed in order to develop new and innovative approaches to measure oxidative stress in order to reveal subtitle differences.

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Appendix 1

Pre-Exercise Medical Questionnaire

The information in this document will be treated as strictly confidential

Name: .....

Date of Birth: ..... Age: .....Sex: .....

Blood pressure: …… Resting Heart Rate: …….

Height (cm): ……..... Weight (Kg): ……....

Please answer the following questions by putting a circle round the appropriate response or filling in the blank.

1. How would you describe your present level of **exercise** activity?
   Sedentary / Moderately active / Active / Highly active

2. Please outline a typical weeks exercise activity

…….....
…….....
…….....

3. How would you describe your present level of **lifestyle** activity?
   Sedentary / Moderately active / Active / Highly active

4. What is your occupation? …….....

5. How would you describe your present level of fitness?
   Unfit / Moderately fit / Trained / Highly trained

6. Smoking Habits Are you currently a smoker? Yes / No
   How many do you smoke …….. per day
   Are you a previous smoker? Yes / No
   How long is it since you stopped? ..... years
   How many did you smoke? ..... per day

7. Do you drink alcohol? Yes / No

If you answered **Yes** and you are male do you drink more than 28 units a week?
Yes / No

If you answered **Yes** and you are female do you drink more than 21 units a week?
Yes / No

8. Have you had to consult your doctor within the last six months? Yes / No
If you answered **Yes**, Have you been advised **not** to exercise?
Yes / No

9. Are you presently taking any form of medication? Yes / No
If you answered **Yes**, Have you been advised **not** to exercise?
Yes / No

10. Do you have a history of fainting during or following exercise? Yes / No
If **Yes**, please provide details.....
.....
.....

11. To the best of your knowledge do you, or have you ever, or have a family history:

a Diabetes?	Yes / No	**b** Asthma?	Yes / No
c Epilepsy?	Yes / No	**d** Bronchitis?	Yes / No
e ✸Any form of heart complaint?	Yes / No	**f** Raynaud's Disease	Yes / No
g ✸Marfan's Syndrome?	Yes / No	**h** ✸Aneurysm / embolism?	Yes / No
I Anaemia	Yes / No		

12. ✸Are you over 45, and with a history of heart disease in your family? Yes / No

13. Do you currently have any form of muscle or joint injury? Yes / No
If you answered **Yes**, please give details…….
……
…… …..

14. Have you had to suspend your normal training in the last two weeks? Yes / No
If the answer is **Yes** please give details……
……..
……..
……

15. ✸ Please read the following questions:

a)	Are you suffering from any known serious infection?	Yes / No
b)	Have you had jaundice within the previous year?	Yes / No
c)	Have you ever had any form of hepatitis?	Yes / No
d)	Are you HIV antibody positive	Yes / No
e)	Have you had unprotected sexual intercourse with any person from an HIV high-risk population?	Yes / No
f)	Have you ever been involved in intravenous drug use?	Yes / No
g)	Are you haemophiliac?	Yes / No

16. As far as you are aware, is there anything that might prevent you from successfully completing the tests that have been outlined to you? Yes / No.

IF THE ANSWER TO ANY OF THE ABOVE IS YES:

a) Discuss with the test administrators or another appropriate member of the department.

b) Questions indicated by (✸) answered yes: Please obtain written approval from your doctor before taking part in the test.

PLEASE SIGN AND DATE AS INDICATED ON THE NEXT PAGE

Participant Signature: …… Date……

Test Administrator:…….. Date……

Supervising staff member..... Date.....

Parent (if minor)…….…….. Date: …….

THIS SECTION IS ONLY REQUIRED FOR RETURN VISITS!

For any future testing sessions it is necessary to verify that the responses provided above are still valid, or to detail any new information. This is to ensure that you have had no new illness or injury that could unduly increase any risks from participation in the proposed physical exercise.

ANSWER THE FOLLOWING QUESTION AT EACH REPEAT VISIT.

Is the information you provided above still correct, and can you confirm that you have NOT experienced any new injury or illness which could influence your participation in this exercise session?

Repeat 1	Yes / No*	Signature:	Date:
*Additional info required:			
Repeat 2	Yes / No*	Signature:	Date:
*Additional info required:			
Repeat 3	Yes / No*	Signature:	Date:
*Additional info required:			

Repeat 4	Yes / No*	Signature:	Date:
*Additional info required:			
Repeat 5	Yes / No*	Signature:	Date:
*Additional info required:			

Appendix 2

Optimisation of voltages in LC-MS for the measurement of 8-oxo-dG.

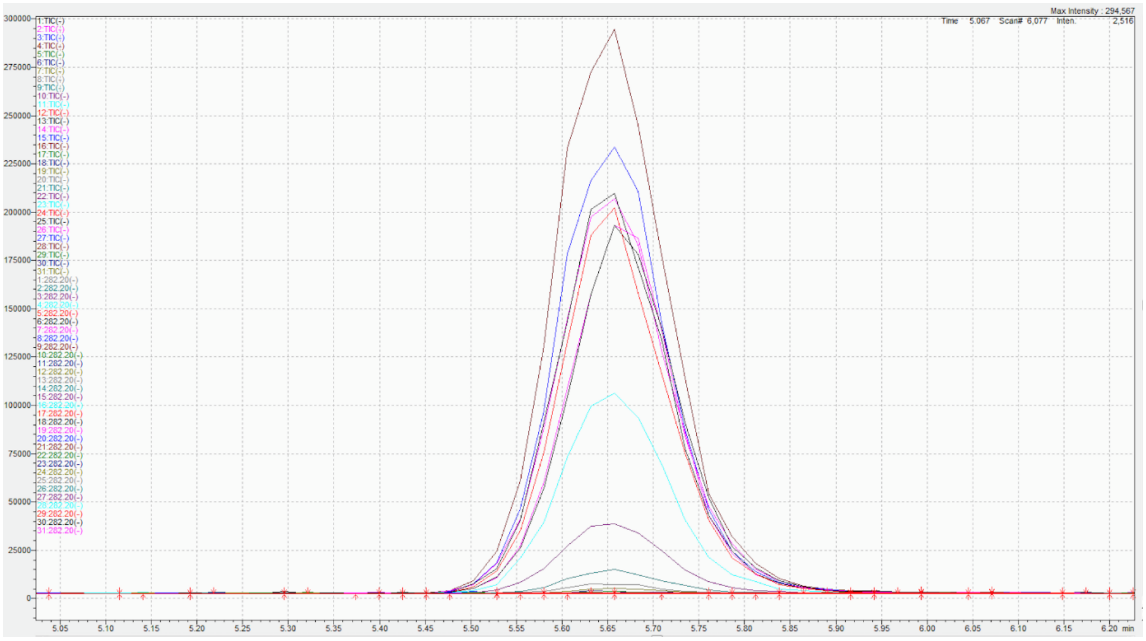

Figure A2.1 DC volt optimisation. Default settings were chosen as the most appropriate to be used for 8-Oxo-dG method after observation of the results.

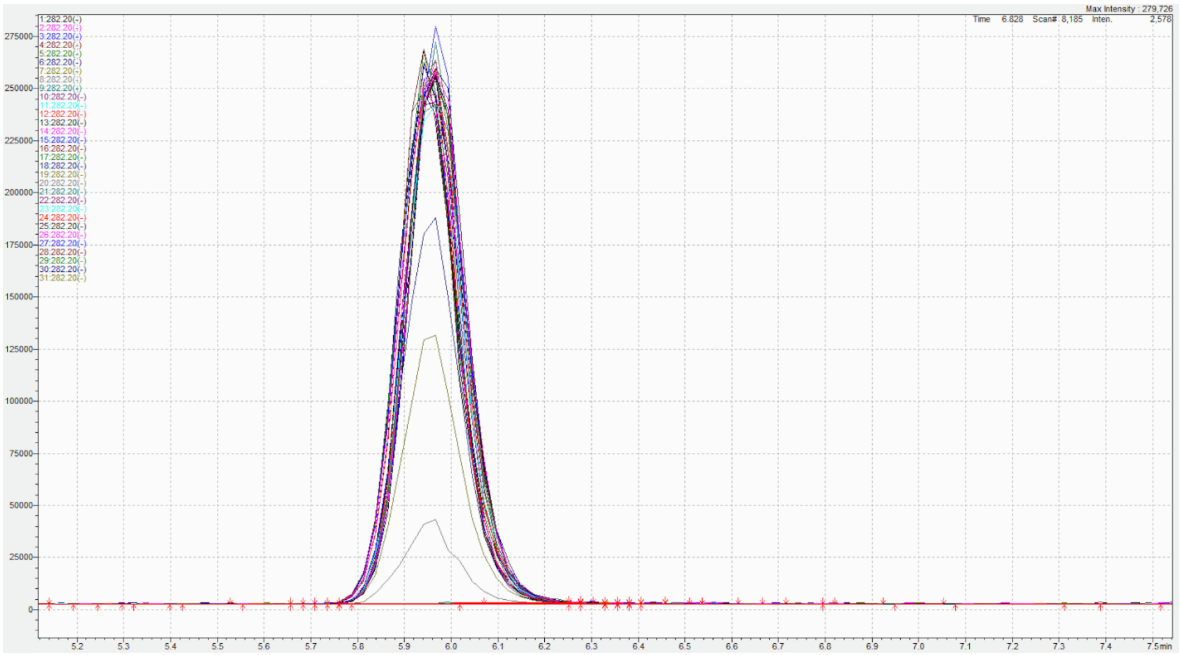

Figure A2.2 DL volt optimisation. Default setting were chosen as the most appropriate to be used for 8-Oxo-dG method after observation of the results.

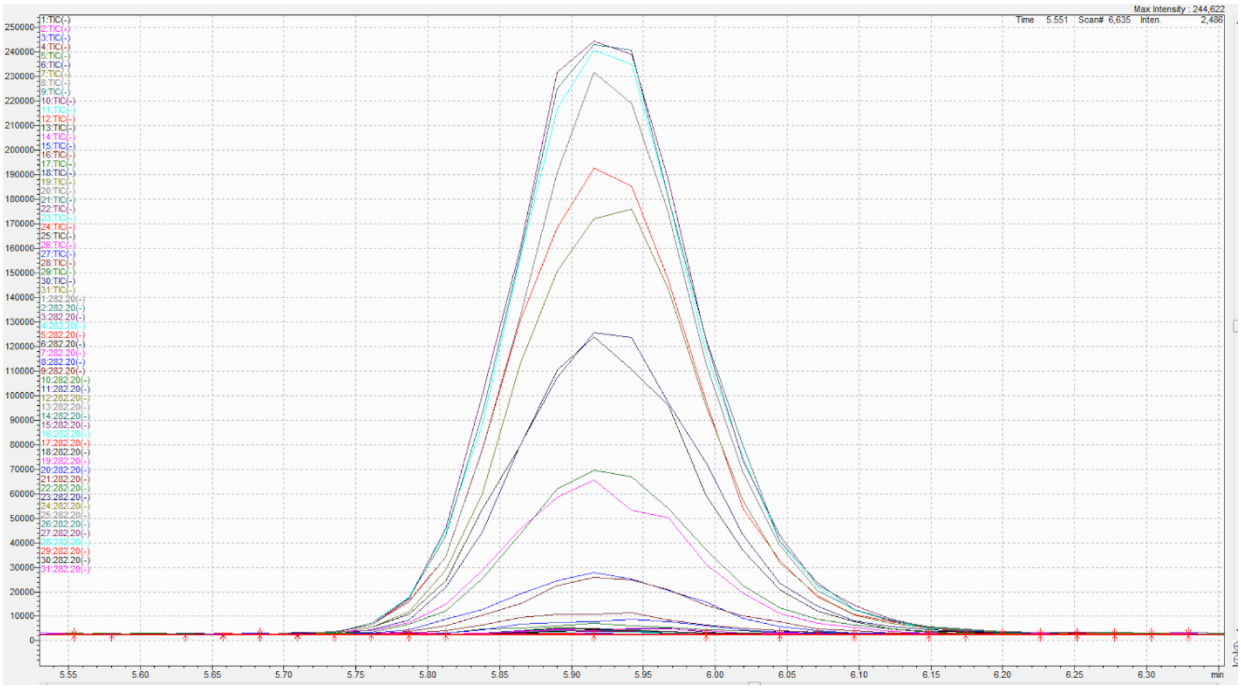

Figure A2.3 RF volt optimisation. Default settings were chosen as the most appropriate to be used for the 8-Oxo-dG method after observation of the results.

Appendix 3

Exercise mimicking of high-intensity intermittent exercise using a C2C12 mouse myoblast cell line to determine oxidative stress levels

A3.1 Introduction

It is widely recognised that muscle is a predominant source of ROS during exercise (Steinbacher and Eckl, 2015, Powers and Jackson, 2008). However, oxidative stress in exercise is commonly measured in blood, due to ease of access of this biological fluid (Powers and Jackson, 2008) . You et al. (2005) suggested that even though the concentrations of the oxidative stress biomarkers examined in blood and muscle can be different, there are indications that similar responses occur between the 2 tissues. However, Fogarty et al. (2013) suggested that the levels of oxidative stress blood and muscle are not associated. As research findings are contradictory, by investigating only blood biomarkers several issues can be raised, as it is not fully established whether an accurate depiction of oxidative damage in the muscle can be signified by examining only the blood (Nikolaidis et al., 2008). Argüelles et al. (2004) described a 5 step process may occur where the oxidised damage components may be transferred to the serum, plasma, urine and breath therefore, the levels of a specific biomarker in plasma or serum will be dependent on the rate of oxidation of the molecules by oxidants (step 1), export of the tissue marker into serum or plasma (step 2), the detoxification and metabolism of the marker (step 3) and the elimination through urine and breath (step 4 and 5). Thus, it is possible that blood may accumulate the amount of oxidative damage, if the damage is excreted from different tissues into the blood (Argüelles et al., 2004). However, in this approach the importance of other factors is not considered. As an example inflammatory processes which may be experienced as a result of exercise, may also induce oxidative stress in the blood (e.g. respiratory burst),

(Sallam and Laher, 2016). Thus, an investigation of the muscle may more accurately and directly demonstrate oxidative stress responses in this tissue compared to indirect assessment through blood tissue.

Due to the compilations in obtaining a muscle sample, as discussed above, exercise-induced oxidative stress studies have mainly used blood is the primary tissue to measure oxidative damage (Powers and Jackson, 2008). Due to this, animal models have been developed for the investigation of oxidative stress (Liu et al., 2000, Gomez-Cabrera et al., 2005, Hollander et al., 2001, Okamura et al., 1997, Hinchcliff et al., 2000). This approach has certain advantages such as homogeneity or better control in design (e.g diet, environmental conditions), (Fisher-Wellman and Bloomer, 2009). However, these models can still exhibit some ethical implications (Ghasemi and Dehpour, 2009). In contrast to such procedures, oxidative stress has also been investigated using cell culture (McClung et al., 2009, Irrcher et al., 2009). Generally, the application of cell culture technique may have some limitations (e.g. fluctuating or elevated oxygen levels, culture shock, pro-oxidant substances or in media), which could result in a less accurate representation of oxidative stress *in vivo* (Halliwell, 2014). However, there are some positive aspects in its application which make this technique appealing. The positive features in use of cell culture is that it requires less ethical considerations, as the use of animals or humans is not required, cell lines are commercially available, the experimental periods can be shorter, it can be easy to culture and there is no effect or influences in culture from other tissues or organs

(Manabe et al., 2012). Thus, cell culture can be a promising methodological application prior to animal or human studies for the analysis oxidative stress in muscle.

Thus, the aim of this study was to implement an exercise mimicking protocol of high-intensity intermittent exercise, using a C2C12 mouse myoblast cell line, to determine oxidative stress levels in muscle.

A3.2 Methods

A3.2.1 Preliminary optimisation

An investigation was conducted (by Dr. Fogarty's group) prior to this study to investigate the hypothesis that an exercise mimicking treatment (containing ionomycin an forskolin) which was described by Kurdiova et al. (2014) induces ROS formation. Accordingly, tests were conducted using dihydroethidium (DHE) assay to optimise the method in order to apply it for the examination of oxidative stress following HIIE. Thus, a range of mimicking protocols were tested (figure A3.1) with results indicating that 3 treatments of 5 minutes provided a reproducible result. This treatment was further examined to determine the dose-response relationship and a dose of 100% of forskolin (4 μM) and ionomycin (0.5 μM) was chosen as the more appropriate (figure A3.2). Thus, the modified method of Kurdiova et al. (2014) was applied.

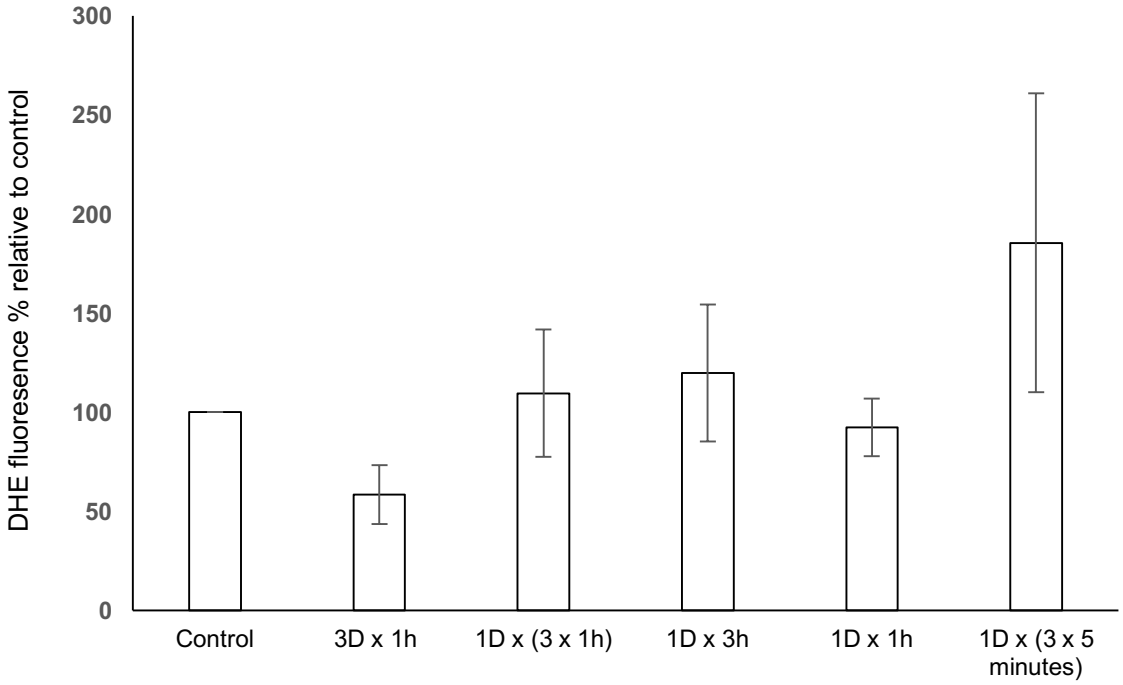

Figure A3.1 DHE fluorescence as a percentage of control (where control is set = 100%) for exercise mimicking treatments. Where 3D x 1hr = 1 hour treatment over a period of 3 days, 1D x (3 x 1h) = 3 treatments for 1 hour each over 1 day, 1D x 3h = 3 hour treatment over 1 day, 1D x 1h = 1 hour treatment over a day, 1D x (3 x 5 minutes) = 3 treatments of 5 minutes over a period of 1 day.

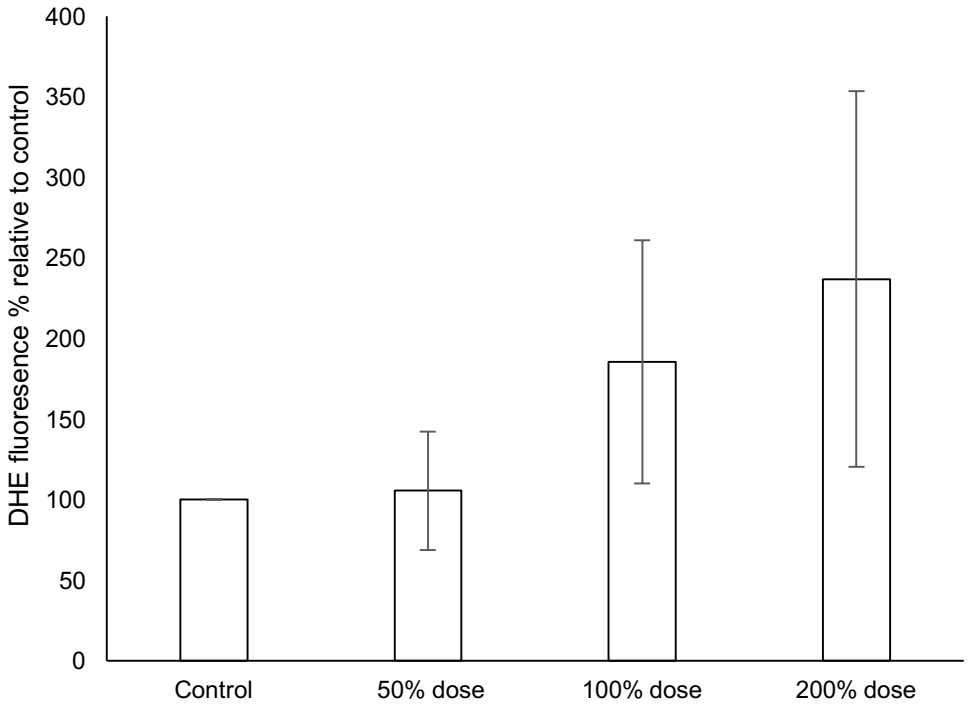

Figure A3.2 Dose-response relationship of forskolin and ionomycin treatments (3 treatments of 5 minutes each) as a percentage of control (where control is set = 100%). Treatments were examined with for a dose of 50%, 100% and 200%.

A3.2.2 Method of mimicking HIIE using a C2C12 cell line

In order to mimic HIIE exercise using a cell line, a protocol from Kurdiova et al. (2014) was applied with the following modifications. A mouse adherent myoblast cell line (C2C12), purchased from Sigma (91031101, Sigma-Aldrich, Dorset, UK), was used for mimicking HIIE in cell culture conditions. Using flasks of a growth area of 75 cm² (T75) purchased from VWR (734-2705, Leicestershire, UK), cells were maintained in growing Dulbecco's modified Eagle's medium (DMEM), containing L-glutamine (L0102, Biowest, Leicestershire, UK) supplemented with 15% fetal bovine serum (F0804, Sigma-Aldrich, Dorset, UK), 1% penicillin (5.000 U / ml) and streptomycin (5mg / ml) (P4458, Sigma-Aldrich, Dorset, UK) and 0.1% amphotericin B (L0009, Biowest, Leicestershire, UK) at 37°C with 5% CO_2. When approximately 80% confluence was reached, cells were passaged by trypsination at a density of 20×10^4 as follows.

Upon reaching approximately 80% confluence, cells were rinsed 3 times with phosphate-buffered saline (PBS), (P4417, Sigma-Aldrich, Dorset, UK) and trypsinised (T3924, Sigma-Aldrich, Dorset, UK) at room temperature until cells were visually observed to be in suspension upon close examination under a microscope. Growing DMEM was added in the cell lysates and contents were centrifuged for 5 minutes at 1600 rcf at room temperature (Mistral 1000, MSE, Leicestershire, UK). Cell pellet was re-suspended in 1 ml DMEM and contents were vigorously mixed using a pipette. Cell counting was assessed in live cells visually under a microscope by the Trypan Blue (T8154, Sigma-Aldrich, Dorset,

UK) method and accompanied with the use of a Neubauer-improved chamber haemocytometer (BC1000, Hawksley, Sussex, UK). `Cell numbers were counted at the 4 large corner squares of the haemocytometer. Following cells were seeded at the required density and left to incubate in 7 ml of growing DMEM. In order to mimic HIIE, C2C12 cells were cultured into rectangular flasks of growth area of T25 cm² (T75) purchased from VWR (734-1712, Leicestershire, UK). Cells were seeded at a density of 66.66×10^3 cells per ml during passages 19 – 21.

Differentiation was induced when approximately 80% confluence was reached by substituting the growing DMEM with differentiation DMEM (containing L-glutamine), (L0102, Biowest, Leicestershire, UK) supplemented with 2% fetal bovine serum (F0804, Sigma-Aldrich, Dorset, UK), 1% penicillin (5.000 U / ml) and streptomycin (5mg / ml) (P4458, Sigma-Aldrich, Dorset, UK) and 0.1 % amphotericin B (L0009, Biowest, Leicestershire, UK).

Cells were observed under a microscope at regular time intervals to examine the morphology and inspect the differentiation process. The process was deemed complete (between 4 – 7 days after the induction of differentiation DMEM), when myoblasts proliferated and fused, forming elongated multi-nuclei myotubes. Subsequently, the experimental procedure to mimic HIIE exercise in a C2C12 cell line followed as described below.

A3.2.3 Experimental procedure for mimicking high-intensity intermittent exercise in C2C12 cell line.

In order to mimic HIIE exercise conditions, cells were treated with a differentiation DMEM supplemented with 4 μM forskolin (1099, Tocris, Abingdon, UK) and 0.5 μM ionomycin (2092, Tocris, Abingdon, UK). Between each exercise mimicking treatment, a washing period was also implemented using 2 ml of differentiation DMEM without forskolin or ionomycin. A control group in which C2C12 cells were treated with differentiation DMEM supplemented with 0.2% DMSO (0231, VWR, Leicestershire, UK) and 0.1% ethanol (20821.330, VWR, Leicestershire, UK) was also incorporated in the experimental procedure, following the same pattern of steps the exercise mimicking group, according the following experimental implementation (figure A3.3):

- 3 times of treatment for 20 minutes each, with washing periods (using differentiation DMEM) of 20 minutes between treatments
- 3 times of treatment for 10 minutes each, with washing periods (using differentiation DMEM) of 10 minutes between treatments
- 3 times of treatment for 5 minutes each, with washing periods (using differentiation DMEM) of 5 minutes between treatments

During each treatment or wash, cells were left to incubate at 37°C with 5% CO_2.

After each exercise mimicking experiment was complete, 1 ml of either methanol (M / 4056 / 17, Fisher Scientific, Loughborough, UK), for the analysis of FOX-1, or PBS (P4417, Sigma-Aldrich, Dorset, UK), for the analysis of SOD and GSH was

added in each flask and cells were scraped using a rubber policeman. Cells lysates (1 ml) were collected and stored at -20°C until further analysis.

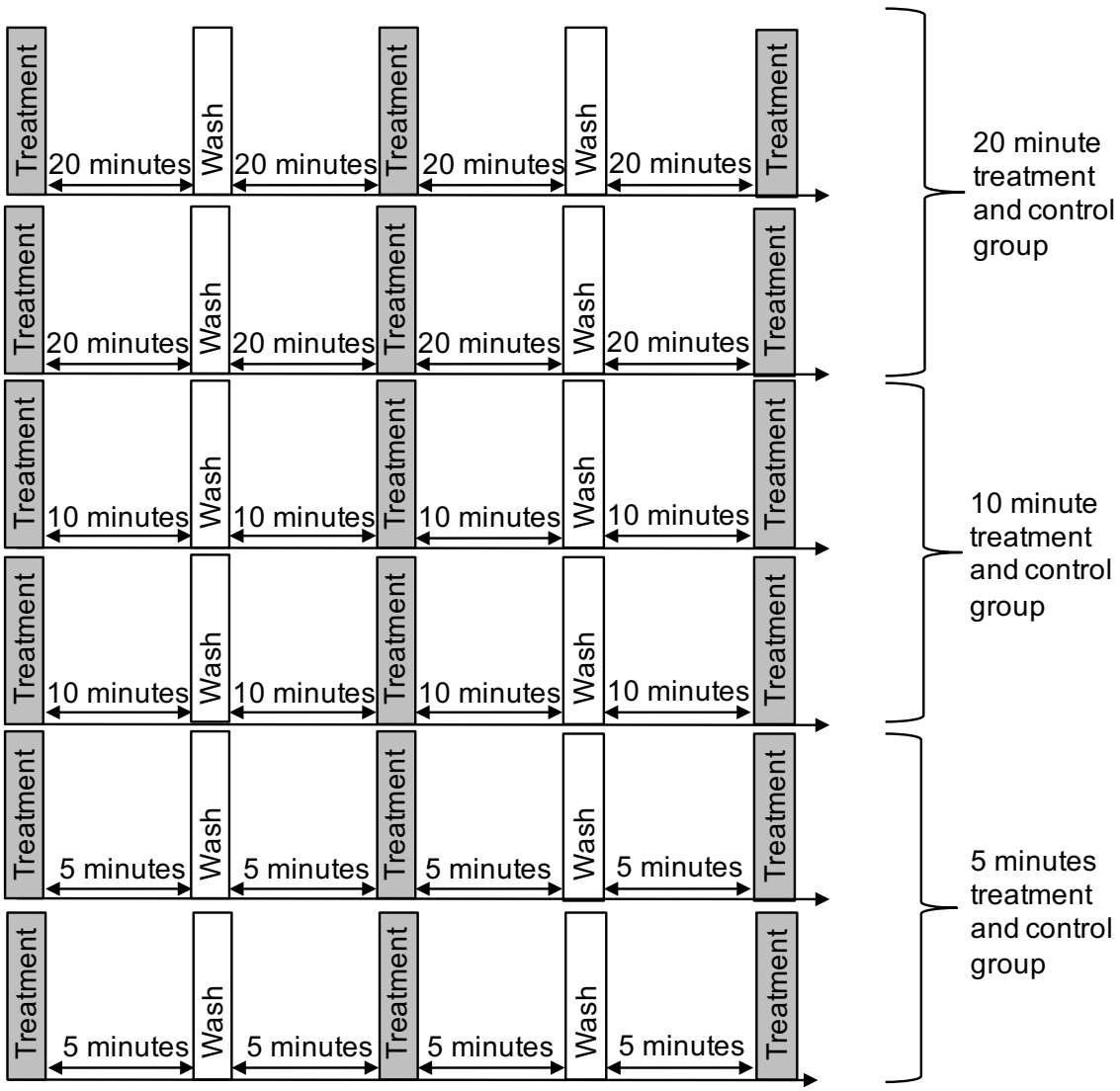

Figure A3.3 Exercise mimicking protocol. C2C12 cells were treated with differentiation DMEM supplemented with 4 μM forskolin and 0.5 μM ionomycin for either 20, 10 or 5 minutes with washing periods, between each treatment, of equal amount. A control group for each protocol was also implemented by supplementing differentiation DMEM with DMSO and ethanol following the same periods of incubation as the exercise mimicking protocol.

A3.2.4 Analysis of FOX-1, SOD and GSH assays for C2C12 lysates.

Lipid hydroperoxide concentrations were measured using the FOX-1 assay as previously described (chapter 2) with the following modifications. As the cell lysates were suspended in methanol, the standard curve was also prepared in the same solution. Cell lysates were centrifuged for 5 minutes at room temperature at 3000 rcf. A total of 20 µl of cell lysates were then added on each well of a 96-well plate along with 180 µl of FOX reagent. Samples were incubated in the dark at room temperature for 30 minutes and absorbance was measured at 560 nm using a plate reader (Tecan, Infinite M200 pro, software: Magellan).

Superoxide dismutase was measured using the SOD assay as previously described in chapter 2 with the following modification. Cell lysates were sonicated for 10 minutes (U100H, Ultrawave, Cardiff, UK) to disrupt any the cell membranes and release the cellular contents. The procedure that followed afterwards followed the same pattern previously described.

Total glutathione (GSH) was measured as previously described in chapter 2 with the following modifications. Cell lysates were sonicated for 10 minutes (U100H, Ultrawave, Cardiff, UK) to disrupt the cell membranes and release the cellular contents. Lysates were then centrifuged at 10000 rcf for 15 minutes at 4°C (17R micro star, VWR, Leicestershire, UK). In 200 µl of lysates 200 µl of metaphosphoric acid were added and after a brief vortex period lysates were centrifuged at 10000 rcf for 2 minutes at 4°C (17R micro star, VWR, Leicestershire, UK). Supernatant

was collected and concentrated by lyophilisation for 24 hours. The residues were reconstituted using 1 / 3 of its original recovered volume that was retrieved after centrifugation and after examining the pH in each sample the protocol followed the same description as previously described.

Calculations to assess the concentration for all the assays was performed as previously described (chapter 2) with the addition of correcting for the protein content, by taking into account the concentration of Bradford assay values, of each average sample.

A3.2.5 Bradford protein assay.

Bradford protein assay was performed in C2C12 cell lysates collected for SOD and GSH assays for the measurement of their protein content and the assessment of protein concentrations. Standards were prepared in a total volume of 1 ml of cell lysis reagent (Cellytic M), (C2978, Sigma-Aldrich, Dorset, UK), supplemented with 10 mg of albumin bovine serum (422361V, VWR Leicestershire, UK) at concentrations ranging from 0 - 1.4 mg / ml. On a clear bottom 96-well plate (Corning, Costar, CLS3599, Sigma-Aldrich, Dorset, UK) a total of 5 µl of standard or cell sample were added on top of 200 µl of Bradford reagent (B6916, Sigma-Aldrich, Dorset, UK). An incubation period in the dark, at room temperature followed and absorbance was measured at a wavelength of 595 nm using a plate reader (Tecan, Infinite M200 pro, software: Magellan).

Due to the methanol content of the C2C12 samples, Bradford assay for FOX-1 assay was adapted as follows. Lysates in methanol were centrifuged and the pellet and supernatant were retained in separate tubes. The pellet was dried to remove excess methanol and then dissolved in 100 µl of 0.1 M sodium hydroxide. Standards in the range of 0 – 14 µl were made in a total volume of 1 ml of sodium hydroxide supplemented with 10 mg of albumin bovine serum.

Analysis of Bradford assay was performed using Microsoft office Excel® (version 15.26) after calculating the blank corrected values of each duplicate well of standards against the known concentrations of the hydrogen peroxide. The y-intercept was set at 0 and Pearson coefficient of determination (R^2) value was examined to indicate the proportion of variance in the samples after they were corrected against the blank. The assay was considered to be successful when R^2 was ≥ 0.98. The average values of each duplicate well of sample were calculated and the concentration of each sample was determined in µg / µl by dividing the average value of each well against the slope. The final concentration was then calculated by multiplying the concentration previously found in µg with the dilution factor to determine the final concentration.

A3.3 Results

A3.3.1 Statistical analysis

Statistical analysis using SPSS to examine normality was performed using Shaphiro Wilk test, $p < 0.05$. When requirements of normal distribution were met, data were analysed using 1 way-ANOVA. Data that did not meet the normal distribution requirements were analysed using Kruskal-Wallis tests. In all the data analysis, statistical significance was indicated by values of $p < 0.05$.

A3.3.2 Lipid hydroperoxides

For all the conditions tested no statistical significance was observed (figure A3.4). All exercise protocols showed similar lipid hydroperoxide concentrations to the control conditions.

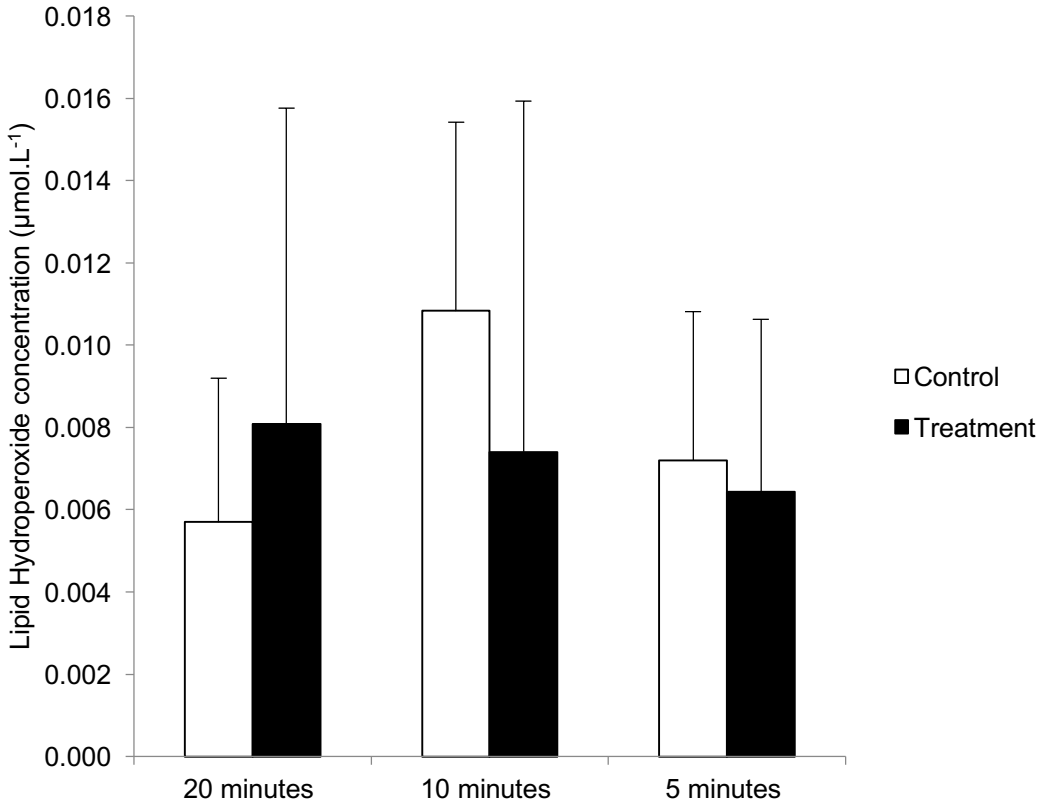

Figure A3.4 Lipid hydroperoxide concentrations ($\mu mol.L^{-1}$) following 3 different HIIE mimicking protocols using a C2C12 cell line (n = 3). Each protocol consisted of 3 treatments of either 20, 10 or 5 minutes interspersed with a corresponding wash out period of equal length of time. Figure represents means with error bars representing standard deviation.

A3.3.3Total glutathione

For all the conditions tested no statistical significance was observed (figure A3.5). All exercise protocols showed similar total glutathione concentrations to the control conditions.

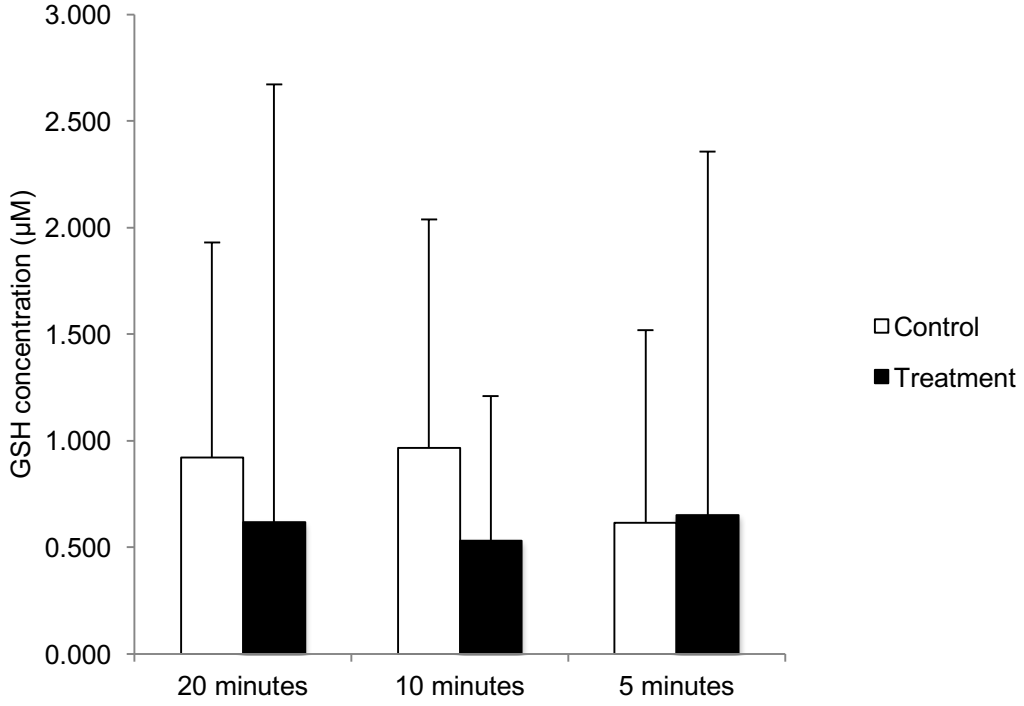

Figure A3.5 Total GSH concentration (µM) following 3 different HIIE mimicking protocols using a C2C12 cell line (n = 3). Each protocol consisted of 3 treatments of either 20, 10 or 5 minutes interspersed with a corresponding wash out period of equal length of time. Figure represents median values with error bars indicating 75% upper percentile.

A3.3.4 Superoxide dismutase activity

For all the conditions tested no statistical significance was observed (figure A.6). All exercise protocols showed similar superoxide dismutase activities to the control conditions.

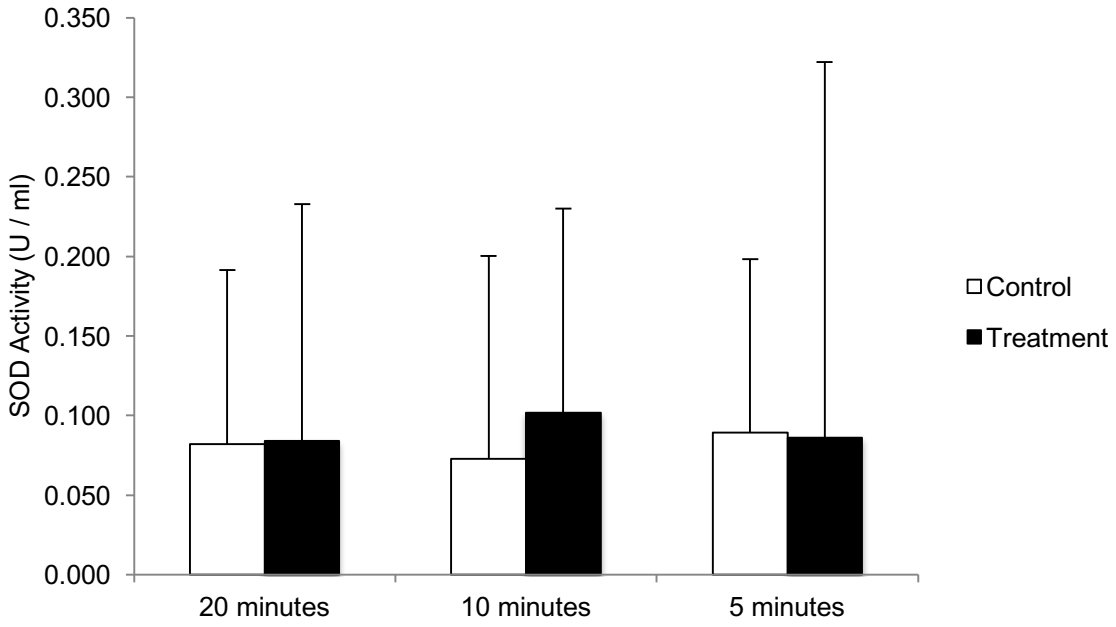

Figure A3.6 Superoxide dismutase activity (U / ml) following 3 different HIIE mimicking protocols using a C2C12 cell line (n = 3). Each protocol consisted of 3 treatments of either 20, 10 or 5 minutes interspersed with a corresponding wash out period of equal length of time. Figure represents median values with error bars indicating 75% upper percentile.

A3.4 Discussion

This study is the first one to examine oxidative stress using a C2C12 cell line following a mimicking protocol of HIIE. The application of a cell line to mimic the impact of exercise-induced oxidative stress can be advantageous as an alternative, non-invasive technique compared to investigations using human or animal models. As the previous studies of this thesis examined blood biomarkers during HIIE, this study served to compliment blood biomarkers by exploring the impact of HIIE in muscle, with the addition of investigating the responses immediately after exercise. Even though a statistical significance was not found, data indicate that oxidative stress was not induced with a HIIE mimicking protocol, which is in agreement with blood biomarkers analysed in previous study of this thesis (chapter 4). However, it is interesting that preliminary investigations using the DHE assay observed increases in ROS.

While research by Fogarty et al. (2013) as well as Radak et al. (1999) showed that the oxidative stress status is increased when muscle biopsies are examined following exercise, the dissimilarities found in comparison to this study could be due to several reasons. The exercise protocols used by the above researchers are of maximum effort and human participants are involved. Thus, the dissimilarities could be due to the intensity of the exercise mimicking protocol used in this study or due to dissimilarities in oxidative stress responses between human, animal, or cell line models. Also a disparity in data has been observed in studies using rats, with evidence indicating that xanthine oxidase production in human muscle is

expressed at lower levels when compared to rats (Gomez-Cabrera et al., 2005, Gomez-Cabrera et al., 2003). Furthermore, studies examining muscle damage in rats have shown differences between males and females, with male rats indicating higher muscle damage (Amelink and Bar, 1986). However, human studies so far indicate either no differences or a slight higher damage in females (Stupka et al., 2000, Clarkson and Hubal, 2001, Rinard et al., 2000). Due to the above, it is also possible that oxidative stress levels could be expressed differently between animal and human studies. Even though the models of cell culture used in studies to investigate oxidative stress have been widely used as an alternative approach, it is important to note that there are some considerations in the use of these methods. Halliwell (2014) reviewed the issues when cell culture techniques are applied to determine oxidative stress and suggested that special consideration should be given to the culture media, which could increase hydrogen peroxide levels, as well as to artefacts caused by oxygen levels in culture. While there might be some issues in the use of the cell culture, it is still a technique widely used by researchers. With careful consideration of the complications of this technique, the method can be used to extrapolate oxidative stress responses during exercise.

As this study was the beginning of establishing a model to determine oxidative stress levels following HIIE in a C2C12 cell line only the immediately post exercise levels of oxidative stress were determined. Also the intermittency was the same in the protocols (3 treatments interspersed with 3 controls) and the duration varied (5, 10 or 20 minutes). Thus, future studies could further develop this method to

include more HIIE mimicking conditions (e.g. lasting several hours or even days) or more severe treatments which could induce higher oxidative stress levels. The use of C2C12 cell line could also be included in further work as an alternative method to determine exercise-induced oxidative stress, without using animal models or muscle biopsies which are invasive and require careful ethical considerations.

A3.5 Conclusion

This research examined HIIE by using a C2C12 mouse myoblast cell line to determine oxidative stress responses immediately after exercise. Results showed no statistical significance in the protocols implemented. However, as this method was the initial work of establishing a model to mimic HIIE using C2C12 cell line, more research is needed to further develop this method.

A3.6 Chapter references

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