Oxidant Stress and Platelet Responsiveness: Implications of body mass index and the modulating effects of diet, acute exercise and selenium supplementation.

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CERTIFICATE OF RESEARCH

This is to certify that, except where specific reference is made, the work described in this thesis is the result of the candidate. Neither this thesis, nor any part of it, has been presented, or is currently submitted, for any degree at any other University.

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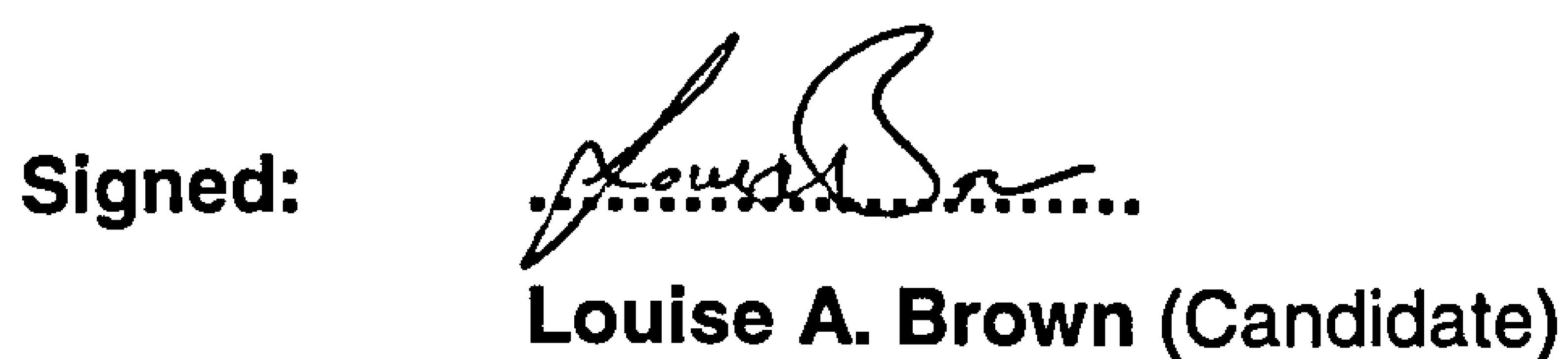

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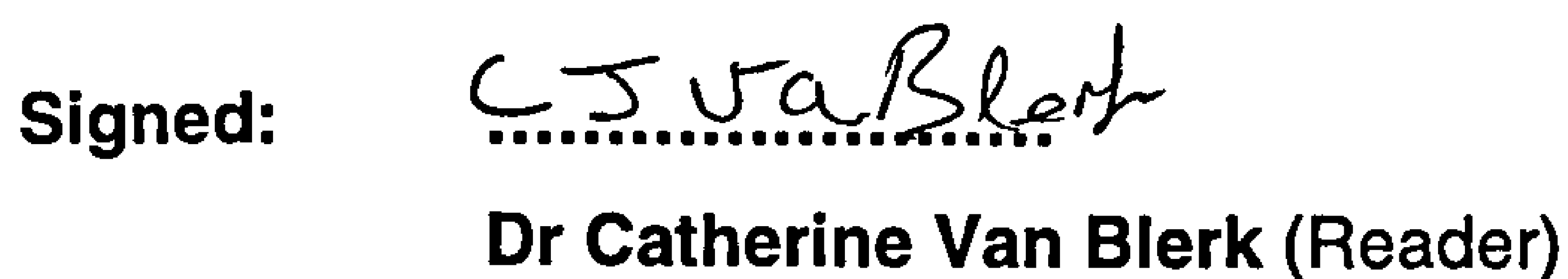

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ABSTRACT

The overall aim of the present study was to investigate the effects of obesity on oxidant stress and platelet responsiveness and examine the modulating effects of both short-term dietary interventions and acute exercise on oxidant stress levels and also the effects of acute exercise on platelet responsiveness. The role of oxidant stress in mediating platelet responsiveness was also studied and selenium supplementation was investigated as a potential antioxidant therapy to decrease oxidant stress levels and improve platelet responsiveness.

This work has shown that LH levels is enhanced in obese individuals compared to normal-weight individuals ($P<0.01$) but no other significant differences were identified between normal-weight, overweight and obese individuals for TAS, SOD and GSH. A multiple regression analysis found the following variables to be predictors of LH levels: positive association with weight, vitamin A and fasting triglycerides and a negative association with fasting glucose. Although overweight individuals were not prone to increased oxidant stress at rest compared to normal-weight individuals, adherence to a short-term low carbohydrate diet or completion of a high-intensity aerobic exercise session, enhanced oxidant stress. For example on the low-carbohydrate diet, LH levels significantly increased at week 2, which decreased by week 4. After four weeks on the low-carbohydrate diet, TAS levels significantly increased and SOD and GSH showed a non-significant increased trend. Following 30-minutes high intensity aerobic exercise LH levels significantly increased which returned to resting levels thirty minutes post-exercise. No other changes in TAS, SOD and GSH was identified. Interestingly this exacerbated exercise induced response in overweight subjects was ameliorated by selenium supplementation. In a double-blind cross-over study, compared to placebo, short-term selenium supplementation (3-weeks) significantly increased plasma selenium levels in both the normal-weight and overweight groups. Consequently, compared to placebo, the overweight group showed a non-significant trend for reduced LH levels at rest, significant reduction following high-intensity exercise and a non-significant trend for reduced LH thirty-minutes post-exercise following selenium supplementation. The normal-weight group did not show any benefit from selenium supplementation on LH levels at rest, following high-intensity exercise and thirty-minutes post-exercise. No significant changes were found in TAS, SOD and GSH pre and post exercise following selenium supplementation in both the normal-weight and overweight groups.

Platelet responsiveness was also studied in several of the above studies to examine the role of oxidant stress in mediating platelet responsiveness. Despite showing a possible association between oxidant stress and platelet responsiveness pre- and post-high-intensity exercise in both normal-weight and overweight subjects, the nature of the association between oxidant stress and platelet aggregation still remains unresolved as no association was found between LH levels and percentage ADP-induced platelet aggregation at rest across normal-weight, overweight and obese groups. In addition although selenium supplementation ameliorated oxidant stress in the overweight group, this did not improve platelet responsiveness. Therefore oxidant stress does not appear to mediate platelet responsiveness (enhance *in vivo* platelet reactivity).

Abbreviations
LH, lipid hydroperoxide; TAS, total antioxidant status; SOD, superoxide dismutase; GSH, reduced glutathione; ADP, adenosine triphosphate.

ABBREVIATIONS

ADP Adenosine diphosphate

AGE Advanced glycosylation end products

ANOVA Analysis of variance

Apo Apoprotein

ApoE$^{-/-}$mice Mice homozygous knockout for the *APoE* gene

ATP Adenosine triphosphate

BF Body fat

BIA Bioelectrical impedance

BMI Body mass index

BP Blood pressure

BTG Beta-thromboglobulin

BTPS Body Temperature, Pressure Saturated

cAMP Cyclic adenosine monophosphate

CAT Catalase

Cl^- Chlorine

CLA Conjugated linolenic acid

ConvD Conventional diet

CRP C-reactive protein

CtrlD Control diet

Cu/ZnSOD Copper-zinc superoxide dismutase

Cu^{2+} Copper ion

CV Coefficient of variation

CVD Cardiovascular disease

DBP Diastolic blood pressure

DTNB 5, 5-Dithiobis (2-nitrobenzoic acid)

E Electron

EDRF Endothelial-derived relaxing factor

EDTA	Di-potassium ethylene diamine tetra-acetic acid
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric state
FFA	Free fatty acids
FFM	Free fat mass
FOX	Ferrous oxidation of xylenol orange
FRAP	Ferric reducing antioxidant power
GAD2	Glutamic acid decarboxylase enzyme
GK	Glycerokinase
GOD	Glucose oxidase
GP	Glycoprotein
GPO	L-α-glycerol phosphate oxidase
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
GSSG	Glutathione disulphide
GTN	Glyceryl trinitrate
H^+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HCHF	High calorie high fat
HCl	Hydrochlorous acid
HCNF	High calorie normal fat
Hct	Haematocrit
HDL	High-density lipoprotein
$HO_2^{\cdot}$	Hydroperoxyl
HOCl	Hypochlorous acid
HPLC	Performance Liquid Chromatography

HR	Heart rate
ICAM – 1	Intracellular adhesion molecule-1
IL-6	Interleukin-6
KCAL	Kilocalorie
LA	α-Lipoic acid
LDL	Low-density lipoprotein
LDLOx	Lipoprotein oxidation
LH	Lipid hydroperoxide
LowCD	Low carbohydrate diet
MDA	Malondialdehyde
Mg^{2+}	Magnesium
MnSOD	Manganese superoxide dismutase
MPO	Myeloperoxidase
MUFA (s)	Monosaturated fatty acid (s)
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NO	Nitric oxide
NO^-	Nitroxyl anion
$NO^{\cdot}$	Nitric oxide radical
$NO_2^{\cdot}$	Nitrogen dioxide radical
$O_2^{\cdot -}$	Superoxide radical
O_2	Oxygen
$OH^{\cdot}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
PAF	Platelet activating factor
PAI-1	Plasminogen activator inhibitor-1
PDEGF	Platelet-derived endothelial growth factor
PDGF	Platelet derived growth factor

PF4	Platelet factor 4
POD	Peroxidase
PON-1	Paranoxase-1
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PUFA (s)	Polyunsaturated fatty acid (s)
RAGE	Specific cell surface receptors
RMR	Resting metabolic rate
RNS	Reactive nitrogen species
$RO^{\cdot}$	Alkoxyl
$ROO^{\cdot}$	Peroxyl radical
ROS	Reactive oxygen species
RPE	Rate of perceived exertion
SBP	Systolic blood pressure
Se	Selenium
SEM	The standard error of measurement
SFA (s)	Saturated fatty acid (s)
SOD	Superoxide dismutase
STPD	Standard Temperature Pressure Dry
TAS	Total antioxidant status
TBA	Thiobarbituric acid
TBAR (S)	Thiobarbituric acid-reactive specie (s)
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TNF-α	Tumor necrosis factor alpha
TRAP	Total radical-trapping antioxidant parameter assay
TSP-1	Thrombospondin-1
VCAM-1	Vascular cell adhesion molecule

VCO_2	Carbon dioxide production
VLCLD	Very low calorie liquid diet
VLDL	Very low density lipoprotein
VO_2	Oxygen consumption
vWf	Willebrand factor
5-HT	5-hydroxytryptamine
8-iso $PGF_{2\alpha}$	F_2 isoprostrane 8-iso prostaglandin $F_{2\alpha}$
cm	Centimetre
g/dL	Grams per decilitre
IU	International Unit
IU/mg	Units per milligram
kg	Kilograms
m	metre
mg	Milligram
micrograms/ml	Micrograms per millilitre
mL	Millilitre
mM	Millimolar
mmHg	Millimetre of mercury
mmol/mL	Millimoles per millilitres
mmol/L	Millimoles per litre or millimolar
$mmol \cdot mol^{-1}$	Micromoles per mole
mol	Unit of material quantity
mg/dL	Milligrams per decilitre
n	Sample number
nmol	Nanomoles
nmol/mL	Nanomoles per millilitres
nmol/mg	Nanomoles per milligram
P	Level of significance

pg	Picogram
pg/mg	Picogram per milligram
pH	Power of hydrogen
pKa	Acid dissociation constant
pO_2	Partial pressure of oxygen
SD	Standard deviation
Sec(s)	Second (s)
SEM	Standard error of the mean
TxB_2	Tromboxane metabolite
U/g Hb	Units per gram of haemoglobin
U/mL	Units per millilitres
yr	Year
µg	Microgram
µg/dL	Micrograms per decilitre
µg/L	Micrograms per litre
µL	Microlitre
µM	Micromole
µmol	Micromoles
µmol/g Hb	Micromoles per gram of haemoglobin
µmol/L	Micromoles per litre
%	Percentage

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Chapter ONE

Introduction

1.0 Introduction

Oxidant stress is an imbalance between the formation of reactive oxygen/nitrogen species and antioxidants (Powers et al., 2004) which is capable of damaging tissues i.e. DNA, lipids and protein (Nicki,1991) which consequently has implications in biological phenomena such as cellular aging, mutagenesis, inflammation, and other pathologies (Alessio, 1994). Oxidant stress is elevated in conditions such as hypertension, hyperinsuliemia and hyperlipidaemia (Vincent and Taylor, 2006).

Obese patients (Yesilbursa *et al.*, 2005; Mohn *et al.*, 2005; Keaney *et al.*, 2003; Ozata *et al.*, 2002; Davi *et al.*, 2002; Dandona *et al.*, 2001; Prázný *et al.*, 1999) have also been shown to have elevated oxidant stress levels but it is unknown if this enhanced stress is also evident in overweight individuals. Effective strategies are being sought to combat oxidant stress, and in overweight/obese patients an obvious solution would be to lose weight. Since overweight/obese individuals often undergo repeated episodes of weight loss via short term changes to dietary intake and/or acute bouts of exercise, both these strategies will be examined independently in terms of their impact on oxidant stress levels. High fat (Slim *et al.*, 1996) and high sugar diets (Faure *et al.*, 1997) and moderate unaccustomed exercise have all been shown to pose an acute oxidant stress (Alessio *et al.*, 2000), but the independent effects of a short-term low carbohydrate diet and acute high intensity exercise on oxidant stress levels in overweight individuals is unknown. Antioxidant therapy (Ashton et al., 1999; Skrha *et al.*, 1999; Manning *et al.*, 2004) has been shown to improve oxidant stress levels but there appears to be no published data to ascertain the effects of selenium supplementation on oxidant stress levels at rest or following acute exercise. Selenium is an essential component of the GSH-Px system (Ladenstein *et al.*, 1979), which functions as part of an antioxidant system and since Olusi *et al* (2002) demonstrated reduced GSH-Px levels in obesity, Se supplementation in overweight/obese individuals

may increase GSH-Px activity (Bortoli *et al.,* 1991; Wilke *et al.,* 1992) and hence reduce oxidant stress levels.

In this thesis, the role of oxidant stress in mediating platelet responsiveness is also investigated. This potential link was examined as a mechanism contributing to an increased risk of cardiovascular disease which is observed in obese individuals. Diabetes mellitus, hypertension and hypercholesterolemia have all been associated with ROS-mediated platelet aggregation (Davi et al., 2003; Minuz et al., 2002; Davi et al., 1997).

1.1 Aims and Objectives

The principle aim of this work is to investigate the effects of BMI on oxidant stress and examine the modulating effects of both short-term dietary interventions and acute exercise and selenium supplementation on oxidant stress levels. In addition the work set out to examine the link between oxidant stress and platelet responsiveness.

The primary objectives may be outlined as follows:

Pilot Studies:

(a) To develop and evaluate PlateletWorks®, a platelet aggregation whole-blood assay

(b) To determine the reliability of the Bioelectrical Impedance Analyser. Bodystat QuadScan 4000.

Study 1

(a) To investigate the effect of BMI (normal-weight, overweight and obese) on lipid hydroperoxide, total antioxidant status, superoxide dismutase and reduced glutathione levels.

(b) To identify physiological and lifestyle factors that may contribute to the normal variability of the overall oxidant stress status.

Study 2

(a) To investigate the effect of BMI (normal-weight, overweight and obese) on ADP-induced platelet aggregation.

(b) To investigate the association between lipid hydroperoxide, total antioxidant status and ADP-induced platelet aggregation in normal-weight, overweight and obese subjects.

Study 3

(a) To investigate the effects of two commercially available diet plans for weight loss i.e. low carbohydrate and conventional diet (Slimming World) versus a control diet on lipid hydroperoxide, total antioxidant status, superoxide dismutase and reduced glutathione.

Study 4

Part 1

(a) To investigate the effects of an acute exercise session of low- and high-intensity on lipid hydroperoxide and total antioxidant status in healthy subjects.

Part 2

(a) To investigate the effect of acute high-intensity exercise on lipid hydroperoxide, total antioxidant status, superoxide dismutase and reduced glutathione and percentage ADP-induced platelet aggregation in normal-weight and overweight subjects.

(b) To investigate the association between lipid hydroperoxide and total antioxidant status and percentage ADP-induced platelet aggregation pre and post acute high-intensity exercise.

Study 5

(a) To investigate the effect of selenium supplementation on lipid hydroperoxide, total antioxidant status, superoxide dismutase, reduced glutathione and percentage ADP-induced platelet aggregation at rest and post high-intensity exercise in normal-weight and overweight subjects.

(b) To investigate the association between lipid hydroperoxide and percentage ADP-induced platelet aggregation pre and post Se supplementation at rest and post high-intensity exercise.

Chapter TWO

Review of Literature

This chapter discusses the main issues relating to this research project, which include obesity, weight management treatment (mainly dietary and exercise interventions) and their corresponding effects on oxidant stress markers, antioxidant status and platelet responsiveness. In addition, nutritional therapy is discussed to identify possible antioxidant-therapy treatments to reduce oxidant stress and improve platelet responsiveness. This literature review also highlights the association between oxidant stress and platelet responsiveness, a known marker for increased cardiovascular risk.

1.0 Obesity

Obesity is defined as a body mass index (BMI) of 30 kg/m^2 or more, where a person's BMI is defined as their weight in kg divided by the square of their height in metres (Health Survey for England, 1999). Overweight is defined as a BMI between 25 and 29.9 kg/m^2 (Health Survey for England, 1999). In 1980 just 6% of men and 8% of women were classified as obese, whereas by 2002 the proportion of the population obese had trebled to 23% of men and 25% of women (16 years to 75+ years). Approximately 55% of the adult population in the UK is overweight or obese (Health Survey for England, 2000).

Obesity has been rated as the sixth most important risk factor contributing to the overall burden of disease worldwide (Ezzati *et al.,* 2002). Obesity causes or exacerbates a large number of health problems, both independently and in association with other diseases (Kopelman, 2000). In particular it is associated with the development of type 2 diabetes mellitus, coronary heart disease, obstructive sleep apnoea, osteoarthritis of large and small joints and increased risk of certain forms of cancer (Kopelman, 2000). Obesity has also been associated with a decrease in life expectancy (Department of

Health, 2004a). The UK Government estimates that a BMI of 25.0 kg/m^2 decreases the life expectancy of English men by 2 years and given the progressive epidemic of obesity, the effect will increase to 5 years by 2050 (Department of Health, 2004a).

Obesity is accompanied by a range of physiological changes, which is largely dependent on the regional distribution of adipose tissue. Generalised obesity causes deterioration to respiratory and cardiac function whilst upper body obesity or intra-abdominal visceral deposition is a major contributor to 'syndrome X' (Lapidus *et al.*, 1994). Syndrome X refers to the clustering of abdominal obesity, hypertriglyceridaemia, reduced high-density lipoprotein (HDL) cholesterol levels, hyperinsulinemia, glucose intolerance and hypertension (Reaven, 1997). In addition to these clustering risk factors, further abnormalities have been added to the metabolic alterations namely, elevated apoprotein (apo) B concentrations and raised plasminogen activator inhibitor-1 (PAI-1) (Kopelman and Grace, 2004). The addition of these biochemical abnormalities rephrased the syndrome as the metabolic syndrome or insulin-resistance syndrome (Kopelman and Grace, 2004).

1.1 Aetiology of obesity

By definition, obesity is caused by an imbalance between energy intake and energy expenditure (Kopelman and Grace, 2004). Excessive caloric intake, or low energy expenditure, or both may explain the development of obesity if the net positive balance is prolonged (Kopelman and Grace, 2004). Although it is assumed that obesity results simply from overeating or a sedentary lifestyle, obesity is regarded as a 'complex disease' because it arises from multifaceted interactions between genetic, physiological, behavioural and environmental factors (Lindpaintner, 1995).

The genetic hypothesis underlying obesity in both animal and human models has allowed immense progress in the understanding of body weight regulation. Monogenic forms of obesity in humans have been characterized by mutations in genes affecting the central pathways of food intake regulation e.g. mutations in the leptin-hypothalamic feedback loop; for example the 'ob' gene may lead to improper coding of leptin resulting in obesity (Andersson, 1996). However, monogenic forms of obesity are rare, so human obesity is commonly considered as a complex polygenic disease involving interactions between multiple genes and the environment (Weinsier *et al.,* 1998). So far more than 600 genes, gene markers and chromosomal regions have been identified (Perusse *et al.*, 2005), which highlights obesity as a 'complex trait' (Nammi *et al.,* 2004). Candidate genes such as adrenergic beta-3 receptor (Arner, 1995), regions regulating the leptin gene (Andersson, 1996), neuromedin B (Bouchard *et al.,* 2004) and glutamic acid decarboxylase enzyme (GAD2) (Boutin and Froguel, 2005), may play a minor role in the development of obesity. Several susceptibility genes may affect energy expenditure, fuel utilization, muscle fibre characteristics and even taste preferences, which could impact on our behavioural responses to the environment. Therefore unprecedented environmental influences together with genetic susceptibility may underpin the obesity prevalence. Weinsier *et al* (1998) highlighted that genetic predisposition may permit obesity but the environment determines if individuals do become obese.

Environment is a major determinant of overweight and obesity, which is primarily related to food intake and physical activity behaviour (James, 1995). Calorie-dense and aggressive food marketing in mass media, supermarket and restaurants and the large portions of food served outside the home, promote high calorie consumption (James, 1995). The high prevalence of a sedentary lifestyle resulting from the

proliferation of labour-saving machinery is also a major environmental factor contributing to the development and maintenance of obesity (James, 1995).

From a behavioural perspective, a number of individual characteristics may place individuals at increased risk of obesity (Rennie *et al.*, 2005). Specific behavioural risk factors may promote or protect against excess weight gain in adults. A number of behavioural factors have been postulated, including diets with a high energy density, large portion sizes, eating patterns, high levels of sedentary behaviour and low levels of physical activity (Rennie *et al.*, 2005).

1.2 Complications of obesity

Obesity is associated with increased morbidity and mortality (Manson *et al.*, 1995). Clear associations have been found between obesity and the risk factors for coronary heart disease, type 2 diabetes mellitus, certain forms of cancer, gallstones, some respiratory disorders and osteo-arthritis (Kopelman, 2000). In addition to the amount of excess weight, upper body obesity (as opposed to lower body obesity) contributes significantly to the risk for metabolic and cardiovascular disease (CVD) (Pi-Sunyer, 2002). Physiologically, upper body obesity is a major contributor to the development of hypertension, elevated plasma insulin concentrations and insulin resistance, hyperglycaemia, hyperlipidaemia, and metabolic alterations (increased LDL particles, elevated apo B concentrations and raised PAI-1 (Pi-Sunyer, 2002). This condition is known as the metabolic syndrome.

1.3 Treatment of obesity

Clinical guidelines have been published in the UK on the management of obesity (National Institute for Clinical Excellence, 2006; Scottish Intercollegiate Guidelines Network, 1996; The Royal College of Physicians of London, 1998, 2003; National Institute of Health, 1998). Several treatment strategies have been identified for weight loss: dietary therapy, physical activity, behavioural therapy, combined therapy, pharmacotherapy and weight loss surgery.

General goals of treatment: are prevention of further weight gain (at the very least), weight loss and long-term maintenance of a lower weight. An initial weight loss of 10% over 6 months is generally recommended (National Institute for Clinical Excellence, 2006; Scottish Intercollegiate Guidelines Network, 1996; The Royal College of Physicians of London, 1998, 2003; National Institute of Health, 1998). After achievement of 10% weight loss, individuals can then move into a maintenance phase programme or attempt additional weight loss, through alterations to the initial weight loss plan. Treatment plans such as dietary intake and physical activity for obesity management will be discussed individually in the next section but generally the scientific evidence suggests that a combination of dietary modification and exercise is the most effective behavioural approach for weight loss (National Institute for Clinical Excellence, 2006; National Institute of Health, 1998).

2.3.1 Dietary intervention

It is common for weight loss programmes to reduce energy intake to 1000-1500 kcal/day to induce weight loss in overweight adults, which has shown to be safe and effective in individuals averaging 90kg before weight loss (American College of Sports

Medicine, 2001). Absolute energy intake is generally adjusted based on body weight to elicit an energy deficit of 500-1000 kcal/day. With this level of deficit a minimum weight loss of 0.5-0.9 kg (1-2 pounds) a week would be realistic. Alternatively, in the severely obese patient, very low calorie diets (VLCD) may be useful under medical supervision (National Institute of Clinical Excellence, 2006; Scottish Intercollegiate Guidelines Network, 1996, 1996; The Royal College of Physicians of London, 1998; National Institute of Health, 1998). VLCDs are defined as energy intake <800 kcal/day and can greatly increase the magnitude and rate of weight loss compared with more conservative reductions in energy intake (Wadden *et al.*, 1997). However due to the low energy value of these diets, they are generally used for relatively short periods of time (12-16 weeks) alongside dietary supplements (American College of Sports Medicine, 2001). Evidence suggests that VLCDs produce a 13kg weight loss over six months (Nammi *et al.*, 2004), but long-term weight loss by VLCDs is not superior to moderate dietary strategies (National Institute of Health, 1998).

Commercial programmes recommend various combinations of macronutrient compositions for weight loss including high-fat, high-protein, and high- and low-carbohydrate diets (American College of Sports Medicine, 2001). However the ideal macronutrient composition for weight management and risk reduction is still debatable, as macronutrient composition dietary treatments are often varied to meet patients' requirements and preferences (Kopleman and Grace, 2004). Furthermore scientific evidence suggests that the level of energy intake has the greatest impact on weight loss short term and that changes in the composition of the diet affects weight loss by ultimately affecting energy intake (Astrup *et al.*, 2000).

The impact of dietary fat restriction on weight loss has received much attention and has been shown to be an effective method in lowering energy density with spontaneous

weight loss (Astrup *et al.*, 2000). A 10% reduction in dietary fat may lead to approximately a 5-6 kg weight loss in the obese (Astrup *et al.*, 2000). However these diets are still often associated with poor compliance (Westerterp *et al.*, 1996) and weight regain in the long term (Toubro and Astrup, 1997). As a result, public interest in alternative diets to encourage weight loss have escalated, sparking particular interest in short term bouts of 'crash' dieting and carbohydrate restriction (Consumer reports, 2002) e.g. Atkins Diet (Atkins, 1992). The low-carbohydrate diet (LowCD) promotes rapid weight loss within a few days or weeks (Atkins, 1992). The LowCD diet contains a high proportion of protein foods, unrestricted use of fat particularly saturated fats and a severe restriction of carbohydrates (Atkins, 1992). But despite the renewed interest, there is little scientific evidence to determine the safety and efficacy of LowCDs on health (Freedman *et al.*, 2001; Blackburn *et al.*, 2001). As most energy in a LowCD is derived from protein and fat, there is considerable concern that such diets will raise lipid levels and increase risk of coronary disease (Blackburn *et al.*, 2001). Furthermore the long-term safety of the LowCD on kidney function and bone health is unknown (Astrup *et al.*, 2004). In addition to possible detrimental effects on health, Foster *et al.*, (2003) highlighted that weight loss initially achieved on the LowCD was not maintained after one year. Such findings, alongside health complications, question the efficacy of long-term LowCDs for weight loss and health improvements.

2.3.2 Physical activity

Physical activity is an important variable component of energy expenditure, representing approximately 20-50% of total energy expenditure (Saris, 1989), and is a way to induce an energy deficit (American College of Sports Medicine, 2001). When physical activity is used in the treatment of obesity, little evidence suggests that exercise produces magnitudes of weight losses that are similar to that achieved with

dietary modification (National Institute of Health, 1998). However in a review by Ross *et al* (2000a), it was shown that when energy deficit is held constant and other factors that affect energy balance are controlled, exercise can induce significant weight loss. For example Ross *et al* (2000b), found that a 700 kcal/day energy deficit produced solely through exercise with energy intake remaining constant resulted in a weight loss of 7.6kg over a 3-month period. Exercise is also important for preserving fat-free mass in exercising obese subjects, which is important because fat-free mass is the best predictor of resting metabolic rate (RMR) and that is the largest contributor to daily energy expenditure (Ravussin and Bogardus, 1992). Independent of weight loss, regular physical activity has several other physiological benefits including reduced blood pressure (BP), improved lipid profile and improved glucose tolerance (Kopelman and Grace, 2004) and improved mental and emotional status (Nammi *et al.,* 2004).

Physical activity guidelines recommend 30 minutes of moderate activity on at least five days a week (Department of Health, 2004b), which is associated with improved fitness and protection from CVD (Pate *et al.,* 1995). However Saris *et al* (2003) highlighted that a longer duration of daily activity (45-60 minutes/daily) is required to maintain lowered weight and prevent weight regain. There is also evidence that the benefits of physical activity can be obtained through brief exercise periods that do not necessarily need to be continuous. For example, three 10-minute periods of moderate intensity aerobic exercise (intensity eliciting a heart rate of 65-75% of peak heart rate attained on a treadmill) is equivalent to a single 30-minute period of exercise (DeBusk *et al.,* 1990).

2.4 Basic concepts of Free-radicals

Oberley (1988) defined free-radicals as harmful by-products of oxidative metabolism, causing molecular damage in living systems. This concept has implications in numerous biological phenomena such as cellular aging, mutagenesis, inflammation, and other pathologies (Alessio, 1994).

The term 'free-radical' can be defined as any atom or molecule that contains an unpaired electron in its outer orbit that can exist independently (Halliwell, 1994; Halliwell and Gutteridge, 1999). As a result, free-radicals can be highly reactive towards cellular targets, although this varies from radical to radical. To achieve stability, free-radicals accept or donate electrons to other molecules. The collective term for a variety of free-radicals and non-radical intermediates is reactive oxygen species (ROS) (Halliwell, 1994). Examples of oxygen (O_2) free-radicals are superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), and peroxyl ($ROO^{\cdot}$) radicals. The nitrogen free-radicals include nitric oxide ($NO^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$). Both oxygen and nitrogen free-radicals can be converted to other non-radical reactive species such as hydrogen peroxide (H_2O_2) and peroxynitrite ($OONO^{-}$).

2.5 Biochemistry of Free-radicals

Free-radicals are formed *in vivo* at rest and exercise as by-products of normal energy metabolism (Jackson, 1995) which represents the most powerful obligatory source of free-radicals in the body (Locatelli *et al.,* 2003). It has been estimated that for every twenty-five O_2 molecules reduced by normal respiration, one free-radical is formed (McCord, 1979). Therefore a rise in metabolism may increase free-radical production, which may inevitably damage surrounding tissues and organs. For example, the rate

of O_2 uptake by the body during exercise may increase by up to approximately 35-fold (Aw *et al.,* 1986) and that O_2 flux through active-whole-muscle tissue may reach 200-fold above resting values (Keul *et al.,* 1972). This paradoxical 'O_2' relationship between an apparently healthy act (exercise) and the occurrence of harmful biological reactions (Kanter, 1998) prompted Jenkins (1993) to state that:

'Elemental and gaseous oxygen presents a conudrum in that it is simultaneously essential for and potentially destructive to human life'.

In addition to the mitochondrial respiratory chain, free-radicals are also formed via activation of neutrophils (Halliwell, 1994), generated in the body in response to electromagnetic radiation from the environment and are acquired directly as oxidizing pollutants such as ozone and $NO_2^{\cdot}$ (Halliwell, 1994). However the majority of free-radicals in biological systems are oxygen-derived free-radicals. The complete reduction of O_2 to water (H_2O) requires four steps and generates further free-radical intermediates and other toxic products during the process (Figure 2.0)

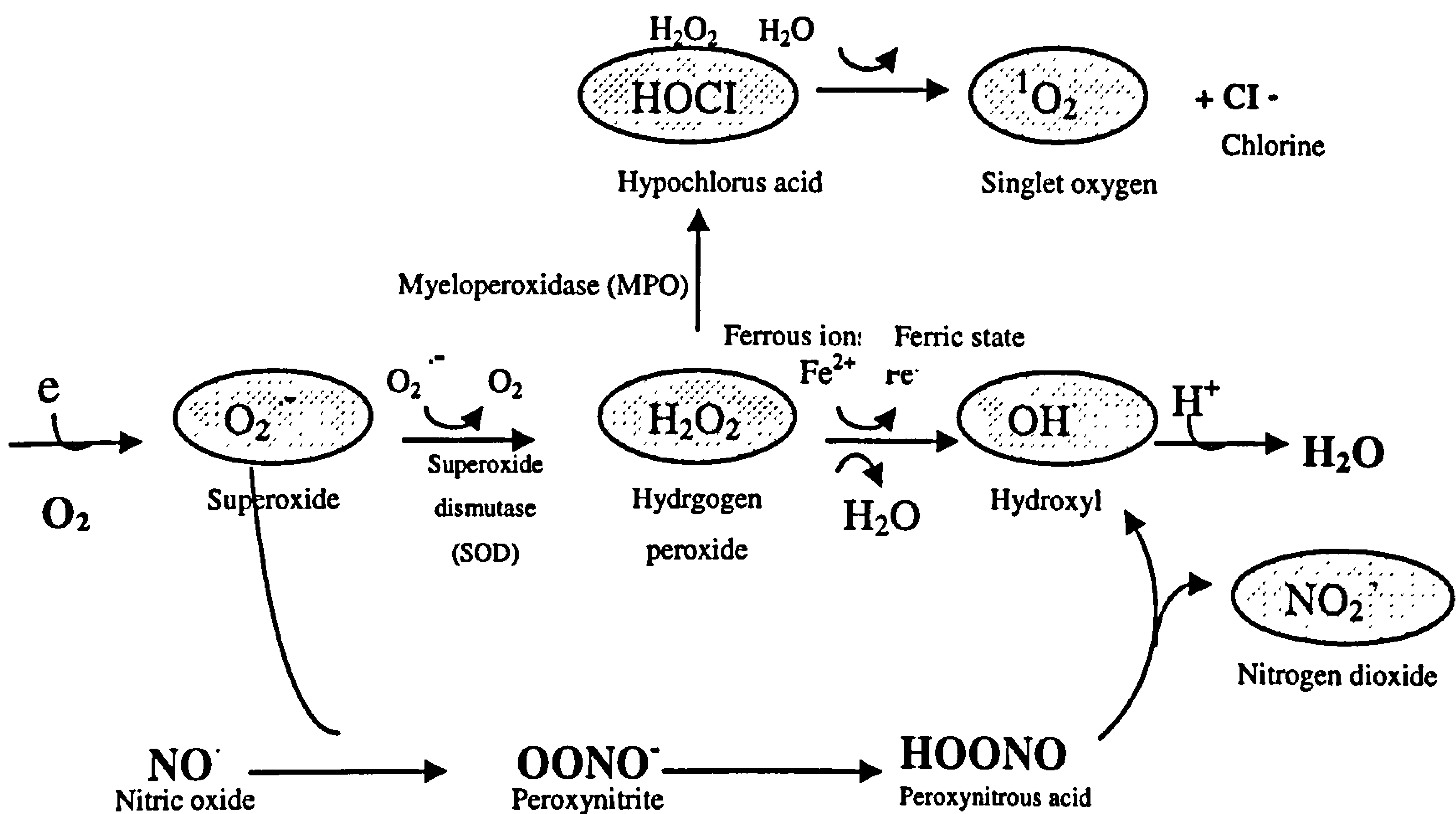


Figure 2.0. Generation of ROS

The first one-electron reduction of O_2 generates the $O_2^{\cdot-}$ anion. Addition of a second electron and two protons to $O_2^{\cdot-}$ will form H_2O_2. Both $O_2^{\cdot-}$ and H_2O_2 are precursors for the production of more powerful oxidants. $O_2^{\cdot-}$ interacts with $NO^{\cdot}$ to form highly reactive nitrogen species, while H_2O_2 reacts with intracellular iron to form $OH^{\cdot}$, that are heavily implicated in cell membrane lipid degradation, protein aggregation and DNA damage. Furthermore H_2O_2 is the substrate for myeloperoxidase (MPO) to produce the chlorinated oxidants. In the presence of chlorine (Cl^-), MPO converts H_2O_2 into hypochlorous acid (HOCl), a powerful compound capable of oxidizing a number of molecules such as lipids and other membranous or intracellular constituents, particularly the thiol groups of membrane proteins. $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$, the first three intermediary molecules in the O_2 reduction pathway will be examined in more detail in the following sections.

2.5.1 Oxygen and its derivatives

All oxygen-derived intermediates are potentially reactive, but they vary in their biological importance. The $O_2^{\cdot-}$ radical is the most important oxygen-derived free-radical because it can lead to the formation of additional ROS (Harris, 1992). Molecular O_2 is actually a di-radical, having two unpaired electrons, located in a different antibonding orbital with the same directional spin. The subsequent effect of this arrangement is that O_2 can only react with non-radicals by accepting a pair of electrons that spin in an anti-parallel manner (McCord, 1979; Young, 1994).

Free-radicals in living organisms include $O_2^{\cdot-}$, hydroperoxyl ($HO_2^{\cdot}$), $OH^{\cdot}$, $ROO^{\cdot}$, alkoxyl ($RO^{\cdot}$) and $NO^{\cdot}$. H_2O_2 and HOCl have no unpaired electrons and by definition are not free-radicals but are powerful oxidants that are involved in the free-radical reactions (Halliwell, 1989, Karlsson, 1997). H_2O_2 is predominately produced via the dismutation

of $O_2^{\cdot-}$ and is able to generate high reactive $OH^{\cdot}$ through interactions with transition metals (Clarkson and Thompson, 2000). $OH^{\cdot}$ is potentially one of the most reactive oxidants in biological systems (Clarkson and Thompson, 2000). Furthermore the $OH^{\cdot}$ readily attack polyunsaturated fatty acids (PUFAs) to initiate lipid peroxidation (Gutteridge, 1995).

2.5.2 Superoxide anion ($O_2^{\cdot-}$)

$O_2^{\cdot-}$ is a commonly known oxygen-centred free-radical species. $O_2^{\cdot-}$ is formed when a single electron enters an O_2 molecule (Pryor, 1986). $O_2^{\cdot-}$ is relatively unreactive with non-radical species in comparison to other radical types, but if it is generated near the site of any biochemical molecule it can be extremely destructive. The chemistry of $O_2^{\cdot-}$ differs greatly depending on its solution environment (aqueous solutions or organic solvents), but in general aqueous-phase reactions are more likely to occur *in vivo* (Halliwell and Gutteridge, 1999). In aqueous solutions $O_2^{\cdot-}$ can act as a base, accepting a proton to form $HO_2^{\cdot}$.

Some of the $O_2^{\cdot-}$ production that occurs *in vivo* appears to be a chemical accident, due to autooxidation reactions and the 'leakage' of electrons from electron-transport chains to O_2 (Fridovich, 1989; Imlay and Fridovich, 1991). Other $O_2^{\cdot-}$ productions appear to be made deliberately e.g. by activated phagocytes (Babior and Woodman, 1990) and to a lesser extent, by different cell types such as fibroblasts and lymphocytes (Murrell *et al.*, 1990, Maly, 1990). $O_2^{\cdot-}$ is also continuously produced by vascular endothelium to neutralise $NO^{\cdot}$ (Young and Woodside, 2001). Under normal conditions, removal of excess $O_2^{\cdot-}$ by the enzyme superoxide dismutase (SOD) is an important physiological antioxidant defence mechanism in aerobic organisms (Fridovich, 1989). However too much SOD (in relation to the activities of H_2O_2 - removing enzymes such as catalase

(CAT) and glutathione peroxidase (GSH-Px) may sometimes be deleterious (Scott *et al.,* 1989). This is because SOD enzymes convert $O_2^{\cdot-}$ into O_2 and H_2O_2 as shown in the following equation;

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

SOD has no other purpose than to dismute $O_2^{\cdot-}$ to H_2O_2 and O_2.

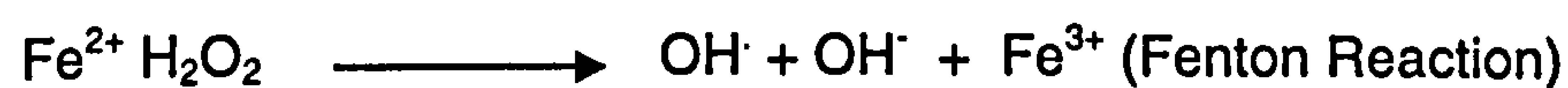
2.5.3 Hydrogen peroxide (H_2O_2)

Any biological system producing $O_2^{\cdot-}$, will produce H_2O_2, as a result of the dismutation reaction. Although SOD is the main method for production of H_2O_2 many enzymes such as irate oxidase, glucose oxidase and D-amino acid oxidase produce H_2O_2 directly by the transfer of two electrons to O_2 (Gutteridge, 1995). H_2O_2 has an uncharged covalent structure (Gutteridge, 1995), a relatively long-lived half-life (Matsuo and Kaneko, 2000), and is treated as a water molecule, which allows it to pass freely through biological membranes (Young and Woodside, 2001). H_2O_2 can therefore act as a passage to transmit free-radical induced damage across cellular compartments (Young and Woodside, 2001). H_2O_2 is a weak oxidant and a weak reducing agent that is relatively stable in the absence of transition metal ions (Gutteridge, 1995). In the presence of transition metal ions (e.g. copper and iron, fenton chemistry), H_2O_2 can lead to the production of $OH^\cdot$, which is an extremely highly reactive free-radical (Slater, 1984; Bast *et al.,* 1991). The redox properties of H_2O_2 and its ability to form highly reactive free-radicals necessitated the evolution of body defence mechanisms. For example H_2O_2 is removed from cells by the action of CAT and GSH-Px (selenium containing) (Gutteridge, 1995).

2.5.4 Hydroxyl radical ($OH^{\cdot}$)

The $OH^{\cdot}$ is probably the final intermediary product to be formed before tissue damage occurs (Lloyd *et al.,* 1997). All ROS also exert most of their pathological effects through $OH^{\cdot}$ formation (Young and Woodside, 2001). This most powerful oxidant when formed has the ability to react immediately and abstract a hydrogen atom from many biological molecules, including carbohydrates, DNA, lipids and thiols at an extremely high rate (Halliwell, 1991; Young and Woodside, 2001). The degradation of these compounds may produce damaged products and a range of secondary organic radicals of variable reactivity (peroxyl, alkoyl and alkyl radicals) (Halliwell and Gutteridge, 1999). Because the $OH^{\cdot}$ is extremely highly reactive and has a short half-life, the radical is unable to react with any molecule beyond 5 molecular diameters from its site of formation (Pryor, 1986; Matsuo and Kaneko, 2000), and therefore $OH^{\cdot}$-induced damage is site specific. The best-characterized biological damage caused by $OH^{\cdot}$ is its ability to stimulate the free-radical chain reaction known as lipid peroxidation (Halliwell, 1991). This occurs when the $OH^{\cdot}$ is generated close to membranes and attacks the fatty-acid side-chains of the membrane phospholipids e.g. PUFA side-chains such as arachidonic acid. In biological systems $OH^{\cdot}$ derives from the less toxic $O_2^{\cdot-}$ and H_2O_2 via the Haber-Weiss and Fenton reactions (Gutteridge, 1995).

$$O_2^{\cdot-} + H_2O_2 \longrightarrow OH^{\cdot} + OH^{-} + O_2 \text{ (Haber-Weiss Reaction)}$$



$$Fe^{2+}\ H_2O_2 \longrightarrow OH^{\cdot} + OH^{-} + Fe^{3+} \text{ (Fenton Reaction)}$$

2.5.5 Reactive Nitrogen Species (RNS)

Reactive Nitrogen Species (RNS) is a term to explain the process of an unpaired electron residing on a nitrogen molecule (Halliwell and Gutteridge, 1999). RNS includes $NO_2^{\cdot}$, nitroxyl anion (NO^-), $NO^{\cdot}$ and $ONOO^-$. The most discussed RNS is $NO^{\cdot}$.

$NO^{\cdot}$ is a free-radical due to the presence of a single unpaired electron (Sen *et al.*, 2000). $NO^{\cdot}$ is produced in some mammalian cells and can influence blood flow, thrombosis and neural activity (Beckman and Koppenol, 1996). $NO^{\cdot}$ has the ability to diffuse between cells and bind to $O_2^{\cdot-}$ to produce the $ONOO^-$ which is not itself a free-radical but has the potential to attack and damage cellular membranes (Sen *et al.*, 2000). This RNS has been reported to have $OH^{\cdot}$ characteristics in that it can damage most molecules in its surrounding location (Beckman and Koppenol, 1996). One-electron reduction of $NO^{\cdot}$ yields the NO^-, which is relatively unreactive and short-lived (Halliwell and Gutteridge, 1999). Given the lack of literature on RNS in health and disease, ROS will be the major species referred to in this thesis.

2.6 Antioxidant and defence system

To prevent the harmful effects of ROS, antioxidant systems, both enzymatic and non-enzymatic, are naturally present in the body to counteract free-radicals (see Table 2.0). Halliwell and Gutteridge (1999) defined antioxidants as;

'any substance which, when present at much lower concentrations than an oxidisable substrate, significantly delays or prevents oxidation of that substrate'

Antioxidants can act at several different stages in an oxidative sequence;

a) Removing O_2 or decreasing local O_2 concentrations

b) Removing catalytic metal ions

c) Removing key ROS such as $O_2^{\cdot-}$ and H_2O_2.

d) Scavenging initiating free-radicals such as $OH^{\cdot}$, $RO^{\cdot}$

e) Quenching or scavenging singlet O_2 ($^1O_2$).

(Gutteridge,1995; Halliwell and Gutteridge, 1999)

Enzymatic	**Non-enzymatic**
Superoxide dismutase (SOD)	Glutathione
Catalase (CAT)	Vitamin E & C
Glutathione peroxidase (GSH-Px)	Selenium and Ferritin
	Transferrin and Albumin
	Carotenoids

Table 2.0 Examples of Antioxidants

Specifically, enzymatic and non-enzymatic antioxidants exist in both the intracellular and extracellular environment and work as complex units to remove different ROS. To provide maximum intracellular protection, these scavengers are strategically compartmentalized throughout the cell.

2.7 Antioxidant enzymes

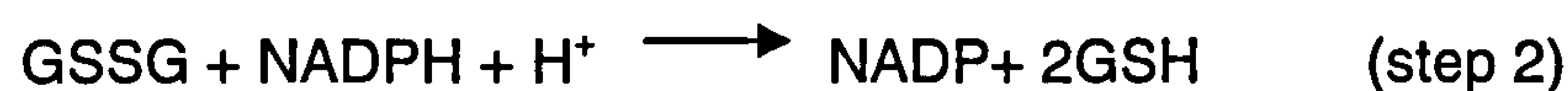
The antioxidant enzymes involved in removing ROS include SOD, CAT and GSH-Px and other peroxidases. These free-radical-scavenging enzymes are the first line of cellular defence against oxidative injury.

It consists of four protein subunits (each processing a haem group) and is found in high concentrations in the peroxisomes of liver and erythrocytes (Halliwell and Gutteridge, 1999). CAT becomes an important antioxidant enzyme when H_2O_2 levels are high (Halliwell and Gutteridge, 1999; Brown, 1993).

2.7.3 Glutathione peroxidase (GSH-Px)

GSH-Px catalyses the decomposition of H_2O_2 and organic peroxides, consuming reduced glutathione (GSH) to form oxidized glutathione, glutathione disulphide (GSSG) (step 1). GSSG is then converted back to GSH by glutathione reductase (step 2).

$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$ (step 1)



$GSSG + NADPH + H^+ \longrightarrow NADP + 2GSH$ (step 2)

GSH-Px is a low-molecular mass thiol compound found intracellularly in most mammalian cells, particularly erythrocytes (Halliwell and Gutteridge, 1999). GSH-Px is thought to be more important than CAT in H_2O_2 detoxification, particularly when it is low in concentration (Halliwell and Gutteridge, 1999; Brown, 1993). This may be because GSH-Px is located near SOD (Halliwell and Gutteridge, 1999; Brown, 1993).

2.8 Non-enzymatic antioxidants

Non-enzymatic antioxidants include, but are not limited to uric acid, albumin, GSH, vitamin E, vitamin C, carotenoids, bilrubin and ubiquinone (Powers *et al.,* 2004). These endogenous nutrients are all important sources of antioxidants obtained from the diet. The most prominent dietary antioxidants are tocopherols, the fat-soluble vitamins (Vitamin E), ascorbate, water-soluble vitamins (vitamin C) and carotenoids (Jackson,

1994). Minerals such as selenium (Se), iron, copper and zinc also possibly interact with every nutrient that affects the pro-oxidant/antioxidant balance of the cell (Jackson, 1994) These trace minerals contribute to the body's antioxidant defence system by acting as co-factors for antioxidant enzymes (Powers *et al.,* 2004). Se is an integral part of GSH-Px (Rotruck *et al.*, 1973) and also appears to support the activity of vitamin E limiting the oxidation of lipids (Burk and Levander, 1999). Copper and iron are also critical components of SOD and CAT, respectively (Jackson, 1994). Other antioxidants such as albumin and other proteins including ceruloplasmin and transferrin, also protect against oxidative injury by binding the transition metal Fe^{2+} (ferrous ion) and Cu^{2+} (copper ion) thereby preventing generation of $OH^{\cdot}$ via the Fenton reaction (Gutteridge, 1995).

2.8.1 Ascorbic acid (Vitamin C)

Vitamin C is a water- soluble vitamin that is widely distributed in mammalian tissues, but is present in relatively high amounts in the adrenal and pituitary glands (Yu, 1994). Vitamin C is a dibasic acid with an enediol group embedded in a five-membered lactone ring. The molecular structure contains two ionising hydrogen atoms that give the compound its acidic character (pKa, 4.25). In aqueous environments, vitamin C is readily oxidised to the ascorbyl radical and further to dehydroascorbic acid, oxalic acid and L-theonic acid as shown in Figure 2.1 (Elmadfa and Koenig, 1996; Tsao, 1997). The ascorbate anion is the predominant form that exists at physiological pH (Yu, 1994).

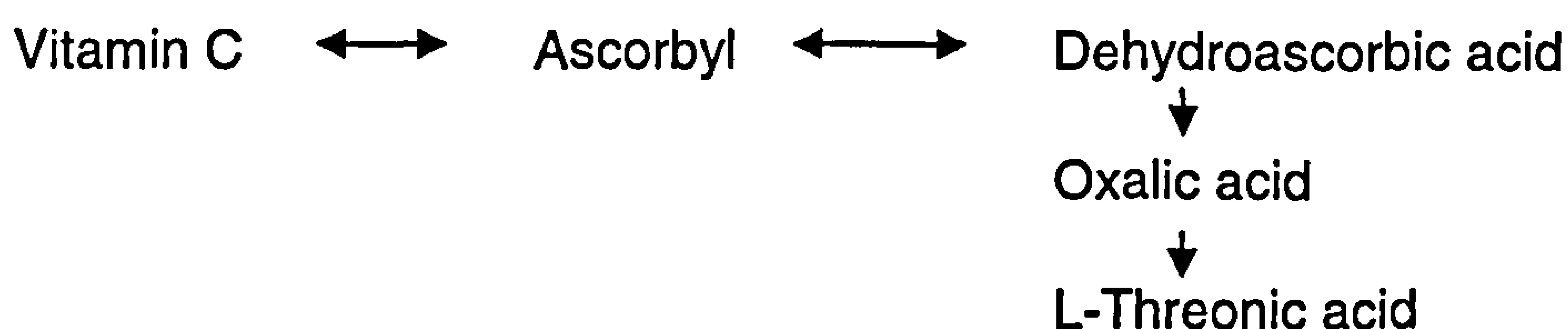


Figure 2.1. Vitamin C degradation properties

The role of vitamin C as an antioxidant is two-fold. Vitamin C can directly scavenge $O_2^{\cdot-}$, $OH^{\cdot}$ and lipid hydroperoxides (LH) (Powers *et al.,* 2004). Additionally vitamin C plays an important role in recycling vitamin E back to its reduced and active state (Packer *et al.,* 1979). In the process of recycling vitamin E, reduced vitamin C is converted to a vitamin C radical which can be regenerated back to its original form by GSH or α-lipoic acid (LA) (Sen and Packer, 2000).

In association with vitamin E, increased cellular concentrations of vitamin C should provide protection against radical-mediated injury (Yu, 1994). However in high concentrations, vitamin C can exert pro-oxidant effects in the presence of transition metals ions. Ascorbic acid has the ability to reduce Fe^{3+} (ferric state) to Fe^{2+} (Sen, 1995). Fe^{2+} is known to be a potent catalyst in the production of free-radicals (Powers *et al.,* 2004) which is important because the majority of hydroxyl radical generation comes from Fenton chemistry. Therefore mega-dose vitamin C supplementation has been questioned by some investigators due to its pro-oxidant potential (Yu, 1994).

2.8.2 α - tocopherol (Vitamin E)

Vitamin E refers to at least eight structural isomers of tocopherols, but among these vitamin E is the best known and possesses the most potent antioxidant activity (Burton and Ingold, 1989; Janero, 1991). From an antioxidant perspective, vitamin E is the primary chain-breaking antioxidant in cell membranes (Burton and Ingold, 1989; Janero, 1991).

Because of its high lipid solubility, vitamin E is associated with lipid-rich structures such as the mitochondrial, sarcoplasmic reticulum and the plasma membranes. Under most dietary conditions, the concentration of vitamin E in tissues is relatively low (e.g. ratio of

vitamin E to lipids in the membrane may range from 1:1000 in red blood cells to 1:3000 in other tissues and organelles, Janero, 1991; Packer, 1991) but continues to have the ability to react directly with most free-radicals (converts $O_2^{\cdot-}$ and $OH^{\cdot}$ to less reactive forms) before they interact with fatty acids (Janero, 1991). If oxidation occurs, vitamin E inhibits propagation by free-radical stabilisation.

Despite vitamin E being an efficient radical scavenger, the interaction of vitamin E with a radical results in a decrease in functional vitamin E and the formation of a vitamin E radical. However, the vitamin E radical can be 'recycled' back to its native state by a variety of other antioxidants (Packer *et al.*, 1979; Burton and Traber, 1990). The ability of vitamin E to serve as an antioxidant is synergistically connected to other antioxidants, such as glutathione, vitamin C and LA, which are capable of recycling vitamin E during periods of oxidant stress (Packer *et al.*, 1979).

2.8.3 Carotenoids

Carotenoids (e.g. α- and β-carotene) are lipid-soluble antioxidants located primarily in biological membranes (Strain and Benzie, 1998). They serve as precursors of vitamin A (Halliwell and Gutteridge, 1999) and it is believed that β-carotene in particular is protected by vitamin E (Strain and Benzie, 1998). Their antioxidant properties come from their structural arrangement consisting of long chains of conjugated double bonds, which permit the scavenging of several ROS, including $^1O_2$, $O_2^{\cdot-}$, and $OH^{\cdot}$ and can also trap $ROO^{\cdot}$ at low pO_2, with a potency as great as vitamin E (Burton and Ingold, 1984; Yu, 1994). Carotenoids display an efficient biological antioxidant activity as evidenced by their ability to reduce the rate of lipid peroxidation induced by radical generating systems (Krinsky and Deneke, 1982). In contrast, carotenoids can function also as a pro-oxidant. Under nonphysiological circumstances, high O_2 tensions have shown

caroteniods to lose its antioxidant capacity (Burton and Ingold, 1989; Palozza *et al.*, 1997).

2.8.4 Selenium (Se)

Se plays a critical role in antioxidant defence as a co-factor for the antioxidant enzyme GSH-Px (Rotruck *et al.*, 1973). GSH-Px is located in both the cytosol and mitochondria of cells and is responsible for removing H_2O_2 and other organic hydroperoxides from the cell (Halliwell and Gutteridge, 1999). Its antioxidant mechanism is outlined in more detail in the antioxidant enzyme section under GSH-Px (see Section 2.8.3). Se also appears to support the activity of vitamin E in limiting the oxidation of lipids (Burk and Levander, 1999). Animal studies indicate that Se and vitamin E tend to spare one another and that Se can prevent some of the damage resulting from vitamin E deficiency in a model of oxidant stress (Burk and Levander, 1999).

2.8.5 Reduced glutathione (GSH)

GSH is the most abundant non-protein thiol source in muscle cells (Meister and Anderson, 1983). GSH is primarily synthesized in the liver and transported to peripheral tissues via the circulation.

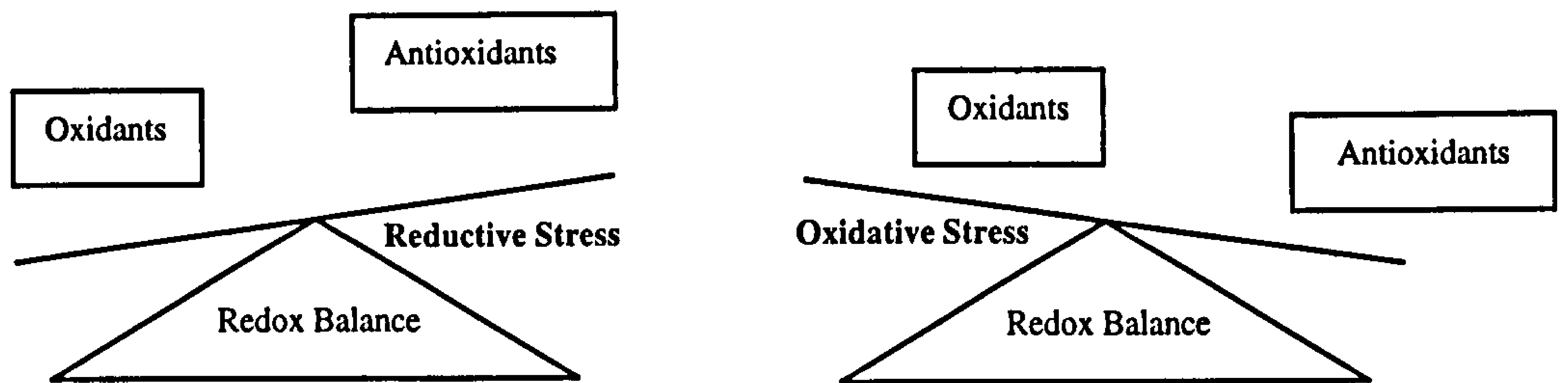
GSH concentration in the cell is variable across organs in the body with the two highest concentrations being in the lens of the eye (10 $mmol \cdot mol^{-1}$) and the liver (5-7 $mmol \cdot mol^{-1}$) (Halliwell and Gutteridge, 1999). Other key organs include lung, kidney and heart containing around 2-3 $mmol \cdot mol^{-1}$ of GSH (Ji, 1995a). Skeletal muscle GSH concentration varies depending on muscle fibre types, for example in rats slow fibres (type 1) contain 600% more GSH than fast fibres (type IIb).

GSH as a cellular antioxidant may directly scavenge a variety of free-radicals by donating a hydrogen atom (Yu, 1994) or may act as a co-substrate with GSH-Px in the elimination of both H_2O_2 and other organic peroxides (Ji *et al.*, 1992). GSH has also been shown to be involved in reducing a variety of cellular antioxidants. For example GSH has been reported to reduce vitamin E radicals that are formed in the chain-breaking reactions with alkoxyl or lipid peroxyl radicals (Packer, 1991). In addition GSH is used to reduce the semidehydroascorbate radical generated during the vitamin C-mediated recycling of vitamin E (Powers and Lennon, 1999). Finally GSH has been reported to reduce LA to dihydrolipolate, which is a powerful antioxidant acting against several radical species (Packer, 1994) and is important in recycling vitamin C (Packer, 1994).

2.9 Oxidant stress

When free-radical formation is greatly increased, or protective antioxidant mechanisms compromised, a state of oxidant stress will result (Powers *et al.*, 2004, figure 2.2). If oxidant stress persists, it will eventually lead to molecular damage and tissue injury (Symons and Gutteridge, 1998). Consequently, oxidant stress has been defined as a disturbance in the balance between production of free-radicals and antioxidant defences, which may lead to tissue injury (Halliwell, 1994). Oxidant stress has the potential to cause damage to critical cellular targets, such as DNA, proteins and lipids (Niki, 1991). The extent of the damage caused to cells depends on the duration, degree of stress and the nature of the system stressed (Halliwell and Chirico, 1993).

Redox Balance and Oxidative Stress



Adapted from Powers *et al.*, (2004)

Figure 2.2. Relationship between oxidants and antioxidants in the determination of cellular redox balance. An increase in oxidants or antioxidants results in a disturbance in cellular redox balance. Oxidant stress occurs when oxidants outnumber the available antioxidants. In contrast, reductive stress occurs when antioxidants out-number the oxidants present in the cell (Powers *et al.*, 2004)

2.10 Lipid peroxidation

Lipid peroxidation is probably the most extensively investigated free-radical-induced process (Halliwell and Gutteridge, 1999). The potential consequences of peroxidation of membrane lipids include: loss of PUFAs, loss of lipid fluidity, altered membrane permeability, effects on membrane-associated enzymes, altered iron transport, release of material from subcellular compartments and the generation of cytotoxic metabolites of LHs (Rice-Evans and Miller, 1994). PUFAs are particularly susceptible to peroxidation and once the process is initiated, it proceeds as a free-radical-mediated chain reaction involving initiation, propagation and termination (see figure 2.3) (Gutteridge, 1995).

Lipid peroxidation is initiated by the attack on a fatty acid or fatty acyl side-chain by any primary free-radical that has sufficient reactivity to abstract a hydrogen atom from a methylene group upon PUFAs (step 1). Since a hydrogen atom in principle is a free-radical with a single paired electron, its removal leaves behind an unpaired electron on the carbon atom to which it was originally attached (step 2). The carbon-centred radical is stabilised by a molecular rearrangement to form a conjugated diene (step 3) followed by reaction with O_2 to give a ROO˙ (step 4). The ROO˙ is capable of abstracting a hydrogen atom from another fatty acid side-chain to form a LH (step 5), but can combine with each other or attach membrane proteins. When the ROO˙ abstracts a hydrogen atom from a fatty acid, the new carbon-centred radical can react with O_2 to form another ROO˙, and so the propagation of the chain reaction of lipid peroxidation can continue. This propagation step, can be amplified with the availability of O_2 and PUFA side-chains (Rice-Evans and Miller, 1994). Hence a single substrate radical may result in conversion of multiple fatty acid side-chains into LHs, leading to oxidative damage in cells, membranes and lipoproteins (Rice-Evans, 1994). The length of the propagation chain before termination depends on several factors, e.g. O_2 concentration and the amount of chain-breaking antioxidants present (Young and McEneny, 2001). LHs are fairly stable molecules, but their decomposition can be stimulated by high temperatures or by exposure to transition metal ions (iron and Cu^{2+}). Decomposition of LHs generate a complex mixture of secondary lipid peroxidation products such as hydrocarbon gases (e.g. ethane and penthane) and aldehydes (e.g. malondialdehyde (MDA) and 4-hydroxynonenal). The fatty acid structure determines the product. Another complexity of the lipid peroxidation process is that the initial abstraction of a hydrogen atom can occur at different points on the carbon chain of the fatty acid. Thus, peroxidation of arachidonic acid for example gives six different LHs as well as cyclic peroxides and other products such as isoprostanes (Halliwell and Gutteridge, 1999). Furthermore, the number of double bonds determines the

susceptibility of a fatty acid to peroxidation (Wagner *et al.*, 1994; Porter *et al.*, 1995). PUFAs are readily attacked by free-radicals and become oxidised into LHs (Symons and Gutteridge,1998), whereas saturated fatty acids (SFAs) with no double bonds and monounsaturated fatty acids (MUFAs) with one double bond are more resistant to peroxidation (Reaven *et al.*, 1991). An adjacent double bond weakens the energy of attachment of the hydrogen atoms present on the next carbon atom. Therefore, the greater the number of double bonds in a fatty acid chain the easier the removal of a hydrogen atom, which is why PUFAs are more susceptible to peroxidation.

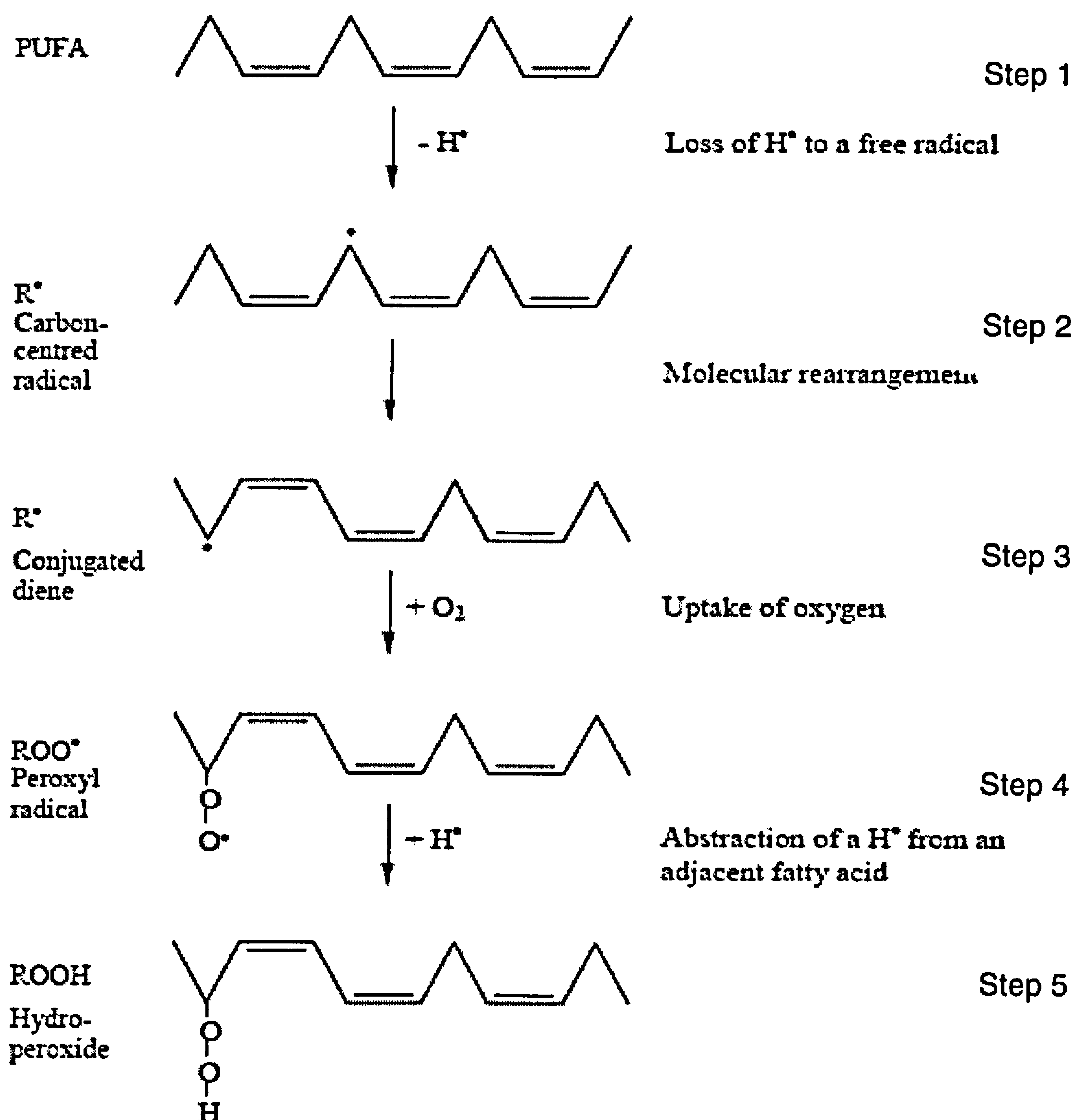


Figure 2.3. Mechanism of lipid peroxidation (modified from Gutteridge, 1995).

2.11 Oxidant Stress in obesity

There is emerging evidence suggesting that obese individuals have concomitant increased free-radical production and depletion of cellular antioxidant defence systems. It has been shown that the balance between pro-oxidant/antioxidant is shifted towards an increased oxidant stress in obese rats (Dobrian *et al.*, 2001; Beltowski *et al.*, 2000; Vincent *et al.*, 1999; Vincent *et al.*, 2001) and obese humans (Yesilbursa *et al.*, 2005; Mohn *et al.*, 2005; Keaney *et al.*, 2003; Ozata *et al.*, 2002; Davi *et al.*, 2002; Dandona *et al.*, 2001; Prázný *et al.*, 1999). Yesilbursa *et al* (2005) found significantly higher MDA levels in non-diabetic obese adults compared to healthy normal-weight controls (2.0±0.77 vs 0.63±0.14nmol/mL). Mohn *et al* (2005) studied the effects of childhood obesity on oxidant stress. In prepubertal children, obese subjects had significantly increased MDA levels compared to normal-weight controls (0.90±0.31 vs 0.45±0.24nmol/mg). Keaney *et al* (2003) found that in more than 2800 men and women, BMI was independently associated with increased levels of urinary concentrations of the F_2 isoprostrane 8-iso prostaglandin $F_{2\alpha}$ (8-iso $PGF_{2\alpha}$), a bioactive product of lipid peroxidation. For example following multivariable regression models, each 5 kg/m^2 was associated with a 9.9% increase in 8-iso $PGF_{2\alpha}$. Overall the multivariate model obtained was able to explain 15.8% of the variability in 8-iso $PGF_{2\alpha}$. The most important contributors to the model were smoking, BMI, gender, glucose, total cholesterol and HDL cholesterol ratio and prevalent CVD that contributed 7.7%, 2.4%, 2%, 2%, 0.5%, 0.6% and 0.3% respectively. Davi *et al* (2002) found that obese women had significantly higher levels of urinary 8-iso $PGF_{2\alpha}$ compared to their non-obese counterparts. In addition, android obese women had significantly higher levels of urinary 8-iso $PGF_{2\alpha}$ (523 (393-685) pg/mg of creatine) than gynoid obese women (275 (220-349) pg/mg of creatine). Both android and gynoid obese women had significantly higher levels of urinary 8-iso $PGF_{2\alpha}$ when compared to non-obese women

(187 (140-225) pg/mg of creatine). Dandona *et al* (2001) found significantly increased plasma of thiobarbituric acid-reactive species (TBARS) (an assessment of lipid peroxidation) in obese adults compared to age-matched controls (1.68±0.17 vs 1.29±0.12µmol/L). Prázný *et al* (1999) concluded that plasma MDA concentration was significantly correlated with BMI in both Type 1 and Type 2 diabetic patients (r=0.68). Ozata *et al* (2002) presented significant evidence that BMI was associated with significantly increased levels of TBARS (obese vs non-obese; 7.77±3.41 vs 3.92±0.93mmol/mL). In animals, Vincent *et al* (1999) demonstrated that in the myocardium of obese Zucker rats markers of oxidant stress were enhanced by obesity. In Wistar rats, Beltowski *et al* (2000) demonstrated increased levels of TBARS by 43% and 52% after following an 8-week high calorie high fat (HCHF) diet and a high calorie normal fat (HCNF) diet, respectively. Body weight gains were 9.3% and 15.2% higher than the control group for HCHF and HCNF diets, respectively. Following a study by Vincent *et al* (2001) it appears that obesity may be associated with enhanced oxidant stress, irrespective of underlying cause. Vincent *et al* (2001) found that obesity induced by either a leptin receptor defect or high-fat feeding was associated with similar oxidative injury levels e.g. the high-fat fed and fatty animals had similar significant elevations in myocardial TBARS in comparison to those of lean controls, 23% and 25% respectively.

Inducing weight loss has been shown to reduce oxidant stress levels providing additional evidence for a cause and effect relationship between obesity and oxidant stress (Yesilbursa *et al.,* 2005; Mohn *et al.,* 2005; Uzan *et al.,* 2004; Kisakol *et al.,* 2002; Davi *et al.,* 2002). Yesilbursa *et al* (2005) found that plasma MDA concentrations reduced significantly following 6-months of Orlistat treatment (anti-obesity drug) in obese patients. After 6-months treatment, the mean weight of the obese patients decreased by 6.8kg and plasma MDA levels were significantly reduced

by weight loss from 2.0±0.77 to 0.89±0.41nmol/mL. Mohn *et al* (2005) studied the effect of a 6 month dietary restriction weight loss programme on prepubertal severely obese children. During the intervention period, children showed a significant reduction in BMI (28.99±2.40 vs 27.34±1.87kg/m^2) which was associated with a significant reduction in MDA levels (0.86±0.43 vs 0.47±0.09nmol/mg). The 6-month change then reversed to baseline levels together with fatness indexes when children followed a further 6-month control diet.

Kisakol *et al* (2002) and Uzan *et al* (2004) both investigated the impact of bariatric surgery on oxidant stress and both concluded a positive effect. Kisakol *et al* (2004) found that a vertical banded gastropasty significantly decreased MDA levels in 22 morbidly obese patients with significant weight loss [pre-op vs 24 weeks post-op; MDA levels: 1.505±0.11 vs 0.712±0.05µmol/L and weight: 123.8±3.65 vs 88.78±2.45kg]. Uzan *et al* (2004) studied the impact of open vs laparoscopic surgery for a gastric band in obese patients. Following 6 months, both surgery types produced similar reductions in both MDA and weight which were all significant. Lastly Davi *et al* (2002) studied the effects of a 12-week calorie restriction diet on urinary 8-iso $PGF_{2\alpha}$ levels in 20 women. Of 20 women with android obesity, 11 achieved successful weight loss (mean weight loss averaged 15.3±10.5kg), which was associated with significant reductions in 8-iso $PGF_{2\alpha}$ levels by 32%. However, despite highlighting evidence for a cause and effect relationship between the obese state and oxidant stress, there is still confusion that the association between oxidant stress and obesity may be related to variables such as obesity-related diseases i.e. hypertension, hyperlipidaemia and hyperinsulinemia. Irrespective of this, several mechanisms have been suggested which may explain the obesity-related oxidant stress:

a) Increased O_2 consumption (i.e. mechanical overload) and subsequent radical production via mitochondrial respiration

b) Diminished antioxidant capacity

c) Increased fat deposition

d) Cell injury causing increased rates of radical formation such as $O_2^{\cdot}$ and $OH^{\cdot}$.

(Vincent *et al.*, 2001)

2.11.1 Mechanical overload

Increased muscle activity can activate metabolic pathways that form free-radicals, including increased electron transport chain activity (Ji, 1995a; Ji, 1996) and conversion of hypoxanthine to urate (Saiki *et al.*, 2001). Increased respiration rates and rapid electron transfer can cause some electrons to leak from the electron transport chain to form $O_2^{\cdot -}$ and H_2O_2 (Ji, 1995a; Ji, 1996) and the conversion of hypoxanthine (during high muscle activity) to urate forms $O_2^{\cdot -}$ (Saiki *et al.*, 2001). Among obese persons, high cell respiration rates and O_2 consumption may be exacerbated in muscle tissue during physical activity due to the additive mechanical load of carrying excessive body weight (Vincent *et al.*, 2004) and mechanical inefficiency (Vincent and Taylor, 2006). For example during the same absolute load-bearing walking activity, obese persons have 38% higher VO_2 (oxygen consumption) values than non-obese persons and these values are correlated with post-exercise LH values (Vincent *et al.*, 2004). Higher resting and post-exercise hypoxanthine and uric acid levels in obese compared to non-obese persons have been identified (Saiki *et al.*, 2001), suggesting that hypoxanthine may be a cause for increased oxidant stress in obese persons (Saiki *et al.*, 2001).

2.11.2 Compromised antioxidant defence

Adequate tissue, dietary, enzymatic and non-enzymatic antioxidant defences are critical to maintain the pro-oxidant/antioxidant balance in tissues (Halliwell and

Gutteridge, 1999). However in obesity, perturbations to antioxidant defences may occur (Vincent and Taylor, 2006). For example several studies suggest that obesity is associated with lower plasma antioxidant concentration (Reitman *et al.,* 2002; Strauss *et al.,* 1999; Decsi *et al.,* 1997; Kuno *et al.,* 1998; Moor De Burgos *et al.,* 1992) and decreased activities of erythrocyte cytoprotective enzymes (Olusi, 2002; Ozata *et al.,* 2002; Beltowski *et al* 2000). Reitman *et al* (2002) demonstrated that levels of fat-soluble antioxidants, plasma carotenoids and vitamin E were significantly lower in patients with severe obesity as compared to normal-weight subjects (0.69±0.32 vs 1.25±0.72micrograms/mL and 24±10 vs 33±14micrograms/mL, respectively). Strauss *et al* (1999) concluded that vitamin E and ß-carotene were significantly lower in obese children compared to their normal-weight counterparts (2.68±0.59 vs 3.17±±0.60μmol/L and 0.22±0.14 vs 0.29±0.17μmol/L, respectively). Strauss *et al* (1999) highlighted that the lower serum antioxidants in obese children remained despite similar self-reported intakes of fruit and vegetable servings between the normal-weight and obese children. Kuno *et al* (1998) and Decsi *et al* (1997) also found significantly lower levels of lipo-proteins and plasma concentrations of vitamin E and ß-carotene in obese children compared to their normal-weight counterparts [Kuno *et al* (1998); 8.77±1.93 vs 13.14±2.73nmol/mg protein and 0.30±0.23 vs 0.61±0.25nmol/mg protein, respectively, and Decsi *et al* (1997); 0.34 (0.13-0.70) vs 0.75 (0.32-1.48) mg/dL and 3.8 (1.4-21.4) vs 7.8 (1.5-38.6) μg/dL, respectively]. Furthermore Kuno *et al* (1998) found that the obese girls also had a higher 'peroxidizability index' (lipid peroxidation per amount LDL) and concluded that the inadequate antioxidants available within the large LDL lipid pool caused the oxidant stress. Similarly, in adults, Moor De Burgos *et al* (1992) found that blood retinol, vitamin E, vitamin C and carotene concentrations were 18-37% lower in obese women than in lean women respectively. Overall they speculated that the altered antioxidant vitamin levels in obesity were due to the redistribution of fat-soluble

vitamins into fatty tissues, leaving fewer antioxidants available for plasma and other essential sites (Reitman *et al.,* 2002).

Activities of the major antioxidant enzymes may also be inadequate in obesity. Olusi (2002) found that erythrocyte Cu/ZnSOD activity and GSH-Px were significantly lower in obesity than in the non-obese state (Cu/ZnSOD: 1005±26 vs 1464±23U/g Hb and GSH-Px: 84.3±6.7 vs 98.4±3.3U/g Hb). Similarly, Ozata *et al* (2002) also reported 75% and 42% lower erythrocyte GSH-Px and Cu/ZnSOD activities in obese men than in non-obese men. In rat models of diet-induced obesity, erythrocyte SOD and GSH-Px activities were reduced by 29-42% in the HFHC group compared with the control animals after the 8-week diet-induced obesity period (Beltowski *et al.,* 2000). Other research have shown that individual antioxidant enzymes were enhanced in obesity (Vincent *et al.,* 2001, Dobrian *et al.,* 2000). Vincent *et al* (2001) found that Cu/ZnSOD activity in the left ventricles of rats was significantly greater in the obese animals compared to lean controls (135 vs 117IU/mg protein). Dobrian *et al* (2000) also reported increased activities of erythrocyte Cu/ZnSOD and GSH-Px after 10 weeks of diet-induced obesity. They attributed the increases in these erythrocyte cytoprotective enzymes to their stimulation by oxidant stress. Olusi (2002) believed the discrepancies in antioxidant enzymes in obesity could be due to the duration of the obesity. For example, in the early days of the development of obesity, antioxidant enzymes may be stimulated whereas chronic obesity continually depletes antioxidant enzymes.

Other measures of antioxidant activity have been used such as total antioxidant status (TAS) and ferric reducing antioxidant power (FRAP). Several studies have shown significantly lower TAS and FRAP values in obese persons than in non-obese individuals (Lopes *et al.,* 2003, Fenkci *et al.,* 2003). For example, FRAP values were 22% lower in obese than in non-obese matched controls (271±15 vs 333±29µmol/L)

(Lopes *et al.*, 2003) and TAS values were moderately lower in obese persons compared to non-obese persons (1.15±0.01 vs 1.30±0.02mmol/L) (Fenkci *et al.*, 2003). More recently, obese children with the metabolic syndrome had significantly lower plasma TAS levels than their non-obese counterparts (1.2±0.4 vs 1.57±0.21mmol/L) (Molnar *et al.*, 2004). Furthermore, lower TAS values were directly related to lower levels of various forms of plasma carotenoids such as vitamin E, vitamin C and ß-carotene (Molnar *et al.*, 2004).

2.11.3 Increased fat deposition

Obesity is characterized by increased dietary fat intake, increased fat storage, excessive free fatty acids (FFA), excessive intracellular triglycerides and dyslipidaemia (Davi *et al.*, 2002; Vincent *et al.*, 2004), which may all contribute to the production of oxidant stress (Vincent and Taylor, 2006).

Dietary lipids influence oxidant stress by providing double bonds in fatty acid chains (Reaven *et al.*, 1991). For example, diets high in PUFAs are readily attacked by free-radicals as a result of their high number of double bonds in a fatty acid chain which makes it easier to remove the hydrogen atom (Symons and Gutteridge, 1998). However fatty diets high in SFAs (no double bonds) and MUFAs (one double bond) are more resistant to peroxidation (Reaven *et al.*, 1991). Basu *et al* (2000) studied the effect of consuming PUFAs in the diet on oxidant stress. In a randomized controlled trial obese middle-aged men were supplemented with 4.2 g day of conjugated linolenic acid (CLA) for one month. Compared to the control group significant increases in 8-iso $PGF_{2\alpha}$ were observed in the supplemented group. The lipid peroxidation parameters (8-iso $PGF_{2\alpha}$) returned to their basal levels at 2 weeks after the cessation of CLA (Basu *et al.*, 2000). Riserus *et al* (2004) studied the effects of consuming a diet added with

CLA (an 18 carbon unsaturated fatty acid with two conjugated double bonds derived from dairy products and consumption of meat from ruminant animals) for four-weeks in obese men. Compared to those who consumed the normal diet, urinary 8-iso $PGF_{2\alpha}$ significantly increased by 50% in the treatment group.

Alternatively, the increased number of lipid molecules present in obesity may simply be an enlarged target for oxidative modification of ROS (Vincent *et al.,* 2001). A comparative, experimental study investigated myocardial tissue in 16-week old lean controls (fa/?, normal diet), obese high-fat fed (Fa/?, 45% dietary fat) and obese fatty (fa/fa, normal diet) Zucker rats. Compared to lean controls, the high-fat fed and fatty animals had similar significant elevations in myocardial TBARS and PEROX (23%, 25% and 29% 45%, respectively), and elevated susceptibilities to oxidant stress *in vitro* following exposure to oxidizing agents. Resting heart work was slightly higher in both the high-fat fed and fatty animals compared to controls. Myocardial lipid content, SOD activities and non-protein thiol (glutathione) levels were significantly elevated in high-fat fed and fatty animals compared to controls. The rate of superoxide formation by isolated papillary muscles *in vitro* did not differ among groups. Regression analysis revealed that the myocardial lipid content contributed most to myocardial lipid peroxidation (R^2=0.76). This observation can be explained by increased PUFAs, promoting lipid peroxidation (Symons and Gutteridge, 1998) since myocardial oxidative injury was not closely associated with heart work, insufficient antioxidant defences or a greater rate of superoxide production (Vincent *et al.,* 2001). Furukawa *et al* (2004) reported that increased oxidant stress in accumulated fat is an important pathogenic mechanism in obesity-associated metabolic syndrome. Furukawa *et al* (2004) demonstrated that, in non-diabetic subjects, fat accumulation closely correlated with the markers of systemic oxidant stress (TBARS and hydrogen peroxide concentration). The findings were reproduced in several mouse models of obesity and production of

ROS increased selectively in adipose tissue, accompanied by augmented expression of NADPH oxidase and decreased expression of antioxidative enzymes (SOD, GSH-Px and CAT) (Furukawa *et al.*, 2004).

Abdominal or visceral adiposity is also linked with increased levels of plasma FFA (Vincent and Taylor, 2006) which elevates blood glucose and produces $NO^{\cdot}$ in smooth vascular and endothelial cells via a protein kinase C mechanism (Inoguchi *et al.*, 2000). FFA can also induce the oxidative respiratory burst in white cells and acutely increase ROS formation in culture (Inoguchi *et al.*, 2000).

Excessive triglycerides may also increase ROS formation via their impact as a mitochondrial adenine nucleotide transporter suppressor. This results in an increase in $O_2^{\cdot-}$ production within the mitochondrial electron transport chain, which in turn decreases intramitochondrial adenine diphosphate. Electrons then accumulate within the electron transport chain and react with adjacent O_2 to form $O_2^{\cdot-}$ (Bakker *et al.*, 2000).

Hypercholesteraemia is associated with enhanced oxidizability of LDL molecules (Vincent and Taylor, 2006). The lag phase of lipid oxidation is shorter in LDL particles from obese individuals and rapid lipid peroxidation occurs in the PUFA of LDL particles (Van Gaal *et al.*, 1998; Ozata *et al.*, 2002).

2.11.4 Cell Injury / low grade inflammation

Obesity is associated with a state of chronic inflammation (Saito *et al.*, 2003) and serum adipokines increase with fat mass, especially visceral fat (Vincent and Taylor, 2006). Inflammation is characterized by inflammatory cytokine expression (interleukin-

6 (IL-6) and tumor necrosis factor alpa (TNF-α), C-reactive protein (CRP) production and increased white blood cell concentration and white cell activity (Vincent and Taylor, 2006). Expansion of the adipose tissue depot in obesity may increase IL-6 and TNF-α which both activate CRP production (indication of vascular inflammation) (Kopp *et al.,* 2003). Elevations of inflammatory molecules (specifically TNF-α) promote oxidant stress by stimulating the expression of atherogenic endothelial adhesion molecules and promoting the attachment and migration of monocytes to macrophages in vessel walls (Lyon *et al.,* 2003). Macrophages themselves also produce interleukins and TNF-α (Vincent and Taylor, 2006). Furthermore increased adhesion molecules may impair the insulin signalling cascade which leads indirectly to glucose dysregulation and hyperglycaemia (Hotamisligil *et al.,* 1994), contributing further to ROS formation (Cosentino *et al.,* 1997). In summary, TNF-α, IL-6 and CRP all cause a shift towards a pro-oxidant environment in obesity (Taylor and Vincent, 2006). However weight loss in humans have shown to attenuate CRP, TNF-α and IL-6 (Kopp *et al.,* 2003).

2.11.5 Other potential sources of increased oxidant stress in obesity

Vincent and Taylor (2006) highlighted several other additional potential sources of increased oxidant stress in obesity. These include hyperglycaemia, hypertension and hyperleptinemia.

2.11.6 Hyperglycaemia

Several oxidative pathways are activated in hyperglycaemia by advanced glycosylation end products (AGE), polyol pathway and glucose autooxidation (Jiang et al., 1990). AGE formed from proteins, lipid and nucleic acids are diabetic precursors which bind to

specific cell surface receptors (RAGE) and lead to postreceptor signalling and further generation of ROS. AGE also activate intracellular transcription factors such as factor-kB which initiates a cascade of intracellular pathways (vascular cell adhesion molecule (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). Activation of these molecules can produce ROS as shown in rodent vessel tissues (Rodriguez-Manas *et al.*, 2003). In addition this accelerates monocytes homing into the endothelium (Evans *et al.*, 2002). Intracellular glucose elevations also stimulate the polyol pathway in which aldose reductase mediates conversion of glucose to sorbitol. Excess sorbitol causes oxidative damage and activates stress genes (Evans *et al.*, 2002). When glucose auto-oxidises itself, oxidants are produced which are similar to $OH^{\cdot}$ and $O_2^{\cdot-}$ (Aronson and Rayfield, 2002). Hyperglycaemia also increases nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and NADPH produces $O_2^{\cdot-}$ especially in the endothelium (Zhang *et al.*, 1999). Finally diet-induced elevations of glucose uptake into adipocytes of obese mice increase ROS formation (Talior *et al.*, 2003).

2.11.7 Hypertension

Hypertension in obesity is a major co-morbidity (Vincent and Taylor, 2006). In the endothelium, there are several enzymatic sources of oxidant generation including NADPH oxidase, xanthine oxidoreductase, NO synthase and intraluminal pressure from hypertension. NADPH oxidase provides the major source of endothelial $O_2^{\cdot-}$, which can be enhanced by the presence of other cytokines and hormones of the renin-angiotensin system (Rajagopalan *et al.*, 1996). In particular concentrations of hormones in the renin-angiotensin system are higher in obese persons (Egan *et al.*, 2001) Under ischaemic conditions, the enzyme xanthine oxidase reacts with O_2 to form $O_2^{\cdot-}$ and H_2O_2 (Kaminski *et al.*, 2002) and increased $O_2^{\cdot-}$ may react with NO to produce $ONOO^-$, which in turn reduces NO bioavailability (Wheatcroft *et al.*, 2003).

Hence these alterations are implicated in endothelial dysfunction and vascular insensitivity. Elevated intra-luminal pressure from hypertension may also stimulate the production of $O_2^{\cdot-}$ and $ONOO^-$ in the vasculature (Frisbee *et al.*, 2002). ROS inhibits calcium-activated K^+ channels and reduces vascular sensitivity as has been found in the arterial tissues of obese Zucker rats (Frisbee *et al.*, 2002). In addition, hypertension itself may increase oxidant formation and excessive renin-angiotensin system hormones may exacerbate this process. Therefore both mechanisms can enhance endothelial dysfunction in obesity.

2.11.8 Hyperleptinemia

Leptin, a polypeptide mediator produced by white adipose tissue acts on hypothalamic centres to regulate food intake and energy expenditure (Vincent and Taylor, 2006). However excessive amounts of leptin have been associated with increased CVD risk in obese persons (Maingrette and Renier, 2003). Leptin may play several roles in obesity-induced oxidant stress by increasing direct production of $OH^{\cdot}$ and H_2O_2, by being a proinflammatory substance and by reducing cellular antioxidant paranoxase-1 (PON-1) (Vincent and Taylor, 2006).

Leptin can directly stimulate production of ROS in cultured endothelial cells (Bouloumie *et al.*, 1999). Following injection with leptin, higher plasma and urine LH, MDA, isoprostane and protein carbonyl content (27-33% higher) were noted in Wistar rats compared to non-treated controls (Beltowski *et al.*, 2003). This was attributed to lower antioxidant defences such as PON-1 (Beltowski *et al.*, 2003). Leptin is a proinflammatory substance and indirectly stimulates production of inflammatory cytokines such as IL-6 and TNF-α, which may then increase NADPH oxidase which generates $O_2^{\cdot-}$. Finally leptin reduces the activity of PON-1 and this is directly related to

increased plasma MDA and LH (Beltowski *et al.*, 2003). Ferretti *et al* (2005) recently reported that PON-1 activity in HDL was lower in obese compared with non-obese individuals (120 vs 475IU/mg protein). In the obese group, low PON-1 activity was accompanied by elevations in LH content in HDL and LDL and was inversely correlated with plasma leptin concentrations (Ferretti et al., 2005).

2.12 Oxidant stress in obesity complications

Vincent and Taylor (2006) and Furukawa *et al* (2004) believe that obesity creates the oxidant conditions of a 'breeding ground' for diseases such as diabetes, hypertension and CVD. For example, oxidant stress in accumulated fat underlies the dysregulation of adipocytokines or adipokines, including PAI-1, TNF-α, resistin, leptin and adiponectin. Increased production of PAI-1 and TNF-α from accumulated fat contributes to the development of thrombosis (Shimomura *et al.*, 1996) and insulin resistance (Hotamisligil *et al.*, 1994; Uysal *et al.*, 1997) respectively, in obesity. In contrast, adiponectin exerts insulin-sensitizing (Yamauchi *et al.*, 2001) and anti-atherogenic effects (Yamauchi *et al.*, 2003) and hence a decrease in plasma adiponectin causes insulin resistance and atherosclerosis in obesity. Furukawa *et al* (2004) demonstrated that ROS increased selectively in white adipose tissue which was accompanied by augmented expression of NADPH oxidase, which is a major source of oxidant stress (Rajagopalan *et al.*, 1996).

Oxidant stress in obesity can be corrected either by improving antioxidant defences, decreasing fat volume, exercise and dietary modification, or a combination of the three. Alternatively the redox state in adipose tissue may be a potentially useful target in new therapies targeted against obesity-associated metabolic syndrome.

2.13 Lifestyle factors affecting oxidant stress

Various lifestyle, nutritional, environmental and genetic factors can induce an abnormal increase in free-radical production and/or a decrease in antioxidant defences causing a negative effect on the oxidant stress balance (Moller *et al.,* 1996). Non-smoking, vitamin and/or trace element supplementation, regular physical activity and limited UV light exposure all contribute to an enhanced antioxidant defence potential, whilst tobacco smoking, high psychologic stress, heavy alcohol drinking and low/moderate vegetable intake, low fruit and little fish consumption contribute to a decreased antioxidant potential (Lesgards *et al.,* 2002).

The following chapters consider the effect of two primary weight management strategies on oxidant stress, namely dietary composition and physical exercise.

2.13.1 Diet composition

Endogenous antioxidant defences are inadequate to scavenge ROS in the body completely so ongoing oxidative damage to DNA, lipids and proteins and other molecules may ultimately contribute to the development of disease (Halliwell, 1996). Diet-derived antioxidants may be particularly important in protecting against these diseases. However the optimal intake of the most commonly diet-derived antioxidants is uncertain, but numerous studies have examined the effects of dietary composition on oxidant stress.

Hyperlipidaemia (Aliev *et al.,* 1998), high sugar (Faure *et al.,* 1997) high protein (Mohanty *et al.,* 2002) and high-fat diets (Slim *et al.,* 1996) have all been shown to induce oxidant stress. Furthermore, the high sucrose content of a high-fat, refined carbohydrate diet may cause postprandial hyperglycaemia (Roberts *et al.,* 2000), which

has been demonstrated recently to increase the generation of $O_2^{\cdot-}$ in human endothelial cells (Cosentino *et al.,* 1997). In support of this, Roberts *et al* (2002) observed that a reduced consumption of fat and refined sugar consumption decreased oxidant stress. Velthuis-te Wierik *et al* (1996) suggested that the quantity and composition of dietary fat may affect fat-soluble anti-oxidative vitamin intake and/or anti-oxidative capacity and lipid peroxidation status. Fats in food serve as a carrier for fat-soluble vitamins. Therefore, reduced-fat foods may adversely affect the availability of fat-soluble vitamins and subsequently, anti-oxidative capacity (Velthuis-te Wierik *et al.,*1996). In addition, increased consumption of PUFAs may enhance lipid peroxidation due to the existence of two or more double bonds within their structure (Symons and Gutteridge, 1998). However both Velthuis-te Wierik *et al* (1996) and Swinburn *et al* (1999) found that the consumption of reduced-fat products did not affect the integrity of the anti-oxidative scavenging capacity, assessed by measuring plasma MDA and anti-oxidative vitamins (ß-carotene and α-tocopherol) and erythrocyte free-radical scavenging enzymes (SOD, GSH and CAT). SFAs have been shown to adversely affect plasma lipids, lipoproteins and haemostatic factors (Mitropoulos *et al.,* 1994) as well as susceptibility to oxidation (Mata *et al.,* 1996). However replacing SFAs with either MUFAs or PUFAs in diets that provide less total fat did not appreciably affect LDL oxidative susceptibility (Schwab *et al.,* 1998) but Berry *et al* (1991) and Reaven *et al* (1991) support the notion that subjects who consumed diets rich in MUFAs demonstrated lower LDL oxidation than subjects who consumed diets rich in PUFAs. However, unfortunately Wolff and Nourooz-Zadeh (1996) noted that the above research findings relating to lipid content and oxidant stress are not entirely straightforward. Although it has been shown that diets high in PUFAs and MUFAs and low in SFAs decrease atherosclerosis risk (Watts *et al.,* 1992), lipoproteins isolated from individuals consuming diets rich in PUFA and MUFA also demonstrated greater pre-disposition to peroxidation than lipoproteins from

individuals given SFAs (Kleinveld *et al.,* 1993). Individual hydroperoxide intake is expected to vary widely but will be largely dependent upon the intake of food containing hydroperoxides generated by pyrolysis such as fatty fried foods (Wolff and Nourooz-Zadeh, 1996). Hydroperoxide intake will also be influenced by the consumption of lard and compound cooking fat (such as baking and frying margarines) which contain high levels of pre-formed hydroperoxides (Wolff and Nourooz-Zadeh, 1996). Margarine has substantially lower levels of hydroperoxide but contains more hydroperoxide than butter, which may be due to the greater amount of processing required in its manufacturing (Wolff and Nourooz-Zadeh, 1996).

Both a reduced or increased protein intake may also affect the oxidant stress balance. For example, dietary deficiency of protein not only impairs the synthesis of antioxidant enzymes but also reduces tissue concentrations of antioxidants, thereby compromising antioxidant status (Sies, 1999; Machilin and Bandito, 1987). According to Fang *et al* (2002) high protein diets lead to increased oxidant stress on the basis of the following considerations. First homocysteine, an independent risk factor for cardiovascular disease, (Boushey *et al.,* 1995) increases endothelial $O_2^{\cdot-}$ production and induces oxidant stress in the vasculature (Wu and Meininger, 2002). Secondly, increasing protein intake has been shown to stimulate generation of ROS and lipid peroxidation in human polymorphonuclear leukocytes and mononuclear cells (Mohanty *et al.,* 2002). Finally, increasing dietary protein intake increases whole-body NO production by constitutive and inducible NOS in rats (Wu *et al.,* 1999).

In addition to studying the effects of short term and long term effects of dietary composition of oxidant stress parameters, it has been shown that immediately following consumption of a high fat meal or glucose load, free-radical production is enhanced (Vogel *et al.,* 1997; Dandona *et al.,* 2001). This suggests that increased ROS in

obesity maybe due to over-eating rather than dietary macro-nutrient consumption or the pathologies associated with obesity.

2.13.2 Calorie restriction

If over-eating increases ROS, under-eating may have the opposite effect. Velthuis-te Wierik *et al* (1996) demonstrated that reduced energy intake and fat intake over 6-months compared to controls contributed to a reduction in MDA levels. Ramsey *et al* (2002) observed that energy restriction, without malnutrition could retard the development of atherosclerosis, due to a reduction in O_2 molecules within the mitochondria, thereby lowering the production of ROS. This is also supported by the studies of Guo *et al* (2002) who demonstrated that calorie restriction retarded the development of atherosclerosis, reduced the levels of lipid peroxidation and decreased the production of ROS in the arterial wall of *ApoE*$^{1-}$mice (mice homozygous knockout for the *APoE* gene). As calorie restriction did not alter the level of plasma cholesterol, the results suggested that reduction of oxidant stress in the arterial wall is a possible mechanism, by which dietary restriction inhibits atherogenesis in *ApoE*$^{1-}$mice. In humans, Dandona *et al* (2001) found significant reductions in concentration of TBARS following four weeks' of energy restriction and weight loss in humans independent of changes in anti-oxidant levels. Mean weight loss was 4.5±2.8kg at week 4 and there was a reduction in plasma TBARS from 1.68±0.17µmol/L at week 0 to 1.47±0.13µmol/L at 4 weeks. It remains unclear whether energy restriction without weight loss or weight loss without energy restriction could reduce oxidant stress. This would identify the independent contributions energy restriction and weight loss has on influencing oxidant stress levels (Fenster *et al.,* 2002).

2.13.3 Acute aerobic exercise session

Acute-exercise-generated oxidant stress has been well documented (Alessio *et al.*, 2000). Whole body O_2 consumption during exercise may increase 15 fold and O_2 flux in skeletal muscle may increase 100-fold (Sen, 1995). Therefore it is conceivable that an increase in metabolism can overwhelm endogenous antioxidant defences to form free-radical species (Ashton *et al.*, 1998). Even moderate exercise may increase ROS production exceeding the capacity of antioxidant defences (Alessio, 1993; Ji, 1993). However Toshinai *et al* (1998) demonstrated that high-intensity exercise (80% VO_{2max} for 22.5 minutes) induced a larger increase in lipid peroxidation compared to moderate exercise (40% VO_{2max} for 45 minutes and 60% VO_{2max} for 30 minutes). Tozzi-Ciancarelli *et al* (2002) demonstrated that strenuous exercise (maximal exercise test on a cycle ergometer), but not moderate exercise (30 minutes at 60% VO_{2max} on a cycle ergometer), resulted in significant increased levels of TBARS and significant decreased levels of TAS [rest vs post-exercise (TBARS: 1.5±0.2 vs 1.4±0.1µmol/L and 1.6±0.2 vs 3.9±0.3µmol/L, respectively; TAS: 1.35±0.04 vs 1.47±0.05U/mL and 1.45±0.03 vs 1.05±0.03U/mL)]. Lovlin *et al* (1987) also demonstrated that higher aerobic exercise intensities caused an increase in oxidant stress. For example exercise eliciting 100% VO_{2max} resulted in a 26% significant increase in plasma MDA, exercise eliciting 70% VO_{2max} resulted in a non-significant reduction in plasma MDA and exercising at 40% VO_{2max} resulted in a 10.3% significant decrease in plasma MDA. A vast majority of exercise-induced oxidant stress has been studied in healthy individuals whilst the effect of certain pathologies on exercise-induced oxidant stress remains unclear.

To date, four studies have investigated the effect of obesity on exercise-induced oxidant stress (Saiki *et al.*, 2001; Vincent *et al.*, 2004; Vincent *et al.*, 2005a; Vincent *et*

al., 2005b). Saiki *et al* (2001) studied 7 obese and 16 normal-weight individuals and observed changes in serum hypoxanthine (marker of free radical production) following aerobic exercise on a treadmill (15 minutes at 70% heart rate reserve). Baseline serum hypoxanthine levels were significantly higher in the obese group than in the normal-weight group (3.46±3.70 vs 1.23±1.16μmol/L). Exercise also induced a pronounced increase in serum hypoxanthine level in the obese group compared with the normal-weight group (43.86±4.56 vs 10.65±6.81μmol/L) despite the obese group achieving a significantly lower peak VO_2 than in the control group (28.16±4.0 vs 37.16±4.7mL/kg/min). Vincent *et al* (2004) assessed LH levels in normal-weight and obese individuals, pre and post aerobic exercise. At rest LH levels were not significantly different between the normal-weight and obese group, but post-exercise LH levels increased by 70% and 62% in the obese and normal-weight, respectively, which was significantly different to pre-exercise LH levels and significantly different between the normal-weight and obese group. Vincent *et al* (2005a) also found significantly increased post-exercise LH levels in obese women compared to normal-weight women following a maximal aerobic exercise session (0.13 vs 0.02 (nmol/mL) (O_2/kg/min). This occurred despite 20% shorter exercise times for the obese than for the normal-weight women. After controlling for body fat percentage and baseline LH, the major contributors to the change in LH levels during exercise were age, peak heart rate and exercise duration. However when exercise loads were matched by relative intensity, LH responses still remained higher in obese than normal-weight persons (0.289 vs 0.054 (nmol/mL) (O_2/kg/min) (Vincent *et al.,* 2005b).

Several mechanisms that may contribute to increased free-radical generation during exercise include O_2 metabolism, anoxia-reoxygenation, mechanical damage to tissues and enhanced inhalation of environmental pollutants (NO_2, ozone etc) (Vincent *et al.,* 2004). The redirection of blood flow during exercise to exercising muscle (reduced

blood supply to other organs: liver, kidney and intestine) then the return of the blood flow to liver, kidney and intestine mimics ischaemia-reperfusion phenomenon. Evidence highlights that ischaemia-reperfusion causes excessive production of free-radicals, leading eventually to extensive tissue damage (McCord, 1988). Physical stress and shearing forces can also cause significant damage to the skeletal muscle. The tissue damage may attract neutrophils, leading to further generation of free-radicals (Weiss and LoBuglio, 1982). Air pollutants can also be a direct source of free-radicals such as NO or an indirect source of free-radicals, such as ozone. Strenuous exercise which leads to hyperventilation can cause increased exposure to environmental pollutant related free-radicals (Singh, 1992). Strenuous exercise is known to stimulate catecholamine secretion and the circulation, which could potentially generate free-radicals in the body through autooxidation, and/or through metal-ion or $O_2^{\cdot -}$ catalyzed oxidation (Jewett *et al.,* 1989) and neutrophil activation (Steensberg et al., 2001).

2.13.4 Exercise training

Although an acute bout of exercise induces oxidant stress (Alessio *et al.,* 2000) regular physical activity can favourably effect the pro-oxidant/antioxidant balance (Fukai *et al.,* 2000). Repeated exposures to the mild oxidant stress, which occurs with exercise training can initiate adaptations to reduce oxidative insult, such as reducing $O_2^{\cdot -}$ production or up-regulating antioxidant enzyme cells (Fukai *et al.,* 2000). Miyazaki *et al* (2001) found that 12 weeks of strenuous aerobic exercise training (running at 80% maximal exercise heart rate for 60 minutes a day, 5 days a week) in untrained normal-weight individuals increased basal activity of erythrocyte antioxidant enzymes and decreased the production of neutrophil $O_2^{\cdot -}$ production following an acute period of exhaustive exercise on a cycle ergometer. Regardless of exercise training, exhaustive

exercise caused a significant increase in the ability of the neutrophils to produce $O_2^{\cdot-}$ and increased levels of TBARS. However, the magnitude of the increase of both neutrophil $O_2^{\cdot-}$ production and TBARS was significantly reduced after training. The activities of SOD and GSH-Px increased by 17.1% and 11.5% at rest, respectively after 12 weeks of intense exercise training but were unaffected by the period of acute maximal exercise. No change in CAT activity was observed with either maximal exercise or endurance training. In summary many of the cardio-protective effects (decreased blood pressure, reduced platelet aggregation and adhesiveness, and increased coronary blood flow (Gielen *et al.,* 2001) of regular exercise can be attributed to adaptations that minimize oxidant stress (Powers *et al.,* 1999) and/or those that increase bioavailable NO (Gielen *et al.,* 2001).

In addition to weight management therapy (dietary composition, calorie restriction and exercise training) reducing oxidant stress levels in obesity, the improvements in oxidant stress levels may be enhanced with antioxidant therapy. The use of antioxidant therapy to reduce oxidant stress is discussed in the following chapters.

2.14 Antioxidant therapy

Many research studies have studied the effect of antioxidant therapy on reducing oxidant stress, following the assumption that the solution to oxidant stress was to increase antioxidant status by administering antioxidants. This hypothesis was supported by studies undertaken by Armstrong and Doll (1975) and Rimm *et al* (1996), who demonstrated that people in higher socioeconomic classes suffered lower rates of CVD compared to lower socioeconomic groups because they ate more fresh fruit and vegetables and they smoked less with the net effect of improving their antioxidant

status. The studies considered below highlight the potential antioxidant therapy can have on reducing oxidant stress in obesity.

2.14.1 Effect of antioxidant therapy on oxidant stress in obesity

Various studies have been undertaken to study the therapeutic use of antioxidants on the primary and secondary prevention of CVD and in summary they revealed an overall lack of benefit (Morris and Carson, 2003). This lack of effect may be explained by several facts such as trial populations which were generally older and/or had advanced stages of disease or had major confounding variables that negated any potential positive supplementation effect. The timing, type and dosage of antioxidant supplementation may not have been optimal for the specific population studied (Morris and Carson, 2003). Furthermore many CVD antioxidant interventions have used endpoints of clinical symptoms of disease i.e. myocardial infarction to assess the effectiveness of antioxidant therapy in reducing disease. This is not suitable for early detection of disease processes and therefore prevention. Heinecke (2001) stressed that future antioxidant supplementation studies should always include biomarkers of oxidant stress.

Although the studies are few in number, they demonstrate the potential antioxidant therapy may have for decreasing oxidant stress levels, which may in turn prevent or delay disease onset. Skrha *et al* (1999) found that vitamin E (600mg daily) administration in obese diabetic patients for 3 months decreased plasma MDA from 3.13±0.68 to 2.87±0.97µmol/L. A similar finding was also observed by Manning *et al* (2004) who found reduced LH levels in obese subjects following 6-months of vitamin E supplementation (3 months 800IU vitamin E/day, 3 months 1200IU vitamin E/day). LH was decreased by 27% at 3-months and by 29% at 6-months following vitamin E

supplementation and the decrease in LH was positively correlated with plasma vitamin E concentrations at the 6-month time point (r = 0.40). Another study also indicated protection against formation of oxidative biomarkers with antioxidant treatment, for example in overweight type II diabetic versus non diabetic contols (Anderson *et al.*, 1999). Twenty diabetics completed an 8-week control period, 8 week treatment period (β-carotene (24mg), vitamin C (1000mg) and vitamin E (800IU) followed by an 8-week control period (subjects were on a weight maintaining diet). Following the treatment period in the diabetic group, lag time for LDL oxidation (measured using copper oxidation at 37°) significantly increased (pre vs post: 27.9±6.81 vs 58.9±8.68minutes) and TBARS formation significantly decreased (pre vs post: 101.5±10.70 vs 70.6±9.75nmol/mg).

Although antioxidant therapy may be suitable therapy to reduce the obesity-associated oxidant stress, there still remains few data on the effects of other antioxidants not mentioned above on oxidant stress, such as Se.

2.15 Selenium (Se)

The essential trace mineral, Se is of fundamental importance to human health (Rayman *et al.*, 2000). As a constituent of selenoproteins it plays both structural and enzymatic roles, in the latter context it is best known as an antioxidant and catalyst for the production of active thyroid hormone (Rayman *et al.*, 2002). Se is the essential metal cofactor for the activity of the antioxidant enzyme, GSH-Px (Rotruck *et al.*, 1973). GSH-Px removes H_2O_2, lipid and phospholipid hydroperoxides and other O_2 derived species (Alissa *et al.*, 2003). If not removed, LH impairs membrane structure and function (Gutteridge and Halliwell, 1990) and causes blood clotting disturbances by decreasing the production of prostacyclin while increasing the production of

thromboxane (Rayman *et al.,* 1996). Furthermore, in the presence of transition metal ions, LH can decompose to give further reactive free-radicals and cytotoxic aldehydes (Diplock, 1994). Such secondary products may initiate more lipid peroxidation, promote atherosclerosis, damage DNA, and metabolically activate carcinogens (Diplock, 1994).

The activity of these selenoproteins depends on adequate dietary content of Se. Se enters the food chain through plants but its bioavailability is variable on a geographical level. For example, Se bioavailability is low in most parts of Europe and in areas of China, soils being a poorer source of Se (MacPherson *et al.,* 1997). Low concentration of Se is associated with Se deficiency diseases such as Keshan disease (cardiomyopathy) and Kashin-Beck disease (deforming arthritis) (Rayman, 2000). In the UK, Se intake is a cause for concern (Rayman, 2002). For example, 22 years ago Se intake in Britain was 60µg/day (Thorn *et al.,* 1978) compared to 34µg/day in 1994 as found in a survey undertaken for Britain's Ministry of Agriculture, Fisheries, and Food (Barclay *et al.,* 1995). The UK reference Se intake for males and females is 75 and 60ug/d respectively (Department of Health, 1991) which current intakes clearly do not meet.

The fall in Se intake may be explained by the drop in imports of Se rich, high protein wheat for breadmaking flour from North America (Rayman, 2002), coupled with changes in breadmaking technology (Rayman, 1997). Parallel reductions in intake have occurred in other European Union countries for similar reasons; added to which, bioavailability of Se may have fallen in areas subjected to acid rain or excessive artificial fertilisation of soils, both of which reduce plant absorption of the mineral (Rayman, 1997).

The current Se intake does not allow for maximal expression of plasma GSH-Px (Duffield *et al.,* 1999). It has been shown that maximum GSH-Px activity plateaus at serum Se levels between 90 and 100ug/l (Pearson *et al.,* 1990). Excessive doses of Se are not required to enhance GSH-Px activity, because there appears to be a level of Se, above which further increases in its availability cause no elevation in enzyme activity i.e. GSH-Px (Pearson *et al.,* 1990).

Increasing Se intake from dietary foods may be achieved by consuming meat, poultry and fish, which make the biggest contribution to Se intake (about 36% in the UK). Breads and cereal are also commonly consumed and make a substantial contribution to Se intake in northern Europe (around 22% in the UK) (UK Ministry of Agriculture Fisheries and Food, 1997). Brazil nuts, kidneys and crab are also sources of Se (Barclay *et al.,* 1995). Other solutions to increase Se intake would be to add Se to the food supply by treating crops with fertiliser containing sodium selenate (Na^2SeO_4) via the process fortification of foods or via Se supplementation (Aro *et al.,* 1998). Se consumed in foods and supplements exists in a number of organic and inorganic forms including selenomethionine (plant and animal sources and supplements), selenocysteine (mainly animal sources), selenate and selenite (mainly supplements) (Young *et al.,* 1982). Bioavailability and tissue distribution depends on the form ingested. For example, selenomethionine is more effective in increasing apparent Se status because it is non-specifically incorporated into proteins (e.g. haemoglobin and albumin) in place of methionine (Thomson *et al.,* 1993). However, it has no catalytic activity and so must be catabolised to an inorganic precursor before entering the available Se pool (Rayman *et al.,* 2000). Selenomethionine is a less-available metabolic source of Se than selenite or selenate, since these only need to be reduced to selenide to provide selenophosphate, the precursor of selenocysteine, the active form of Se in selenoproteins (Allan *et al.,* 1999). Despite this, organic forms of Se (high

Se yeast) are often preferred in interventions because they are less acutely toxic (Institute of Medicine, 2000) but they may be more toxic during long-term consumption owing to non-specific retention of Se as selenomethionine in body proteins, rather than its excretion (Rayman *et al.*, 2000).

2.15.1 Effects of selenium supplementation on oxidant stress

To date, no study has examined the effects of administering Se supplements on oxidant stress in obese individuals, but Olusi *et al* (2002) has shown that obese individuals have reduced GSH-Px activity (which may be due to reduced Se intake levels) and so may benefit from Se supplementation as a potential to reduce the obesity-associated oxidant stress. Several investigators have examined the effect of Se supplementation on oxidant stress in other population groups as discussed in the next chapter.

Bortoli *et al* (1991) studied the effects of 30-days Se supplementation (4 x 16.5μg inorganic Se and 5.0mg Vitamin E) in twenty elderly women. Following 30-days Se supplementation, plasma Se levels significantly increased from 73.2±15.9 to 95.7±21.8μg/L which decreased to 78.5±8.9μg/L, 30-days post supplementation. GSH-Px increased from 35.9±7.2 to 41.4±3.5U/g Hb, which increased further to 42.7±9.2Ul/g Hb, 30-days post Se supplementation. MDA levels showed insignificant changes during the Se supplementation period but 30-days post Se supplementation, MDA significantly decreased to a level lower than pre Se supplementation (baseline vs 30-days post-Se supplementation: 4.3±0.6 vs 3.3±0.3μmol/L). Wilke *et al* (1992) noted improvements in oxidant stress status in phenylketonuric (PKU) children (at risk of Se deficiencies) who were given a daily sodium selenite (0.13μmol Se/kg/day) supply for 6-months. Compared to controls, PKU children had significantly lower plasma and

erythrocyte Se, significantly lower plasma and erythrocyte GSH-Px and significantly higher plasma MDA. Following Se supplementation, compared to control values plasma Se and GSH-Px values normalised after 1-month, erythrocyte Se after 2-months, and erythrocyte GSH-Px and plasma MDA after 4-months. The relatively slower biochemistry correction in red cells compared to plasma may be due to red cell turnover times (Wilke *et al.,* 1992). Sarada *et al* (2002) examined the role of Se in reducing hypoxia-induced oxidant stress (at high altitudes ROS are continuously generated as a consequence of low O_2 partial pressure). In a study by Sarada *et al* (2002), twenty four male Sprague-Dawley rats were divided into four groups: control (normoxia), group II (hypoxia), group III (normoxia plus Se) and group IV (hypoxia plus Se). The rats being exposed to hypoxic stress were placed in a decompression chamber for 6-hours daily for one week. Compared to the control group, hypoxia (group II) significantly increased plasma MDA production (2.4±0.5 vs 4.0±0.4nmol/mL), significantly decreased plasma GSH (21.2±1.1 vs 11.9±1.0nmol/mL), significantly decreased blood GSH-Px, significantly decreased plasma protein and significantly decreased plasma Se content. However Se supplementation in group IV reversed the trend. Compared to group II, group IV demonstrated a significant decrease in MDA (4.0±0.4 vs 1.7±0.7nmol/mL) and subsequent significant increase in plasma GSH levels (11.9±1.0 vs 25.8±9.7nmol/mL). Similarly blood GSH-Px, plasma protein and plasma Se content all significantly increased in the Se supplemented hypoxia group (group IV) compared with hypoxia alone (group II). These results indicated that Se could decrease lipid peroxidation during hypoxia.

However, no beneficial effect of Se supplementation was found by Portal *et al* (1995) who performed a double-blind cross-over Se supplementation study on lipid peroxidation markers in cystic fibrotic children. Similar to obese patients, cystic fibrosis patients have been shown to have increased lipid peroxidation markers (Wilke *et al.,*

1990), as a result of increased production of ROS mediated by infections or a defect in antioxidant defences (Portal *et al.,* 1995). Portal *et al* (1995) assessed twenty seven cystic fibrosis children who were given 2.8µg of sodium selenite per kg per day for 5-months and 5-months with a placebo control and inversion of treatment periods. Simultaneously, 17 healthy children were also investigated as control subjects. Although Se status was similar in both the control and cystic fibrosis children, cystic fibrosis children had significantly higher lipid peroxidation markers (organic hydroperoxides) (122.6±23.3 vs 171.5±54.4µmol/L). However organic hydroperoxides were normalized at 12-months on either Se or placebo treatment. After the initial treatment, Se supplementation significantly increased both plasma Se concentration and GSH-Px- activity whilst the placebo group demonstrated a significant reduction in plasma Se concentration and no significant changes in GSH-Px activity [baseline vs first treatment: Se concentration (Se group, 0.83±0.17 vs 1.11±0.18µmol/L and placebo group, 0.78±0.14 vs 0.67±0.13µmol/L), GSH-Px concentration (Se group, 269.8±40 vs 340.4±77µmol/L and placebo group, 260±45 vs 291.4±67µmol/L)]. However compared to month 5, following the second treatment period, Se supplementation significantly increased plasma Se concentration but decreased GSH-Px activity whilst in the placebo group plasma Se concentration significantly decreased and GSH-Px activity decreased. These results indicated that improvement of lipid peroxidation markers in cystic fibrosis was not related to the Se supplementation. The decrease in organic hydroperoxide levels observed following Se treatment may be linked to the improvement of the biological indices of Se status as reported in studies by Bortoli *et al* (1991) and Wilke *et al (*1990). However this relationship is inconsistent with data observed in the placebo group since organic hydroperoxide levels normalized in spite of a reduced plasma selenium concentration. It may be possible that the variable organic hydroperoxide levels in healthy subjects was a result of seasonal variations or the interpretation of the data may have been limited a result of inferences in the organic

hydroperoxide marker, despite complying with the criteria of good analytic practice. Alternatively, the reduced organic hydroperoxide levels in the placebo group may be a placebo effect which may have been influenced by change in patient behaviour due to inclusion in the clinical protocol (Portal *et al.*, 1995).

Overall Se supplementation may have an important role as a potent antioxidant effect but its effects on oxidant stress in obesity is unknown. When considering Se requirements, the following factors should be borne in mind; the form of selenium ingested affects the response of the selenoenzymes (Brown *et al.*, 2000), the concentration of some selenoenzymes is affected more than others by scarce selenium supply owing to the hierarchy of selenoprotein expression (Behne *et al.*, 2000), there is a significant variation between individuals in the extent of the response of the selenoenzymes to Se supplementation so Se requirements between individuals in the same population may differ (Institute of Medicine, 2000) and adaptation to low Se intake can occur by sparing excretion (Thomson *et al.*, 1993).

In addition to studying the impact of Se supplementation on oxidant stress it seemed appropriate to incorporate other possible potential benefits of Se supplementation on preventing disease such as CVD. For example, oxidants may influence the process of thrombus formation by interfering at multiple steps within the 'cascade of thrombogenesis' (Ambrosio *et al.*, 1997). Of particular interest is the association between oxidants and platelet aggregation. In the following chapters the importance of haemostasis and platelet aggregation is discussed which is followed by a discussion of the possible association between obesity and platelet aggregation and ROS mediated platelet aggregation.

2.16 Haemostasis and platelets

Haemostasis protects the integrity and ensures the balance of blood components by preventing excessive blood loss through damaged vessel walls (Horne, 2005). The normal haemostatic system is extremely complex but exquisitely well regulated. Interrelationships exist between responses of the vasculature; circulating platelets, coagulation proteins and fibrinolytic mechanisms, which all serve to limit blood loss, preserve tissue perfusion and stimulate local repair processes (Troy, 1988). Following pathologic stimuli, natural inhibitors of coagulation and fibrinolysis modulate the above systems to prevent uncontrolled thrombosis or haemorrhage, although some of the constituent proteins involved in the above mechanisms also have additional roles in inflammation, angiogenesis and tissue repair (Horne, 2005). Platelets play a central role in the process of thrombus formation (Hoak, 1988), as well as playing an important role in atherogenesis (Rabbani and Loscalzo, 1994) and the progression of atherosclerotic lesions (Kamath *et al.*, 2001).

2.16.1 The platelet

Platelets are small anucleate cells derived from human bone marrow megakaryocytes (George, 2000). Each megakaryocyte releases about 4000 platelets on maturation. The normal platelet count in blood is between 150 x 10^9 and 400 x 10^9/l, the average life span being around 7-10 days (George, 2000).

Platelets have a complex internal structure of membranes. Platelets reveal an open canalicular system comprising invaginated plasma membrane, thus increasing the effective platelet surface many-fold. The membrane of phospholipids promotes the

cascade of coagulation by converting coagulation factor X to Xa and prothrombin to thrombin (George, 2000).

In the platelet interior are numerous granular bodies including dense granules, alpha-granules, lyosomes and glycogen. Dense granules are packed with 5-hydroxytryptamine (5-HT), noradrenaline, adenosine triphosphate (ATP), adenosine diphosphate (ADP) and calcium all of which have a profound effect on other platelets and vasomotor responses i.e. contraction and relaxation of the blood vessels. (George, 2000) The alpha granules contain a range of proteins, including growth factors, coagulation proteins and platelet-specific proteins that influence the adhesion of the platelets to each other and to the endothelium. These proteins include platelet-derived growth factor (PDGF), platelet-derived endothelial growth factor (PDEGF), fibrinogen, Von Willebrand factor (vWF), factor F, bironectin, beta-thromboglobulin (BTG), thrombospondin-1 (TSP-1) and the platelet factor 4 (PF4) (George, 2000).

Platelets have a complex cytoskeleton which is involved in the initial changes in platelet shape associated with platelet activation and also the movement and release of the internal granules and their contents (George, 2000). The platelet plasma membrane contains many glycoproteins which serve as receptors for agonists that initiate platelet adhesion and platelet aggregation (McEver, 1990). Specific glycoprotein (GP) receptors that react with aggregating factors, clotting factors and inhibitors include:

- GP Ia – that facilitates adhesion to collagen exposed by trauma
- GP Iib and IIIa (integrin) – attach the platelet to subendothelial vWF; GP Iib – IIIa also binds fibrinogen to promote platelet aggregation
- GP Ib – that also binds to vWF.

2.16.2 Platelet function

The main function of platelets is the formation of mechanical plugs during the normal haemostatic response to vessel wall injury, following platelet activation. The platelet surface has various receptors, which activate platelets following diverse stimuli, with equally diverse platelet responses (mediated by the binding of various stimulants to specific platelet receptors). For example, the occupation of ADP receptors by ADP leads to the transformation of specific proteins (GP IIb/IIIa complexes) into a form that binds fibrinogen (Seiss, 1989). Platelet response may be categorized by 'reversible or irreversible' platelet responses (Seiss, 1989). Reversible platelet responses include adhesion, shape change and reversible aggregation and the irreversible platelet response includes release action, and secondary reversible aggregation (Seiss, 1989). Platelet adhesion and a change in shape are the initial physiological responses leading towards the development of a haemostatic plug or thrombus (Sixma *et al.*, 1991).

Vascular injury causes brief vasoconstriction, which is then followed by platelet plug formation, then coagulation (Horne, 2005). Constriction or narrowing of the lumen of the arterioles, minimizes both the flow of blood to the wounded area and the loss of blood from the wound. Platelets then quickly adhere to the injury site because they come in contact with sub-endothelial collagen and vWF (Horne, 2005). As a result of adhesion, platelets become activated and change their characteristics by extending many projections that enable them to recruit additional platelets to the primary haemostatic plug (Roberts *et al.*, 2001). Thromboxane A_2 and ADP release play a major role in the activation of nearby platelets. The occupancy of these agonists on receptors (the agonists are released from damaged vessel walls) on the platelet plasma membrane, initiate a sequence of events, namely shape change, adhesion of the platelets to the vessel wall, the release of granule contents, activation of

neighbouring plug and a transitory local vasoconstriction (Rand *et al.*, 2003). Following primary haemostasis, the matrix of platelet and other blood components temporarily arrests the bleeding. However the plug is fragile and could be easily dislodged from the vessel wall at this stage, so platelets form links to become more stable, which is induced by a number of agents such as ADP, collagen and thrombin (Kinlough-Rathbone, 1977). The linking of platelets via fibrinogen brings about platelet aggregation. Simplistically, vWF and fibrinogen bind to receptors on one platelet and crosslink to the other platelets by binding onto receptors on the latter (McManama *et al.*, 1986). Once platelet aggregates are formed, there is a tendency for the fibrin threads to be laid on them to form a clot. The addition of fibrin to the primary haemostatic plug creates the secondary haemostatic plug (Roberts *et al.*, 2001). This process is facilitated by platelets using several mechanisms such as the platelet membrane phospholipids which potentiate the intrinsic pathway of coagulation to form thrombin from prothrombin by activated factor X (Hemker *et al.*, 1983). PF4 is a platelet release reaction which possesses fibrinogen and P-selectin and which result in platelet-leukocyte interaction leading to fibrin deposition by the leukocytes to form a thrombus (Palabrica *et al.*, 1992). The entire process is contained at the site of vessel injury by anticoagulant proteins (antithrombin and activated protein C) which prevent the reactions from spreading (Simmonds *et al.*, 1998). When the vessel injury has healed, the secondary haemostatic plug is no longer needed and is broken down and removed by additional components of the haemostatic system, known as fibrinolysis (Horne, 2005)

2.17 Haemostatic risk factors for vascular disease

The importance of the involvement of platelets in the early development of atherosclerosis has received differing degrees of support (Miller and Bruckdorfer, 2005)

but evidence for the involvement of platelet activity in the terminal stages of CVD is compelling (Miller and Bruckdorfer, 2005).

According to Tsiara *et al* (2003) and Broadley *et al* (2003), platelet aggregation and platelet activation are central processes in the pathophysiology of CVD. Platelets have been found to be engulfed within macrophages (Sevitt, 1986) and there is evidence of episodic platelet involvement during plaque development (indicated by fluorescent antibodies to platelet antigens which identify platelet emboli). Platelet activity is also increased in individuals following myocardial infarction and a low-dose aspirin for the suppression of platelet activation has been shown to be effective as a prevention for myocardial infarction and stroke (Antiplatelet Trialists' Collaboration, 1994). However Elwood *et al* (2001) found that platelet aggregation did not provide any predictive power for myocardial infarction in a cohort of 2000 men.

2.18 Platelets in obesity

Obese subjects are predisposed to CVD and furthermore independent of the degree of obesity, abdominal obesity strongly correlates with established risk factors for CVD (Coleman *et al.,* 1992). In addition to factors such as hypertriglyceridaemia, hypercholesterolaemia, low HDL cholesterol and impaired glucose tolerance, haemostatic abnormalities in obese individuals have been identified as a cause for increased CVD risk (Avellone *et al.,* 1994). For example, obesity has been associated with higher fibrinogen levels (Avellone *et al.,* 1994), increased Factor VII (Avellone *et al.,* 1994) and higher baseline tissue plasminogen activator and higher PAI-1 levels (Landin *et al.,* 1990). In addition, platelet aggregation has been shown to be enhanced in obesity (Davi *et al.,* 2002; Haszon *et al.,* 2003). For example, Davi *et al* (2002) found that obesity increased 11-dehydrothromboxane B_2 (a marker of *in vivo* platelet

activation), which was further enhanced in individuals with android obesity. It was concluded that a potential mechanism between CVD mortality and gross obesity was via enhanced thromboxane-dependent platelet activation. However in this particular study it should be noted that the authors did not directly measure platelet aggregation. Haszon *et al* (2003) concluded that obese children demonstrated increased platelet aggregation (measured using collagen as an agonist with a laser rheoaggregometer) which contributed to the development of hypertension and to the promotion of vascular damage. However Juhan *et al* (1980) found that platelet activity was not related to body weight and displayed no correlation with excess fat mass. However, platelet activity was significantly increased in cases where obesity predominated in the upper body (hyperandroid obesity). Alternative measures of platelet activity such as mean platelet volume have been shown to be higher in obese individuals (Coban *et al.,* 1992), which may also be an indicator of platelet activation (Park *et al.,* 2002).

Current literature discussed on the association between platelet activity and obesity is non-existence despite platelet activity playing an important part in the complex 'cascade of thrombogenesis'. The lack of literature may be due to the problems associated with measuring platelet activity. Provided a robust measure of platelet activity can be identified, platelet activity could be assessed in high risk populations to ascertain possible risk factors for increased platelet activity i.e. increased oxidant stress. This may potentially lead to new founding mechanisms and thus the development of future treatments to reduce the risk of CVD in high risk population groups. A possible treatment may be antioxidant therapy if a close association between oxidants and platelet aggregation is present.

2.19 Association between oxidant stress and haemostasis risk factors

At the vascular endothelial level, oxidant attack disrupts endothelial integrity, which disturbs the dynamic interaction between the endothelial cell surface and mechanisms aimed at inhibiting or promoting thrombus formation (Ambrosio *et al.*, 1997). Disrupted activities include the coagulation cascade, platelet function and fibrinolysis system. In normal circumstances the endothelial cells can inhibit thrombus via coagulation by synthesizing various substances such as thrombomodulin, tissue-factor pathway inhibitor, prostacyclin and PAI-1 or by activating fibrinolysis (Ambrosio *et al.*, 1997). Oxidants may also impair anti-thrombotic properties more directly via the breakdown of endothelial-derived relaxing factor (EDRF). Endothelial cells release EDRF in the vascular lumen, which exerts anti-platelet effects via stimulation of guanylate cyclase (Mellion *et al.*, 1981), which counteracts platelet aggregation. $O_2^{\cdot-}$ have been implicated in the breakdown of EDRF (Ambrosio *et al.*, 1997). *In vitro*, the half-life of EDRF is significantly shortened in the presence of $O_2^{\cdot-}$ and conversely, SOD has been demonstrated to prolong EDRF half-life (Rubanyi and Vanhoutte, 1986; Gryglewski *et al.*, 1986).

Unwanted intravascular thrombus formation is usually inhibited by an important component of the coagulation pathway, 'tissue factor' (TF) which is suppressed in endothelial cells and under tight control because the endothelium is in contact with circulating blood (Jaffe, 1991). TF forms a complex with coagulation factors VII and VIIa, allowing enzymatic activation of factors X and IX, the substrates for factor VIIa, ultimately leading to the generation of thrombin and the potential to trigger intravascular thrombus formation (Pawashe *et al.*, 1994). Exposure to O_2 radicals has been shown to significantly increase in TF mRNA levels, which has been indicated in coronary artery occlusion and reperfusion in rabbits (Golino *et al.*, 1996). Consequently

Ambrosio *et al* (1997) speculated that O_2 radical-mediated TF expression by endothelial cells, with its attendant activation of the extrinsic coagulation pathway, may have important consequences as it might impact on the pathophysiology of post-ischemic reperfusion. In addition to inducing TF expression in endothelial cells, ROS could promote intravascular thrombus formation by interfering with mechanisms that normally inhibit activation of the coagulation pathway (Ambrosio *et al.*, 1997). Lipid peroxides have been shown to increase thrombin production and slow down the rate of thrombin decay (Barrowcliffe *et al.*, 1975). Other key anti-thrombotic factors have been shown to be susceptible to oxidant-mediated inactivation i.e. plasminogen activator (Lawrence *et al.*, 1986), thrombodulin (Glaser *et al.*, 1992) and tissue factor pathway inhibitor (TFPI) (Golino *et al.*, 1995).

Platelets, which play a major role in thrombus formation are a prime target for oxidants produced or released in the vascular lumen and at the same time are also capable of endogenous generation of oxidants (Finazzi-agro *et al.*, 1982; Del Principe *et al.*, 1991). Previous research investigating the effects of oxidants on platelet aggregation is misleading. Some investigators have described an inhibitory effect of oxidants on platelets and others have reported that oxidants enhanced platelet aggregation (Ambrosio *et al.*, 1997). These discrepancies may be the result of differences in experimental protocols. Relationships between oxidant stress and platelet aggregation, to date, have demonstrated that H_2O_2 plays an inhibitory role when platelets are exposed (Ambrosio *et al.*, 1997). According to Ambrosio *et al* (1997) $O_2^{\cdot-}$ demonstrated a lack of effect on platelet aggregation. Other studies have shown $O_2^{\cdot-}$ as having an pro-aggregatory role, but this finding was found after platelets were washed in buffer which lacks the endogenous scavengers usually present in plasma (Ambrosio *et al.*, 1997). However this effect has been shown to be lost when platelets

are resuspended in plasma which indicates that the effects of $O_2^{\cdot-}$ on platelets may have limited pathophysiological relevance.

Several mechanisms are associated with the complex effects of oxidants on platelets. For example, adenylate cyclase and guanylate cyclase both control platelet aggregation. Oxidant exposure to plasma resuspended platelets had no effect on basal cAMP (Ambrosio *et al.*, 1994). However exposure to H_2O_2 caused an impaired aggregation alongside a 10-fold increase in platelet concentration of cGMP (Ambrosio *et al.*, 1994). Cyclooxygenase, a key enzyme of prostanoid metabolisms, is also affected by oxidants, mainly H_2O_2. For example arachidonic acid-dependent aggregation was almost completely suppressed in the presence of CAT (Ambrosio *et al.*, 1994). Therefore low level production of H_2O_2 seems necessary to promote thromboxane synthesis, and hence platelet aggregation in response to arachidonic aid stimulation (Ambrosio *et al.*, 1997). Another possible mechanism by which oxidants may influence platelet aggregation is through potentiation of the effects of platelet-activating factor (PAF). PAF is an auta-coid released by platelets and other cell types e.g. endothelium and leukocytes, which acts at low concentrations on platelets. O_2 radicals may enhance platelet aggregation through local increases in PAF concentrations secondary to reduced breakdown of PAF (Ambrosio *et al.*, 1997). For example $O_2^{\cdot-}$ administration in dogs indicated that PAF-mediated aggregation of platelets resuspended in plasma, reduced significantly whilst plasma acetylhydrolase activity was preserved (Yao *et al.*,1993). Finally, platelet aggregation is influenced by NO availability (Chen *et al.*, 1996). Impaired NO degraded by O_2 derived free-radicals have been shown to enhance platelet aggregation (Chen *et al.*, 1996).

In summary, ROS may affect thrombus formation within the vasculature through several mechanisms. Oxidants may enhance the activity of the extrinsic coagulation

cascade, leading to thrombin formation (via effects in stimulation of tissue factor activity and inhibition of fibrinolytic pathways) (Ambrosio *et al.,* 1997). In addition, oxidants may have a complex effect on platelets. For example H_2O_2 may inhibit aggregation (Ambrosio *et al.,* 1994) whilst $O_2^{\cdot-}$ may inactivate EDRF and PAF-acetylhydrolase and enhance thrombin formation via TF (Ambrosio *et al.,* 1997). However the net effects of oxidants on intravascular thrombosis is dependent on the integrity of the endothelium as well as on oxidant-mediated alterations of other major players in thrombosis, such as endothelial-derived relaxing factor and coagulation factors (Ambrosio *et al.,* 1997).

Although there is a complex relationship between oxidant stress and platelet aggregation, weight management or antioxidant therapy may have a indirect effect on reducing platelet aggregation through reducing oxidant stress, potentially reducing the risk of CVD.

Chapter THREE

General Methodology

3.0 Introduction

This chapter outlines the full details of equipment used and testing procedures used in the studies described in this thesis.

3.1 Haematological measurements

3.1.1 Blood sampling

Blood sampling was carried out by several technicians with previous experience and qualifications in the methods employed. To standardise blood handling and minimise inter-subject analytical variation, the same technicians were used for each subject. All venous blood sampling was completed after a 12 h fast because it has been shown that diet may adversely affect several blood borne metabolites, in particular plasma lipids and lipoproteins (Pronk *et al.,* 1993). Blood was obtained from a forearm ante-cubital vein (venous vein) and from finger-tip (arterialised capillary blood). The equations of Dill and Costill (1974) were used to correct and control for exercise induced plasma volume shifts.

3.1.2 Collection of venous blood

Each subject assumed a supine position and a tourniquet was fixed above the distal region of the subject's bicep (Bachorik, 1982). Venous blood samples were drawn after sterilising the forearm with a sterilised swab saturated with 70% (v/v) isopropyl alcohol (Medi Swab, Smith and Nephew, UK), using the syringe method. For platelet aggregation blood samples the tourniquet was released and blood was collected. The first 5ml of blood for platelet aggregation was discarded.

After immediate venous blood collection, blood was transferred to some or all of the following tubes (depending on the study)

- di-potassium ethylene diamine tetra-acetic acid (K_2EDTA) and mixed thoroughly.
- Serum separation tube (allowed to clot at room temperature for 30 minutes before centrifugation) and mixed thoroughly.
- Plain tubes containing sodium citrate as anti-coagulant (assessment of haemoglobin (Hb), haematocrit (Hct) and packed cell volume and platelet aggregation).
- Blood (200µl of EDTA) placed into a plain tube for assessment of GSH.

EDTA and serum separation tubes were then centrifuged at 3000rpm for 10 minutes. Plasma and serum were removed using a 1ml pipette and transferred to 1.5 ml plastic vials. Plasma and serum aliquots were stored at -70°C and -20°C before biochemical analysis.

3.1.3 Collection of arterialised capillary blood

The volunteer's finger-tip (usually index finger) was wiped clean with a sterilised swab saturated with 70% (v/v) isopropyl alcohol (Medi Swab, Smith and Nephew, UK) and then the sample site was punctured using a sterile stainless lancet (Lancet, Sheffield, UK). Subsequent blood was wiped clean with medical grade cotton wool. To obtain a blood sample, gentle pressure was applied to the site using the thumb and index finger. Arterialised capillary blood was used to measure glucose, Hct and Hb.

3.1.4 Packed cell volume (PCV)

A 75mm (59μl) volume heparinised capillary tube (Hawksley and Sons Limited, Sussex, UK) was used to collect arterialised capillary blood from the subject's finger-tip. An air bubble free sample was then sealed at the distal end with cristaseal (Hawkley and Sons Limited, Sussex, UK) and carefully inserted into a micro Hct centrifuge (Hawkley and Sons Limited, Sussex, UK) with the sealed end facing outwards. The capillary sample was immediately centrifuged at 11,800 revolutions per minute (rpm) for four minutes and the subsequent packed erythrocytes were measured using a Hawksley Micro Hct Reader (Hawksley and Sons Limited, Sussex, UK). The value expressed in $L.L^{-1}$ of whole blood was subsequently corrected by 1.5% for plasma trapped between erythrocytes (Dacie and Lewis, 1968). Packed cell volume was measured in duplicate. The intra- and interassay variation for packed cell volume using a Hawksley Micro Hct Reader are 1% and <5%, respectively.

3.1.4.1 Packed cell volume (PCV): PlateletWorks®

The PlateletWorks® machine (see Section 3.1.14.2) for assessment of platelet aggregation also provided a full blood count including a measure of Hct and Hb. The intra- and interassay variation for packed cell volume using PlateletWorks are 7% and 8%, respectively.

3.1.5 Haemoglobin (Hb)

The concentration of Hb in whole blood was measured photometrically following the method outlined by Vanzetti (1966). The procedure involves the release of

haemoglobin from haemolysed erythrocytes by sodium deoxycholate. Hb is converted to methemoglobin by sodium nitrite, which together with sodium azide forms azidemethemoglobin. The absorbance was subsequently measured at 570 and 880nm. This method has been validated against the established haemiglobincyanide (HiCN) method. Following calibration with an optical interference filter (Hb = 13.7 g.dL-1 (8.7mmol.L-1) ± 0.3g.dL-1 (0.2mmol.L-1), arterialised capillary blood was collected from the finger tip (10μl) in a microcuvette (HemoCue B-Haemoglobin, Sheffield, UK). The microcuvette was inserted into the photometer (HemoCue B-Hemoglobin, Sheffield, UK) and a digital result was obtained in approximately 25 seconds. The intra- and interassay variation for haemoglobin using HemoCue are 5.5% and 5.6%, respectively.

3.1.5.1 Haemoglobin (Hb): PlateletWorks®

The PlateletWorks® machine (Section 3.1.14.2) for assessment of platelet aggregation also provided a full blood count including a measure of Hb. The intra- and interassay variation for haemoglobin using PlateletWorks® are 7% and 8%, respectively.

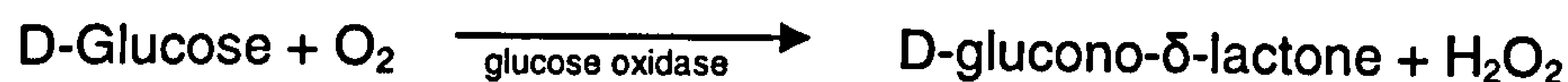
3.1.6 Glucose analysis

Glucose was measured in either of two ways, which was dependent on the location of the research study. At the exercise physiology laboratories in Bedford, glucose was measured immediately following blood collection using a YSI 2300 Stat Plus Glucose and L-Lactate analyser. At the Centre for Obesity Research, plasma was stored in the freezer for later analysis using the glucose oxidase assay method.

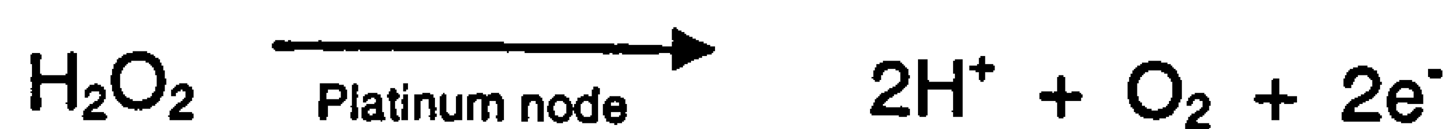
3.1.6.1 YSI 2300 Stat Plus Glucose and L-lactate analyser (YSI Incorporated)

This was a commercial analyser which measured glucose in whole blood samples (25µl). The YSI incorporates immbolized enzyme technology. Specific to glucose measurement, the enzyme glucose oxidase is immbolized between two membrane layers, polycarbonate and cellulose acetate. The glucose substrate and O_2 molecules are oxidised in the presence of glucose oxidase as it enters the membrane layers of polycarbonate, producing H_2O_2, which passes through the cellulose acetate to a platinum electrode, where the H_2O_2 is oxidized. The resulting current is proportional to the concentration of the glucose substrate. Results were expressed as mmol/L.

Glucose measurement



H_2O_2 measurement

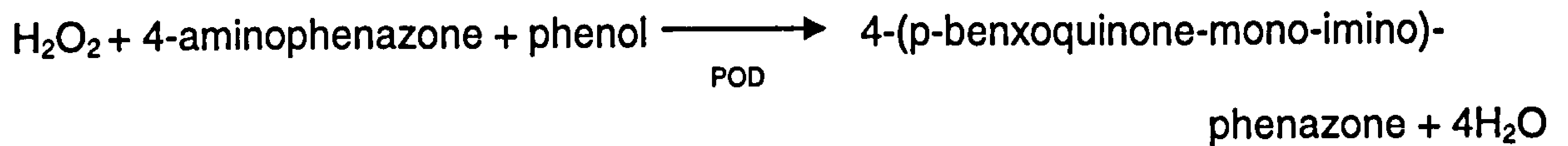


The intra- and interassay variation for glucose using YSI are $<3\%$ and 5%, respectively.

3.1.6.2 Glucose oxidase assay (enzymatic calorimetry)

Plasma glucose was measured using the glucose oxidase method described by Barham and Trinder (1972). Glucose is oxidized to gluconate by atmospheric oxygen in the presence of glucose oxidase (GOD). The indicator 4-aminophenazone and

phenol are oxidized by the H_2O_2 formed in the presence of peroxidase (POD). The test principle of the glucose oxidase method is shown below:



The intensity of the colour (red - violet quinoneimine dye) produced is proportional to the glucose concentration in the sample and colour intensity was measured at 546 nm. The glucose reagent was supplied by Randox Ltd, UK. Results were expressed as mmol/L. The intra- and interassay variation for glucose using the glucose oxidase assay are 1.8% and 2.6%, respectively.

3.1.7 Fructosamine analysis

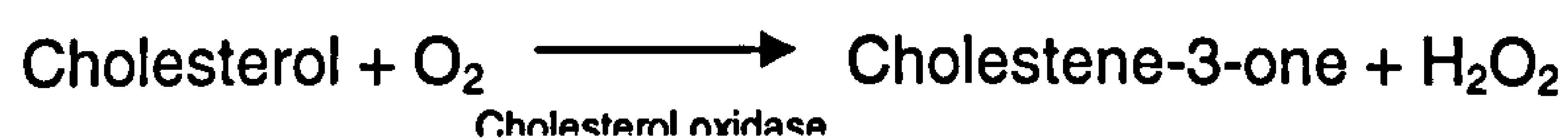
Fructosamine is a time-averaged indicator of blood glucose levels and is used to assess the glycaemic status of diabetics (Armbruster, 1987). The concentration of glycated proteins such as glycohaemoglobin, glycoalbumin or glycated total protein is generally recognized to be valuable in evaluating the glycaemic status of diabetic patients.

In the present studies serum fasting fructosamine concentration was measured according to the colorimetric assay method described by Johnson *et al* (1983). Fructosamine from the sample is present in an alkaline medium (reagent solution at pH 10.3) in the enol form, which reduces nitrotetrazolium-blue (NBT) to a formazan compound. The rate of formation of formazan is directly proportional to the

concentration of fructosamine presence in the sample. The rate of reaction was measured at 546nm. The fructosamine reagent kit was supplied by Roche Diagnostics, UK. Results are expressed as mmol/L. The intra- and interassay variation for fructosamine are 0.9% and 2.9%, respectively

3.1.8 Total cholesterol analysis

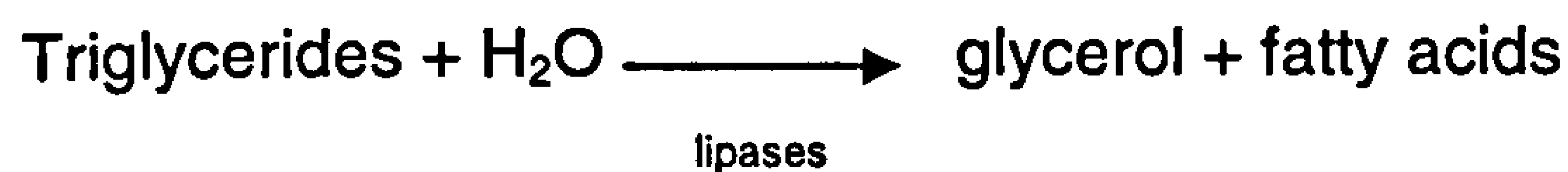
Plasma cholesterol concentrations were measured by the method of Richmond (1973), Roeschlau *et al* (1974) and Trinder (1969). Cholesterol esters were first hydrolyzed into cholesterol and fatty acids in the presence of cholesterol esterase. Free cholesterol was then oxidized into cholestenone by atmospheric O_2 in the presence of cholesterol oxidase, which also produces H_2O_2. This latter compound then oxidised an incorporated indicator 4-aminoantipyrine and phenol to quinoneimine and H_2O in the presence of POD.

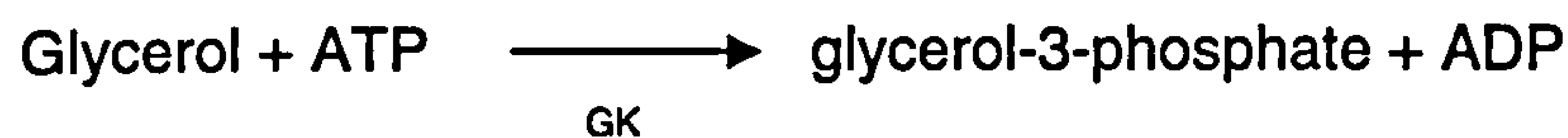


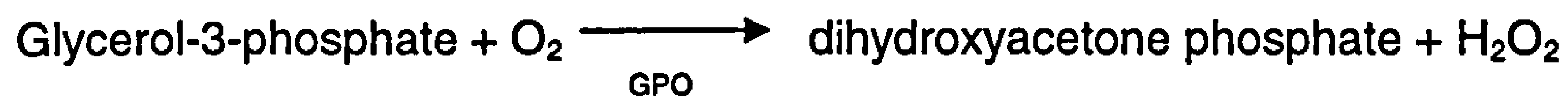
The intensity of the colour (blue coloured complex) produced was proportional to the cholesterol concentration in the sample and colour intensity was measured at 546 nm. The cholesterol reagent kit was supplied by Randox Ltd, UK and results were expressed as mmol/L. The intra- and interassay variation for cholesterol are 2.11% and 2.52%, respectively.

3.1.9 Triglycerides analysis

Plasma triglycerides were measured using the method described by Tietz (1990), Jacobs and Vandemark (1960) and Koditschek and Umbreit (1969). The triglycerides were first hydrolysed into glycerol and FFA by lipase. The glycerol was then phosphorylated into L-α-glycerol phosphate by glycerokinase (GK) and ATP in the presence of magnesium (Mg^{2+}) ions. The glycerol phosphate was then oxidised by atmospheric oxygen into hydroxyacetone phosphate and H_2O_2 in the presence of L-α-glycerol phosphate oxidase (GPO).







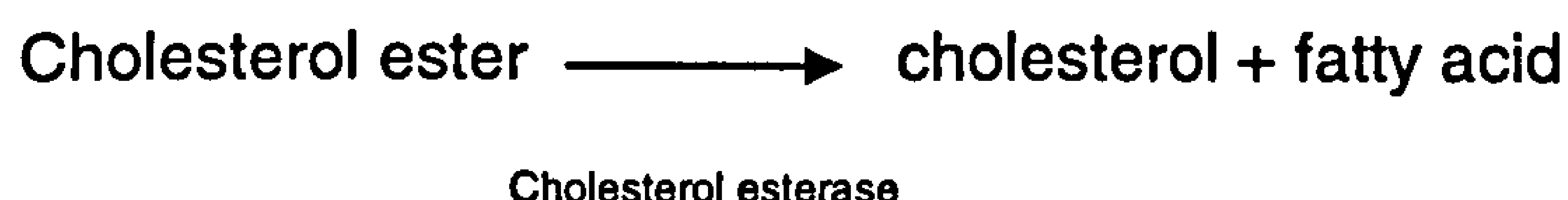
Finally, H_2O_2 oxidised an incorporated indicator 4-aminophenazone and 4 chlorophenol to quinoneimine, HCl and H_2O in the presence of POD. The blue coloured complex was measured at 500nm. The triglycerides kit was supplied by Randox Ltd, UK and results were expressed as mmol/L. The intra- and interassay variation for triglycerides are 2.32% and 3.55%, respectively.

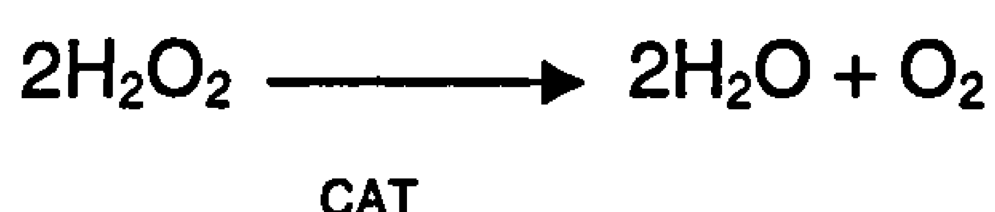
3.1.10 High density lipoprotein (HDL) cholesterol analysis

Plasma HDL concentrations were measured by the method of Izawa (1990). The assay consists of two distinct reaction steps:

1) *Elimination of chylomicron, VLDL-Cholesterol and LDL Cholesterol by cholesterol esterase, cholesterol oxidase and subsequently CAT.*

The enzyme cholesterol esterase catalyses the cleavage of cholesterol esters into cholesterol and the corresponding fatty acid. In the presence of O_2, free cholesterol is oxidized by means of cholesterol oxidase to produce cholesternone and H_2O_2. The non-HDL-derived peroxidase is then scavenged by the enzyme CAT to O_2 and H_2O.





2) *Specific measurement of HDL-Cholesterol after release of HDL-Cholesterol*

An inhibitor of CAT and a surfactant releases specifically HDL-C to produce cholestenone and H_2O_2. The H_2O_2 converts the reduced form of the indicator 4-(4-dimethylaminophenyl)-5-methyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-imidazole dihydrochoride into a blue (oxidized) form, provided POD is present. The concentration of the blue dye is proportional to the concentration of HDL in the sample and the colour

intensity was measured at 500nm. The HDL kit was supplied by Randox Ltd, UK and results were expressed as mmol/L.

HDL + CAT inhibitor ⟶ Cholestenone + H_2O_2

$2H_2O_2$ + *4-AA + *HDAOS ⟶(POD) Quinone pigment + $4H_2O$

*Key 4-AA = 4 - Aminoantipyrine

HDAOS = N – (2 – hydroxy – 3 – sulfopropyl) – 3.5 – dimethoxyaniline

The intra- and interassay variation for HDL are 2.24 and 3.4% , respectively.

3.1.11 Low density lipoprotein (LDL) cholesterol estimation

Often LDL is calculated using the empirical equation of Friedewald *et al* (1972), which incorporates primary measures of total cholesterol, triglycerides and HDL cholesterol. In this study LDL concentration of the samples were estimated by the Friedewald equation which is shown below. Results were expressed as mmol/L.

$$\text{LDL Cholesterol} = [\text{Total cholesterol}] - [\text{HDL cholesterol}] - \frac{\text{Triglycerides}}{2.2}$$

3.1.12 Lipid peroxidation measurements

Lipid peroxidation is a repetitive process whereby PUFA molecules are degraded to a variety of end products as highlighted in Section 1.11. The following technique was used to determine peroxidation of lipids in human blood.

3.1.12.1 Serum lipid hydroperoxide (LH)

Hydroperoxides in biological systems can be measured by High Performance Liquid Chromatography (HPLC) coupled with electrochemical determination, chemiluminescence, activation of cyclooxygenase and various methods that incorporate thiobarbituric acid (TBA). The TBA methods are most widely used, but are also criticised on grounds of both their ambiguity and underestimation of extent of LHs (Nourooz-Zadeh *et al.,* 1994). There are two simple and reliable spectrophotometric methods in which LHs can be determined *in vivo.* Both methods are known as the 'FOX' (Ferrous Oxidation of Xylenol orange) assays and are differentiated by the terms FOX 1 and FOX 2.

Aqueous phase LHs were measured using the methods of Wolff (1994); FOX 1 assay, which is best suited for the determination of small levels of H_2O_2 in aqueous samples. At low pH (dilute acid) hydroperoxides oxidise Fe^{2+} to Fe^{3+} and the resultant Fe^{3+} are used as an indirect measure of hydroperoxide content, which can be detected by ferric-sensitive dyes. A blue-purple coloured complex is produced with the selective binding of xylenol orange to the Fe^{3+}. The absorption could then be measured at 560nm.

$$Fe^{3+} + XO \longrightarrow \text{blue-purple complex (560nm)}$$

The LH assay method was as followed:

Serum (90µl) was incubated with 10µl CAT for 30 minutes at room temperature. To this solution 900µl FOX reagent 1 (250µmol/L ammonium ferrous sulphate, 100µmol/L xylenol orange, 100µmol/L sorbitol, 25µmol/L sulphuric acid (H_2SO_4), was added and incubated for a further 30 minutes at room temperature in the dark. Standard solutions were prepared using 0-5.0µmol/L of H_2O_2, which were then incubated for 30 minutes

with the FOX 1 reagent after which the sample was centrifuged in a Beckman microfuge for 5 minutes to remove any flocculated material. The absorbance of the supernatant was read spectrophotometrically (U-2001, Hitachi, England) at 560nm against the standard curve that was linear over the range 0-5μmol/L. Plasma samples were spiked with the enzyme CAT (Sigma, Dorset) to discriminate between authentic hydroperoxides reacting with Fe^{2+} and H_2O_2 in the sample. All chemicals were purchased from Sigma-Aldrich (Poole, Dorest) unless otherwise stated.

This assay may yield higher LH values compared to other methods because of the presence of iron ions in the assay (Young and McEneny, 2001). The FOX 1 assay was used in preference to the FOX 2 assay, following personal communication with Dr Jane McEneny, (Queen's Belfast University). The FOX 1 assay had been found to be very reproducible with intra- and interassay co-variances of less than 5%, whilst the FOX 2 assay was less reliable. In this thesis, the intra- and interassay variation for LH are 2% and 4%, respectively.

3.1.13 Determination of antioxidant capacity (non enzymatic and enzymatic)

This section describes the various procedures and techniques employed to determine various antioxidant activity in venous blood.

3.1.13.1 Total Antioxidant Status (TAS)

It is impractical to measure all the potentially biologically active antioxidants in human samples, but because there is evidence that antioxidants can work simultaneously (Halliwell and Gutteridge, 1999), the concept of a 'global' assessment of antioxidant

capacity has proved attractive for use in clinical research. Several methods have been developed, which may take one of two main approaches; either the quenching or delayed production of a stable, measurable radical species, or the reductant properties of the antioxidants against a radical cation or a metal ion (Schlesier *et al.,* 2002). An example of the former is the TRAP assay (Total radical-trapping antioxidant parameter assay) and the latter the FRAP assay (Ferric reducing/antioxidant power assay) (Benzie and Strain, 1996). Because these methods use different reaction principles, the same antioxidant can produce different contributions to each assay. Vitamins that can contribute to TAS are ascorbate, α-tocopherol, and β-carotene. One major drawback of TAS methods is the variable contribution of common plasma constituents, particularly albumin and urate. Changes in circulation of these molecules due to changes in renal function can alter values without reflecting changes in antioxidant concentration. However this problem may be overcome by the use of the 'antioxidant gap', a derived value, which subtracts the trolox-equivalence of albumin and urate from the measured TAS (Miller *et al.,* 1997). Although measures of TAS are unable to detect changes in individual antioxidants, they can be used as a tool to assess redox status and may be useful in providing information on the absorption and bioavailability of the nutritional complex (Ghiselli *et al.,* 2000).

In the present studies, the FRAP method was used to assess TAS (Benzie and Strain, 1996). This test measures the ferric reducing ability of plasma. At low pH, when a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the Fe^{2+} form, an intense blue colour with an absorption maximum at 593nm develops.

The TAS method is as follows with some modification to the protocol of Benzie and Strain, (1996). Freshly prepared FRAP reagent (300µL) [300mmol/litre acetate buffer,

pH 3.6 (3.1gC2H3NaO2·3H2O and 16ml C2H4O2 per litre of buffer solution; 10mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40mmol/L HCl; 20mmol/L FeCl3·6H2O)]. Working FRAP reagent was prepared as required by mixing 25ml acetate buffer, 2.5ml TPTZ solution and 2.5ml FeCl3·6H2O solution and was warmed to 37°C which was then read at 593nm. Sample (10μL) was then added to the FRAP reagent along with 30μL H_2O. Absorbance readings were taken after two minutes. The change in absorbance (ΔA593nm) between the final reading (two minutes) and the initial reading was calculated for each sample and related to ΔA593nm of a Fe^{2+} standard solution tested in parallel. Benzie and Strain (1996) studied the FRAP reaction for up to 8 minutes, but selected 4-minute readings for calculations. The readings after 2 minutes were used in the present studies as it was found that the FRAP reactions in plasma samples reached an end-point around this time. The intra- and interassay variation for total antioxidant status are 1% and 3%, respectively.

3.1.13.2 Superoxide dismutase (SOD)

SOD was first identified and purified from red cells by McCord and Fridovich (1969). It has since been detected in a large number of tissues and its purpose is essential to the red cell for the detoxification of $O_2^{\cdot -}$ and plays a protective role similar to that of the GSH-GSH Px system.

In the present studies, SOD activity was assessed using a method developed by Winterbourn *et al* (1975). The assay is based on the method of Beauchamp and Fridovich (1971) and depends on the ability of the enzyme to inhibit the reduction of nitroblue tetrazolium (NBT) by $O_2^{\cdot -}$, which is generated by the reaction of photoreduced riboflavin and O_2.

SOD was measured as described below:

Blood was collected in EDTA tubes and plasma and the majority of white cells were removed. The red cells were washed twice with saline (0.9% w/v), then haemolyzed by adding approximately 1.5 volumes of water.

Hb concentrations were measured and samples adjusted to 10gm/100 ml. A chloroform-ethanol extract was prepared (0.5ml hemolysate, 3.5ml ice-cold distilled water, 1.0ml ethanol and 0.6ml chloroform). When preparing the chloroform-ethanol extract, the solution was mixed at each addition and shaken for 1 minute. Tubes were then centrifuged for 10 minutes at 3000 rpm.

Another stock solution was prepared ($^{M}/15$ phosphate buffer, pH 7.8, 0.1M EDTA containing 1.5mg of sodium cyanide per 100ml; 0.12mM riboflavin (4.5mg per 100ml) stored cold in a dark bottle; and 1.5mM NBT (12.3mg per 10 ml), stored cold (5°C). For each sample to be assayed, six tubes were set up containing 10, 20, 40, 60, 80 and 500µl red cell extract, plus 0.2ml EDTA/NaCN, 0.1ml NBT, 0.05ml riboflavin (added last after tubes had been brought to ambient temperature, 20-22°C) and phosphate buffer to give a total volume of 3ml. Three tubes containing no red cell extract was also included per test run. The tubes were then placed in a light box to receive uniform illumination for a standard period of 12 minutes. Optical densities of the tubes were then measured using a spectrophotometer at 560nm.

Results were expressed as units of SOD per gram of Hb. One unit is defined as the amount of enzyme causing half of the maximum inhibition of NBT reduction. This unit depends on the conditions of the assay, namely light intensity, riboflavin and NBT concentrations. Percentage inhibition is calculated from each optical density and the

value in the absence of enzyme and plotted against the amount of red cell extract (up to 80µl). The tube containing 500µL extract gives maximum inhibition (maximum inhibition is also given by 200µL of extract from normal cells, so 500µL is well in excess of the minimum requirement). The amount of extract (VµL) which gives half this inhibition (1 unit) is determined from the graph plotted above. Since the extract is equivalent to a 1 Gm per 100ml Hb solution, the red cell enzyme activity is

$$\frac{100{,}000}{V} \text{ units per gram of Hb}$$

The red cell SOD activity in healthy adults is between 2,400 to 3,700 units per gram of Hb with a mean value of 2,900 units. The intra- and interassay variation for superoxide dismutase are 4% and 5.5%, respectively

3.1.13.3 Reduced glutathione (GSH)

GSH – a tripeptide consisting of glutamic acid cysteine and glycine acts as a substrate for the enzyme GSH-Px. As such it is an important component of the body's intracellular antioxidant defences. In particular, GSH functions to protect cellular proteins from thiol oxidation. When challenged with oxidative stress, intracellular GSH rapidly oxidises to GSSG. Oxidised GSSG produced intracellularly is converted back to GSH by glutathione reductase, requiring NADPH as a cofactor.

In the present studies, GSH was measured using a method developed by Beutler (1975). Viritually all of the nonprotein sulfhydryl compounds of red cells are in the form of GSH so the assay incorporates 5, 5'-Dithiobis (2-nitrobenzoic acid) (DTNB) as a

disulfide compound to readily reduce sulfhydryl compounds to form a highly coloured yellow anion (Beutler, 1975).

GSH was measured using the method described below:

Whole blood (EDTA) (200µL) was added to 2.0ml of distilled water. Lyzate (200µL) was then removed and stored at –20°C for estimation of Hb. Precipitating solution (3ml) (one hundred millilitres containing 1.67g of glacial metaphosphoric acid, 0.2g of disodium EDTA and 30g of sodium chloride) was added to the remaining 2ml of haemolyzate. After standing for 5 minutes the mixture was filtered through medium grade of filter paper and was stored in eppendorf tubes at –20°C.

During analysis, 2.0ml of filtrate was added to 8ml of 0.3 M Na_2HPO_4 solution in a cuvette. The absorbance was read at 412nm against a blank (prepared by adding 2ml of 2:5 water diluted precipitating solution to 8ml of phosphate solution). A second optical density reading was taken after 1ml of DTNB reagent (20 milligrams of DTNB per 100 ml of 1% sodium citrate solution) had been added to the blank cuvette and the cuvette containing filtrate.

Results are expressed as GSH in micromoles / gram of Hb. The calculation used was as follows:

$$\frac{C}{1000} = \frac{(OD_2 - OD_1)}{13{,}600} \times E_1 \times \frac{11}{2} \times \frac{5}{2} \times \frac{100}{Hb}$$

$$C = \frac{(OD_2 - OD_1) \times E_1 \times 101}{Hb}$$

E1 is the molar extinction coefficient of the yellow anion produced when GSH interacts with DTNB. However this value changes when different light path and band widths are used for analysis. For example a band width greater than 6 nm may produce a lower extinction coefficient. The extinction coefficient can be calculated by performing a GSH determination and reading an aliquot in a spectrophotometer with a narrow slit in a 1 cm cuvette to obtain the extinction value D_1. An aliquot of the same sample is then read in the system being calibrated, obtaining a second optical density reading D_2.

$$E1 = \frac{D_1}{D_2}$$

OD_1 is the optical density measured at 412nm before the addition of DTNB solution, and OD_2 is the optical density after the addition of DNTB.

The red cells of GSH activity in healthy adults is approximately 6.57±1.04µmoles of GSH / g Hb. The intra- and interassay variation for reduced glutathione are 5% and 6%, respectively.

3.1.13.4 Selenium

Selenium concentrations in plasma were determined by a Varian model SprectrAA-880 Zeeman graphite furnace atomic absorption spectrometer (GFAAS, Varian, UK) with software SpecrAA-880 following the method of Knowles and Brodie (1988). In brief, plasma samples were diluted 1/10 by using a sample diluent. The sample diluent was prepared by dissolving 0.56g of ascorbic acid (DBH/AnalaR g, Grade, UK), 0.88ml of TritonX-100 (BDH, UK), 1ml of Antifoam B and 0.1ml of hydrochloric acid, made up to 1.0L with deionized water. The 20µl of plasma sample was injected into the graphite furnace with a modifier solution (500mg palladium, 0.05% Triton X-100 and 0.1%

Antifoam B). By using the auto sampler facility of the GFAAS the calibration curve was prepared from the stock solution of selenium (1mg/L) by mixing the appropriate volume. The hallow cathode lamp from selenium was operate at 20mA and absorbance was measured at 196nm. The analysis was performed in duplicate and results expressed as µg/L. Reagents were supplied by Sigma-Aldrich, UK. The intra- and interassay variation for total antioxidant status are 1% and 3%, respectively.

3.1.14 Platelet aggregation assessment

Platelet aggregation was assessed by several methods in a preliminary experiment to determine a suitable protocol to assess platelet function (see methodology section .10). The two platelet aggregation methods include platelet aggregometry and PlateletWorks®, which are both explained in detail below.

3.1.14.1 Platelet Aggregometry

Platelet aggregometry is the 'gold-standard' for assessment of platelet function, which measures platelet aggregation in the presence of an agonist by optical methods. In general there are four independently operated aggregation channels. Platelet poor plasma (PPP) is inserted into the machine and platelet aggregation is started at 0%. This activates the printer to record platelet aggregation and then the platelet rich plasma (PRP) is placed into the machine. The reagent (agonist, ADP) is then added to the PRP and platelet aggregation is stimulated which is recorded and printed until aggregation is completed.

Platelet aggregation was measured using the method of Born (1962).

Whole blood (4.5ml) was added to 0.5ml sodium citrate in a plain plastic tube which was centrifuged for 10 minutes at 1000 rpm to obtain PRP. The remaining blood was centrifuged for a further 10 minutes at 3000 rpm to obtain PPP.

PRP (200µL) was pipetted into an aggregation cuvette and placed in an incubation block for 3 minutes. PPP (200µL) was pipetted into an aggregation cuvette and placed in the aggregometer to set '100%' transmission. After 3 minutes, the PRP cuvette (including magnetic stir bar) was inserted into the aggregometer to set 0% transmission. When the platelet aggregometer was zeroed, the stable baseline was obtained with PRP for 30 seconds to 1 minute before the agonist was added to record the platelet aggregation response.

For the assessment of platelet aggregation, several ADP concentrations were used. The ADP stock solutions were prepared on the day of testing using stock solutions equivalent to 10mM, which were kept stored at -20°C. Final ADP concentrations of 20, 15 and 10µM were used.

Results for platelet aggregation tests were calculated for percent maximum following 4 minutes. The intra- and interassay variation for platelet aggregation using a platelet aggregometer are 8.7% and <10%, respectively.

3.1.14.2 PlateletWorks®

Plateletworks® methodology is an adaptation of platelet aggregometry, which is extremely simple, inexpensive and quick to perform. This two-step method involved using an automated cell counter to measure total platelet count in whole blood and then re-determines the number of platelets on a second sample that has been exposed

to a known platelet agonist. The agonist stimulates the platelets which are functional to aggregate in clumps, and they will therefore be counted as platelets in the second sample. The difference in the platelet count between samples one and two provides a direct measurement of platelet aggregation and is reported as percent aggregation as per the following equation.

$$\frac{\text{Baseline Platelet Count} - \text{Agonist Platelet Count}}{\text{Baseline Platelet Count}} \times 100\% = \%\ \text{Aggregation}$$

Whole blood (1ml) was added to 4 plain plastic tubes each containing 111µL sodium citrate, and varying degrees of ADP concentration added to give a final concentration of 20µM, 15µM and 10µM or 0µM. The ADP stock solutions were prepared on the day of testing using stock solutions equivalent to 10mM, which were kept stored at -20°C.

The baseline tube (no agonist) was inverted 3-5 times to mix the whole blood with the anticoagulant which was then run on PlateletWorks® and platelet count recorded. The remaining tubes with varying ADP concentrations were inverted 3-5 times and run on PlateletWorks® and platelet count recorded. The percentage aggregation was then calculated. The intra- and interassay variation for platelet aggregation using PlateletWorks® are 7.2% and <10%, respectively.

3.2 Anthropometric measurements

Several different techniques were used for anthropometric measurements, which were dependent on the location of the research studies. However all these measurements are described below.

3.2.1 Height

Each subject was instructed to remove footwear prior to height measurement. Height was measured to the nearest 0.1cm using a wall-mounted stadiometer in the Exercise Physiology laboratories at De Montfort University in Bedford and a free-standing stadiometer at the Centre for Obesity Research, Luton and Dunstable Hospital.

3.2.2 Weight

Each subject was instructed to wear light clothing and remove footwear prior to weight measurement. Weight was measured to the nearest 0.1kg using a free-standing stadiometer (Seca model 713, Cophenhagen, Denmark) in the Exercise Physiology laboratories at De Montfort University in Bedford and a Tanita weighing scale at the Centre for Obesity Research, Luton and Dunstable Hospital.

3.2.3 Waist Circumference

Waist circumference was measured at a point midway between the costal margin and the iliac crest and in line with the mid-axilla (WHO, 1995). The tape was pulled taut and a measurement to the nearest 1mm was taken in mid-expiration with subjects standing straight with feet together, arms hanging loosely, and looking straight ahead.

3.2.4 Body fat

3.2.4.1 Bioelectrical Impedance (BIA)

BIA measurements were carried out with the subject lying in a supine position on a flat, nonconductive bed by using a multifrequency tetrapolar technique (QuadScan 4000; Bodystat, Douglas, United Kingdom). The Bodystat QuadScan 4000 unit has 4 electrodes. Two electrodes were placed on the right wrist with one just proximal to the third metacarpophalangeal joint (positive) and one on the wrist next to the ulnar head (negative). Two electrodes were placed on the right ankle with one just proximal to the third metatarsophalangeal joint (positive) and one between the medial and lateral malleoli (negative). Multifrequency (5, 50, 100, and 200 kHz) currents were introduced from the positive leads and travelled throughout the body to the negative leads. Body fat percentage was calculated using the manufacturer's software. It is known that resistance depends positively on fat content of the body and negatively on total body fluids (Donadio *et al.*, 2005). In addition, reactance, which expresses the capacitance of the body, is positively dependent from the number of cell membranes. Impedance is also low in the presence of high volumes of body fluids. In particular, impedance to the passage of a high-frequency current depends on total body fluids, whereas impedance to introduction of a low-frequency current depends only on extracellular fluids (Donadio *et al.*, 2005).

The intra- and interassay variation for body fat using bioelectrical impedance are 1.18% and <5%, respectively.

3.2.4.2 BodPod

Whole body air displacement plethysmography was performed using the BodPod body composition system (Life Measurement Instruments, California, USA) according to the manufacturer's instructions and recommendations. Subjects wore a tight fitting swimsuit and swimming cap. The procedure involved a volume calibration with and without a 50 litre metal cylinder. Subjects entered the BodPod and sat inside the anterior chamber (450 litres), which was connected to a rear measuring chamber (300 litres) via oscillating diaphragms (used to induce pressure changes in the anterior chamber), and breathed normally (relaxed tidal breathing). The recommended procedure, consisting of two measurements of body volume (50 seconds each), was adopted and when, occasionally, body volumes differed by more than 150 ml, the system required that a third measurement be performed. The final result reported by the BodPod instrumentation was the mean of the two (or the two closest) measurements.

In summary the operating principles of whole body air displacement plethysmography technique is described below. In air-displacement plethysmography, the body volume is measured indirectly by the volume of air which is displaced inside an enclosed chamber (plethysmograph). Human body volume is therefore measured when a subject sits inside a chamber and displaces a volume of air equal to his or her body volume. Body volume is calculated indirectly by subtracting the volume of air remaining inside the chamber when the subject is inside from the volume of air in the chamber when it is empty. The air inside the chamber is measured by applying relevant physical gas laws. Boyle's Law states that at a constant temperature, volume (V) and pressure (P) are inversely related: $P_1 / P_2 = V_2 / V_1$. However because body volume is affected by adiabatic conditions created by the subject's presence (this warmer air,

approximately 37°C, is more compressible than the ambient air), the BodPod software applies certain corrections to the thoracic gas volume (TGV; litres) and air next to the skin (using the surface area artefact, SAA; litres) to adjust to isothermal conditions to obtain each subject's actual body volume (Dempster *et al.,* 1995). The final body volume calculation in the BodPod is as followed:

$$Vb_{corr}\ (L) = Vb_{raw}\ (L) - SAA\ (L) + 40\%\ V_{TG}\ (L)$$

where Vb_{corr} is the body volume corrected for SAA (the effect of isothermal air near the skin's surface is estimated by calculating a surface area artefact) and V_{TG} (the average amount of air in the lungs during normal tidal breathing, thoracic gas volume, which is measured or predicted).

Once body mass (M) and Vb_{corr} are known, the principles of densitometry are applied (Behnke et al., 1942; Siri, 1961). Body density is calculated as M/Vb_{corr} and body density (D_b) is then inserted into a standard formula for estimating percentage body fat based on a 2-compartment model, such as the models of Siri equation.

$$\%\ \text{body fat} = (495/D_b) - 450 \qquad \text{Siri equation}$$

The intra- and interassay variation for body fat using BodPod are 6.7% and 7.8%, respectively.

3.3 Cardiovascular measurements

3.3.1 Heart rate

Two methods of calculating heart rate were utilised. One method involved calculating resting heart rate by palpating the radial artery at the wrist. Pulse was counted for 20 seconds, then multiplied by three to give beats/min. The other method involved using a

Polar heart rate transmitter belt, worn over the chest. The transmitter belt detects the electrocardiogram of the heart and sends an electromagnetic signal to either the Polar wrist receiver or Metamax 3B CPX system (if being used) where heart rate information appears.

3.3.2 Blood pressure

Systemic arterial blood pressure (BP-mmHg) was measured in the brachial artery using a mercury sphygmomanometer and stethoscope. Systolic blood pressure was noted when clear muffling of repetitive tapping sounds (Korotkoff) became apparent and diastolic pressure was noted when the repetitive sounds (Korotkoff) diminished (Tortora and Grabowski, 1996).

3.4 Respiratory measurements

3.4.1 Douglas Bag method

Off-line gas analysis was determined using the 'gold standard', Douglas Bag method. Subjects breathed through a large 2-way non-returnable breathing valve. Expired air was directed into a Douglas bag through plastic tubing and a two-way stopcock valve. The collection of expired gas was hand timed using chronography to the nearest whole breath for a specific time period. Samples of expired air were dried using 97% anhydrous calcium sulfate ($CaSO_4$) crystals and presented to a paramagnetic O_2 and infra-red CO_2 analyzer for the determination of percentage O_2 and CO_2. Before use, the analysers were calibrated using specialised gas mixtures. Computation of O_2 uptake and CO_2 production corrected to STPD [STDP correction means conversion of the measuring environment BTPS (Body Temperature, Pressure Saturated) to STPD

(Standard Temperature, Pressure Dry) environment] was computed using the Haldane transformation equation (Wasserman *et al.*, 1994).

3.4.2 On-line gas analyser

On-line VO_2 was measured using a Cortex gas analysis system (Metamax 3B, Birmingham, UK), which provided real-time breath-by-breath VO_2. Subjects breathed through a rubber mask attached to a DVT volume transducer, which was attached to the MetaMax Portable CPX system via a sample line. The MetaMax 3B was calibrated before each test using the Hans Rudolph 3 litre syringe for ventilation volume and standard gases of known O_2 and VCO_2 concentration. Respiratory parameters were converted from BTPS to STPD. The measured and calculated data was also smoothed (every two points) before obtaining numerical printouts.

3.5 Treadmill

The treadmill used at De Montfort University in Bedford was the PowerJog JX200 and at the Centre for Obesity Research, Luton and Dunstable Hospital, the Marquette 2000. Both treadmills were serviced and calibrated regularly.

3.6 Rating of perceived exertion (RPE)

Rating of perceived exertion (RPE) was estimated using Borg's Scale (1973). A numeric value which ranges from 6-20 could be selected to indicate a perceived rate of exertion from 'very very light' to 'maximum'.

3.7 Temperature and humidity regulation

Room temperature was measured using a wall-mounted temperature and humidity gauge (Thermo-Hydro), which measured temperature and humidity to the nearest 0.1°C and 0.1% respectively. Barometric pressure was obtained from a wall-mounted barometer.

3.8 Dietary analysis

In the present studies, subjects were asked to record the quantity and type of food and beverage they consumed over several days or a week. This data was analysed using a computerised software package (Compeat, Nutrition Systems, Carlson Bengston Consultants Limited).

3.9 Physical activity questionnaire

The Baecke questionnaire (Baecke *et al.*, 1982) was used as a short questionnaire for the measurement of habitual physical activity. The questionnaire was self-administered although if necessary help from an observer was provided. In summary this questionnaire contained 13 questions with three distinct dimensions; physical activity at work, sport during leisure centre and other physical activity during leisure time. The sum of all these dimensions allow calculation of a total physical activity score which refers to habitual physical activity. Each evaluation on the questionnaire allows the calculation of four different physical activity scores; a total physical activity score ($Btot_{0,...60}$), a sport-related physical activity sub- score ($Bsport_{0,...60}$), a leisure-time-related physical activity sub-score ($Bleisure_{0,...60}$) and an occupation-related physical activity sub-score ($Boccup_{0,...60}$).

3.10 Preliminary experiment to develop and evaluate PlateletWorks® (1)

3.10.1 INTRODUCTION

The platelet aggregometer is the 'gold' standard method for the assessment of platelet aggregation (Born, 1962). But its methodology is highly labour intensive, expensive and the reproducibility of results is dependent on controlling many factors; temperature, pH, aggregometer speed, platelet count, sample volume and venepuncture technique (White and Jennings, 1999). This necessitated the development of several whole blood assays, such as PlateletWorks® which is an adaptation of platelet aggregometry and is extremely simple, inexpensive and quick to perform (Lau et al., 2002). Its use in exercise physiology per se is a relatively new phenomenon. With this in mind a preliminary experiment was completed to supplement the validity of platelet aggregation data reported in this thesis.

This study assessed platelet aggregation techniques; platelet aggregometry and PlateletWorks® and established whether PlateletWorks® was a cost-effective, quick and reproducible measure of platelet aggregation, which could be used to predict clinical outcomes for research purposes.

3.10.2 METHODOLOGY

Subject Characteristics

Ten (n=10) apparently healthy male and females (n=5/5, respectively) were recruited from the Luton and Dunstable Hospital to participate in the present study (see table 3.0 for subject characteristics). Ten subjects were chosen as it had previously been

suggested that the generation and application of data on biological specimens could be obtained from a relatively small group of subjects over a reasonably short period of time (Fraser and Harris, 1989). The subjects had no known physician-diagnosed diseases or ailments as assessed by a medical history questionnaire. Subjects with hypertension, with or without hypertensive treatments, dyslipidaemia or CVD, any of those taking any antioxidant supplementations, nonsteroidal anti-inflammatory drugs or lose-dose aspirin and smokers were excluded from the study. Written informed consent was obtained from all the subjects after they were given a full explanation of the study. The research was given ethical approval by Bedfordshire Local Research Ethics Committee.

Table 3.0. Subject characteristics

Characteristic	
Age, yr	33 ± 3.1
BMI, kg/m^2	25.4 ± 1.1
Weight, kg	74.1 ± 3.1

Values expressed as the mean±SEM. BMI, body mass index.

Experimental design

This study was broken up into two stages.

Stage 1: Familiarisation and developmental

Stage 2: Validity and reliability

Stage 1

Three of the ten subjects recruited visited the Centre for Obesity Research at the beginning of the study to enable the familiarisation and development of platelet aggregation methods to take place. Subjects attended the centre between 9-12am and were instructed to fast for 10-12 hours and refrain from exercise, caffeine and alcohol

for 48 hours before the study visit. Subjects were also told to maintain their usual dietary regimen.

Stage 2

Following the development stage, all ten subjects visited the Centre for Obesity Research on 3 separate occasions over a 3-week period. Subjects attended the centre between 8-10am and were instructed to fast for 10-12 hours and refrain from exercise, caffeine and alcohol for 48 hours before the study visit. Subjects were also told to maintain their usual dietary regimen.

Anthropometric measures

During visit one at stage two, height was measured without shoes using a stadiometer. Weight was measured using a Tanita weighing scale (see methodology section 3.2.1 and 3.2.2).

Venous blood sampling

At each visit, in the supine position, blood samples (exactly 13.5ml) were collected from an antecubital vein using the syringe method (see methodology section 3.1). During venepuncture the tourniquet was released and the first 5ml of blood for platelet aggregation was disposed of.

PLATELET AGGREGATION METHODS

Platelet aggregometry

Platelet aggregation was measured using the method of Born (1962), as described in the methodology section 3.1.15.1.

PlateletWorks®

Platelet aggregation was measured using a modified version of the manufacturer's guidelines, as described in the methodology section 3.1.15.2.

Statistical Analysis

Values reported as mean±SEM. SEM was appropriate because it demonstrates how liable to error the mean is (e.g. the mean value). The Shapiro-Wilk test confirmed that the data was normally distributed. The agreement between the two platelet aggregation methods was assessed using correlation analysis, Pearson, Kendal tau and Spearman rho and a statistical measure of agreement as reviewed by Bland and Altman (1986). The Bland and Altman approach allows for calculation of bias (mean of the individual differences between estimates) and the limits of agreement (+2 SD from the mean bias). Repeated measures ANOVA was used to test the significance of differences in % platelet aggregation between time periods. The data was analysed with two way (AxB) mixed analysis of ANOVA which incorporated one between (PlateletWorks® vs platelet aggregometer) and one within (time: Wk 1 vs Wk 2 vs Wk 3) subject factor. Precision data are reported as mean (± SD) with coefficient of variation. P-values < 0.05 were regarded as statistically significant. Analyzes of data was performed using a computer software package (SPSS for Windows, Version 13.0).

3.10.3 RESULTS

Stage 1

Some minor changes were made to the PlateletWorks® methodology. The PlateletWorks® methodology uses two PlateletWorks® tubes (EDTA baseline tube (0.024mL of 7.5 % K_3 EDTA solution (1.80mg) and an ADP tube (approximately 20μM ADP) to which 1.0mL fresh whole blood was added. In this study, four plain tubes with 111μL sodium citrate (3.8% w/v) and varying concentrations of ADP (final concentration of 20, 15, 10 and 0μM) were set up to obtain a detailed ADP-stimulated platelet aggregation response.

Stage 2

PlateletWorks® vs Platelet aggregometer

Platelet aggregation in whole blood samples was determined using PlateletWorks® and compared with aggregation using PRP on the aggregometer at three ADP-induced platelet aggregation concentrations. Table 3.1 presents the established reference range of platelet aggregation at three different ADP concentrations (Final concentration of ADP was 10, 15 and 20μM). It was demonstrated that the reference range for platelet aggregation at ADP-induced platelet aggregation 20μM was 81±3.0% for the platelet aggregometer and 81±1.3% for PlateletWorks®. At ADP concentration of 15μM, platelet aggregation was 69.03±3.03% for the platelet aggregometer and 68.1±4.1% for PlateletWorks®. At ADP concentration of 10μM, platelet aggregation was 60.87±3.68% and 53.1±5.3% for PlateletWorks®.

Regression analysis was performed on the ADP agonist data to evaluate the relationship between the two test modalities, namely platelet aggregometer and PlateletWorks® (r=0.64). The results from this analysis are shown in figure 3.0. Since correlation coefficients measure the strength of the relationship and not the agreement between them, these data were also analyzed using the non-parametric statistical analyzes of both Kendall tau (r=0.506) and Spearman rho (r=0.682) (see table 3.2).

Table 3.1. Established reference range of platelet aggregation using platelet aggregometer and PlateletWorks® at various ADP-induced platelet aggregation concentrations.

Method		% Platelet aggregation	
	N	Mean	SEM
Platelet aggregometer			
10 μM	30	60.87	3.68
15 μM	30	69.03	3.03
20 μM	30	81.17	3.31
PlateletWorks®			
10 μM	30	53.1	5.3
15 μM	30	68.1	4.1
20 μM	30	81.2	1.3

Values expressed as the mean±SEM. 10, 15 and 20μM, Final concentrations of ADP-induced platelet aggregation. For further details see text

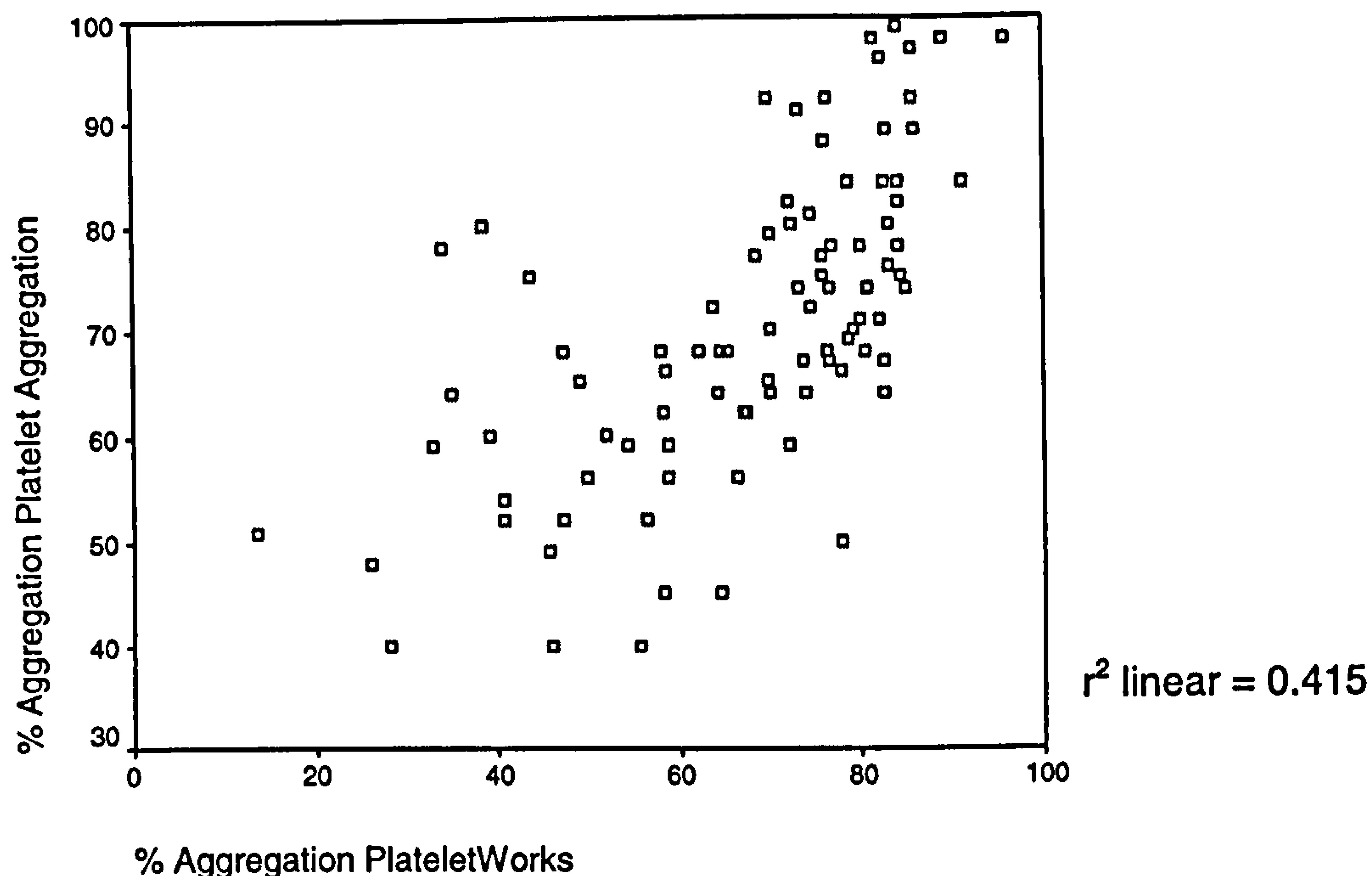


Figure 3.0. Regression analysis to evaluate the relationship between two test modalities, namely platelet aggregometer and PlateletWorks®. Results were analyzed using Spearman rank correlation test. For further details see text.

Table 3.2. Comparative analyzes to compare platelet aggregometer and PlateletWorks® for ADP-induced platelet aggregation.

Agonist	N	Pearson ®	Spearman rho	Kendall tau
ADP	90	0.64*	0.682*	0.506*

ADP, platelet-induced agonist. Results analyzed using Pearson correlation test, Spearman rank correlation test and Kendall tau coefficient test and r values are given in the Table. (*, P<0.01). For further details see text.

Using the Bland and Altman approach, figure 3.1 shows a comparison of platelet aggregation measured by a platelet aggregometer and PlateletWorks®. The mean difference was –2.9% with a 95% confidence interval between –29.7 to 23.9%. There was a clear tendency for the Plateletworks® to under-report platelet aggregation, by an

average of –2.9%. Despite this, the 95% confidence interval for the lower limit of agreement was from –34.5% to –24.9% and the 95% confidence interval for the upper limit of agreement was 19.1 to 28.7%.

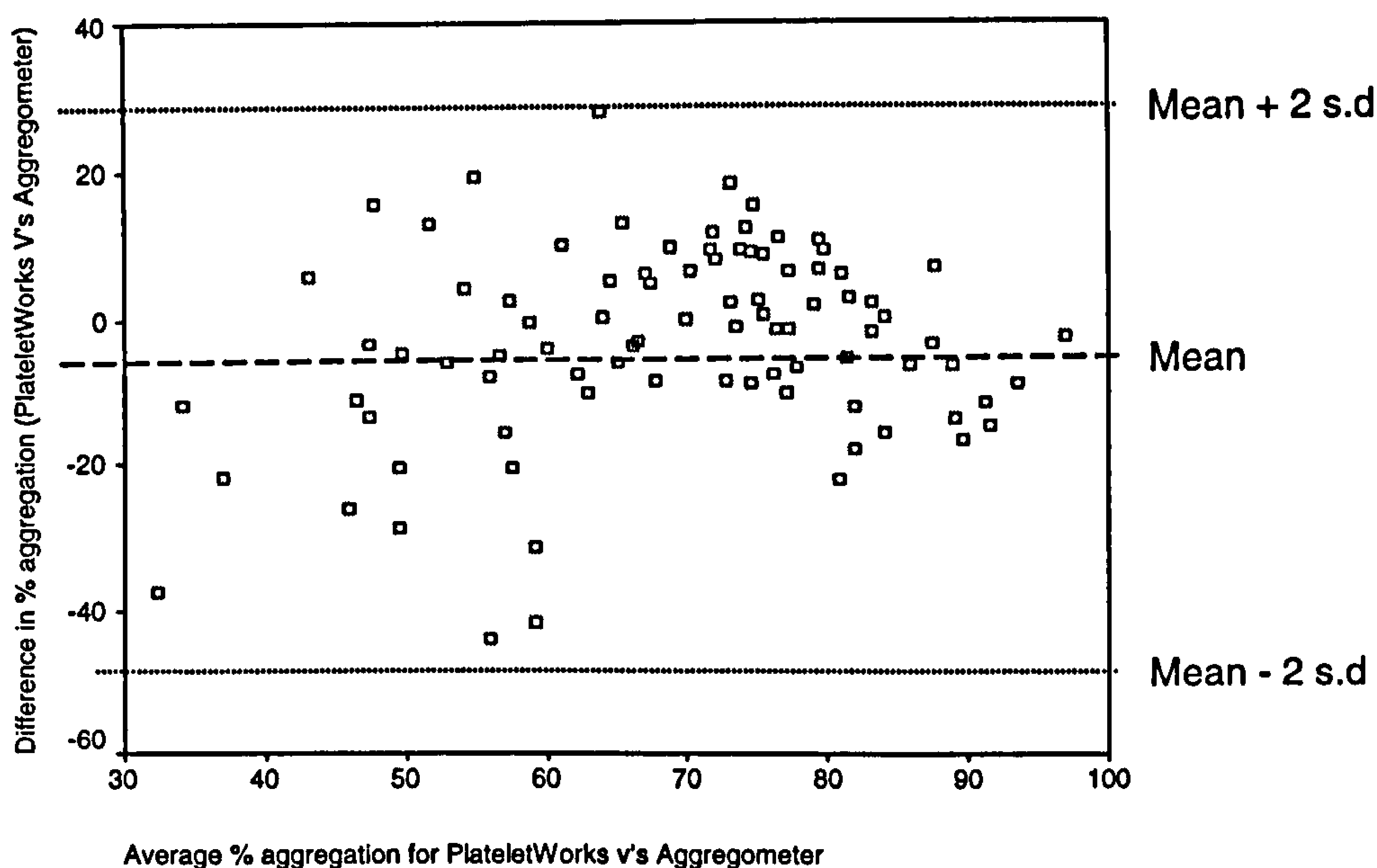


Figure 3.1. Scatter plot (Bland-Altman plot) of PlateletWorks® ADP-induced platelet aggregation minus platelet aggregometer ADP-induced platelet aggregation (vertical axis) against mean of PlateletWorks® and platelet aggregometer ADP-induced platelet aggregation. The dashed horizontal line represents the mean difference and the dotted lines represent the 95% limits of agreements (n=90). For further details see text.

Table 3.3. Intra- and interassay for ADP-induced platelet aggregation using two test modalities, namely platelet aggregometer and PlateletWorks®.

Method	Sample	% Platelet aggregation		CV (%)	
	N	Mean	SD	Mean	SD
Platelet aggregometer	90	70.36	13.5	8.66	5.03
PlateletWorks®	90	67.45	16.76	7.20	6.86

Values expressed as the mean±SD. Reproducibility of % platelet aggregation analyzed using the coefficient of variation (CV %). For further details see text.

Intra- and interassay was tested for ADP-induced platelet aggregation (%) using the platelet aggregometer and PlateletWorks®. Intrassay was 67.5±16.8% for PlateletWorks® and 70.4±13.5% for the platelet aggregometer. The interassay was 8.7 (SD 5.03)% for the platelet aggregometer and 7.2 (SD 6.86)% for PlateletWorks® and CV of % platelet aggregation ranged from 1.2 to 22% and 0.6 to 35.2% respectively (see table 3.3). There was no evidence of significant differences in % platelet aggregation between time periods for both the platelet aggregometer and PlateletWorks® (ANOVA, P>0.05) (see table 3.4).

Table 3.4. Analysis of variance of ADP-induced platelet aggregation for two test modalities, namely platelet aggregometer and PlateletWorks®.

Method		Sum of squares	Df	Mean Square	F	P Value
Platelet Aggregometer	Between groups	36.16	2	18.08	0.86	0.92
	Within groups	18252.47	87	209.80		
	Total	18288.62				
PlateletWorks®	Between groups	125.27	2	62.63	0.21	0.81
	Within groups	25611.64	87	294.39		
	Total	25736.90	89			

Results analyzed using repeated measured ANOVA and P values are given in the Table. For further details see text.

3.10.4 DISCUSSION

This study was designed to assess the platelet aggregation techniques, platelet aggregometry and PlateletWorks® and to establish whether the PlateletWorks® was a cost-effective, quick and reproducible measure of platelet aggregation, which could be used to predict clinical outcomes for research purposes.

Platelets have an increasingly well-defined role in haemostasis both in health and disease e.g. coronary artery thrombosis (Ruggeri, 2002) and other CVDs, including stroke and micro- or macro-angioplastic complications due to diabetes mellitus (Michelson, 2002). In addition platelets also have a role in the underlying atherosclerotic process (Ruggeri, 2002). Platelet function tests have been studied extensively in CVD as a means to predict both clinical outcomes and to monitor treatment therapies.

Primary haemostasis may be assessed in two ways: 1) by measuring platelet count and 2) by testing platelet function (platelet aggregation) (Lau *et al.,* 2002). Platelet aggregometers are the 'gold standard' for assessment of platelet aggregation (Born, 1962) but as an assay has several limitations. For example, platelet aggregometers, use large volumes of blood and a complex, lengthy analytical process. Furthermore PRP aggregometry is limited by the concentration of platelets (only samples of $\geq 50 \times 10^3/\mu L$ platelets can be tested) and also it cannot detect micro-aggregates of <100 platelets (Storey *et al.,* 1998). These limitations may be overcome by a new test platform, PlateletWorks®, which directly evaluates platelet aggregation using whole blood. In addition to its rapid turn-around time, it provides a full haematology profile (including platelet count), which is extremely useful in the clinical research

environment. Lau *et al* (2002) summarised PlateletWorks® as a novel-bedside assay of platelet function suitable for the clinical environment.

Despite PlateletWorks® not being studied extensively, this study supports the finding of Lau *et al* (2002) in that the PlateletWorks® system may be used as an alternative to platelet aggregometry. In this study, the mean difference between the two methods was –2.9% with a 95% confidence interval –29.7 to 23.9%. The reference range for platelet aggregation following 20μM ADP-induced platelet aggregation was 70-96% for platelet aggregometry and 76-89% for PlateletWorks®. Similarly Lau *et al* (2002) found that the reference range for the platelet aggregation response to 20μM ADP induced platelet aggregation was 80-97% with PlateletWorks®. This study showed that at lower ADP concentrations, platelet aggregation decreased compared to ADP-induced platelet aggregation at 20μM. For example at ADP concentrations of 15μM and 20μM, platelet aggregation was 69.03±3.03% and 60.87±3.68% respectively for the platelet aggregometer and 68.1±4.1% and 53.1±5.3% respectively for PlateletWorks®.

Intra- and interassay was tested for ADP-induced platelet aggregation for each platelet function method. Intraassay (% aggregation) was 67.5±16.8% for PlateletWorks® and 70.4±13.5% for the platelet aggregometer. The interassay was 8.7 (SD 5.03)% for the platelet aggregometer and 7.2 (SD 6.86)% for PlateletWorks®. The slight variation observed may be attributed to natural, diurnal physiological processes during the test period or a change in factors which may compromise the sample, for example temperature, pH, aggregometer stir speed, platelet count, sample volume and venepuncture technique all affect platelet aggregation (White and Jennings, 1999). The anticoagulant used will also affect platelets, for example in this study sodium citrate was used rather than EDTA as recommended by the PlateletWorks®

manufacturer's guidelines. EDTA has been shown to cause falsely low platelets counts known as platelet agglutination, irrespective of the presence or absence of any disease process (George, 2000).

3.10.5 CONCLUSION

To conclude, PlateletWorks® has demonstrated utility in the clinical / research setting to provide information on overall primary haemostasis (platelet aggregation and platelet count) by using a whole blood sample, which is devoid of long laborious techniques, excessive blood volume and the limitations associated with PRP. To confirm PlateletWorks® as a clinical research tool, future research should carry out large scale clinical studies using PlateletWorks® to assess primary haemostasis between population groups and following clinical treatment known to reduce platelet aggregation levels. Furthermore, further studies should investigate whether PlateletWorks® has the potential to diagnose inherited platelet irregularities (Lau *et al., 2002).*

3.11 Preliminary experiment to determine the reliability of the Bioelectrical Impedance (Bodystat QuadScan 4000) (2)

3.11.1 INTRODUCTION

Body composition assessment is often an integral part of obesity research since basic descriptive information on body composition is linked to energy intake, physical activity, energy metabolism and incidence of chronic disease (Brodie *et al.*, 1998). BIA is a widely used method to assess body composition (National Institute of Health, 1994). The analysis determines the electrical impedance of body tissue, which provides an estimate of total body water. From this, fat-free mass (FFM) and body fat (BF) can be estimated (Ackmann and Seitz, 1984). Several other techniques, measure body composition such as hydrodensitometry, skinfold calipers and DEXA (Brodie *et al.*, 1998). Some body composition techniques are restricted to research institutes with high capital, but low cost body composition systems such as bioelectrical systems can be applied rapidly and non-invasively for research purposes and clinical practice.

It is recognised that the measurement of bioelectrical impedance is influenced by other factors that should either be controlled or reported. These include the menstrual cycle (Mitchell *et al.*, 1993), skin temperature (Caton *et al.*, 1988), exercise-induced dehydration (Brodie *et al.*, 1991), prior food (Fogelholm *et al.*, 1993) and different positions (Pinilla *et al.*, 1992). If such features are controlled the prediction errors to calculate body fat are 3-5% (Brodie *et al*, 1998). Most studies report that the impedance method is reliable and valid (van Marken-Lichtenbelt, 1994), which is extremely important because poor reliability may reduce the precision of a single measurement and the ability to track changes in measurements in experimental studies may be reduced (Zemel *et al.*, 1996).

The purpose of this preliminary study was to assess the reliability of the BodyStat QuadScan 4000 for assessment of body composition.

3.11.2 METHODOLOGY

Subject characteristics

Six (n=6) healthy male and females (n=2/4) were recruited from the Department of Sport Sciences, De Montfort University (see table 3.5 for subject characteristics). All subjects had previously been involved in studies involving the BIA and so were familiar with all the experimental procedures. Written informed consent was obtained from all the subjects after they were given a full explanation of the study. All subjects were fully informed that they were free to withdraw from participation at any time. The study was approved by De Montfort University Research Ethics Committee.

Table 3.5. Subject characteristics

Characteristic	
Age, yr	23.7 ± 3.2
BMI, kg/m^2	24.1 ± 1.0
Weight, kg	72.0 ± 4.9
Body fat, kg	17.8 ± 3.1

Values expressed as the mean ± SEM. BMI, body mass index.

Experimental design

Subjects visited the Exercise Physiology laboratories on three separate days over one week between 9-12am and were instructed to fast for 10-12 hours beforehand and

refrain from exercise, caffeine and alcohol for 48 hours before the experimental period began. Subjects were also told to maintain their usual dietary regimen.

Anthropometric measurements.

Height and weight measurements are described in detail in the methodology chapter (section 3.2.1, 3.2.2, 3.2.3). Body composition was analyzed using the BIA (BodyStat® Quadscan 4000) which is outlined in the methodology chapter (section 3.2.4.1).

BIA measurements

Subjects rested in a supine position for 5-10 minutes before assessment of body composition. Three measurements of body composition were made in each subject. This was repeated twice and the average between the two measurements were obtained.

Statistical Analysis

Values reported as mean±SEM. SEM was appropriate because it demonstrates how liable to error the mean is (e.g. the mean value) The Shapiro-Wilk test confirmed that the data was normally distributed. The significance of differences in BIA measurements between time periods was analyzed using one-way ANOVA which incorporated one within (time: Wk 1 vs Wk 2 vs Wk 3) subjects factor. Precision data are reported as mean (± SD) with coefficient of variation. P-values < 0.05 were regarded as statistically significant. Analyzes of data was performed using a computer software package (SPSS for Windows, Version 13.0).

3.11.3 RESULTS

Results for the reproducibility tests and analysis of variance for BIA are shown in table 3.6 and Table 3.7, respectively.

Table 3.6. Intra- and interassay variation for body fat (kg) using the BIA method.

	Sample			CV (%)	
Equipment	N	Mean	SD	Mean	SD
BIA (Body fat, kg)	18	17.80	7.69	1.18	1.17

Values expressed as the mean±SD. Reproducibility of body fat (kg) analyzed using the coefficient of variation (CV%). For further details see text.

Intra- and interassay was tested for body fat (kg) using the bioelectrical impedance. Intraassay was 17.80±7.69kg and the interassay was 1.18 (SD 1.17)kg and ranged from 0.17 to 3.0%. There was no evidence of significant differences in body fat levels between time periods when using BIA (ANOVA, P>0.05) (see table 3.7)

Table 3.7. Analysis of variance of body fat (kg) for BIA method.

Parameter		Sum of squares	Df	Mean Square	F	Sig.
BIA	Between groups	0.214	2	0.107	0.002	0.998
	Within groups	887.284	15	59.152		
	Total	887.497	17			

Results analyzed using repeated measured ANOVA and P values are given in the Table. For further details see text.

3.11.4 DISCUSSION

This study was designed to assess the reproducibility of the BIA (BodyStat QuadScan 4000) for assessment of body composition.

This study found that the BodyStat QuadScan 4000 was highly reproducible. This is crucial for single measurement precision and for the ability to track changes in measurements during experimental studies. In this study many factors associated with influencing the measurement of bioelectrical impedance were controlled, such as menstrual cycle (Mitchell *et al.*, 1993), skin temperature (Caton *et al.*, 1988), exercise-induced dehydration (Brodie *et al.*, 1991), prior food (Fogelholm *et al.*, 1993) and different positions (Pinilla *et al.*, 1992). According to Brodie *et al* (1998), controlling for the above factors whilst using a BIA, obtain a prediction error to calculate body fat of around 3-5%. In this study the BIA possessed a day-to-day variation of around 0.3–3% (ANOVA, P>0.05). In general most studies report that the BIA method is reliable and valid (van Marken-Lichtenbelt, 1994) but highlight that caution should be used with single frequency BIA in a clinical setting (Tagliabue *et al.*, 1992). In this study, the multiple frequency BIA was used, which is able to differentiate between total and extracellular fluid compartments in the body (Chumlea and Guo, 1994). Multi-frequency BIA increases the value of assessing clinical and nutritional status (Brodie *et al.*, 1998) and has been shown to produce significant improvements in the prediction of body water (Cornish *et al.*, 1994). However the application of the BIA to special groups must also be interpreted with caution. For example BIA tends to overestimate fat in the lean and underestimate fat mass in the obese (Brodie *et al.*, 1998). This is because the geometric proportions of obese individuals compared with leaner individuals are such that a greater proportion of body mass and body water is accounted for by the trunk in relation to the extremities; the trunk however contributes a relatively minor

amount to total body impedance. However the advantages of the BIA include speed of operation, portability, simplicity and lack of intrusion (Brodie *et al.*, 1998).

3.11.5 CONCLUSION

In conclusion, the BIA method to assess body composition was sufficiently reproducible for research purposes. However, for BIA assessments, prior to and during, measurement conditions must be standardized.

Chapter FOUR

Study 1

Lipid peroxidation and antioxidant status at rest in healthy normal-weight, overweight and obese subjects

4.0 INTRODUCTION

Oxidant stress in obesity may be an important pathogenic mechanism in the obesity-associated metabolic syndrome (Furukawa *et al.*, 2004) which includes co-existence of several risk factors for atherosclerosis, including hyperglycaemia, dyslipidaemia and hypertension. Oxidant stress has also been shown to play a critical role in the pathogenesis of various diseases such as cancer, CVD and diabetes (Niki, 2000).

Several research studies have reported that obesity is associated with increased oxidant stress in humans (Yesilbursa *et al.*, 2005; Mohn *et al.*, 2005; Keaney *et al.*, 2003; Ozata *et al.*, 2002; Davi *et al.*, 2002; Dandona *et al.*, 2001; Prázný *et al.*, 1999) i.e. increased free-radical production and/or depleted cellular antioxidant defence systems (Powers *et al.*, 2004). The mechanisms suggested that may underlie the obesity-associated oxidant stress include increased VO_2 (i.e. mechanical overload) and subsequent radical production via mitochondrial respiration, diminished antioxidant capacity, increased fat deposition and cell injury causing increased rates of radical formation such as $O_2^{\cdot}$ and $OH^{\cdot}$ (Vincent *et al.*, 2001). In addition, hyperglycaemia, hypertension and hyperleptinemia are also possible sources of increased oxidant stress in obesity (Vincent and Taylor, 2006). It remains unknown if the obesity-associated oxidant stress is a cause and effect relationship or of it is due to the obesity-related diseases i.e. hypertension, hyperlipidaemia, hyperleptinemia and hyperglycaemia (Facchini *et al.*, 2000).

Lifestyle factors also influence oxidant stress levels in obese subjects (Moller *et al.*, 1996). Non-smoking, vitamin and/or trace element supplementation, regular physical activity and limited UV light exposure also contributes to an increased antioxidant defence potential, whilst tobacco smoking, high psychological stress, heavy alcohol

drinking and low/moderate vegetable, low fruit and little fish consumption contribute to a decreased antioxidant potential (Lesgards *et al.*, 2002).

This study investigated the levels of LH, TAS, SOD and GSH in normal-weight, overweight and obese subjects. This study also sought to identify physiological and lifestyle factors that may contribute to the normal variability of the overall oxidant stress status. The evaluation of oxidant stress, taking into account physiological factors, nutritional habits and lifestyle factors, may be useful in preventative medicine as a precocious diagnosis to identify healthy subjects who are at risk of oxidant stress-associated diseases.

4.1 METHODOLOGY

Subject Characteristics

The study group consisted of 90 subjects who were sex-matched between groups [30 normal-weight (BMI: 23.4±0.46kg/m^2), 30 overweight (BMI: 26.6±0.36kg/m^2) and 30 obese (BMI: 33.2±2.82kg/m^2) healthy male and females (n=24/66)] (see table 4.0 for subject characteristics). Volunteers were invited to take part in the study by local advertisement. An inclusion criterion was age between 18 and 50 years old and BMI between 20 and 40 kg/m^2. Subjects with a history of diabetes, cardiovascular or cerebrovascular disease, hepatic or renal disease, tobacco abuse, or those on hormone replacement therapy were excluded. In addition subjects were excluded if they were hypertensive (with or without treatment), taking treatment for dyslipidaemia, taking any antioxidant supplementations or a smoker. Written informed consent was obtained from all the subjects after they had been given a full explanation of the study. The research was given ethical approval by Bedfordshire Local Research Ethics Committee.

Table 4.0. Subject characteristics

Characteristic	Normal-Weight (NW) (n=30) (m/f; 8/22)	Overweight (OW) (n=30) (m/f; 8/22)	Obese (O) (n=30) (m/f; 8/22)	P Value NW vs OW	P Value NW vs O	P Value OW vs O
Age, yr	30.83 ± 1.65	34.67 ± 1.56	37.93 ± 1.91	.18	<.05	.06
Body mass, kg	64.55 ± 1.26	77.85 ± 1.66	91.66 ± 2.11	<.001	<.001	<.001
BMI, kg/m^2	22.82 ± 0.25	26.90 ± 0.21	33.03 ± 0.51	<.001	<.001	<.001
Body fat, kg	16.79 ± 1.04	23.45 ± 1.12	35.97 ± 1.48	<.001	<.001	<.001
Waist, cm	75.42 ± 1.14	87.59 ± 1.49	101.56 ± 2.00	<.001	<.001	<.001
Systolic BP, mmHg	114.67 ± 1.97	124.23 ± 2.43	127.93 ± 2.97	<.01	<.001	.55
Diastolic BP, mmHg	74.50 ± 1.33	81.03 ± 1.75	86.03 ± 1.65	<.01	<.001	<.05
Fasting glucose, mmol/L	5.89 ± 0.29	5.57 ± 0.24	5.73 ± 0.33	.30	.22	.63
Fructosamine, mmol/L	210.00 ± 10.67	223.33 ± 8.30	241.35 ± 13.06	.33	.07	.25
Total cholesterol, mmol/L	4.36 ± 0.24	5.01 ± 0.22	5.46 ± 0.29	<.05	<.01	.29
HDL-cholesterol, mmol/L	1.88 ± 0.11	1.57 ± 0.12	1.33 ± 0.12	<.05	<.001	.10
LDL-cholesterol, mmol/L	2.23 ± 0.23	3.01 ± 0.23	3.59 ± 0.25	<.02	<.001	.07
Triglycerides, mmol/L	0.76 ± 0.05	0.95 ± 0.07	0.99 ± 0.07	.10	<.05	.53

Values expressed as the mean±SEM. BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Results were compared using a Mann Whitney U-Test and P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

Experimental design

Subjects visited the Centre for Obesity Research on one occasion between 9-12am and were instructed to fast for 10-12 hours and refrain from exercise, caffeine and

alcohol intake for 48 hours before the study visit. Subjects were also asked to maintain their usual dietary pattern.

Anthropometric measurements

Height was measured without shoes using a stadiometer. Weight and body composition was assessed using the BodPod and waist measurements were also taken (see methodology section 3.2.1, 3.2.2, 3.2.3 and 3.2.4.2).

Cardiovascular measurements

Heart rate was measured at the radial artery and blood pressure was measured using a mercury sphygmomanometer (see methodology section 3.3).

Food diary

Dietary intake was monitored using a 7-day weighed food diary, which was subsequently analyzed for macro- and micro-nutrient content using dietary analysis software, Compeat (see methodology section 3.8).

Physical activity

Subjects completed a Baecke Physical activity questionnaire which was used to assess physical activity levels (see methodology section 3.9).

Venous blood sampling

Blood samples were collected following a 10-12hr fast using the syringe method (see methodology section 3.1). Blood samples were divided between EDTA and serum tubes. Samples were centrifuged (3000 rpm for 10 minutes), and then divided and stored at both –70°C and -20°C. For preparation of SOD and GSH assays, see methodology section 3.1.14.2 and 3.1.14.3, respectively.

Biochemical measurements included LH, TAS, SOD and GSH as described in the methodology chapter (section 3.1.12.1, 3.1.13.1, 3.1.13.2, 3.1.13.3). In addition fasting plasma glucose, plasma fructosamine, plasma cholesterol, plasma LDL, plasma HDL and plasma triglycerides was measured (see methodology section 3.1.6, 3.1.7, 3.1.8, 3.1.9, 3.1.10 and 3.1.11, respectively).

Statistical Analysis

Values reported as mean±SEM. SEM was appropriate because it demonstrates how liable to error the mean is (e.g. the mean value). The Kolmogorov-Smirno test confirmed that the data was not normally distributed so was analyzed by non-parametric methods to avoid assumptions about the distribution of the measured variables. The Kruskal Wallis analysis of variance test was used to compare groups and the Mann-Whitney *U*-Test was used to evaluate the differences between two independent groups. Associations between parameters were assessed using the Spearman rank correlation test. For multiple regression analysis, LH was log-transformed. Multiple regression analysis was conducted after examination of potential effect modifiers and variables were included if they were significantly associated with LH levels, $P<0.05$. Differences were considered statistically significant at $P<0.05$. Statistical analysis was performed using a computer software package (SPSS for Windows, Version 13.0).

4.2 RESULTS

Subject characteristics for the normal-weight, overweight and obese groups are shown in table 4.0. Body mass, body fat, waist circumference and resting diastolic blood pressure all progressively increased significantly ($P<0.01$) with higher BMI values. Other significant differences between groups with increasing BMI values include

increased resting systolic blood pressure (normal-weight vs overweight, P<0.01 and normal-weight vs obese, P<0.001), increased cholesterol (normal-weight vs overweight, P<0.05 and normal-weight vs obese, P<0.01), decreased HDL concentration (normal-weight vs overweight, P<0.05 and normal-weight vs obese, P<0.001), increased LDL concentration (normal-weight vs overweight, P<0.02 and normal-weight vs obese, P<0.001), increased triglycerides (normal-weight vs obese, P<0.05) and increased age (normal-weight vs obese, P<0.02). Fasting blood glucose and fructosamine levels were similar in all subjects.

Table 4.1 highlights the mean values for habitual physical activity and energy intake for the normal-weight, overweight and obese groups. Percentage carbohydrate intake progressively decreased with higher BMI values (Kruskall Wallis test, P<0.001) with the overweight and obese group demonstrating significantly lower percentage carbohydrate intake when compared to the normal-weight group (P<0.01 and P<0.001, respectively) [normal-weight vs overweight vs obese (48.41±0.80 vs 43.43±1.39 vs 43.19±1.19%)]. However no differences between groups were found when carbohydrate intake was expressed in grams. Percentage fat intake increased progressively with higher BMI values (Kruskall Wallis test, P<0.001) with the overweight and obese group demonstrating significantly increased percentage fat intake when compared to the normal-weight group (P<0.01 and P<0.001, respectively) [normal-weight vs overweight vs obese (31.25±0.73 vs 35.56±1.30 vs 37.14±0.99%)]. However when fat intake was expressed in grams, fat intake was only significantly greater in the obese group compared to the normal-weight group [normal-weight vs obese (60.42±2.94 vs 80.01±6.83g)]. Saturated, polyunsaturated and monosaturated fat intake expressed in grams all progressively increased with higher BMI values (Kruskall Wallis test, P<0.001, P<0.01, P<0.02, respectively) with the overweight and obese group demonstrating significantly increased saturated, polyunsaturated and

monosaturated fat intake when compared to the normal-weight group [overweight vs obese (P<0.001, P<0.02, P<0.02 and P<0.001, P<0.01, P<0.01, respectively)].

The mean values for LH, TAS, SOD and GSH for the normal-weight, overweight and obese groups (classified by BMI) are given in table 4.2. LH progressively increased with higher BMI values (Kruskall Wallis test, P=0.025) with the obese group demonstrating significantly higher LH levels when compared to the normal-weight group (0.86±0.05 vs 0.68±0.03µmol/L, P<0.01) but the increased level was not statistically significant when compared to the overweight group (0.86±0.05 vs 0.79±0.06µmol/L, P>0.05) (see table 4.2 and figure 4.0). No other significant differences in TAS, SOD and GSH were identified between groups for BMI (Kruskal Wallis test, P=0.70, P=0.93, P=0.14 respectively).

Further analyses was also carried out to identify the impact of waist circumference on LH, TAS, SOD and GSH levels (see table 4.3). Current guidelines suggest a cut-off of greater than 102 cm in men and 88 cm in women on the basis of causing increased risk of many metabolic risk factors such as hyperinsulaemia and hyperlipidaemia (Han *et al.*, 1995). Interestingly LH levels above the cut-off waist circumference guidelines were significantly higher compared to those below the cut-off waist circumference guidelines (0.84±0.04 vs 0.74±0.04µmol/L, P<0.05). However no significant differences in TAS, SOD and GSH were identified between these two groups.

Table 4.1. Mean values for habitual physical activity and dietary intake between normal-weight, overweight and obese groups.

Variable	Normal-Weight (NW) (n=30)	Overweight (OW) (n=30)	Obese (O) (n=30)	P value NW Vs OW	P value NW vs O	P value OW vs O
Baecke questionnaire	55.58 ± 2.13	53.83 ± 1.86	50.73 ± 1.69	.50	.06	.22
Total energy intake, kcals	1680.50 ± 68.80	1739.55 ± 79.06	1815.92 ± 86.56	.60	.27	.74
Carbohydrate, %	48.41 ± 0.80	43.43 ± 1.39	43.19 ± 1.19	<.01	<.001	.62
Carbohydrate, g	217.29 ± 10.56	198.29 ± 10.52	205.39 ± 12.19	.21	.57	.54
Fat, %	31.25 ± 0.73	35.56 ± 1.30	37.14 ± 0.99	<.01	<.001	.25
Fat, g	60.42 ± 2.94	70.99 ± 4.61	80.01 ± 6.83	.06	<.01	.44
Protein, %	16.92 ± 0.39	16.50 ± 0.58	16.64 ± 0.56	.22	.10	.99
Protein, g	69.62 ± 2.82	71.16 ± 3.85	73.52 ± 3.26	.72	.38	.54
Saturated fat, g	19.10 ± 1.52	25.40 ± 1.43	26.69 ± 2.01	<.001	<.001	.88
Polyunsaturated fat, g	8.45 ± 0.48	11.63 ± 0.94	12.21 ± 0.93	<.05	<.01	.62
Monosaturated fat, g	16.59 ± 0.97	21.25 ± 1.54	22.23 ± 1,57	<.05	<.01	.58
Alcohol, %	2.95 ± 0.41	4.23 ± 0.66	3.74 ± 0.77	.25	.98	.42
Alcohol, g	7.48 ± 1.10	10.70 ± 1.66	9.25 ± 1.87	.26	.81	.42
Selenium, g	37.51 ± 2.70	39.61 ± 3.04	42.42 ± 2.55	.77	.19	.16
Vitamin A, g	608.62 ± 63.62	745.60 ± 128.68	674.58 ± 73.43	.91	.68	.51
Vitamin C, g	79.26 ± 7.21	72.76 ± 6.42	77.93 ± 9.13	.57	.85	.91
Vitamin D, g	3.37 ± 0.55	2.94 ± 0.38	3.17 ± 0.30	.95	.26	.32
Vitamin E, g	6.54 ± 0.53	7.12 ± 0.54	7.47 ± 0.58	.45	.29	.68

Values expressed as the mean±SEM. Results were compared using a Mann Whitney U-Test and the P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

Table 4.2. Mean values for LH, TAS, SOD and GSH between normal-weight, overweight and obese groups.

Variable	Normal-Weight (NW) (n=30)	Overweight (OW) (n=30)	Obese (O) (n=30)	P value NW Vs OW	P value NW vs O	P value OW vs O
LH, µmol/L	0.68 ± 0.03	0.79 ± 0.06	0.86 ± 0.05	.26	<.01	.17
TAS, mmol/L	678.51 ± 31.22	698.51 ± 49.73	676.41 ± 55.53	.98	.43	.51
SOD, U/g Hb	3137.24 ± 161.74	3187.61 ± 170.49	3198.18 ± 178.72	.59	.92	.91
GSH, µmol/g Hb	14.31 ± 1.71	15.07 ± 1.64	11.2 ± 1.90	.80	.19	<.05

Values expressed as the mean±SEM. LH, lipid hydroperoxide; TAS, total antioxidant status; SOD, superoxide dismutase; GSH, reduced glutathione. Results were compared using a Mann Whitney U-Test and the P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

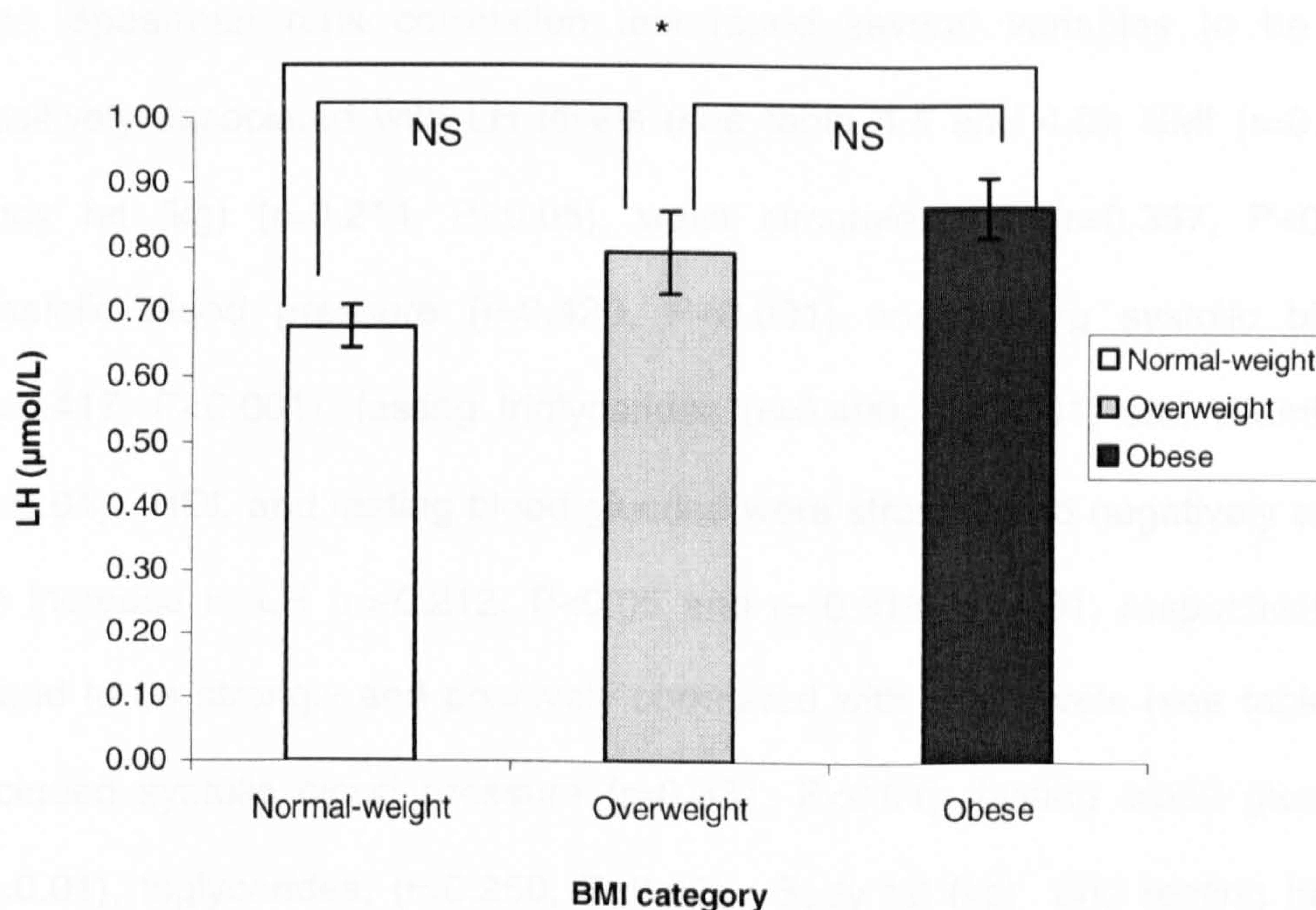


Figure 4.0. LH levels (µmol/L) in normal-weight, overweight and obese groups (classified by BMI). Results (mean±SEM) were compared using Mann-Whitney U Test. (*, P<0.01, NS: not significant). For further details see text.

Table 4.3. Mean values for LH, TAS, SOD and GSH according to waist circumference guidelines [cut-off points, reduced CVD risk (<88cm for women and <102cm for men) and increased CVD risk (>88cm for women and >102cm for men)].

Variable	Reduced CVD risk waist circumference (n=52)	Increased CVD risk waist circumference (n=38)	P-Value
LH, µmol/L	0.74 ± 0.04	0.84 ± 0.04	.025
TAS, mmol/L	692.28 ± 29.87	673.792 ± 48.58	.70
SOD, U/g Hb	3246 ± 128.91	3076.28 ± 148.96	.93
GSH, µmol/g Hb	14.80 ± 1.23	11.76 ± 1.71	.14

Values expressed as the mean±SEM. LH, lipid hydroperoxide; TAS, total antioxidant status; SOD, superoxide dismutase; GSH, reduced glutathione. Results were compared using a Mann Whitney U-Test and P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

The Spearman rank correlation test found several variables to be strongly and positively associated with LH levels (see table 4.4 and 4.5): BMI (r=0.320, P<0.01), body fat (kg) (r=0.214, P<0.05), waist circumference (r=0.357, P<0.001), resting diastolic blood pressure (r=0.420, P<0.001) and resting systolic blood pressure (r=0.417, P<0.001), fasting triglycerides (r=0.490, P<0.001) and vitamin A (r=0.272, P<0.01). HDL and fasting blood glucose were strongly and negatively associated with an increase in LH (r=-0.212, P<0.05 and r=-0.316, P<0.01, respectively). Variables found to be strongly and positively correlated with TAS levels (see table 4.4 and 4.5) included systolic blood pressure (r=0.331, P<0.01), fasting blood glucose (r=0.326, P<0.01), triglycerides, (r=0.250, P<0.02). Body fat (kg) and resting heart rate were strongly and negatively associated with an increase in LH (r=-0.227, P<0.05 and r=-0.212, P<0.05, respectively). As shown in table 4.5, vitamin C was shown to be strongly and positively correlated with SOD levels but vitamin A and triglycerides levels were shown to be strongly and negatively correlated with SOD levels (-0.270, P<0.01

and -0.288, P<0.01, respectively). GSH levels were found to be strongly and positively correlated with a number of variables including: fasting glucose (r=0.561, P<0.001), physical activity levels (r=0.332, P<0.001), total energy intake (kcals) (r=0.299, P<0.01), polyunsaturated fat (r=0.277, P<0.01), carbohydrates (r=0.372, P<0.001), protein (r=0.282, P<0.007), selenium (r=0.323, P<0.01) and vitamin E (0.372, P<0.001). However the following variables were shown to be strongly and negatively associated with GSH: body fat (kg) (r=-0.345, P<0.001), total cholesterol (r=-0.360, P<0.001), LDL-cholesterol (r=-0.307, P<0.01).

A multiple linear regression analysis was performed to quantify further the relationship between LH levels, subject characteristics, biochemical status, dietary intake and level of physical activity (see table 4.6). A significant model emerged ($F_{4,85}$=10.667, P<0.001) which revealed that 30.3% of the variance in LH was explained by the following variables; weight (standardized beta, 0.250, P<0.05), fasting glucose (standardized beta, -0.276, P<0.01), triglycerides (standardized beta, 0.234, P<0.05), vitamin A (standardized beta, 0.258, P<0.01).

Table 4.4. Correlation coefficients for the relationship between LH, TAS, SOD, GSH and BMI, body fat, waist circumference, blood pressure, fasting glucose, fructosamine and lipid profile in healthy subjects.

Variable	LH	TAS	SOD	GSH
	Regression coefficients (P Value)			
Weight, kg	.387 (.000)	.164 (.123)	-.093 (.386)	.025 (.812)
BMI, kg/m^2	.320 (<.01)	-.031 (.774)	-.043 (.689)	-.139 (.192)
Body fat, kg	.214 (<.05)	-.227 (.031)	.088 (.411)	-.345 (.001)
Waist circumference, cm	.357 (<.001)	.068 (.527)	-.206 (.051)	-.052 (.627)
Resting HR, beats/minute	.025 (.81)	-.212 (.045)	.118 (.268)	-.173 (.103)
Diastolic BP, mmHg	.420 (<.001)	.084 (.430)	-.092 (.388)	-.109 (.304)
Systolic BP, mmHg	.417 (<.001)	.331 (.001)	-.068 (.526)	.088 (.410)
Fasting glucose, mmol/L	-.316 (<.01)	.326 (.002)	-.124 (.244)	.561 (.000)
Fructosamine, mmol/L	-.119 (.437)	.087 (.570)	-.002 (.992)	-.138 (.366)
Total cholesterol, mmo/L	.165 (.12)	-.197 (.063)	.043 (.690)	-.360 (.000)
HDL-cholesterol, mmo/L	-.212 (<.05)	.081 (.449)	-.131 (.218)	.083 (.435)
LDL-cholesterol, mmo/L	.164 (.12)	-.197 (.063)	.094 (.380)	-.307 (.003)
Triglycerides, mmo/L	.490 (<.001)	.250 (.017)	-.288 (.006)	-.025 (.817)
TAS, mmo/L	.161 (.129)	1.000 (.)	-.231 (.029)	.477 (.000)
SOD, U/g Hb	-.129 (.224)	-.231 (.029)	1.000 (.)	-.080 (.456)
GSH, μmol/g Hb	-.059 (.580)	.477 (.000)	-.080 (.456)	1.000 (.)
LH, μmol/L	1.000 (.)	.067 (.531)	-.110 (.304)	-.049 (.649)

BMI, body mass index; HR, heart rate; BP, blood pressure; TAS, total antioxidant status; SOD, superoxide dismutase; GSH, reduced glutathione and LH, lipid hydroperoxide. Results were analyzed using the Spearman rank correlation test and P values are given in the Table. For further details see text.

Table 4.5. Correlation coefficients for the relationship between LH, TAS, SOD and GSH and lifestyle variables; physical activity levels, energy intake, dietary macro- and micro-nutrient composition in healthy subjects.

Variable	LH	TAS	SOD	GSH
	Regression coefficients (P Value)			
Baecke questionnaire	-.019 (.856)	.041 (.701)	-.115 (.280)	.332 (.001)
Total energy intake, kcals	-.021 (.842)	.272 (.010)	.096 (.370)	.299 (.004)
Fat, g	-.001 (.990)	.155 (.143)	.075 (.482)	.137 (.197)
Saturated, g	.087 (.415)	-.072 (.500)	.122 (.253)	-.025 (.813)
Poly, g	-.030 (.781)	.162 (.128)	-.018 (.864)	.277 (.008)
Mono, g	-.001 (.991)	.115 (.280)	.040 (.707)	.078 (.464)
Carbohydrate, g	-.023 (.827)	.216 (.041)	.096 (.370)	.372).000)
Protein, g	.059 (.581)	.348 (.001)	.092 (.390)	.282 (.007)
Alcohol, g	.098 (.358)	.322 (.002)	-.199 (.059)	.092 (.388)
Selenium, g	.166 (.118)	.269 (.010)	-.004 (.968)	.323 (.002)
Vitamin A, g	.272 (<.01)	-.007 (.945)	.196 (.064)	.032 (.768)
Vitamin C, g	-.045 (.675)	.143 (.178)	.243 (.021)	.203 (.055)
Vitamin D, g	.152 (.152)	.341 (.001)	-.270 (.010	.205 (.053)
Vitamin E, g	.036 (.733)	.206 (.051)	-.062 (.565)	.375 (.000)

g, weight. Results were analyzed using the Spearman rank correlation test and P values are given in the Table. For further details see text.

Table 4.6. Determinants of LH level in healthy subjects.

Variable	Beta	P value
Fasting glucose, mmol/L	-0.276	.003
Vitamin A, mmol/L	0.258	.007
Weight, kg	0.250	.014
Triglycerides, mmol/L	0.234	.023
Adjusted r^2	0.303	

Results were analyzed using the multiple regression analysis model (Enter model) and standardized beta and P values are given in the Table. For further details see text.

4.3 DISCUSSION

This study investigated the levels of LH, TAS, SOD and GSH in normal-weight, overweight and obese subjects. It is the first study to identify the impact of being overweight on oxidant stress parameters. This study also sought to identify physiological and lifestyle factors that may contribute to the normal variability of the overall oxidant stress status.

This study clearly identified that the level of LH was markedly higher in the obese group compared to the normal-weight group. The overweight group did not show a significant increase in LH levels when compared to the normal-weight group. No other consistent relationships between BMI, TAS, SOD and GSH were reported. Further analyses also showed that LH levels was markedly higher above the cut-off waist circumference guidelines for increased risk of CVD (women, >88cm and men, >102cm) compared to below the cut-off waist circumference guidelines. No other relationships between waist circumference, TAS, SOD and GSH were found.

In comparison to other research studies investigating obesity-associated oxidant stress, this study found similar findings. Increased markers of lipid peroxidation in obesity have been frequently observed in other human studies (Yesilbursa *et al.,* 2005; Keaney *et al.,* 2003; Ozata *et al.,* 2002; Davi *et al.,* 2002; Dandona *et al.,* 2001; Prázný *et al.,* 1999). Yesilbursa *et al* (2005), Dandona *et al* (2001) and Ozata *et al* (2002), recorded significantly higher levels of MDA and TBARS, respectively in obese adults compared to normal-weight controls [obese vs normal-weight (Yesilbursa *et al.,* 2005; MDA, 2.0±0.77 vs 0.63±0.14nmol/ml, Dandona *et al.,* 2001; TBARS, 1.68±0.17 vs 1.29±0.12μmol/L and Ozata *et al.,* 2002; TBARS, 7.77±3.41 vs 3.92±0.93mmol/ml)]. Keaney *et al* (2003) and Prázný *et al* (1999) concluded that BMI was independently

associated with increased levels of urinary concentrations of 8-iso $PGF_{2\alpha}$ in healthy individuals, and plasma MDA concentration in both Type I and Type II diabetics ($r=0.68$, $P<0.001$), respectively. Similarly, Davi *et al* (2002) found that obese women had high levels of urinary 8-iso $PGF_{2\alpha}$ compared to non-obese counterparts, which was also significantly higher in android (523 (393-685) pg/mg of creatine) than gynoid (275 (220-349) pg/mg of creatine) obesity compared to non-obese women (187 (140-225) pg/mg of creatine). The findings in this study also supports that android obesity increases LH levels because LH levels above the cut-off waist circumference guidelines for increased risk of CVD were significantly higher compared to those below the cut-off waist circumference guidelines (0.84±0.04 vs 0.74±0.04μmol/L, $P<0.05$). These findings are also confirmed by Keaney *et al* (2003) who found in a community-based cohort, the Framingham Heart Study, a strong association between markers of oxidant stress and both BMI and waist-hip ratio, implicating adiposity as the main factor for increased oxidant stress. On the contrary, Vincent *et al* (2004, 2005) highlighted no significant difference in both plasma TBARS and lipid hydroperoxide levels at rest between non-obese and obese counterparts. However the BMI levels in each of these studies were 24.9±2.5kg/m^2 (non-obese) vs 33.3±2.3kg/m^2 (obese) and 22.0±0.9kg/m^2 (non-obese) vs 28.0.3±1.2kg/m^2 (obese), respectively. This shows that the studies undertaken by Vincent *et al* (2004, 2005) did not include subjects with morbid obesity hence LH levels in the obese group were not significantly higher than the non-obese group. Although Vincent *et als* (2005) study did not intend to identify the impact of being overweight on oxidant stress levels (objective of the study was to identify the impact of obesity on exercise-induced oxidant stress), the study supports the finding that overweight subjects do not have significantly enhanced LH levels compared to normal-weight subjects. Interestingly, all those studies that show a significant increased LH response in obese subjects compared to normal-weight subjects have included subjects with a mean BMI of around 33-40kg/m^2 (Yesilbursa *et al.,* 2005;

Keany *et al.*, 2003; Ozata *et al.*, 2002; Davi *et al.*, 2002; Dandona *et al.*, 2001; Prázný *et al.*, 1999).

This study did not highlight any major significant differences in TAS, SOD and GSH levels between normal-weight, overweight and obese groups or between gynoid and android obesity, but other research studies have suggested otherwise. Olusi (2002) found that erythrocyte Cu/ZnSOD activity and GSH-Px were significantly lower in obesity than in the non-obese state (Cu/ZnSOD: 1005±26 vs 1464±23U/g Hb and GSH-Px: 84.3±6.7 vs 98.4±3.3U/g Hb). Similarly, Ozata *et al* (2002) also reported 75% and 42% lower erythrocyte GSH-Px and Cu/ZnSOD activities in obese men (n=76) than in non-obese men (n=24). In rat models of diet-induced obesity, erythrocyte SOD and GSH-Px activities were reduced by 29-42% in the HFHC group compared with the control animals after the 8-week diet-induced obesity period (Beltowski *et al.*, 2000). Other research have shown that individual antioxidant enzymes were enhanced in obesity (Vincent *et al.*, 2001, Dobrian *et al.*, 2000). Vincent *et al* (2001) found that Cu/ZnSOD activity in the left ventricles of rats was greater in the obese animals compared to lean controls (135 vs 117 IU/mg protein, $P<0.05$). Dobrian *et al* (2000) also reported increased activities of erythrocyte Cu/ZnSOD and GSH-Px after 10 weeks of diet-induced obesity. They attributed the increases in these erythrocyte cytoprotective enzymes to their stimulation by oxidant stress. Olusi (2002) believed the discrepancies in antioxidant enzymes in obesity could be due to the duration of the obesity. For example, in the early days of the development of obesity, antioxidant enzymes may be stimulated whereas chronic obesity continually depletes antioxidant enzymes. Other antioxidant measures such as TAS and FRAP have been shown to be lower in obese persons compared to non-obese persons (Lopes *et al.*, 2003; Fenkci *et al.*, 2003). For example, FRAP values were 22% lower in obese than in non-obese matched controls [obese vs non-obese (271±15 vs 333±29µmol/L, $P<0.05$) (Lopes *et*

al., 2003)] and TAS values were moderately lower in obese persons compared to non-obese persons [obese vs non-obese (1.15±0.01 vs 1.30±0.02mmol/L, P<0.001) (Fenkci *et al.,* 2003)]. It is unknown why this study did not demonstrate any significant differences between the normal-weight, overweight and obese groups for TAS, SOD and GSH levels. We can only speculate that it is possible that the obese group were averaging a transitional phase from developmental obesity to chronic obesity.

Several mechanisms have been suggested to explain obesity-associated oxidant stress, which include increased O_2 consumption, compromised antioxidant defence, cell injury/inflammation and increased fat deposition (Vincent *et al* 2001). In addition, hyperglycaemia, hypertension and hyperleptinaemia are also possible sources of increased oxidant stress in obesity (Vincent and Taylor, 2006). However, whether obesity-associated oxidant stress is a cause and effect relationship or due to obesity-related diseases i.e. hypertension, hyperlipidaemia, hyperleptinaemia and hyperglycaemia (Facchini *et al* 2000) remains unclear. Since all the subjects in this study were healthy (free of obesity-related diseases), it is possible that obesity in this study or other unknown factors caused increased oxidant stress. However, this study did not intend to identify the primary pathways linking obesity with increased oxidant stress levels.

The study did set out to identify physiological and lifestyle factors that may contribute to the normal variability of the overall oxidant stress status in healthy subjects. To date, it is thought that only two studies (Keaney *et al.*, 2003; Lesgards *et al.,* 2002) have investigated the relationships between intrinsic, lifestyle and environmental factors and oxidant stress parameters. Since LH levels were shown to be significantly greater in the obese group compared to both the normal-weight and overweight-group, it seemed important to discuss potential physiological and lifestyle factors that may contribute to

the normal variability of LH levels in healthy subjects. Increased LH levels were positively associated with BMI, body fat, waist circumference, resting systolic blood pressure, resting diastolic blood pressure, triglycerides and vitamin A. In contrast, fasting glucose and HDL were negatively correlated with LH levels. Calorie intake, dietary composition intake and physical activity levels were not shown to correlate with LH levels. However, Lesgards *et al* (2002) assessed lifestyle factors in relation to overall antioxidant capacity and emphasised that non-smoking, vitamin and mineral supplementation, regular physical activity, high fruit consumption were all positively related to the individual antioxidant capacity. Keaney *et al* (2003) identified the clinical conditions associated with oxidant stress. In an age-adjusted model, increased urinary creatinine-indexed 8-epi-$PGF_{2\alpha}$ was positively associated with female sex, hypertension treatment, smoking, diabetes, blood glucose, body mass index and a history of cardiovascular disease. Age and total cholesterol were negatively correlated with urinary creatinine-indexed 8-epi-$PGF_{2\alpha}$. The main differences in findings in this study compared to Keaney and colleagues and Lesgards and colleagues include the negative association between fasting blood glucose and LH levels and the lack of association between lifestyle and nutritional intake with LH levels. The variables found to be associated with LH in this study are discussed below, alongside other observational and interventional studies.

This study and others (Yesilbursa *et al.*, 2005; Keaney *et al.*, 2003; Ozata *et al.*, 2002; Davi *et al.*, 2002; Dandona *et al.*, 2001; Prázný *et al.*, 1999) have confirmed that a high BMI, waist circumference and body fat levels are associated with increased oxidant stress levels. The possibility of the obesity-associated oxidant stress has already been discussed. Both a high level of diastolic and systolic blood pressure was shown to be positively associated with increased LH levels in this study. However although Reckelhoff *et al* (2000) found a positive association between hypertension and LH in

hypertensive rats, this was not supported by Keaney *et al* (2003) in healthy men and women. But the lack of association between LH and hypertension in the study by Keaney *et al* (2003) may be a result of a third of subjects receiving antihypertensive therapy, contributing to the discrimination of BP as a correlate of oxidant stress (Keaney *et al.,* 2003). Other research suggests that the association of hypertension with oxidant stress only applies to certain causes of hypertension, such as those with high renin levels (Laursen *et al.,* 1997) or salt sensitivity (Somers *et al.,* 2000).

In this study triglyceride levels were positively associated with LH levels. Similar findings were found by Bae *et al* (2001) who investigated the effect of postprandial hypertriglyceridemia in healthy individuals on endothelial function as related to enhanced oxidant stress, by measurement of vascular endothelial function and leukocyte $O_2^{\cdot -}$ production. In response to hypertriglyceridemic stimuli (high fat meal), $O_2^{\cdot -}$ formation by leucocytes correlated with the degree of hypertriglyceridemia. In addition Hiramatsu and Arimori (1998) found that monocytes and polymorphonuclear cells released more $O_2^{\cdot -}$ when exposed to plasma from hypertriglyceridaemic patients.

Vitamin A was positively associated with LH levels in this study which supports epidemiological observational studies that low levels of vitamins A, E and C are associated with increased oxidant stress and CVD (Meagher, 2003; Dagenais *et al.,* 2000). However Block *et al* (2002) observed a significant inverse association between plasma vitamin A levels and malondialdehyde and F_2-isoprostanes in healthy adults. Other investigators have also reported that plasma vitamin A is inversely associated with smoking (Ayaroi *et al.*, 2000; Food and Nutrition Board, Institute of Medicine, 2000). According to Chertow (2004) under some conditions, a vitamin may have oxidant effects. Giving too much of one vitamin in an altered redox environment may change its effect from an antioxidant to a pro-oxidant (Olson, 1996). Very little cross-

sectional population research on vitamin A and oxidative damage has been carried out, so arising to conclusions regarding vitamin A levels and oxidant stress is difficult. In view of this it would seem prudent not to supplement diets with vitamin A or carotene unless levels are pathologically low (Chertow, 2004).

Research has indicated that hyperglycaemia is associated with increased levels of 8-epi-PGF$_2\alpha$ (Keaney *et al.*, 2003; Davi *et al.*, 1999; Gopaul *et al.*, 1995) and LHs (Nourooz-Zadeh *et al.*, 1995). Similarly, urinary levels of 8-epi-PGF$_2\alpha$ are increased in patients with type I and II diabetes and decrease significantly with aggressive control of hyperglycemia (Davi *et al.*, 1999). Several mechanisms have been proposed to explain the link between hyperglycaemia and lipid peroxidation. Glucose may combine directly with LDL phospholipids or apo B lysine groups to form advanced glycation end products that facilitate lipid peroxidation (Bucala *et al.*, 1993). In addition, auto-oxidation of glucose and nonenzymatic glycation of proteins may also generate $O_2^{\cdot-}$ (Baynes, 1991). Hyperglycaemia also induces the enzymatic production of $O_2^{\cdot-}$ through activation of NAD(P)H oxidase in vascular cells (Inoguchi *et al.*, 2000). In this study, fasting blood glucose was negatively associated with LH levels of which is puzzling because hyperglycaemia is a well-known oxidant (Keaney *et al.*, 2003; Davi *et al.*, 1999; Gopaul *et al.*, 1995). However because the majority of subjects in this study had normal fasting glucose levels, it is possible that we lost the discriminatory value of fasting blood glucose as a correlate of oxidant stress.

HDL levels was also found to be negatively associated with LH levels which may be a result of an underlying mechanism linking obesity to oxidant stress, itself in turn contributing to obesity-related disease. For example, it appears that the formation of oxidised cholesterol as a result of lipid peroxidation leads ultimately to a decrease in HDL production (Ozata *et al.*, 2002).

Finally, it was surprising that no association was found between LH levels, dietary intake and physical activity. Lesgards *et al* (2002) concluded that regular physical activity and high fruit consumption was positively related to individual antioxidant capacity. It is speculated that no association was found because the study population across the normal-weight, overweight and obese groups did not record wide differences in physical activity and nutritional habits. This could be because the 7-day food diary and physical activity questionnaire did not reflect true eating and lifestyle habits of the subjects.

To define the relationship further between LH and indices of physical and biochemical factors, a multiple regression analysis was performed. The results demonstrated that 30.3% of the variance in LH could be explained by the following variables, namely weight (standardized beta, 0.250, $P<0.05$), fasting glucose (standardized beta, -0.276, $P<0.01$), triglycerides (standardized beta, 0.234, $P<0.05$) and vitamin A (standardized beta, 0.258, $P<0.01$). All these (weight, fasting glucose, triglycerides and vitamin A) have been discussed above in relation to their effects on LH levels. Further research should aim to examine other potential factors which could lead to enhanced lipid peroxidation in obesity.

4.4 CONCLUSION

This study is the first to identify that LH levels are not enhanced in subjects who are overweight compared to normal-weight subjects. Similar to other research LH levels were markedly higher in the obese group compared to the normal-weight group. In addition, independent of BMI, LH levels were enhanced in subjects who had a waist circumference above the cut-off waist circumference guidelines for increased risk of CVD compared to below the cut-off waist circumference guidelines. The above changes could not be related to either TAS, SOD or GSH because these were reported to remain unchanged. Following multiple regression analysis, 30.3% of the variance in LH could be explained by a negative association with fasting plasma glucose, and positive associations with dietary vitamin A intake, weight and plasma triglycerides. Care should be taken when interpreting the associations between LH and vitamin A and LH and fasting blood glucose as other studies have shown a negative association between LH and vitamin A and a positive association between LH and fasting blood glucose.

Chapter FIVE

Study 2

Platelet responsiveness in healthy normal-weight, overweight and obese subjects: Association with lipid peroxidation and total antioxidant status.

5.0 INTRODUCTION

Central obesity is a risk factor for atherosclerotic CVD (Grundy *et al.*, 2002; McGill *et al.*, 2002), which interferes with a balance between blood coagulation and fibrinolysis, leading to increased thrombogenesis or platelet aggregation (Davi *et al.*, 2002; Haszon *et al.*, 2002; Juhan *et al.*, 1980). For example Davi *et al* (2002) found that gynoid obesity (n=25) increased 11-dehydrothromboxane B_2 compared to non-obese control values (n=24) ($P<0.001$), which was even greater in android obesity (n=24) when compared to non-obese subjects ($p<0.001$) (gynoid: 610 (421-759) android: 948 (729-1296) and non-obese 215 (184-253) pg/mg of creatine). Furthermore, android obesity had significantly higher 11-dehydrothromboxane B_2 when compared to gynoid obesity ($p<0.001$). Haszon *et al* (2002) investigated platelet aggregation (measured using collagen as an agonist with a laser rheoaggregometer) in hypertensive non-obese and obese children and age-sex matched non-obese and obese children. Hypertensive children in the non-obese and obese group and the normotensive obese children showed increased platelet aggregation. A significant positive correlation was also observed between the BMI values and percentage platelet aggregation in overweight children with or without hypertension ($r=0.501$, n=35, $P<0.01$). Juhan *et al* (1980) studied platelet aggregation in 81 obese subjects in relation to fat mass and fat distribution. Results of this work showed that platelet activity was not related to body weight and displayed no correlation or a slightly negative one to fat mass excess. However, platelet activity was significantly increased in cases where obesity predominated in the upper body. Alternative measures of platelet activity such as mean platelet volume have been shown to be higher in obese subjects (Coban *et al.*, 1992), which may also be an indicator of platelet activation (Park *et al.*, 2002).

Various risk factors for atherosclerosis and cardiovascular thrombosis such as diabetes mellitus, hypertension and hypercholesterolemia are associated with ROS-mediated platelet aggregation (Davi *et al.,* 2003; Minuz *et al.*, 2002; Davi *et al.,* 1997) which suggests that increased oxidant stress in obesity may contribute to persistent platelet aggregation. In support of this, Davi *et al* (2002) noted a significant correlation between urinary excretion rate of 8-iso $PGF_{2\alpha}$ and 11-dehydro-TxB_2 in gynoid and android obesity (r=0.61, P<0.001). Furthermore following multiple regression analysis, CRP level was found to predict the highest rates of both lipid peroxidation and platelet activation. To characterise the cause and effect of obesity on 8-iso $PGF_{2\alpha}$ and 11-dehydro-TxB_2 further, Davi *et al* (2002) examined the effects of a short-term weight loss program on changes in urinary 8-iso $PGF_{2\alpha}$ and 11-dehydro-TxB_2 in 20 android obese women. Following successful weight loss (15.3±10.5kg) the rates of 8-iso $PGF_{2\alpha}$ and 11-dehydro-TxB_2 excretion were significantly reduced, by 32% and 54% respectively. Changes in urinary 11-dehydro-TxB_2 excretion correlated with the amount of weight loss (r=0.67, P=0.02) and the values of thromboxane metabolite excretion fell within the reference range by the end of the study. Therefore, increased oxidant stress in obesity maybe a mechanism linking obesity-associated oxidant stress with platelet activity. Apart from Davi *et al* (2002), no other study has observed an association between oxidant stress and platelet aggregation in obesity.

Limited evidence is available on the relationship between obesity, platelet aggregation and oxidant stress which may be due to the problems associated with measuring platelet aggregation. For example 'the gold standard measure of platelet aggregation' is platelet aggregometry which is both labour intensive and expensive (Lau *et al.*, 2002). In addition the reproducibility of results is dependent on controlling many factors: temperature, pH, aggregometer stir speed, platelet count, sample volume and venepuncture technique (White and Jennings, 1999). In this study, a new direct

measure of platelet aggregation is used. PlateletWorks® is an adaptation of platelet aggregometry and measures platelet aggregation in whole blood, which is devoid of long laborious techniques. The PlateletWorks® has been evaluated by Lau *et al* (2002) who found that this novel-bed-side assay of platelet function was extremely suitable for the clinical environment (acute care settings of percutaneous coronary intervention and cardiopulmonary bypass). In addition PlateletWorks® was evaluated against platelet aggregometry in a preliminary experiment as part of this thesis, which demonstrated that PlateletWorks® was a cost-effective, quick, valid and reproducible measure of platelet aggregation.

This study aimed to identify the effect of BMI (normal-weight, overweight, obese) on platelet count and percentage ADP-induced platelet aggregation. In addition the study aimed to identify whether increased oxidant stress in obesity was a determinant of platelet aggregation levels in obesity.

5.1 METHODOLOGY

Subject Characteristics

The study group consisted of 45 subjects who were sex-matched between groups [15 normal-weight (BMI: 22.4±0.34kg/m^2), 15 overweight (BMI: 25.5±0.20kg/m^2) and 15 obese (BMI: 31.8±1.22kg/m^2) healthy male and females (n=18/27)] (see table 5.0 for subject characteristics). Volunteers were invited to take part in the study by local advertisement. An inclusion criterion was age between 18 and 50 years old and BMI between 20 and 40kg/m^2. Subjects with a history of diabetes, cardiovascular or cerebrovascular disease, hepatic or renal disease, tobacco abuse, or those on hormone replacement therapy were excluded. In addition subjects were excluded if they were

hypertensive (with or without treatment), taking treatment for dyslipidaemia, taking any antioxidant supplementations or a smoker. Written informed consent was obtained from all the subjects after they had been given a full explanation of the study. The research was given ethical approval by Bedfordshire Local Research Ethics Committee.

Table 5.0. Subject characteristics

Characteristic	Normal-weight (NW) (n=15) (m/f; 6/9)	Overweight (OW) (n=15) (m/f; 6/9)	Obese (O) (n=15) (m/f; 6/9)	P Value NW vs OW	NW vs O	OW vs O
Age, yr	33.20 ± 2.40	36.47 ± 2.34	35.53 ± 2.05	.33	.519	.663
BMI, kg / m^2	22.38 ± 0.34	25.48 ± 0.20	31.75 ± 1.22	<.001	<.001	<.001
Body mass, kg	62.2 ± 1.60	73.7 ± 2.14	91.8 ± 4.09	<.001	<.001	<.001
Body fat, kg	17.79 ± 1.39	20.88 ± 1.76	33.58 ± 3.15	.213	<.001	<.01
Waist, cm	74.98 ± 1.32	82.44 ± 2.31	99.11 ± 2.92	<.02	<.001	<.001
Systolic BP, mmHg	111.93 ±2.27	123.13 ± 3.25	128.73 ± 3.19	<.01	<.001	.309
Diastolic BP, mmHg	74.2 ± 2.10	79.93 ± 2.41	83.67 ± 1.51	.177	<.01	<.05
Fasting glucose, mmol/L	6.80 ± 0.41	5.80 ± 0.35	7.49 ± 0.65	.110	.709	<.05
Fructosamine, mmo/L	226.34 ± 18.58	220.92 ± 11.79	202.38 ± 13.83	.487	.251	.457
Total cholesterol, mmo/L	4.45 ± 0.24	5.19 ± 0.36	4.95 ± 0.23	.146	.178	.950
HDL-cholesterol, mmol/L	1.72 ± 0.10	1.63 ± 0.14	1.37 ± 0.16	.520	.068	.350
LDL-cholesterol, mmol/L	2.41 ± 0.25	3.18 ± 0.33	3.05 ± 0.26	.120	.110	.950
Triglycerides, mmol/L	0.70 ± 0.05	0.83 ± 0.07	1.17 ± 0.19	.130	<.02	.237

Values are expressed as the mean±SEM. BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Results were compared using a Mann Whitney U-Test and P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

Experimental design

Subjects visited the Centre for Obesity Research on one occasion between 9-12am and were instructed to fast for 10-12 hours and refrain from exercise, caffeine and alcohol intake for 48 hours before the study visit. Subjects were also told to maintain their usual dietary regimen.

Anthropometric and cardiovascular measurements and venous blood sampling.

Height, weight and waist measurements are described in detail in the methodology chapter (section 3.2.1, 3.2.2, 3.2.3). Body composition was assessed using the BodPod which is outlined in the methodology chapter (section 3.2.3.1). Cardiovascular measurements and venous blood sampling are described in the methodology chapter (section 3.3, 3.1.1 and 3.1.2).

Biochemical measurements

LH, TAS, SOD and GSH were performed as described in the methodology chapter (section 3.1.12 and 3.1.13). Other biochemical measures included, fasting plasma glucose, plasma fructosamine, plasma cholesterol, plasma LDL, plasma HDL and plasma triglycerides as described in the methodology chapter (section 3.1.6.1, 3.1.7, 3.1.8, 3.1.9, 3.1.10 and 3.1.11). ADP-induced percentage platelet aggregation was also measured using PlateletWorks®, which is outlined in the methodology chapter (section 3.1.14.2). Final concentrations of ADP used were 10 and 20μM.

Statistical Analysis

Values reported as mean±SEM. SEM was appropriate because it demonstrates how liable to error the mean is (e.g. the mean value). The Kolmogorov-Smirno test

confirmed that the data was not normally distributed so was analyzed by non-parametric methods to avoid assumptions about the distribution of the measured variables. The Kruskal Wallis analysis of variance test was used to compare groups and the Mann-Whitney *U*-Test was used to evaluate the differences between two independent groups. Associations between parameters were assessed by the Spearman rank correlation test. P-values < 0.05 were regarded as statistically significant. Analyzes of data was performed out using a computer software package (SPSS for Windows, Version 13.0).

5.2 RESULTS

Subject characteristics for the normal-weight, overweight and obese groups are shown in table 5.0. Body mass and waist circumference all progressively increased significantly (P<0.05) with higher BMI values. Body fat (kg) was significantly greater in the obese group when compared to both the normal-weight (P<0.001) and overweight (P<0.01) groups but no significant difference in body fat (kg) was found between the normal-weight and overweight group. Blood pressure increased incrementally with higher BMI values but significant differences were only identified in resting diastolic blood pressure between the normal-weight and obese, (P<0.01) and overweight and obese (P<0.05) group and in resting systolic blood pressure between the normal-weight and overweight, (P<0.01) and normal-weight and obese (P<0.001) group. Other significant differences between groups with increasing BMI values included increased triglycerides (normal-weight vs obese, P<0.02) and increased fasting blood glucose values (overweight vs obese, P<0.05). All subjects had similar fructosamine levels, total cholesterol, LDL and HDL levels.

Mean values for LH, TAS, SOD and GSH for the normal-weight, overweight and obese groups are shown in table 5.1. LH increased with higher BMI values with the obese group demonstrating an increased trend towards higher LH levels compared to the normal-weight group [obese vs normal-weight (0.97±0.12 vs 0.67±0.58μmol/L, P=0.078)] and overweight group [obese vs overweight (0.97±0.12 vs 0.68±0.49μmol/L, P<0.078)]. TAS levels were also increased with higher BMI values with the obese group demonstrating significantly higher TAS levels when compared to the normal-weight group [normal-weight vs obese (661.42±34.24 vs 817.67±57.93mmol/L, P<0.05)]. No other significant differences were noted between groups in TAS, SOD and GSH.

Table 5.1. LH, TAS, SOD and GSH values observed in the normal-weight, overweight and obese groups.

Variable	Normal-Weight (NW) (n=15)	Overweight (OW) (n=15)	Obese (O) (n=15)	NW Vs OW	NW Vs O	OW vs O
LH, μmol/L	0.67 ± 0.58	0.68 ± 0.49	0.97 ± 0.12	.756	.078	.078
TAS, mmol/L	661.42 ± 34.24	699.67 ± 48.29	817.67 ± 57.93	.604	<.05	.101
SOD, U/g Hb	3427.22 ± 259.35	3507.67 ± 247.95	2890.86 ± 175.77	.738	.206	.105
GSH, μmol/g Hb	15.33 ± 1.72	16.55 ± 2.25	16.67 ± 1.58	.648	.868	.967

Values are expressed as the mean±SEM. LH, lipid hydroperoxide, TAS, total antioxidant status, SOD, superoxide dismutase, GSH, reduced glutathione. Results were compared using a Mann Whitney U-Test and P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

The mean values for platelet count and percentage ADP-induced platelet aggregation for the normal-weight, overweight and obese groups are given in table 5.2 and figure

5.0. No significant differences were noted between normal-weight, overweight and obese groups in platelet count. But the degree of percentage ADP-induced platelet aggregation decreased with increasing BMI values at both ADP final concentrations of 10 and 20μM. ADP-induced platelet aggregation with 20μM ADP significantly decreased the degree of platelet aggregation in the obese group when compared to both the normal-weight and overweight group [obese vs normal-weight (80.07±2.18 vs 89.33±0.89%, $P<0.001$) obese vs overweight: (80.07±2.18 vs 85.47±1.51%, $P<0.05$)]. ADP-induced platelet aggregation with 10μM ADP produced a significant decrease in the degree of platelet aggregation in the obese group when compared to the normal-weight group and a decreased non-significant trend when compared to the overweight group [obese vs normal-weight (65.27±2.66 vs 75.2±2.21%, $P<0.05$) obese vs overweight (65.27±2.66 vs 72.53±2.29%, $P=0.064$)].

Further analyses was also carried out to identify the impact of waist circumference on platelet count and percentage ADP-induced platelet aggregation (see table 5.3). Current guidelines suggest a cut-off greater than 102cm in men and 88cm in women on the basis of causing increased risk of many metabolic risk factors such as hyperinsulaemia and hyperlipidaemia (Han *et al.*, 1995). ADP-induced platelet aggregation with 20μM ADP produced a significant decrease in the degree of platelet aggregation above the cut-off waist circumference guidelines compared to below the cut-off waist circumference guidelines (81.27±2.28 vs 86.80±1.02%, $P<0.05$). However no significant differences were found between the two groups for percentage ADP-induced platelet aggregation with 10μM ADP and platelet count.

The Spearman rank correlation test found that both LH and TAS were not associated with either platelet count and ADP-induced platelet aggregation (final ADP concentration, 10μM and 20μM) (see table 5.4).

Table 5.2. Platelet count and percentage ADP-induced platelet aggregation (final ADP concentration, 10μM and 20μM) in the normal-weight, overweight and obese groups.

Variable	Normal-Weight (NW) (n=15)	Overweight (OW) (n=15)	Obese (O) (n=15)	NW s OW	NW Vs O	OW vs O
Platelet count	224.40 ± 13.2	205.57 ± 12.47	230.00 ± 10.54	.455	.819	.229
10μM	75.27 ± 2.21	72.53 ± 2.29	65.27 ± 2.66	.454	<.05	.064
20μM	89.33 ± 0.89	85.47 ± .1.51	80.07 ± 2.18	.076	<.001	<.05

Values are expressed as the mean±SEM. 10 and 20μM, Final concentrations of ADP-induced platelet aggregation. Results were compared using a Mann Whitney U-Test and P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

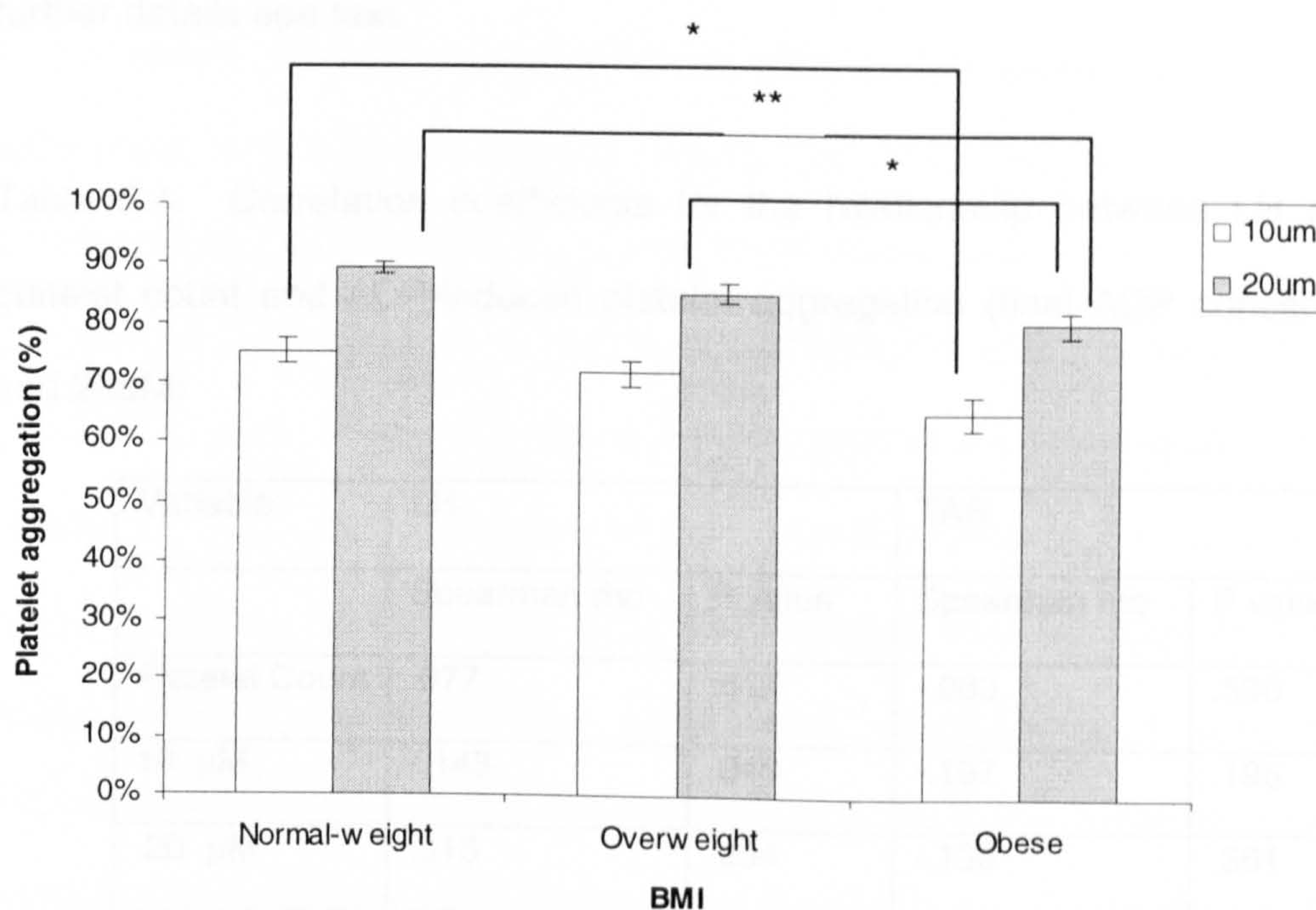


Figure 5.0. Percentage ADP-induced platelet aggregation (10μM and 20μM) in the normal-weight, overweight and obese groups. Results (mean±SEM) were compared using Mann-Whitney U Test. (*, P<0.05, **, P<0.01). For further details see text.

Table 5.3. Platelet count and percentage ADP-induced platelet aggregation according to waist circumference guidelines [cut-off points, reduced CVD risk (<88cm for women and <102cm for men) and increased CVD risk (>88cm for women and >102cm for men)].

Variable	Reduced CVD risk waist circumference (n=30)	Increased CVD risk waist circumference (n=15)	NW Vs OW
Platelet count	215. 08 ± 9.3	229.80 ± 9.46	.263
10μM	73.10 ± 1.66	66.87 ± 2.79	.162
20μM	86.80 ± 1.02	81.27 ± 2.28	.027

Values are expressed as the mean±SEM. 10 and 20μM, Final concentrations of ADP-induced platelet aggregation. Results were compared using a Mann Whitney U-Test and P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

Table 5.4. Correlation coefficients for the relationship between LH and TAS with platelet count and ADP-induced platelet aggregation (final ADP concentration, 10μM and 20μM)

Variable	LH		TAS	
	Spearman rho	P value	Spearman rho	P value
Platelet Count	.077	.617	-.083	.590
10 μM	-.143	.348	-.197	.195
20 μM	.013	.934	-.139	.361

0 and 20μM, Final concentrations of ADP-induced platelet aggregation. Results were analyzed using the Spearman rank correlation test and P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

5.3 DISCUSSION

It is well established that the risk of CVD is increased with the degree of obesity and abdominal obesity (Coleman *et al.*, 1992) and oxidant stress-mediated platelet aggregation is a mechanism through which obesity may affect CVD morbidity and mortality (Davi *et al.*, 2002). This study examined the effect of BMI (normal-weight, overweight and obese) on platelet count and percentage ADP-induced platelet aggregation. In addition the study investigated whether increased oxidant stress in obesity is a determinant of percentage ADP-induced platelet aggregation.

The study group consisted of 45 healthy subjects normal-weight, overweight and obese subjects. Although not significant, LH levels were higher in the obese group compared to the normal-weight group and overweight group. TAS levels were significantly higher in the obese group compared to the normal-weight group but no other significant differences were noted between groups in TAS, SOD and GSH. It was surprising that LH levels were not significantly higher in the obese group, but this could be attributed to variable LH levels as a result of sampling handling and/or storage duration (Södergren et al., 1998). For example Södergren *et al* (1998) found that the storage of plasma at -70°C was associated with a variable degree of loss of detectable plasma hydroperoxides. In this study, data was collected over a period of 6-months so the initial samples collected may have been affected by a loss of detectable hydroperoxides. Alternatively, the reduced LH levels in the obese group may be due to a reduced number of morbid obese subjects as their inclusion is more likely to increase mean LH levels (Yesilbursa *et al.*, 2005; Keany *et al.*, 2003; Ozata *et al.*, 2002; Davi *et al.*, 2002; Dandona *et al.*, 2001; Prázný *et al.*, 1999).

No differences in platelet count was identified between the normal-weight, overweight and obese groups. Percentage ADP-induced platelet aggregation decreased with increasing BMI values at both ADP final concentrations of 10 and 20μM with the obese group demonstrating significantly reduced ADP-induced platelet aggregation compared to the normal-weight group. These results suggest reduced platelet *in vitro* aggregability in obese subjects, perhaps due to reduced sensitivity of receptor sites to specific agonists. The decreased sensitivity to ADP in this study is an expression of enhanced *in vivo* platelet reactivity, consequent to a release of intraplatelet aggregating substances causing a state of platelet exhaustion (Tozzi-Ciancarelli *et al.*, 1997). Further analyses also showed that percentage ADP-induced platelet aggregation with 20μM ADP was significantly reduced in subjects with a greater waist circumference (above the cut-off waist circumference guidelines for increased risk of CVD, women, >88cm and men, >102cm) compared to subjects with a waist circumference below the cut-off waist circumference guidelines. No other significant relationships between waist-circumference and platelet count and percentage ADP-induced platelet aggregation with 10μM ADP were found. Correlation analysis found no significant association between LH and percentage ADP-induced platelet aggregation and TAS and percentage ADP-induced platelet aggregation, which suggests that both LH and TAS are not determinants of platelet aggregation.

This is the first study to demonstrate the impact of a range of BMI groups and waist circumference (normal-weight, overweight obese) on percentage ADP-induced platelet aggregation. In particular the impact of being overweight on obesity has not been studied and it is reassuring that being overweight does not enhance *in vivo* platelet reactivity. The finding that obesity does enhance platelet aggregation is in agreement with several other studies (Juhan *et al.*, 1980; Davi *et al.*, 2002; Haszon *et al.*, 2003). Juhan *et al* (1980) found that platelet activity was significantly related to android

obesity. Davi *et al* (2002) found that obesity increased 11-dehydrothromboxane B_2 (a marker of *in vivo* platelet activation), which was further enhanced in subjects with android obesity [non-obese vs gynoid obesity vs android obesity (187 (140-225) vs 275 (220-349) vs 523 (393-685)] (non-obese vs gynoid obesity ($P<0.001$), non-obese vs android obesity ($P<0.001$) and gynoid obesity vs android obesity ($P<0.001$). Haszon *et al* (2003) concluded that both normal-weight and obese hypertensive and obese nonotensive children demonstrated increased platelet aggregation (measured using collagen as an agonist with a laser rheoaggregometer) which contributed to the development of hypertension and to the promotion of vascular damage. Haszon *et al* (2003) also found a significant correlation between BMI vales and platelet aggregation in overweight children with and with out hypertension ($r=0.51$, $n=35$, $P<0.01$).

The increased platelet aggregation in obesity may be explained by the reduced sensitivity towards antiaggregating agents (Trovati *et al.*, 1994; Trovati *et al.*, 1996; Vinik *et al.*, 2001). For example insulin has been shown to reduce platelet responses to agonists by activating the NO/cyclic nucleotide pathway, but because insulin responses are often blunted in obese subjects (Trovati *et al*, 1995), platelet responses to agonists are increased. Obese subjects also demonstrate platelet resistance to the NO donor glyceryl trinitrate (GTN) (Anfossi *et al.*, 1998a) and to adenosine (Anfossi *et al.*, 1998b) and show an impaired platelet deposition to collagen when submitted to euglycaemic insulin infusion *in vivo* (Westerbacka *et al.*, 2002). The resistance to the agents noted above act via the anti-aggregating cyclic nucleotides, cAMP and cGMP, the main mediators of platelet anti-aggregation (Geiger, 2001). They act via specific protein kinases (Waldman *et al.*, 1987; Butt *et al.*, 1994) to block several steps of the agonist-induced elevation of cytosolic Ca^{2+} (Yamanishi *et al.*, 1983; Kawahara *et al.*, 1984), which is a basic mechanism of platelet activation (Rink and Sage, 1990). It is known that plasma levels of β-thromboglobulin, platelet factor-4, P-selectin, and the

surface expression of lysosomal or granule proteins also play a role in platelet activation (Haas *et al.*, 1999) but the interaction between these factors and obesity has not yet been extensively studied and elucidated.

Despite observing increased *in vivo* platelet reactivity and LH levels in obesity, this study found no association between oxidant stress and percentage ADP-induced platelet aggregation suggesting that oxidant stress does not mediate platelet aggregation. This was surprising because other research studies support that oxidant stress participates in the regulation of platelet activation (Krotz *et al.*, 2004). Oxidant stress-mediated platelet aggregation has been found in several settings of risk factors for atherosclerosis and cardiovascular thrombosis, including diabetes mellitus, hypertension and hypercholesterolemia (Davi *et al.*, 2003; Minuz *et al.*, 2002; Davi *et al.*, 1997). Davi *et al* (2003) examined whether 8-iso-PGF_2 formation (index of *in vivo* lipid peroxidation) correlates with the rate of urinary 11-dehydro-TXB_2 (index of *in vivo* platelet activation) excretion in newly diagnosed diabetic children and adolescents and children and adolescents with longerstanding disease. Newly diagnosed diabetic patients had significantly higher urinary 8-iso-PGF_2 excretion and urinary 11-dehydro-TXB_2 excretion than patients with longerstanding disease ($P<0.05$). In addition a statistically significant linear correlation was found between 8-iso-PGF_2 and 11-dehydro-TXB_2 excretion rates in both groups of diabetic patients (newly diagnosed and longerstanding disease groups) ($r=0.73$, $P=0.0001$ and $r=0.70$, $P=0.001$, respectively). Davi *et al* (2003) concluded that the enhanced lipid peroxidation and platelet activation in newly diagnosed diabetic patients may be related to an acute imflammatory response. For example Davi *et al* (2003) found that patients with the shortest duration of disease and with the highest IL-6 had the highest rates of *in vivo* lipid peroxidation and platelet activation. However IL-6 variability is only likely to explain a small percentage of the variability in lipid peroxidation and platelet activation as other

inflammatory mediators are likely also to be triggers of enhanced lipid peroxidation and platelet activation such as including tumor necrosis factor-α (TNF- α), interleukin (IL)-1ß (Davi *et al.*, 2003). Minuz *et al* (2002) examined whether oxidant stress is enhanced resulting in persistent platelet activation in patients with renovascular disease (RVD) which is a rare form of secondary hypertension. Lipid peroxidation as reflected by urinary 8-iso-PGF_2 excretion and platelet activation as reflected by urinary 11-dehydro-TX_2 excretion, were both significantly enhanced in hypertensive patients with RVD compared to a control group and patients with essential hypertension. A relationship between 8-iso-PGF_2 and 11-dehydro-TXB_2 was also observed in hypertensive patients with RVD ($rs=0.48$; $n=20$; $P=0.032$). Minuz *et al* (2002) attributed the biochemical link between lipid peroixidation and platelet activation to activation of the renin-angiotensin system. Davi *et al* (1997) found a statistically significant correlation between the rates of excretion of 8-epi-$PGF_2\alpha$ and 11-dehydro-TXB_2 in hypercholesterolemic patients who demonstrated significantly enhanced levels of 8-epi-$PGF_2\alpha$ and 11-dehydro-TXB_2 compared to control subjects. Lastly, Davi *et al* (2002) noted a significant correlation between urinary excretion rate of 8-iso $PGF_{2\alpha}$ and 11-dehydro-TxB_2 in gynoid and android obesity ($r=0.61$, $P<0.001$). To characterise the cause and effect of obesity further on 8-iso $PGF_{2\alpha}$ and 11-dehydro-TxB_2 further, Davi *et al* (2002) examined the effects of a short-term weight loss program on changes in urinary 8-iso $PGF_{2\alpha}$ and 11-dehydro-TxB_2 in 20 android obese women. Following successful weight loss (15.3±10.5kg) the rates of 8-iso $PGF_{2\alpha}$ and 11-dehydro-TxB_2 excretion were significantly reduced, by 32% and 54% respectively. Changes in urinary 11-dehydro-TxB_2 excretion correlated with the amount of weight loss ($r=0.67$, $P=0.02$) and the values of thromboxane metabolite excretion fell within the reference range by the end of the study. Therefore, increased oxidant stress in obesity maybe a mechanism linking obesity-associated oxidant stress with platelet activity. In contrast, this study failed to demonstrate a potential biochemical link between platelet

aggregation and lipid peroxidation measured as ADP-induced platelet aggregation and lipid hydroperoxides, respectively. The conflicting findings may be attributed to methodological differences such as measurement of ADP-induced platelet aggregation which may measure different aspects of platelet activation and may differ in terms of sensitivity compared to the markers of platelet activation used in plasma in the above studies (Kamath *et al.*, 2001). Possible variations in LH levels due to sampling handling and storage conditions (Södergren *et al.*, 1998) may also have affected the identification of a biochemical link between lipid peroxidation and platelet aggregation.

Several *in vitro* experimental approaches have been used to investigate the specific role for distinct ROS on platelets. Platelets exposed directly to H_2O_2 have caused divergent results. Inhibited ADP-dependent platelet activation (Ambrosio *et al.*, 1994), enhanced collagen-dependent platelet activation and enhanced arachidonic acid (AA)-dependent platelet activation (Practio *et al.*, 1992) have all been observed. The discrepancy in results may be caused by different antioxidant capacities of the buffers used in the studies (Krotz *et al.*, 2004). Platelets exposed to $O_2^{\cdot-}$ have shown a reduction in the threshold for platelet activation to thrombin, collagen, ADP or AA and induced spontaneous aggregation (Handin *et al.*, 1977; Krotz *et al.*, 2002; Salvemini *et al.*, 1989; De la Cruz *et al.*, 1992). $O_2^{\cdot-}$ also reacts with platelet or endothelium-derived NO to $OONO^-$. The decreased bioavailability of NO is of particular importance for vascular thrombosis since NO is a potent inhibitor of platelet activation (Krotz *et al.*, 2004). $OONO^-$ has also been shown to produce a dual effect on platelets. It activates platelets in normal buffer and inhibits them when in plasma (Moro *et al.*, 1994; Brown *et al.*, 1998). In addition to exogenously derived ROS affecting the regulation of platelet activation, recent data also suggests that the platelets themselves generate ROS. The release of $O_2^{\cdot-}$ and other ROS by activated platelets was first observed by Marcus (1977). The platelet isoform of NAD(P)H-oxidase has been shown to produce $O_2^{\cdot-}$ and

enhance the recruitment of platelets by inactivating a platelet ectonicleotidase, thereby increasing the bioavailability of ADP (Krotz *et al.*, 2002). A variety of other enzymes are capable of producing ROS in platelets such as NO synthase (Wolin *et al.*, 2002) and phospholipase A2 (Caccese *et al.*, 2000).

5.4 CONCLUSION

Percentage ADP-induced platelet aggregation progressively decreased with increasing BMI values but only obese subjects had significantly decreased ADP-induced platelet aggregation compared to normal-weight control subjects (expression of enhanced *in vivo* platelet reactivity). Waist circumference above the cut-off guidelines for increased CVD risk also showed a tendency for decreased percentage ADP-induced platelet aggregation levels when compared to a waist circumference below the cut-off guidelines for reduced CVD risk. The *in vitro* altered platelet behaviour observed in obesity, may be considered an expression of exhausted platelet reactivity to *in vivo* stimulation and maybe the underlying cause for increased risk of CVD (Davi *et al.*, 2002). Various risk factors for atherosclerosis and cardiovascular thrombosis such as hypercholesterolemia, diabetes mellitus and hypertension are associated with oxidant stress-mediated platelet aggregation (Davi *et al.*, 2003; Minuz *et al.*, 2002; Davi et al., 1997), which suggests that increased oxidant stress in obesity may contribute to persistent platelet aggregation. However this study does not support the biochemical link between oxidant stress and platelet aggregation when measuring lipid hydroperoxide levels and percentage ADP-induced platelet aggregation.

Chapter SIX

Study 3

The effects of short-term dietary intervention for weight loss on lipid peroxidation and total antioxidant status.

6.0 INTRODUCTION

Obesity treatment typically promotes long-term adherence to a well-balanced weight loss promoting diet, which emphasises restriction of fat intake (British Nutrition Foundation, 2003). However these diets are often associated with modest weight loss (Brehm *et al.*, 2003), poor compliance (Westerterp *et al.*, 1996), and weight regain in the long term (Toubro and Astrup, 1997) and there has been a concomitant increased trend in the use of short term bouts of 'crash' dieting and carbohydrate restriction e.g. the Atkins Diet (Atkins, 1992) which typically promotes rapid weight loss in the first few days or weeks (Atkins, 1992). Possible mechanisms accounting for the increased weight loss on the LowCD include the simplicity of the diet (restricting food choices) (Foster *et al.*, 2003), alterations in central satiety factors (Foster *et al.*, 2003), dietary adherence (Foster *et al.*, 2003), glycogen depletion (Astrup *et al.*, 2004) and associated water loss (Astrup *et al.*, 2004).

The LowCD derives large proportions of calories from fat and protein (Atkins, 1992), which may potentially have a detrimental impact on the risk of CVD (Blackburn *et al.*, 2001). Increased consumption of saturated fat has been linked to an adverse lipid profile (Law, 2000), glucose intolerance (Marshall *et al.*, 1997) and obesity (Bray and Popkin, 1998). However improvements in conventional CVD risk factors have been found on the LowCD such as triglyceride and HDL-cholesterol concentration and insulin sensitivity (Astrup *et al.*, 2004) which may be attributed to weight loss. The effects of a LowCD on more novel CVD risk factors such as oxidant stress as yet is unknown (Hayden and Reaven, 2000) but it is important to explore these as conventional CVD risk factors do not fully explain the observed rates of coronary heart disease (Reaven, 1997).

Since the early 1990's, it has been accepted that oxidant stress plays a critical role in the pathogenesis of endothelial dysfunction (Loscalzo, 2003). Systemic processes that invoke endothelial dysfunction include stress-induced activation of intracellular oxidative signalling (Cai and Harrison, 2000), with secondary oxidative modulation of low density lipoprotein oxidation (LDLOx) (Witztum, 1994) and NO bioavailability (Williams *et al.,* 2002). These processes may be modulated by factors such as obesity (Williams *et al.,* 2002), weight loss (Higashi *et al.,* 2003) and dietary composition (macronutrient and micronutrient intake) (Roberts *et al.,* 2002).

Several research studies have reported that obesity was associated with increased oxidant stress in humans (Yesilbursa *et al.,* 2005; Mohn *et al.,* 2005; Keaney *et al.,* 2003; Ozata *et al.,* 2002; Davi *et al.,* 2002; Dandona *et al.,* 2001; Prázný *et al.,* 1999) i.e. increased free-radical production and/or depleted cellular antioxidant defence systems (Powers *et al.,* 2004). The proposed mechanisms that may underlie the obesity-related oxidant stress include, increased O_2 consumption (i.e. mechanical overload) and subsequent radical production via mitochondrial respiration, diminished antioxidant capacity, increased fat deposition and cell injury causing increased rates of radical formation such as $O_2^{\cdot-}$ and OH^{-} (Vincent *et al.,* 2001). In addition, hyperglycaemia, hypertension and hyperleptinemia are also possible sources of increased oxidant stress in obesity (Vincent and Taylor, 2006). These abnormalities have been shown to be reversible with weight loss (Davi *et al.,* 2002; Vincent *et al.,* 2001). The macronutrient composition of diets may also influence the oxidant–antioxidant balance For example, high-fat (Slim *et al.,* 1996) and high-sugar diets (Faure *et al.,* 1997) have been shown to induce oxidant stress, whilst a reduced fat and reduced sugar intake reduces oxidant stress (Roberts *et al.,* 2002). The quantity and composition of dietary fat may also affect fat-soluble anti-oxidative vitamin intake and/or anti-oxidative capacity and lipid peroxidation status (Velthuis-te Wierik *et al.,* 1996). Fat in food serves as a carrier for fat-soluble vitamins, so reduced-fat foods

may adversely affect the availability of fat-soluble vitamins and potentially reduce anti-oxidative capacity (Velthuis-te Wierik *et al.,* 1996). In addition to the long-term effects of dietary intervention, it has been shown that following an acute high fat meal or glucose load, free-radical production is enhanced (Vogel *et al.,* 1997; Dandona *et al.,* 2001). The independent contributions of changes in dietary macro-nutrient composition, energy restriction and weight loss on reducing oxidant stress, to date remain unclear (Fenster *et al.,* 2002).

The present studies aimed to investigate the effects of two commercially available diet plans for weight loss i.e. LowCD and ConvD (Slimming World) versus a control diet (CtrlD) on LH, TAS, SOD and GSH in healthy overweight women.

6.1 METHODOLOGY

Subject Characteristics:

Thirty (n=30) overweight women participated in the study (see table 6.0 for subject characteristics). Inclusion criteria were: age between 18 and 50 years and BMI between 25 and 33kg/m^2. Exclusion criteria included recent compliance (minimum 2 weeks) to a dietary regimen for weight loss within the last 3-months, clinically significant physician-diagnosed illnesses such as diabetes or hypertension, physician prescribed hypotensive or lipid-lowering medications, taking medications affecting body weight such as Orlistat and Sibutramine, or any form of antioxidant therapy, and pregnancy or lactation. Written informed consent was obtained from all the subjects after they had been given a full explanation of the study. The research was approved by De Montfort University Ethics Committee.

Table 6.0. Subject characteristics.

Characteristic	LowCD (N = 12)	ConvD (N = 12)	CtrlD (N = 6)	LowCD vs ConvD	LowCD vs CtrlD	ConvD vs Ctrld
Age, yr	39.8 ± 2.75	41.5 ± 1.86	36.8 ± 3.29	.882	.763	.525
Body mass, kg	84.4 ± 2.91	84.1 ± 2.96	75.6 ± 2.68	.940	.088	.112
BMI, kg/m^2	30.2 ± 0.84	31.2 ± 0.80	28.7 ± 1.26	.412	.481	.192
Body fat, kg	32.3 ± 2.16	32.6 ± 2.14	26.1 ± 2.01	.923	<.05	.075
Waist circumference, cm	95.6 ± 3.58	96.6 ± 3.31	89.3 ± 5.60	.984	.558	.475
Systolic BP, mmHg	125.2 ± 3.87	123.6 ± 3.95	114.7 ± 3.70	.847	.104	.236
Diastolic BP, mmHg	84.6 ± 3.16	84.2 ± 2.96	76.8 ± 2.90	.869	.170	.344
Total cholesterol, mmol/L	5.7 ± 0.53	5.3 ± 0.50	5.1 ± 0.59	.789	.209	.542
LDL cholesterol, mmol/L	3.60 ± 0.42	3.59 ± 0.41	3.14 ± 0.45	.916	.116	.068
HDL cholesterol, mmol/L	1.68 ± 0.24	1.16 ± 0.22	1.54 ± 0.26	.173	.604	.586
Triglycerides, mmol/L	0.8 ± 0.11	0.9 ± 0.10	1.0 ± 0.11	.362	.282	.847
Fasting Glucose, mmol/L	4.71 ± 0.19	4.76 ± 0.18	4.64 ± 0.21	.121	.264	.300

Values expressed as the mean±SEM. BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Results were compared using a Scheffe multiple comparison test and P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

Experimental design

The volunteers were randomly assigned to follow either the LowCD (Atkins Diet), ConvD (Slimming World Diet) or CtrlD (Control). On the LowCD, volunteers were instructed to follow an *ad libitum* diet with a maximum intake of 20g carbohydrate/day, which induced ketosis (as shown in all volunteers on the LowCD by using a urinary

ketone kit). After 2 weeks of dieting, volunteers increased their intake of carbohydrate to 40-60g/d if self-testing of urinary ketones continued to indicate ketosis. Volunteers following the LowCD were also instructed to take a daily multivitamin (Holland & Barrett Multivitamin, ABC Plus) as recommended in the Atkins Diet. On the ConvD, volunteers followed the Slimming World Diet, which meets the guidelines of the Balance of Good Health (British Nutrition Foundation, 2003) i.e. high carbohydrate (60% of total kcal intake) / low fat (<30% of total kcal intake) and moderate protein (12% of total kcal intake) content. Volunteers following the CtrlD were instructed to continue their usual dietary regime.

The study was conducted over a 7-week period. All subjects completed a 2-week baseline period, 4-week weight loss programme and a 1-week post-diet follow-up. The subjects attended the research laboratory weekly on the same day until the study was completed. During each visit, volunteers had blood samples taken, underwent anthropometric assessments and volunteers following the LowCD and ConvD received additional dietary advice.

Anthropometric and cardiovascular measurements and venous blood sampling

Height, weight and waist measurements are described in detail in the methodology chapter (section 3.2.1, 3.2.2, 3.2.3). Body composition was analyzed using the BIA (BodyStat® Quadscan 4000) which is outlined in the methodology chapter (section 3.2.4.1). Cardiovascular measurements and venous blood sampling are described in the methodology chapter (section 3.3, 3.1.1 and 3.1.2).

Food diary

Dietary intake was monitored using a 3-day weighed food diary, which was subsequently analyzed for macro-micronutrient content using dietary analysis software, Compeat (methodology chapter, section 3.8).

Biochemical measurements

LH, TAS, SOD and GSH were performed as described in the methodology chapter (section 3.1.12 and 3.1.13). Other biochemical measures included, fasting plasma glucose, plasma fructosamine, plasma cholesterol, plasma LDL, plasma HDL and plasma triglycerides as described in the methodology chapter (section 3.1.6.1, 3.1.7, 3.1.8, 3.1.9, 3.1.10 and 3.1.11).

Statistics

Values reported as mean±SEM. SEM was appropriate because it demonstrates how liable to error the mean is (e.g. the mean value or the mean change or improvement). The Kolmogorov-Smirno test confirmed that the data was normally distributed. Data was analyzed using ANOVA with repeated measures which is an appropriate method for detecting differences between groups, time effects and interactions. A two way (AxB) mixed analysis of variance (ANOVA) was used which incorporated one between group (groups: LowCD vs ConvD cs CtrlD) and one within (time: baseline vs Wk 1 vs Wk 2 vs Wk 4) subjects factor. When significant F values were noted, post hoc analyzes were performed with a Scheffe multiple comparison test. Paired t-tests were used to detect specific differences within groups at certain time points during the study. Relationships between dietary intake and biochemical parameters were examined using Pearson's test of linear correlation. P-values < 0.05 were regarded as

statistically significant. Analyzes of data were carried out using a computer software package (SPSS for Windows, Version 13.0).

6.2 RESULTS

Subject characteristics for the LowCD, ConvD and CtrlD group are shown in table 6.0. The majority of parameters were similar between the three groups at the beginning of the study; age, BMI, waist circumference, blood pressure, fasting lipid profile and fasting glucose, except for body fat levels. Body fat was significantly higher in the LowCD group when compared to the CtrlD group (32.3±2.16 vs 26.1±2.01kg, $P<0.05$), however there was no significant difference in body fat between the ConvD and CtrlD group (32.6±2.14 vs 26.1±2.01kg).

Weight loss and Changes in Body Composition

Following the 4-week dietary intervention both the LowCD and ConvD groups demonstrated significant weight loss [LowCD vs ConvD (mean, -4.88±1.28kg, $P<0.001$ vs. –3.14±1.76kg, $P<0.001$, respectively)] with the LowCD achieving a greater weight loss when compared to the ConvD ($P=0.01$). Despite advice to maintain their habitual dietary regimen and physical activity, the CtrlD group did lose weight over the 4-weeks but this was not statistically significant (-1.2±0.82kg, $P>0.05$). In terms of body composition, following the 4-week dietary intervention both the LowCD and ConvD groups demonstrated significant mean fat loss [LowCD vs ConvD (-2.34±0.47kg, $P<0.001$ vs -3.6±0.58kg, $P<0.001$)]. Although the ConvD resulted in the greatest fat loss over 4-weeks, this was not significantly different when compared to fat loss in the

LowCD group (P>0.05). The CtrlD group demonstrated no significant change in fat mass (-0.89±0.96kg, p>0.05) (see table 6.1).

Table 6.1. Body weight and body fat during the study period (baseline and week 1, 2 and 4 of the dietary intervention period) on the LowCD, ConvD and CtrlD.

Group	Baseline	Week 1	Week 2	Week 4	P group	P time	P interact
Weight (kg)							
LowCD	84.4±2.78	81.6±2.86***### ††	81.4±2.93* ###	79.5±2.89*** ### ††			
ConvD	84.0±2.96	82.6±2.90* ###	82.0±2.87###	80.9±2.74###	0.001	0.000	0.000
CtrlD	75.6±3.99	75.5±4.20	74.7±4.14*	74.4±4.23			

Group	Baseline	Week 1	Week 2	Week 4	P group	P time	P interact
Total Body Fat (kg)							
LowCD	32.2±1.74	31.0±1.82#	30.3±1.88###	30.0±1.78###			
ConvD	32.6±2.14	31.3±2.15##	30.1±2.02###	29.0±1.93* ###	0.135	0.000	0.032
CtrlD	26.1±2.20	25.5±2.62	25.3±3.00*	25.2±2.76			

Values expressed as the mean±SEM. LowCD, low carbohydrate diet, ConvD, Conventional diet, CtrlD, control diet. Results were analyzed using ANOVA with repeated measures. P values < 0.05 were considered significant. For further details see text.

* P<0.05, *** P<0.001 denotes values compared to CtrlD group

# P<0.05, ## P<0.01, ### P<0.001 denotes values compared to baseline levels.

†† P<0.01 denotes difference between LowCD group and ConvD group.

Food Intake

Food intake data is summarized in table 6.2. Mean total daily calorie intake was significantly reduced on both the ConvD and LowCD by approximately one-third versus baseline values [baseline vs 4-week diet (ConvD (1848±127 vs 1229±69kcal), LowCD (1438±89 vs 1032±70kcal), P<0.01)], but did not change significantly on the CtrlD [baseline vs 4-week diet (1491±366 vs 1612±203kcal, P>0.05)]. Mean fat intake (percentage of total calorie intake) was significantly lower (-24%) on the ConvD [baseline vs 4-week diet (38.5±1.7 vs 29.1±1.2%, P<0.001)] and % fat intake higher (+55.2%) on the LowCD [baseline vs 4-week diet (37.1±2.1 vs 57.6±2.1%, P<0.001)]. Fat intake was unchanged in the CtrlD. When expressed in grams, mean fat intake was significantly lower on the ConvD [baseline vs 4-week diet (80.5±7.59 vs 40.4±3.25g, P<0.001) but did not show any significant changes in both the LowCD and CtrlD. Mean carbohydrate intake (percentage of total calorie intake) was significantly higher (+13.6%) on the ConvD [baseline vs 4-week diet (43.8±1.5 vs 49.8±1.2%, P<0.01)] and was significantly reduced (-77%) on the LowCD [baseline vs 4-week diet (41.4±2.8 vs 9.2±1.5%, P<0.01)], but unchanged on the CtrlD. When expressed in grams, carbohydrate intake was significantly reduced following the LowCD [baseline vs 4-week diet (160.2±16.14 vs 24.3±3.56g, P<0.001)] and the ConvD [baseline vs 4-week diet (213.9±14.15 vs 162.6±9.75g, P<0.05)] but the degree of reduction was greater following the LowCD compared to the ConvD (-85% vs -24%). No changes in carbohydrate intake expressed in grams was found on the CtrlD. Mean protein intake (percentage of total calorie intake) increased on the ConvD (+23%) [baseline vs 4-week diet (15.3±0.50 vs 18.9±0.71%, P<0.01)] and on the LowCD (+78%) [baseline vs 4-week diet (18.4±1.2 vs 32.8±1.8%, P<0.01)]. Mean protein intake (percentage of total calorie intake) was unchanged on the CtrlD. When expressed in grams, mean protein intake decreased on the ConvD [baseline vs 4-week diet (69.8±4.14 vs

57.2±2.43g, P<0.05)], increased on the LowCD [baseline vs 4-week diet (66.0±5.51 vs 81.1±6.26g, P<0.05)] and was unchanged on the CtrlD. Over the course of the four-week dietary intervention, the main between group differences in dietary intake expressed in grams were significantly greater fat intake on the LowCD compared to the ConvD (P<0.001), significantly lower carbohydrate intake on the LowCD compared to the ConvD (P<0.001) and CtrlD (P<0.001) and significantly greater protein intake on the LowCD when compared to the ConvD (P<0.001). No significant differences in fat intake and protein intake was found between the LowCD and CtrlD.

Table 6.2. Dietary composition at baseline and mean values for the four-week dietary intervention periods on the LowCD, ConvD and CtrlD.

	Group	Baseline	Diet period	P group	P time	P interact
KCAL	LowCD	$1438.1 \pm 88.53^{\#}$	$1031.6 \pm 70.38^{\#\#*}$			
	ConvD	$1847.8 \pm 126.58^{\dagger}$	$1229.2 \pm 69.21^{\#\#*}$	P<0.011	0.002	P<0.012
	CtrlD	1490.6 ± 366.19	1611.8 ± 203.20			
FAT (%)	LowCD	37.1 ± 2.08	$57.6 \pm 2.06^{\#\#\#\dagger\dagger\dagger**}$			
	ConvD	38.5 ± 1.68	$29.1 \pm 1.24^{\#\#\#}$	P<0.001	0.035	P<0.001
	CtrlD	36.6 ± 1.70	34.0 ± 1.94			
FAT (g)	LowCD	58.3 ± 3.93	$66.7 \pm 5.85^{\dagger\dagger}$			
	ConvD	80.5 ± 7.59	$40.4 \pm 3.25^{*\#\#\#}$	0.806	P<0.01	P<0.001
	CtrlD	69.6 ± 9.13	62.6 ± 10.33			
CARBOHYDRATE (%)	LowCD	41.4 ± 2.75	$9.2 \pm 1.45^{\#\#\#***\dagger\dagger\dagger}$			
	ConvD	43.8 ± 1.51	$49.8 \pm 1.12^{\#}$	P<0.001	P<0.001	P<0.001
	CtrlD	50.1 ± 2.07	48.0 ± 2.05			

CARBOHYD RATE (g)	LowCD	160.2 ± 16.14	$24.3 \pm 3.56^{***\#\#\#\dagger\dagger\dagger}$			
	ConvD	213.9 ± 14.15	$162.6 \pm 9.75^{*\#}$	P<0.001	P<0.001	P<0.001
	CtrlD	220.9 ± 29.22	202.5 ± 20.99			
PROTEIN (%)	LowCD	18.4 ± 1.21	$32.8 \pm 1.76^{\#\#\#***\dagger\dagger\dagger}$			
	ConvD	15.3 ± 0.50	$18.9 \pm 0.71^{\#\#\#}$	P<0.001	P<0.001	P<0.001
	CtrlD	15.1 ± 0.82	15.8 ± 0.88			
PROTEIN (g)	LowCD	66.0 ± 5.51	$81.1 \pm 6.26^{\#\dagger\dagger}$			
	ConvD	69.8 ± 4.14	$57.2 \pm 2.43^{\#}$	.284	0.918	P<0.01
	CtrlD	66.1 ± 11.24	63.3 ± 8.31			

Values expressed as the mean±SEM. LowCD, low carbohydrate diet, ConvD, Conventional diet, CtrlD, control diet. Results were analyzed using ANOVA with repeated measures. P values < 0.05 were considered significant. For further details see text.

* P<0.05, ** P<0.01, *** P<0.001 denotes values compared to CtrlD group

$^{\#}$ P<0.05, $^{\#\#}$ P<0.01, $^{\#\#\#}$ P<0.001 denotes values compared to baseline levels.

† P<0.05, †† P<0.05, ††† P<0.001 denotes difference between LowCD group and ConvD group.

Figures 6.0, 6.1, 6.2 and 6.3 show the results for lipid peroxidation and antioxidant status measures as LH, TAS, the intracellular antioxidant defence GSH and the free-radical scavenging enzyme SOD.

In comparison to baseline levels, subjects following LowCD demonstrated variable LH levels. An initial non-significant trend for increased levels at week 1 was apparent

(P=0.08), followed by a significant increase at week 2 (P<0.05), which returned to near baseline levels at week 4 (P>0.05). LH levels did not change significantly on the ConvD (P>0.05) but on the CtrlD there were slight fluctuations e.g. LH levels increased at week 1 (P<0.05) but this returned to baseline level at week 2 (P>0.05).

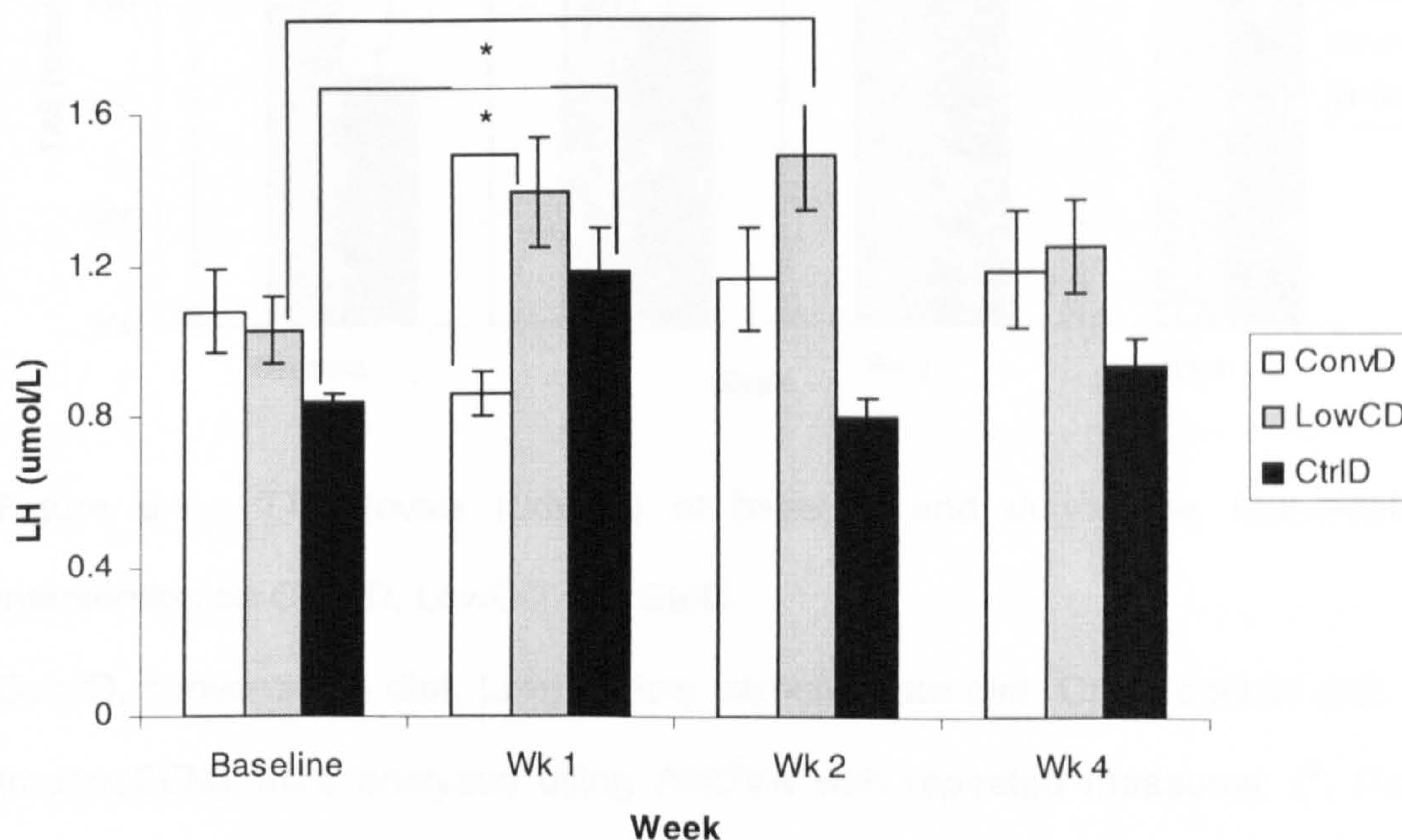


Figure 6.0. LH levels (μmol/L) at baseline and during the four-week dietary intervention on ConvD, LowCD and CtrlD.

ConvD, conventional diet, LowCD, low carbohydrate diet, CtrlD, control diet. Results (mean±SEM) were analyzed using ANOVA with repeated measures. (*, P<0.05). For further details see text.

In comparison to baseline levels, subjects following LowCD demonstrated increased TAS during the four-week intervention period (week 1, P<0.05; week 2, P<0.05 and week 4, P<0.001). No changes in TAS were found during the four-week intervention period in the ConvD. In the CtrlD, TAS significantly increased compared to baseline level at week 2 of the four week intervention period (P<0.05), but returned to near baseline levels at week 4.

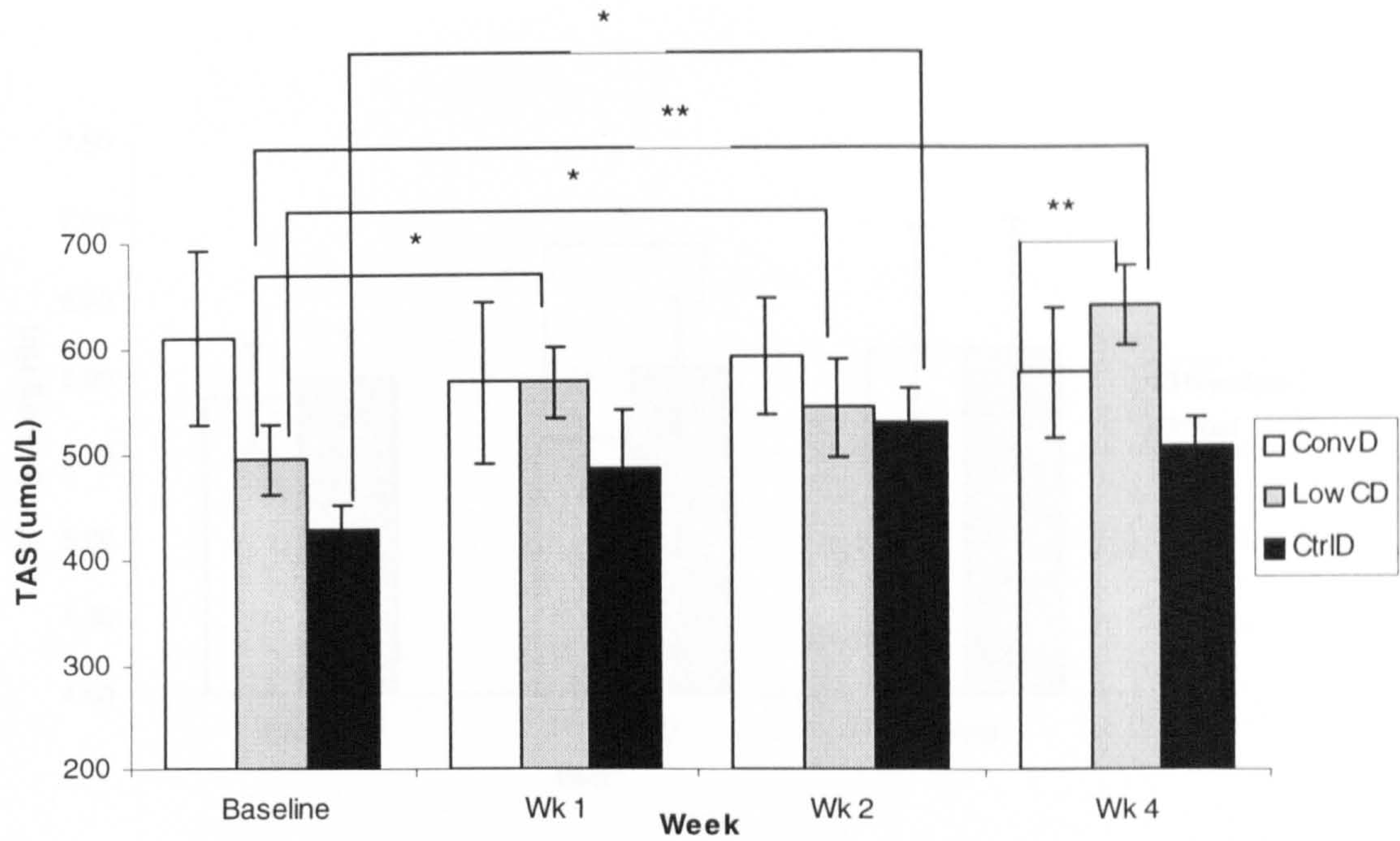


Figure 6.1. TAS levels (µmol/L) at baseline and during the four-week dietary intervention on ConvD, LowCD and CtrlD.

ConvD, conventional diet, LowCD, low carbohydrate diet, CtrlD, control diet. Results (mean±SEM) were analyzed using ANOVA with repeated measures. (*, P<0.05, **, p<0.001). For further details see text.

No significant changes were noted in GSH levels and SOD activity following 4-weeks of dietary intervention in all diet groups. However there were non-significant trends for higher GSH and SOD levels in the LowCD group following the 4-week dietary intervention when compared to baseline level (P = 0.066 and P=0.075 respectively).

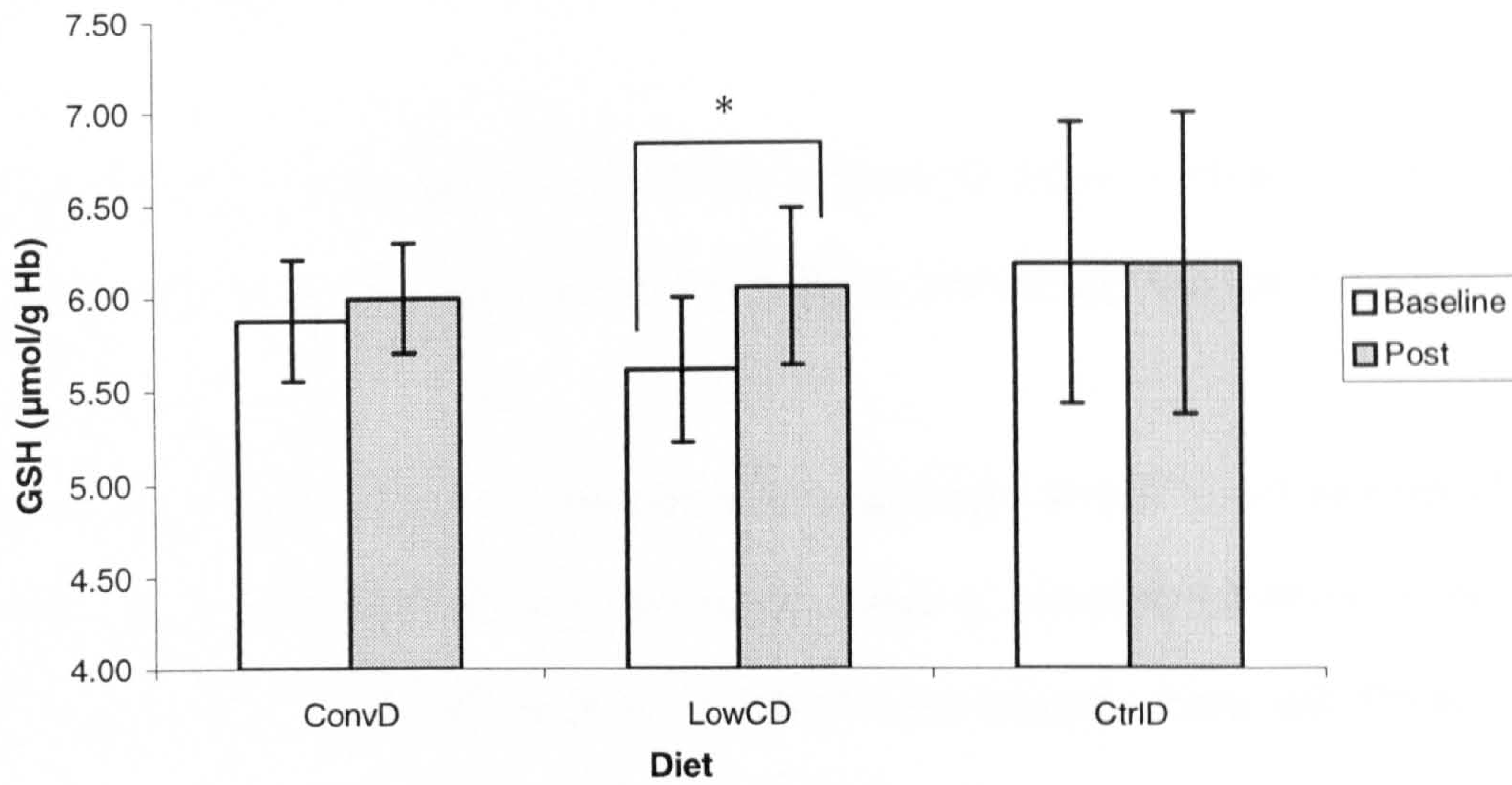


Figure 6.2. GSH levels (μmol/g Hb) at baseline post four-week dietary intervention on ConvD, LowCD and CtrlD.

ConvD, conventional diet, LowCD, low carbohydrate diet, CtrlD, control diet. Results (mean±SEM) were analyzed using ANOVA with repeated measures. (*, P=0.06). For further details see text.

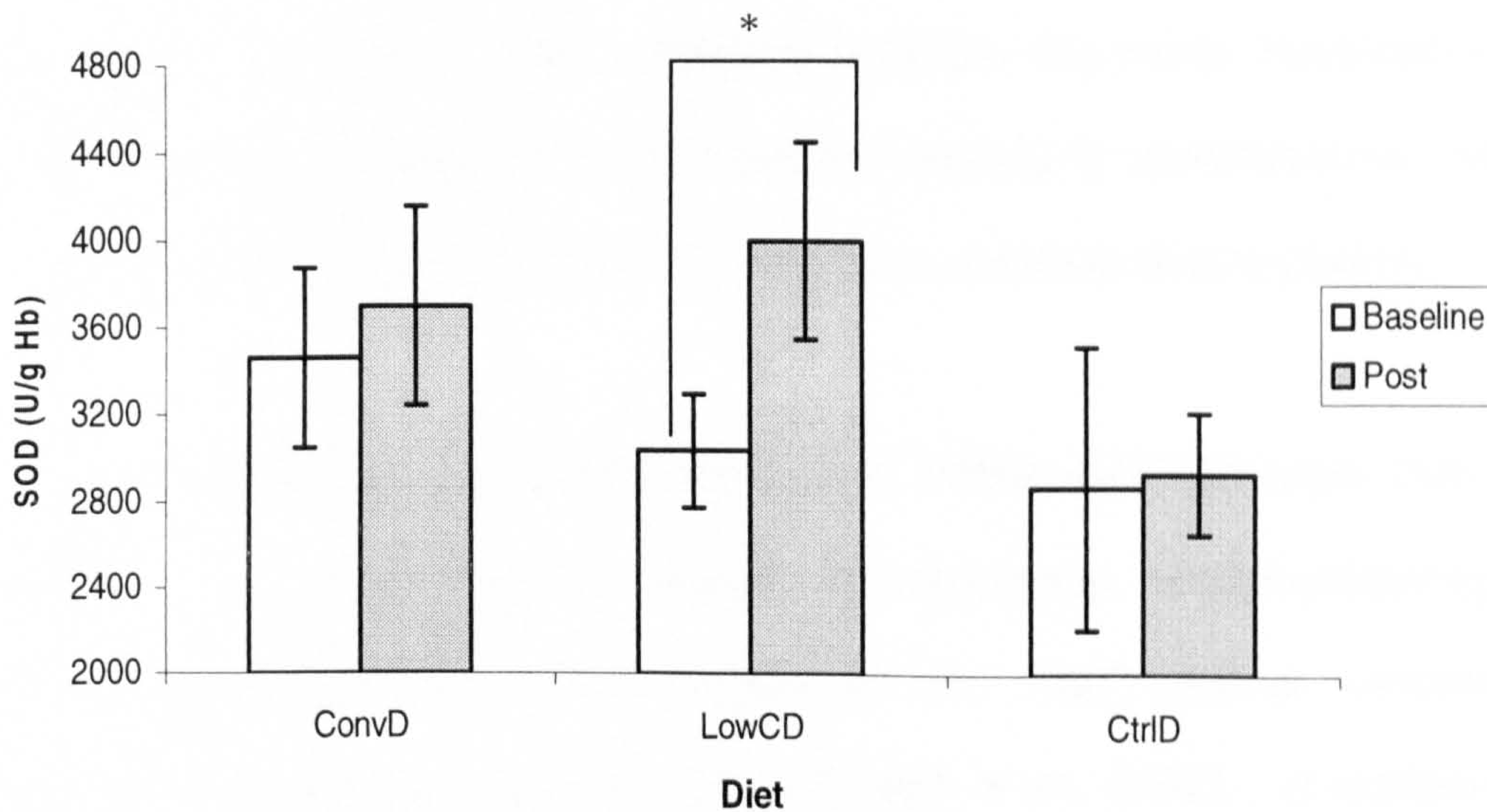


Figure 6.3. SOD levels (U/g Hb) at baseline post four-week dietary intervention on ConvD, LowCD and CtrlD.

ConvD, conventional diet, LowCD, low carbohydrate diet, CtrlD, control diet. Results (mean±SEM) were analyzed using ANOVA with repeated measures. (*, P=0.08). For further details see text.

6.3 DISCUSSION

This study investigated the short-term effects of either a LowCD or ConvD, versus a control group, on LH, TAS, SOD and GSH in overweight healthy women.

Increased consumption of saturated fat has been linked to an increased risk of CVD because of associations with increased plasma concentrations of lipids (Law, 2000), glucose intolerance (Marshall *et al.*, 1997) and obesity (Bray and Popkin, 1998). The LowCD typically has a high proportion of saturated fat consumption (Atkins, 1992). Despite this, a recent study indicated that both the LowCD and ConvD may have favourable effects on lipoprotein subfractions and inflammatory processes over a 6-month period, so the effects of dietary intervention on cardiovascular risk factors may be more complex (Astrup *et al.*, 2004). However such improvements may be in response to decreased calorie consumption (Roberts *et al.*, 2002; Velthuis-te Wierik *et al.*, 1996). In light of these conflicting findings, this study investigated whether the LowCD adversely affected related factors relating to cardiovascular health such as oxidant stress parameters, which to date have not been widely studied.

Evidence suggests that high-fat (Slim *et al.*, 1996) and high-sugar diets (Faure *et al.*, 1997), can induce oxidant stress, by producing radical-producing substances (polyunsaturated fatty acids) (Vogel *et al.*, 1997) and/or reduced antioxidant substances such as vitamin E and C (Vogel *et al.*, 1997). In addition to long-term dietary effects, it has been shown that following an acute high fat meal or glucose load, free-radical production is enhanced (Vogel *et al.*, 1997; Dandona *et al.*, 2001, respectively). This can make studying the effect of macro-nutrients on oxidant stress difficult as it is unknown whether observed increases in ROS were due to excess calorie consumption or metabolic alterations associated with obesity (Fenster *et al.*,

2002). Evidence suggests that reduced energy intake (reduced fat products) contributes to a reduction in MDA level (Velthuis-te Wierik *et al.*, 1996), and energy restriction (without malnutrition) retards the development of atherosclerosis, due to a reduction in O_2 molecules within the mitochondria, thereby lowering ROS (Ramsey *et al.*, 2000). The independent contributions of changes in dietary intake (macro and micro-nutrient composition), energy restriction and weight loss on reducing oxidant stress, still remains unclear (Fenster *et al.*, 2002).

At the beginning of the study all thirty overweight women had similar baseline subject characteristics. For example age, BMI, waist circumference, blood pressure, fasting lipid profile and fasting glucose were not significantly different between each diet group when randomised. However body fat was significantly higher in the LowCD when compared to the CtrlD group (32.3±2.16 vs 26.1±2.01kg, P<0.05). Surprisingly, there were no significant differences in body fat between ConvD and CtrlD (32.6±2.14 vs 26.1±2.01kg), despite the mean and standard deviation of the ConvD being similar to the LowCD. Following the 4-week dietary intervention both the LowCD and ConvD groups demonstrated significant weight loss (-4.88±1.28kg, P<0.001 vs. -3.14±1.76kg, P<0.001, respectively) with the LowCD achieving a greater weight loss when compared to the ConvD (P=0.01). In terms of body composition, following the 4-week dietary intervention both the LowCD and ConvD groups demonstrated significant fat loss (-2.34±0.47kg, P<0.001 vs -3.6±0.58kg, P<0.001, respectively). Although the ConvD resulted in the greatest fat loss over 4 weeks, this was not significantly different when compared to fat loss on the LowCD (P>0.05). It is possible that the greater weight loss on the LowCD is a result of greater glycogen depletion (Astrup *et al.*, 2004) and associated water loss (Astrup *et al.*, 2004).

Following the four week dietary intervention in the LowCD, TAS increased significantly at week 4, LH significantly increased at week 1 and 2 and decreased at week 4 and both SOD and GSH showed a non-significant increased trend at week 4. These changes could reflect initial increased susceptibility to free-radical production, induced perhaps by the change in macronutrient content (Slim *et al.*, 1996; Velthuis-te Wierik *et al.,* 1996) [e.g. increased fat and/or protein content], followed by compensatory elevations in antioxidant enzyme levels, to help protect tissue against potential oxidant damage. However antioxidant concentration changes should be interpreted with caution, as this might not indicate tissue damage (Packer, 1997). For example according to Cooper *et al* (2002) a rise in plasma antioxidant levels might enhance the antioxidant defences in blood, but could possibly impair defences at the sites from which they are mobilized. It is worth noting that the above changes in oxidant stress markers may have been further enhanced if subjects were not taking a daily multivitamin. A daily multivitamin may have reduced the oxidant stress response seen on the LowCD (Anderson et *al.*, 1999).

The observed adverse effects of the LowCD on oxidant stress and lipid peroxidation indicates that further investigation is required (both short-term and long-term). It would not seem prudent to recommend a diet for inducing weight loss to improve cardiovascular health if the diet has an adverse effect on oxidant stress and the subsequent development of atherosclerosis. The present data does not support the notion that calorie restriction may be the major modulator of the pro-oxidant/antioxidant balance as increased oxidant stress susceptibility in the LowCD group was observed despite achieving the greatest weight loss in comparison to both ConvD and CtrlD groups. However, despite not finding a correlation between dietary fatty acid intake and oxidant stress in this study, the quantity and composition of dietary fat intake could influence fat-soluble anti-oxidative vitamin intake and also modulate anti-oxidative

capacity and lipid peroxidation status. For example, SFAs have been shown to adversely affect plasma lipids, lipoproteins and haemostatic factors (Mitropoulos *et al.*, 1994) as well as susceptibility to oxidation (Mata *et al.*, 1996). Berry *et al* (1991) and Reaven *et al* (1991) support the notion that subjects who consume diets rich in MUFAs demonstrate lower LDL oxidation than subjects who consume diets rich in PUFAs. But according to Schwab *et al (*1998) replacing SFAs with either MUFAs or PUFAs in diets that provide less total fat did not appreciably affect LDL oxidative susceptibility. In addition, Velthuis-te Wierik *et al* (1996) found that in healthy normal-weight subjects, neither the difference in energy intake, or change in dietary fat composition affect MDA and antioxidant status levels. Research findings therefore suggest that relating lipid content and oxidant stress is not entirely straight forward (Wolff and Nourooz-Zadeh, 1996). Although it has been shown that diets high in PUFAs and MUFAs and low in SFAs decrease atherosclerosis risk (Watts *et al.*, 1992), lipoproteins isolated from individuals consuming diets rich in PUFA and MUFA also demonstrate greater pre-disposition to peroxidation than lipoproteins from individuals given SFAs (Kleinveld *et al.*, 1993). Wolff and Nourooz-Zadeh (1996) suggested that lipid hydroperoxide intake is influenced by the consumption of lard and compound cooking fat (such as baking and frying margarines) which contain high levels of pre-formed hydroperoxides. Therefore individual hydroperoxide intake is expected to vary widely amongst individuals and will be largely dependent upon the intake of food containing hydroperoxides generated by pyrolysis such as fatty fried foods (Wolff and Nourooz-Zadeh, 1996). This information was unrecorded in the diet diaries in this study so the above can only be suggested as a cause for changes in LH and TAS status in the LowCD.

Changes in protein intake could modulate anti-oxidative capacity and lipid peroxidation status. According to Fang *et al* (2002) high protein diets lead to increased oxidant

stress. High protein diets, increase homocysteine levels, which is an independent risk factor for CVD, (Boushey *et al.*, 1995). Homocysteine increases endothelial $O_2^{\cdot-}$ production and induces oxidant stress in the vasculature (Wu and Meininger, 2002). Increasing protein intake has also been shown to stimulate generation of ROS and lipid peroxidation in human polymorphonuclear leukocytes and mononuclear cells (Mohanty *et al.*, 2002) and increase whole-body NO production by constitutive and inducible NOS in rats (Wu *et al.*, 1999).

The overall glycaemic index and glycaemic load (product of glycaemic index of a specific food and its carbohydrate content) of diets has also been found to be independent risk factors for cardiovascular events (Liu *et al.*, 2000), so may be important contributors modulating anti-oxidative capacity and lipid peroxidation status. Direct evidence from studies in both normal subjects and those with diabetes show that induced hyperglycaemia (Ceriello *et al.*, 1998a) or meal intake and its attendant increase in glucose (Ceriello *et al.*, 1998b; Ursini *et al.*, 1998) can increase oxidant stress and reduce antioxidant defences. The increase in oxidant stress was significantly greater after meals that produced a greater degree of hyperglycaemia (Ceriello *et al.*, 1999). Hu *et al* (2006) supported a direct relation between dietary glycaemic index and markers of oxidant stress, MDA and 8-iso $PGF_{2\alpha}$ by assessing dietary glycaemic index and glycaemic load in the form of a food-frequency questionnaire and measuring plasma MDA and 8-iso $PGF_{2\alpha}$ in 292 healthy adults. The observed rise in plasma MDA and 8-iso $PGF_{2\alpha}$ from the lowest to the highest quartile of glycaemic index was comparable to the differences in those concentrations found between normal-weight and overweight subjects (Block *et al.*, 2002). This data suggests that chronic consumption of high-glycaemic index foods may lead to chronically elevated oxidant stress. Therefore increasing dietary intakes of low-glycaemic index foods, such as most fruit and vegetables, dairy products and whole

gains may be beneficial in terms of reduced oxidant stress. Although the consumption of high- and low-glycaemic index foods was not measured in this study, it can be speculated that it is unlikely that the changes in LH and TAS on the LowCD is related to the consumption of chronic consumption of high-glycaemic index carbohydrates because the total carbohydrate intake on this diet was only about 9.1% of energy intake, which is very low. Further research is needed to investigate the effects of low- and high-glycaemic index on oxidant stress, particularly in relation to commercial diets.

This study was designed to focus on short term effects of dietary factors on oxidant stress but there is a need to study both lipid peroxidation and TAS status following a LowCD of a longer duration. Since the study was conducted in a realistic context as opposed to a strict but perhaps 'artificial' laboratory environment, it was not possible to isolate the independent effects of altered calorie intake and changes in dietary composition on the pro-oxidant/antioxidant balance. Consequently future research should also be undertaken to examine the effect of diet composition variation, whilst controlling for total calorie intake, on the pro-oxidant/antioxidant balance.

6.4 CONCLUSION

In conclusion, the above study demonstrated that short-term consumption of a high fat / high protein / low carbohydrate diet can predispose individuals to increased oxidant stress. Short-term adherence to a ConvD did not demonstrate such potential deleterious effects. A greater understanding is needed of the individual contributions of body weight and composition, energy restriction and diet composition to lipid peroxidation and TAS. Further research should concentrate therefore on the influence of longer-term dietary intervention on the integrity of the pro-oxidant/antioxidant balance.

Chapter SEVEN

Part (1)

Preliminary experiment (3)

Changes in lipid peroxidation and total antioxidant status in response to an acute session of low-intensity and high-intensity exercise

Part (2)

Study 4

The effects of acute high-intensity exercise between normal-weight and overweight subjects on lipid peroxidation, total antioxidant status and platelet responsiveness

The intention of the preliminary study was to identify an exercise intensity which predisposes healthy individuals to enhanced oxidant stress levels. This could then be applied to future research studies to assess the impact of obesity on exercise-induced oxidant stress and the effect of antioxidant therapy on reducing exercise-induced oxidant stress. Although other researchers have examined the effect of various exercise intensities on oxidant stress markers, this study was carried to ensure an exercise intensity was selected which elicited the oxidant stress makers used in this thesis such as lipid hydroperoxide and total antioxidant status.

PART ONE – Preliminary Experiment

7.0 INTRODUCTION

Physical exercise is associated with a significant increase in VO_2 both at the whole-body level and at skeletal muscle (Ji, 1996). Maximal VO_2 is increased 10-15 fold during exercise in relation to a resting period and a small fraction of O_2 is converted into intermediate oxidative products (Ji, 1996; Alessio, 1993) causing biochemical changes and tissue damage (Ji, 1992; Ji, 1995a). The increased oxidative state observed during exercise could be produced by increased free-radical formation or reduced activity of antioxidant enzymes (Ji, 1995a).

During exercise primary sources of radical production in skeletal muscle are the mitochondria, xanthine oxidase, NAD(P)H oxidase and the production of NO by NO synthase (Davies *et al.*, 1982; Jackson *et al.*, 1985; Reid *et al.*, 1992a, b; Borzone *et al.*, 1994; O'Neil *et al.*, 1996; Jackson, 1998). Secondary sources of free-radical production during exercise include autoxidation of catecholamines, radical generation by phagocytic white cells and radical formation due to the disruption of iron-containing proteins (Jackson, 1998; Halliwell and Gutteridge, 1999). However the mitochondria is

the primary source of free-radical production in skeletal muscle. While 95-98% of O_2 consumption by skeletal muscle results in the formation of ATP and H_2O, the remaining 2-5% of the O_2 undergoes one electron reduction to produce $O_2^{\cdot -}$ (Jackson, 1998; Halliwell and Gutteridge, 1999). If the increased production of radicals is not balanced by the antioxidant capacity, subsequent reactions may give rise to other ROS such as H_2O_2 and $OH^{\cdot}$.

Since physical activity protects against the development of CVD and modifies CVD risk (Wannamethee and Shaper, 1998), a regular exercise programme is desirable (National Institute of Health, 1996). However this presents a biochemical paradox as regular exercise is necessary to obtain good cardio-respiratory fitness, but at the same time an acute exercise session may be theoretically harmful (Cutler, 1984; Hooper, 1989).

Ajmani *et al* (2003) found that in healthy individuals (n=14), a maximal graded exercise test (modified Balke test) caused significant increases in plasma LH levels [pre vs post-exercise (6.5±2.0 to 7.9±1.9μM, P<0.0001)]. Similarly, Lawson *et al* (1997) studied 5 subjects and found an increase in MDA levels by 60% from a baseline of 3.28±1.74 to peak levels of 5.18±1.91nmol/mL (P<0.01) following the modified Bruce exercise treadmill protocol. In addition, SOD activity increased significantly by 256% from a baseline value of 2.37±1.27 to 8.47±319U/mL (P<0.01). Some investigators have failed to observe any signs of exercise-induced oxidant stress (Margaritis *et al.*, 1997; Witt *et al.*, 1992), which could be due to a number of reasons. The use of different test subjects (training status, disease state, age and gender) might influence the findings of different studies. A range of different exercise protocols (high- and low-intensity aerobic exercise) have also been used to study exercise-induced oxidant stress. Only high-intensity exercise regimes appear to increase free-radical production enough to

overwhelm antioxidant defences. For example, Lovlin *et al* (1987) demonstrated that maximal exercise, eliciting 100% VO_{2max} resulted in a 26% increase in plasma MDA ($P<0.005$), exercise eliciting 70% VO_{2max} resulted in a non-significant reduction in plasma MDA ($P>0.05$) and exercising at 40% VO_{2max} resulted in a 10.3% decrease in plasma MDA ($P<0.05$). Tozzi-Ciancarelli *et al* (2002) demonstrated that strenuous exercise (maximal exercise test on a cycle ergometer), but not moderate exercise (30 minutes at 60% VO_{2max} on a cycle ergometer) resulted in increased levels of TBARS [rest vs post-exercise (TBARS: moderate (1.5±0.2 vs 1.4±0.1μmol/L, $P>0.05$) and strenuous (1.6±0.2 vs 3.9±0.3μmol/L, $P<0.05$) and decreased TAS levels [rest vs post-exercise (TAS: moderate (1.35±0.04 vs 1.47±0.05U/mL, $P>0.05$) and strenuous (1.45±0.03 vs 1.05±0.03U/mL, $P<0.05$)].

This study investigated the effects of an acute session of low- and high-intensity exercise on LH and TAS responses in healthy subjects.

7.1 METHODOLOGY

Subject Characteristics

The study group consisted of eleven (n=11) apparently healthy male and females (n=9/2) who were recruited from De Montfort University, Department of Sport Sciences (see table 7.0 for subject characteristics). Subjects with a history of diabetes, cardiovascular or cerebrovascular disease, hepatic or renal disease, tobacco abuse, or those on hormone replacement therapy were excluded. In addition subjects were excluded if they were hypertensive (with or without treatment), taking treatment for dyslipidaemia, taking any antioxidant supplementations or a smoker. Written informed consent was obtained from all the subjects after they had been given a full explanation of the study. The research was given ethical approval by Bedfordshire Local Research Ethics Committee.

Table 7.0. Subject characteristics

Characteristic	Mean ± SEM
Age, yr	21.3 ± 0.30
BMI, kg/m^2	25.3 ± 1.25
Body mass, kg	77.1 ± 4.42
Body fat, kg	16.3 ± 1.89

Values expressed as the mean±SEM. BMI, body mass index.

Experimental design

Subjects visited the exercise physiology laboratories at De Montfort University, Bedford on two separate occasions between 9-11am over a 2-week period. For each visit, subjects were instructed to fast for 10-12 hours and refrain from exercise, caffeine and alcohol for 48 h before the study visit. Subjects were also asked to maintain their usual dietary pattern.

During week 1 anthropometric measures were taken which was followed by an exercise session. During week 2 an alternative exercise session was carried out. In both weeks, prior to and immediately post each exercise session a venous blood sample was obtained from a forearm antecubital vein and arterialised capillary blood from a finger-tip.

Exercise session

The exercise testing protocol utilised a motorised treadmill (see methodology section 3.5). For each subject, two exercise sessions were completed (30 minutes walking at 40% or 70% heart rate reserve). The order of the two exercise sessions were randomised and separated by 14 days. The Karvonen method was used to estimate heart rate reserve: % heart rate reserve = [(hr max (220-age) – resting heart rate) x (%

heart rate reserve intensity/100) + resting heart rate. Heart rate was monitored throughout the exercise session.

Anthropometric and cardiovascular measurements and venous blood sampling

Height, weight and waist measurements are described in detail in the methodology chapter (section 3.2.1, 3.2.2, 3.2.3). Body composition was analyzed using the BIA (BodyStat® Quadscan 4000) which is outlined in the methodology chapter (section 3.2.4.1). Cardiovascular measurements are described in the methodology chapter (section 3.3). Venous blood sampling (blood only collected in plain serum tube) and arterialized blood samples for assessment of plasma volume were collected which are described in the methodology chapter (section 3.1.1, 3.1.2, 3.1.3, 3.1.4 and 3.1.5).

Biochemical measurements

LH and TAS were performed as described in the methodology chapter (section 3.1.12.1 and 3.1.13.1). Haematocrit was measured using a Hawksley Micro Hct Reader and Haemoglobin using a HemoCue B-Haemoglobin photometer which are described in the methodology chapter (section 3.14 and 3.1.5). Haematocrit and Haemoglobin were measured to calculate the change in plasma volume (Dill and Costill, 1974).

Statistical Analysis

Values reported as mean±SEM. SEM was appropriate because it demonstrates how liable to error the mean is (e.g. the mean value or the mean change or improvement). The Shapiro-Wilk test confirmed that the data was not normally distributed so was analyzed by non-parametric methods to avoid assumptions about the distribution of the measured variables. The Wilcoxon signed-rank test for paired data was used to

determine the significance of differences of LH and TAS between pre- versus post-exercise among both the low- and high-intensity exercise sessions. Differences were considered statistically significant at P<0.05. Statistical analysis was performed using a computer software package (SPSS for Windows, Version 13.0).

7.2 RESULTS

LH levels only increased significantly following high-intensity exercise [rest vs post-exercise (0.73±0.04 vs 0.88±0.04μmol/L, P<0.02)] which was also shown to be significantly higher than the LH level post-exercise in the low-intensity group [high-intensity vs low-intensity (post-exercise, 0.88±0.04 vs 0.75±0.03μmol/L, P<0.05)] (figure 7.0). TAS increased significantly following low-intensity exercise [rest vs post-exercise (1046.6±64.87 vs 1128.54±70.84mmol/L, P<0.05)] (figure 7.1) and decreased slightly following high-intensity exercise but this was not significant [rest vs post-exercise (1120.2±80.22 vs 1088.8±64.82mmol/L, P>0.05)].

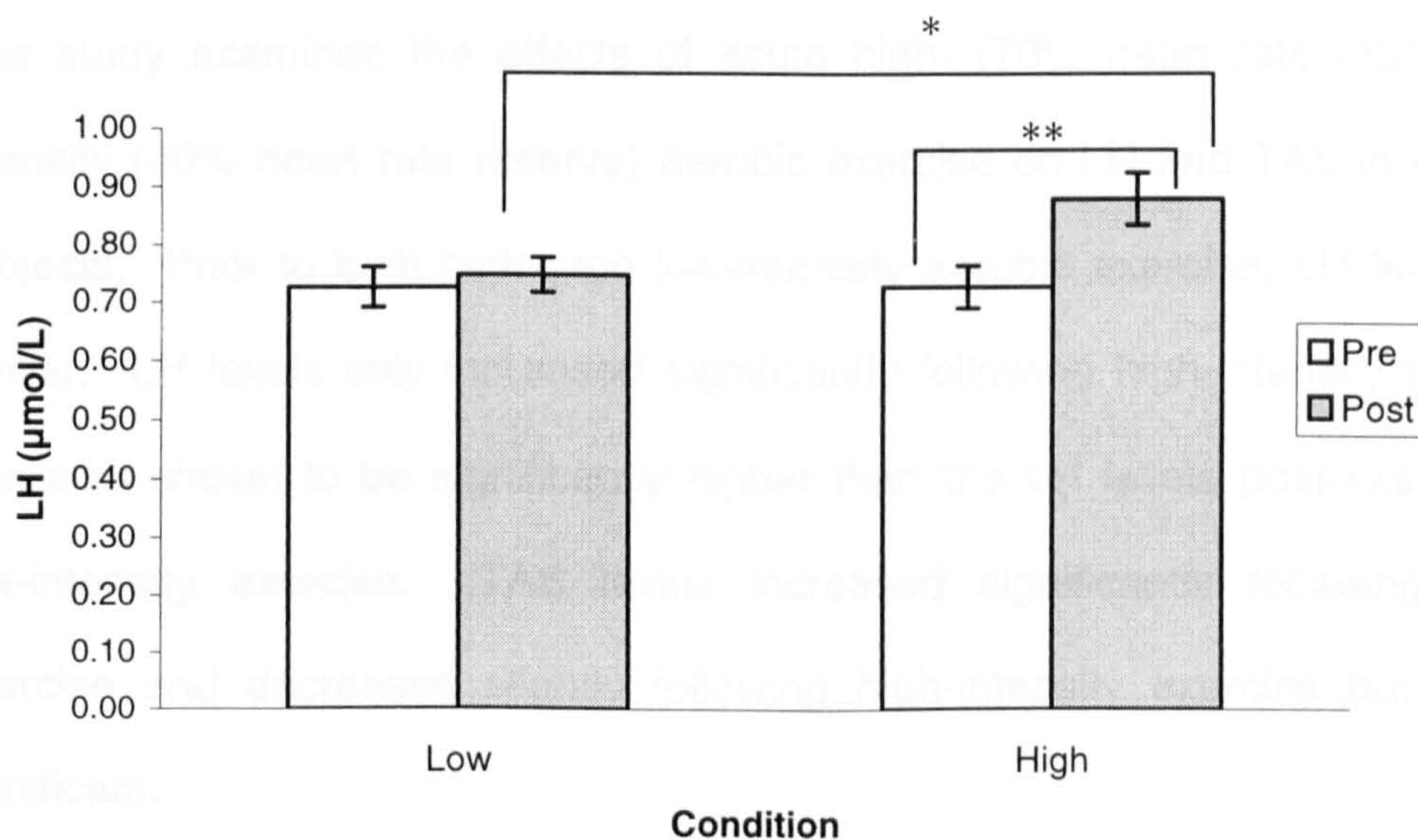


Figure 7.0. LH levels (μmol/L) pre and post acute aerobic exercise (low- and high-intensity). Results (mean±SEM) were analyzed using the Wilcoxon signed-rank test. (*, P<0.05, **, P<0.02). For further details see text.

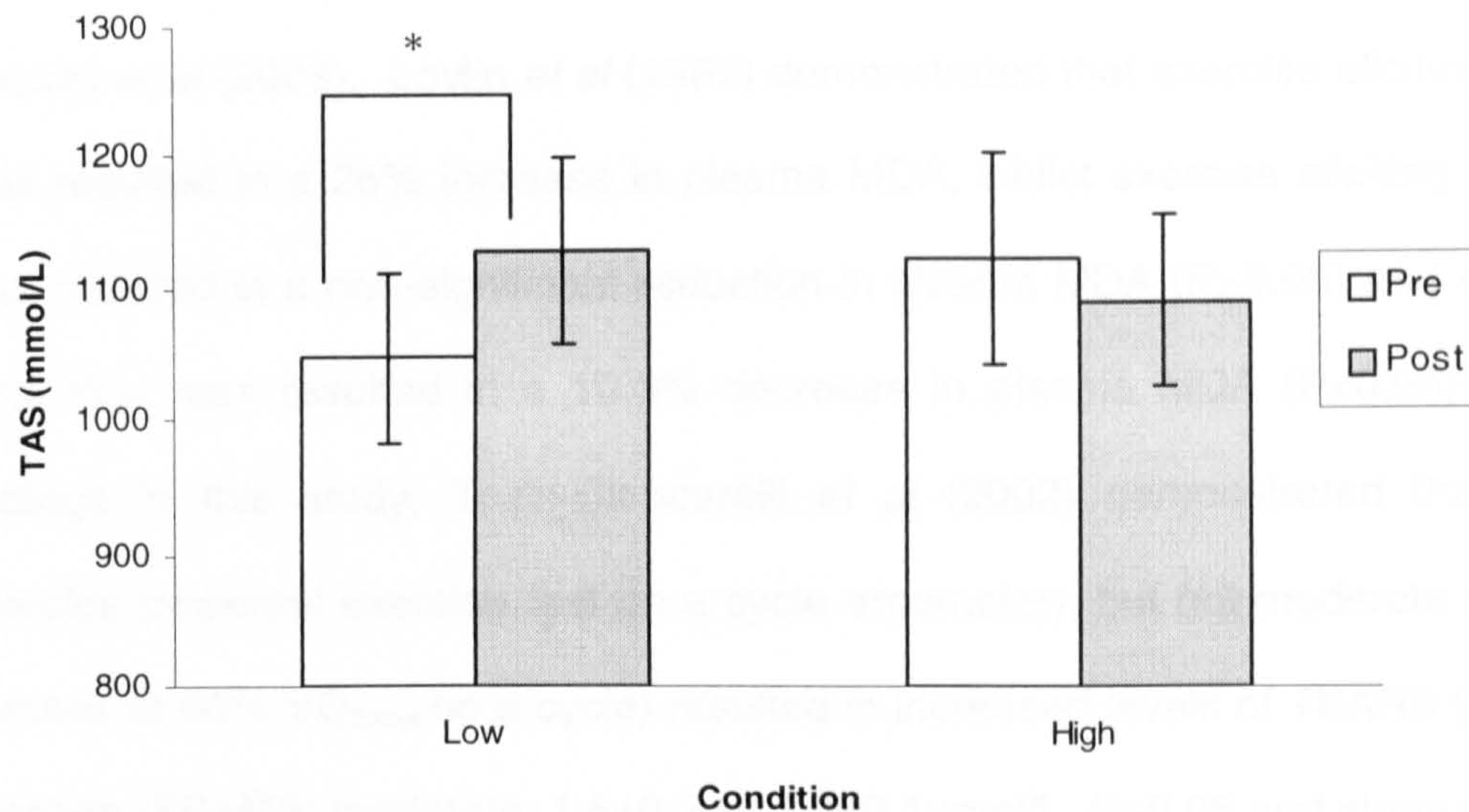


Figure 7.1. TAS levels (mmol/L) pre and post acute aerobic exercise (low- and high-intensity). Results (mean±SEM) were analyzed using the Wilcoxon signed-rank test. (*, P<0.05). For further details see text.

7.3 DISCUSSION

This study examined the effects of acute high- (70% heart rate reserve) and low-intensity (40% heart rate reserve) aerobic exercise on LH and TAS in eleven healthy subjects. Prior to both high- and low-intensity aerobic exercise, LH levels were both similar. LH levels only increased significantly following high-intensity exercise which was also shown to be significantly higher than the LH levels post-exercise following low-intensity exercise. TAS levels increased significantly following low-intensity exercise and decreased slightly following high-intensity exercise but this was not significant.

In comparison to other research studies investigating exercise-induced oxidant stress, this study found similar findings. For example both Ajamini *et al* (2003) and Lawson *et al* (1997) observed a significant increase of 22% (P<0.0001) and 60% (P<0.01) in LH and MDA respectively, following a maximal graded exercise stress test. This study

found a 21% increase in LH levels post high-intensity exercise which is similar to Ajamini *et al* (2003). Lovlin *et al* (1987) demonstrated that exercise eliciting 100% VO_2 max resulted in a 26% increase in plasma MDA, whilst exercise eliciting 70% of VO_2 max resulted in a non-significant reduction in plasma MDA (P>0.05) and exercising at 40% VO_2 max resulted in a 10.3% decrease in plasma MDA (P<0.05). Similar to findings in this study, Tozzi-Ciancarelli *et al* (2002) demonstrated that strenuous exercise (maximal exercise test on a cycle ergometer), but not moderate exercise (30 minutes at 60% VO_{2max} on a cycle) resulted in increased levels of TBARS [rest vs post-exercise (TBARS: moderate, 1.5±0.2 vs 1.4±0.1μmol/L, P>0.05 and strenuous, 1.6±0.2 vs 3.9±0.3μmol/L, P<0.05)]. The findings in this study at low-intensity exercise and those of Tozzi-Cianarelli *et al* (2002) at moderate-intensity exercise did not observe a reduction in LH levels which was found by Lovlin *et al* (1987). The difference in exercise-induced oxidant stress findings between studies may be explained by methodological differences. For example factors such as training status, age and gender of subjects and different exercise protocols could all play a role (Cooper *et al.*, 2002). High-intensity exercise appears to be most consistent factor in provoking increases in free-radical production that overwhelms antioxidant defences (Poulsen *et al.*, 1996). However if oxidative reactions are not elevated directly after exercise, the absence of signs of oxidative stress does not necessarily imply that oxidative damage has not occurred because oxidative reactions may occur hours (Koyama *et al.*, 1999) or even days (Hartmann *et al.*, 1998) after the end of exercise.

In this study, mean TAS values increased significantly following low-intensity exercise (P<0.05) and also decreased slightly following high-intensity exercise but this did not achieve significance. This suggests that a greater oxidant production and antioxidant usage occurred during high-intensity exercise, as the high-intensity exercise group had lower TAS levels post-exercise compared with the low-intensity exercise group

(P>0.05). Similarly Tozzi-Ciancarelli *et al* (2002) also demonstrated that strenuous exercise (maximal exercise test on a cycle ergometer), but not moderate exercise (30 minutes at 60% VO_{2max} on a cycle) resulted in decreased levels of TAS [rest vs post-exercise (TAS: moderate, 1.35±0.04 vs 1.47±0.05U/mL, P>0.05 and strenuous, 1.45±0.03 vs 1.05±0.03U/mL, P<0.05)]. However, Ashton *et al* (1998) found that an exhaustive cycling test increased TAS levels post-exercise (P>0.05) which were similar to the findings of Maxwell *et al* (1993), who showed an increase in plasma TAS levels following 60-minutes of box-stepping exercise. This highlights that the significance of exercise-induced changes in levels of antioxidants post-exercise in relation to oxidant stress is difficult to determine. Oxidant stress could cause a primary decrease in antioxidants, whilst mobilization from secondary sources elsewhere in the body might result in an apparent increase in TAS (Cooper *et al.,* 2002). It has been shown fairly consistently that the GSH:GSSG ratio in the blood decreases with exercise (Dufaux *et al.*, 1997; Viguie *et al.*, 1993), whereas plasma levels of vitamins C and E tend to increase (Gleeson *et al.*, 1987; Duthie *et al.*, 1990; Pincemail *et al.*, 1988). Mobilization of vitamin E has been suggested as a mechanism to explain the changes in vitamin E secondary to oxidative stress; for example, Elsayed *et al* (1990) suggested that vitamin E is mobilized from other tissues to the lungs in response to ozone exposure. In addition, exercise-associated oxidant stress (Ji, 1995b) has shown changes in the distribution of vitamin E, which suggests that some tissues deliver vitamin E. Therefore although changes in oxidation state or concentration of antioxidants can point to impaired antioxidant defences, they do not necessarily indicate tissue damage (Packer, 1997) and it is unclear to what extent they influence oxidant stress. A rise in antioxidant levels might enhance the antioxidant defences in blood, but could possibly impair defences at the sites from which they are mobilized (Cooper *et al.,* 2002).

High-intensity exercise could increase LH in several ways, but the primary source is the mitochondria. As O_2 consumption increases with exercise intensity, the rate of ROS

production increases (Jackson, 1998; Halliwell and Gutteridge, 1999), particularly if the increased production of radicals is not balanced by the antioxidant capacity. Secondary sources of free-radical production include autoxidation of catecholamines, radical generation by phagocytic white cells and radical formation due to the disruption of iron-containing proteins (Jackson, 1998; Halliwell and Gutteridge, 1999). Exercise-induced neutrophilia is another possible source of oxidant stress post aerobic exercise. Neutrophils generate $O_2^{\cdot-}$, reduced plasma vitamin C and uric acid and create oxidant stress (Quindry *et al.*, 2003). Elevations in inflammatory cytokines such as tumor-necrosis factor-α also occur after aerobic exercise (Sen, 1999), which initiate a rapid rise in endogenous oxidants as an essential step in postreceptor signal transduction (Reid and Li, 2001). An alternative mechanism by which exercise may promote free-radical production involves ischaemia-reperfusion. Strenuous exercise is associated with transient tissue hypoxia in several organs as blood is shunted away to cover the increased blood supply required in active skeletal muscles and the skin (Cooper *et al.*, 2002). Strenuous exercise can also cause muscle fibres to undergo relative hypoxia as oxygen supply cannot match the energy requirements (Koyama *et al.*, 1999). Re-oxygenation post-exercise, can also be associated with the production of ROS (Packer, 1997; Koyama *et al.*, 1999).

7.4 CONCLUSION

This study found that thirty-minutes of high-intensity exercise and not low-intensity exercise produced a significant increase in LH levels in healthy subjects. With regards to total antioxidant status, levels increased following thirty-minutes low-intensity exercise, but decreased following thirty-minutes high-intensity exercise. This suggests that a greater oxidation production and antioxidant usage during high-intensity aerobic exercise predisposes subjects to increased oxidant stress.

PART TWO – Study 4

7.5 INTRODUCTION

Obese subjects are prone to increased oxidant stress levels at rest (Yesilbursa *et al.*, 2005; Mohn *et al.*, 2005; Keany *et al.*, 2003; Ozata *et al.*, 2002; Davi *et al.*, 2002; Dandona *et al.*, 2001; Prázný *et al.*, 1999) and four reports of exercise-induced oxidant stress in obesity suggest increase susceptibility to oxidant damage following high-intensity exercise (Saiki *et al.*, 2001; Vincent *et al.*, 2004; Vincent *et al.*, 2005a; Vincent *et al.*, 2005b). Possible mechanisms for the increased exercise-induced oxidant stress include enhanced oxygen metabolism, anoxia-reoxygenation, mechanical damage to tissues and increased inhalation of environmental pollutants (NO_2, ozone etc) (Vincent *et al.*, 2004). This could have long-term implications as increased oxidant stress is associated with reduced contractile function, arrhythmias and muscle fatigue (Kukreja and Hess, 1992; Vincent *et al.*, 1999; Yu, 1994) and is implicated in pathologies such as diabetes, atherosclerosis and obesity (Bouloumie *et al.*, 1999; Gackowksi *et al.*, 2001; Johnson, 2002; Kennedy and Lyons, 1997; Vincent *et al.*, 1999).

Following an acute exercise session, platelet aggregation may also be increased, which potentially increases the risk of developing atherothrombotic vascular disease (Grundy *et al.*, 2002; McGill *et al.*, 2002). Previous studies have shown that a single bout of moderate-intensity exercise does not cause platelet activation (Chicarro *et al.*, 1994) or tends to desensitize platelets (Wang *et al.*, 1994) and that strenuous-intensity exercise may favour (Kestin *et al.*, 1993) platelet aggregation. Although it has been shown that obesity increases platelet aggregation (Davi *et al.*, 2002), it is unknown if obesity also increases exercise-induced platelet aggregation. It could be speculated that platelet aggregation during exercise should be enhanced in obesity when

compared to normal-weight subjects. Furthermore, exercise-induced oxidant stress in obesity may be a pivotal cause for increased exercise-induced platelet aggregation. For example, evidence suggests that not only does exogenously derived ROS affect the regulation of platelet activation, but platelets themselves also generate ROS (Krotz *et al.*, 2004).

This study investigated the effect of acute high-intensity aerobic exercise on LH, TAS, SOD, GSH and percentage ADP-induced platelet aggregation in normal-weight and overweight subjects. The association between LH, TAS and ADP-induced platelet aggregation during acute high-intensity aerobic exercise was also studied.

7.6 METHODOLOGY

Subject Characteristics

The study group consisted of 20 subjects who were sex-matched between groups [10 normal-weight (BMI: 23.6±0.53kg/m^2) and 10 overweight (BMI: 28.4±0.71kg/m^2) healthy male and females (n=10/10)] (see table 7.1 for subject characteristics). Volunteers were invited to take part in the study by local advertisement. An inclusion criterion was age between 18 and 50 years old and BMI between 20 and 35kg/m^2. Subjects with a history of diabetes, cardiovascular or cerebrovascular disease, hepatic or renal disease, tobacco abuse, or those on hormone replacement therapy were excluded. In addition subjects were excluded if they were hypertensive (with or without treatment), taking treatment for dyslipidaemia, taking any antioxidant supplementations or a smoker. Written informed consent was obtained from all the subjects after they had been given a full explanation of the study. The research was given ethical approval by Bedfordshire Local Research Ethics Committee.

Table 7.1. Subject characteristics

Characteristic	Normal Weight NW (n=10) (m/f; 5/5)	Overweight OW (n=10) (m/f; 5/5)	P-Value NW vs OW
Age, yr	27.90 ± 2.19	31.40 ± 1.98	.251
Body mass, kg	67.78 ± 3.03	82.50 ± 3.86	<.01
BMI, kg/m^2	23.55 ± 0.53	28.40 ± 0.71	<.001
Body fat, kg	17.93 ± 1.33	28.50 ± 3.08	<.01
Waist, cm	77.89 ± 1.99	93.50 ± 2.94	<.001
Systolic BP, mmHg	117.70 ± 3.06	126.70 ± 3.94	.088
Diastolic BP, mmHg	73.10 ± 1.43	82.80 ± 1.28	<.001
Fasting glucose, mmol/L	6.51 ± 0.45	5.87 ± 0.44	.327
Fructosamine, mmo/L	182 ± 8.25	195 ± 7.52	.28
Total cholesterol, mmo/L	4.60 ± 0.54	4.24 ± 0.28	.856
HDL-cholesterol, mmol/L	1.40 ± 0.13	1.23 ± 0.10	.322
LDL-cholesterol, mmol/L	2.09 ± 0.19	2.28 ± 0.21	<.05
Triglycerides, mmol/L	2.10 ± 0.83	2.39 ± 0.56	.466

Values expressed as the mean±SEM. BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Results were compared using a One-way ANOVA and P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

Experimental design

Subjects visited the Centre for Obesity Research on two separate occasions between 9-11am over a 2-week period and were instructed to fast for 10-12 hours and refrain from exercise, caffeine and alcohol for 48 hours before the study visits. Subjects were also asked to maintain their usual dietary pattern.

During week 1 anthropometric measures, a venous blood sample and VO_{2max} test were completed.

During week 2, following a 15-minute supine rest a venous blood sample was taken. Subjects were then asked to exercise on a motorised treadmill. The exercise session adopted the following protocol

- 5-minutes warm up (40% VO_{2max})
- The subject was then required to perform a constant workload corresponding to 70% VO_{2max} for 30-minutes.

Heart rate was monitored throughout the exercise sessions. A venous blood sample was taken, immediately post and 30-minutes post-exercise.

Anthropometric and cardiovascular measurements and venous blood sampling

Height, weight and waist measurements are described in detail in the methodology chapter (section 3.2.1, 3.2.2, 3.2.3). Body composition was assessed using the BodPod, which is outlined in the methodology chapter (section 3.2.4.2). Cardiovascular measurements are described in the methodology chapter (section 3.3). Venous blood samples were collected as described in the methodology chapter (section 3.1.1 and 3.2.2).

Maximal aerobic exercise test

The maximal exercise test incorporated the Bruce Protocol (Bruce, 1972). O_2 uptake was assessed using the MetaMax 3B®. Maximal aerobic capacity was determined when the subject met two of the following criteria:

- Within 10 beats of maximal age predicted heart rate (220 – age)
- Respiratory quotient greater than 1.1
- Rating of perceived exertion >17

Biochemical measurements

LH, TAS, SOD and GSH were performed as described in the methodology chapter (section 3.1.12.1, 3.1.14.1, 3.1.14.2 and 3.1.14.3). Other biochemical measures included, fasting plasma glucose, plasma fructosamine, plasma cholesterol, plasma LDL, plasma HDL and plasma triglycerides as described in methodology chapter (section 3.1.6, 3.1.7, 3.1.8, 3.1.9, 3.1.10 and 3.1.11). Percentage ADP-induced platelet aggregation was measured using PlateletWorks®, which is outlined in the methodology chapter (section 3.1.14.2). Final concentrations of ADP used for platelet aggregation were 10 and 20 μM. Haematocrit and haemoglobin was also measured using PlateletWorks® which is also outlined in the methodology chapter (section 3.1.4.1 and 3.1.5.1). Haematocrit and haemoglobin were measured to calculate the change in plasma volume (Dill and Costill, 1974).

Statistical Analysis

Values reported as mean±SEM. SEM was appropriate because it demonstrates how liable to error the mean is (e.g. the mean value or the mean change or improvement). The Kolmogorov-Smirno test confirmed that the data was normally distributed. The data was analyzed using analysis of variance (ANOVA) with repeated measures, which is appropriate for detecting differences between groups, time effects and interactions. A two-way (AxB) mixed ANOVA was used which incorporated one between (exercise intensity: low intensity vs high intensity) and one within (time: rest vs post-exercise) subjects factor. When significant F values were noted paired t-tests were used to detect specific differences within groups at certain time points during the study. To detect differences between groups, one-way ANOVA was incorporated with one within (time: rest vs post-exercise) subject factor. Associations between parameters were assessed using the Pearson correlation test. Differences were considered statistically

significant at P<0.05. Statistical analysis was carried out using a computer software package (SPSS for Windows, Version 13.0).

7.7 RESULTS

Subject characteristics for the normal-weight and overweight groups are shown in table 7.1. Body mass, BMI, body fat and waist circumference, DBP and LDL concentration were all significantly higher in the overweight group compared to the normal-weight group (P<0.001, P<0.01, P<0.001, P<0.001, P<0.05, respectively). All subjects had similar SBP, fasting glucose, fructosamine, cholesterol, HDL and fasting triglycerides.

Figures 7.2 and 7.3 demonstrate LH and TAS levels pre- and post-exercise in the normal-weight and overweight groups, respectively. At rest there was no difference in LH between the groups [normal-weight vs overweight (0.64±0.06 vs 0.77±0.09μmol/L, P>0.05). However following high-intensity aerobic exercise, LH levels increased significantly in the overweight group (P<0.05) compared to the normal-weight group (P>0.05) (percentage increase in LH levels post high-intensity exercise, overweight vs normal-weight, +24.6% vs +12.5%). Thus the LH level in the overweight group was significantly higher than the normal-weight group post-exercise [overweight vs normal-weight (0.96±0.09 vs 0.72±0.06μmol/L, P<0.05)]. Although thirty-minutes post-exercise LH levels had declined, they were not as significantly different from both pre and post LH levels. No significant differences in TAS levels were noted between the normal-weight and overweight group pre- and post-exercise, and pre- and post-exercise within each group.

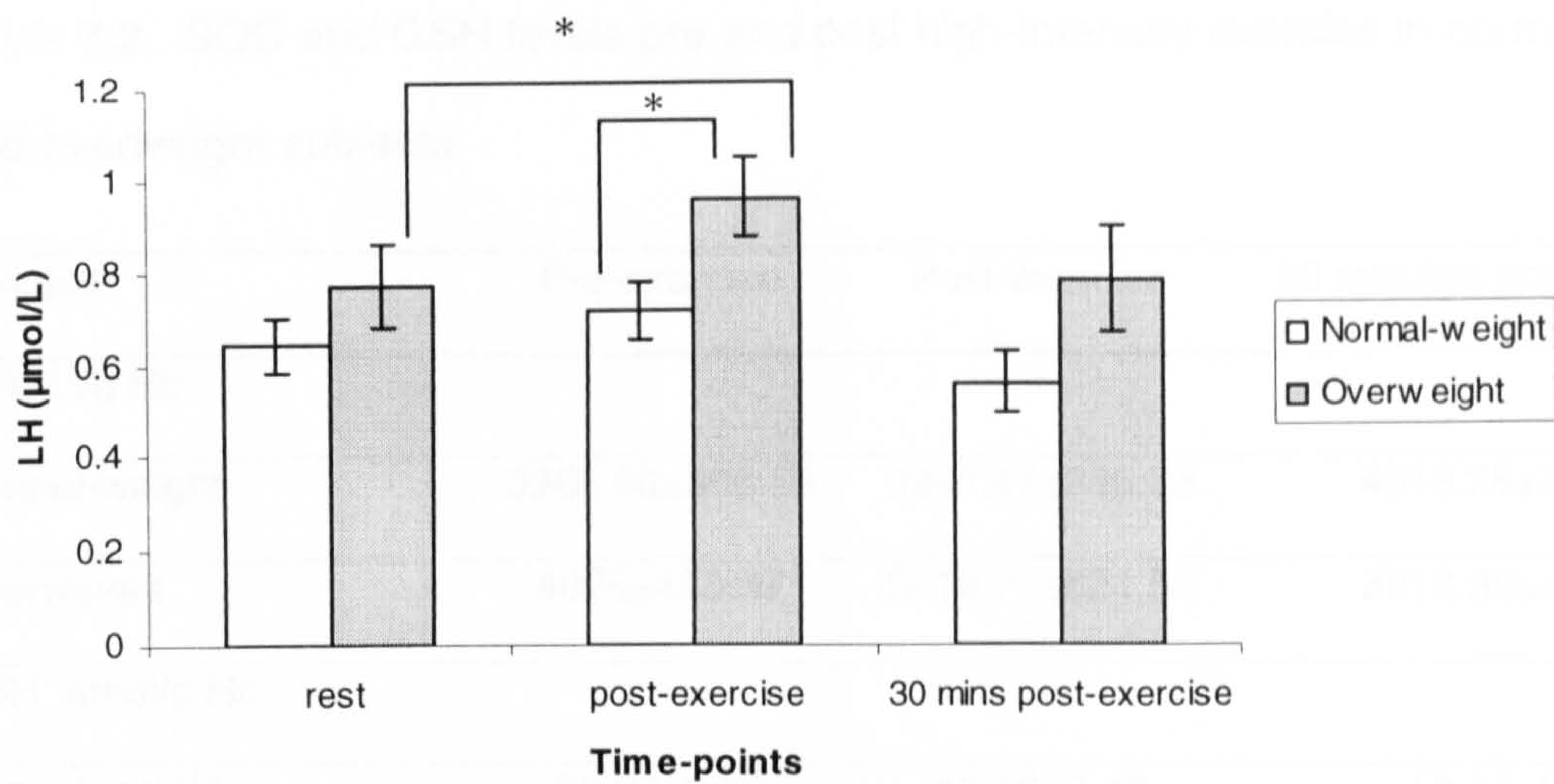


Figure 7.2. LH levels (µmol/L) pre and post high-intensity exercise in normal-weight and overweight subjects. Results (mean±SEM) were analyzed using one-way ANOVA and paired t-tests (*, P<0.05). For further details see text.

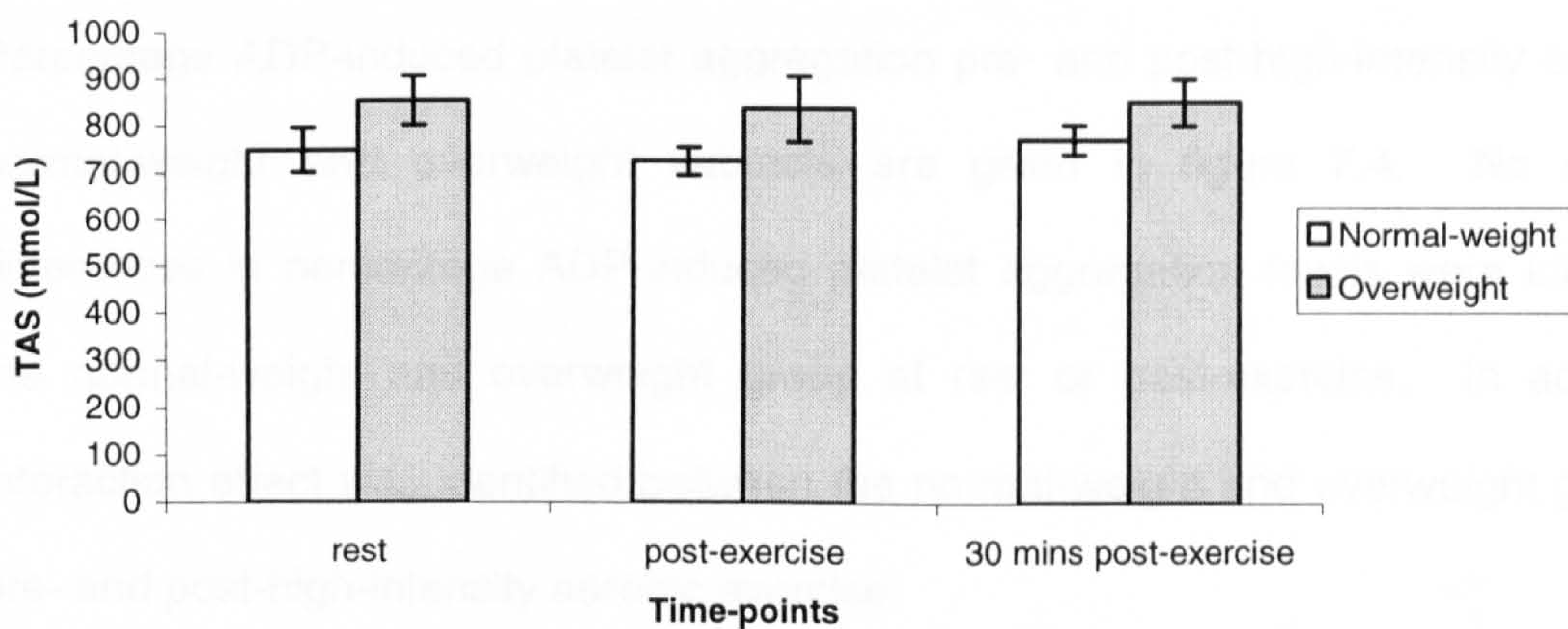


Figure 7.3. TAS levels (mmol/L) pre and post high-intensity exercise in normal-weight and overweight subjects. Results (mean±SEM) were analyzed using one-way ANOVA. For further details see text.

SOD and GSH levels pre- and post-high-intensity aerobic exercise in normal-weight and overweight subjects are given in table 7.2. SOD and GSH levels were not significantly different in the normal-weight and overweight group at rest or post-exercise. In addition, no interaction effect was identified between the normal-weight and overweight group and pre- and post-high-intensity aerobic exercise.

Table 7.2. SOD and GSH levels pre and post high-intensity exercise in normal-weight and overweight subjects.

Variable	Pre-exercise	Post-exercise	30 minutes post-exercise
SOD U/g Hb			
Normal-weight	3965.90±306.23	3967.47±336.23	4013.28±325.31
Overweight	4076±453.37	5018.71±621.52	3912.39±422.46
GSH μmol/g Hb			
Normal-weight	20.86±3.00	12.85±2.43	18.15±3.15
Overweight	16.62±1.78	16.74±5.14	27.6±8.98

Values expressed as the mean±SEM. SOD, superoxide dismutase; GSH, reduced glutathione. Results were analyzed using a one-way ANOVA. For further details see text.

Percentage ADP-induced platelet aggregation pre- and post-high-intensity exercise in normal-weight and overweight subjects are given in figure 7.4. No significant differences in percentage ADP-induced platelet aggregation levels were identified in the normal-weight and overweight group at rest or post-exercise. In addition no interaction effect was identified between the normal-weight and overweight group and pre- and post-high-intensity aerobic exercise.

The association between LH and percentage ADP-induced platelet aggregation pre- and post-exercise is shown in figure 7.5. For both ADP-induced platelet aggregation concentrations (10 and 20μM) an increase in LH level was associated with decreased percentage platelet aggregation, indicating enhanced *in vivo* platelet reactivity. Pearson correlation found that at 20μM, percentage ADP-induced platelet aggregation with LH was $r=-0.329$, $P<0.01$ and at 10μM, the percentage ADP-induced platelet aggregation with LH was $r=-0.240$, $P=0.065$.

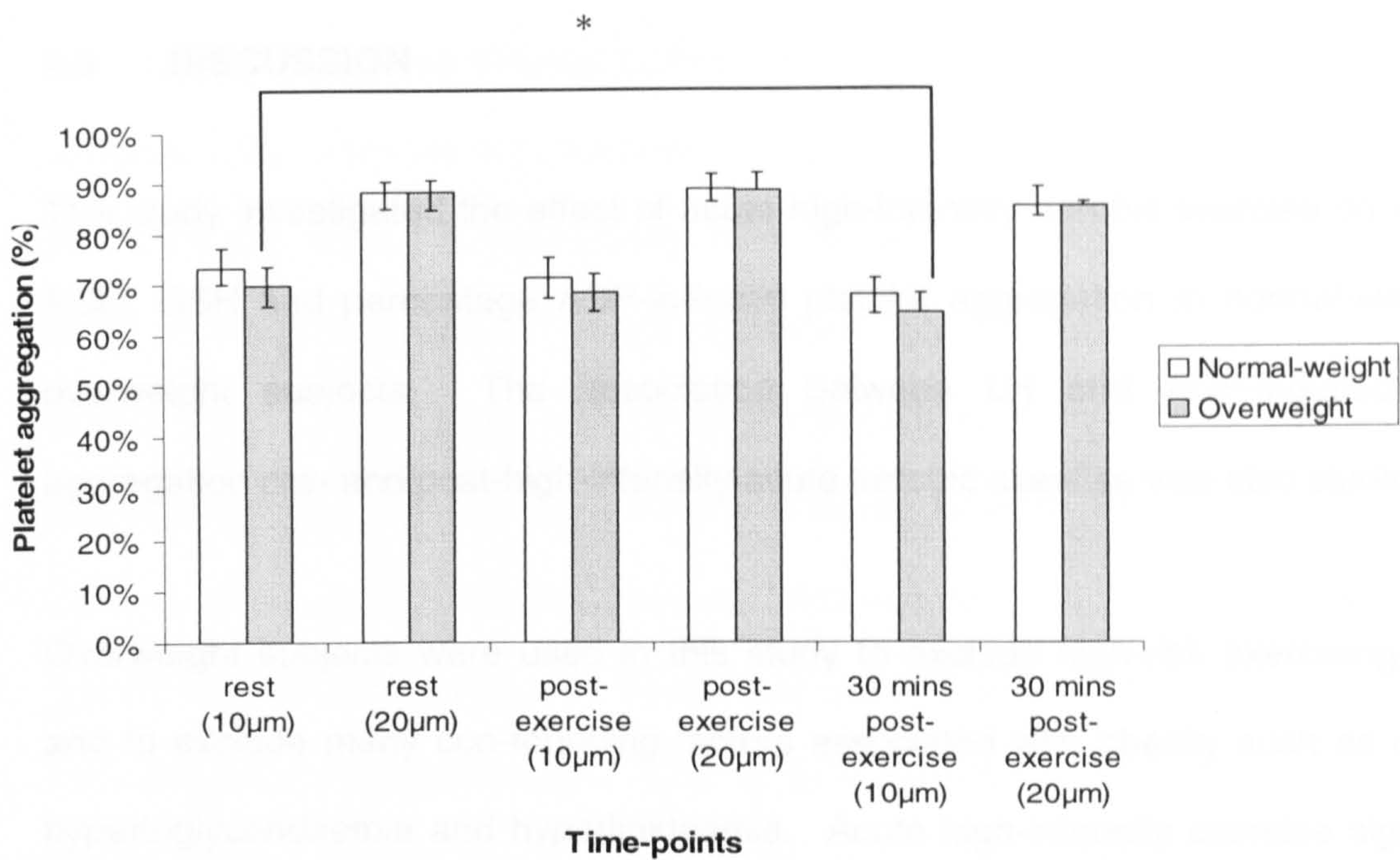
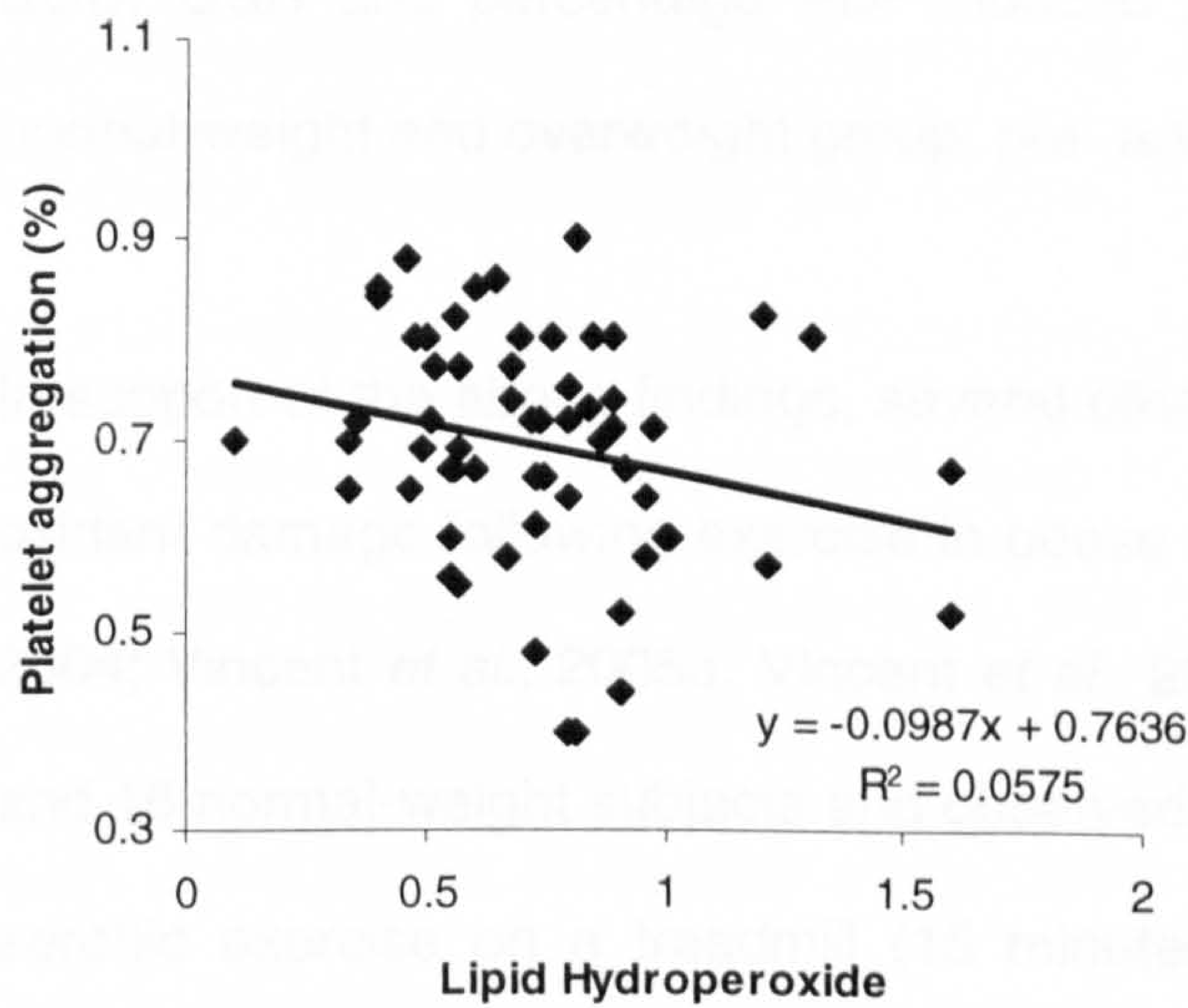


Figure 7.4. Percentage ADP-induced platelet aggregation (20μM and 10μM) pre and post high-intensity exercise in normal-weight and overweight subjects. Results (mean±SEM) were analyzed using one-way ANOVA and paired t-tests. (*, P<0.02). For further details see text.

a) Final ADP concentration 10μM



b) Final ADP concentration 20μM

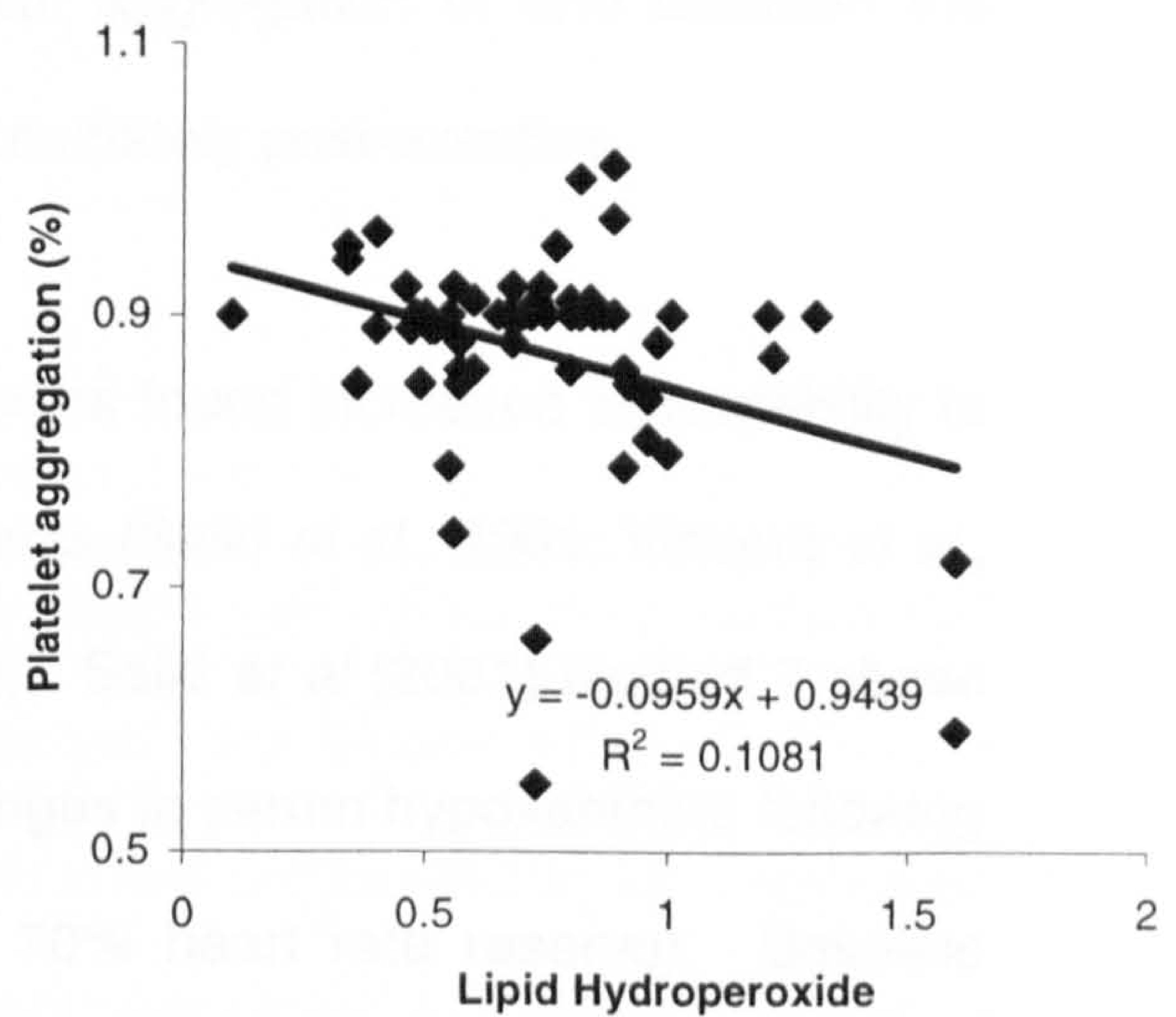


Figure 7.5. Correlation coefficients for the relationship between LH and percentage ADP-induced platelet aggregation [final ADP concentration 10μM (a) and 20μM (b)]. For further details see text.

7.8 DISCUSSION

This study investigated the effect of acute high-intensity aerobic exercise on LH, TAS, SOD, GSH and percentage ADP-induced platelet aggregation in normal-weight and overweight subjects. The association between LH and ADP-induced platelet aggregation pre- and post-high-intensity acute aerobic exercise was also studied.

Overweight subjects were used in this study to exclude high-risk exercising patients and to exclude many con-founding factors associated with obesity such as diabetes, hypertriglyceridaemia and hyperlipidaemia. Acute high-intensity exercise significantly increased LH levels in the overweight group, which returned to pre-exercise levels thirty-minutes post-exercise. No significant changes in LH were identified following high-intensity exercise in the normal-weight group. Interestingly, although no significant difference was identified in LH levels pre-exercise between the normal-weight and overweight group, post-exercise there was a significant difference in LH levels between the two groups. No other significant changes were identified in TAS, SOD, GSH and percentage ADP-induced platelet aggregation in and between the normal-weight and overweight group, pre- and immediately post-exercise.

In support of the above findings, several other studies found increased susceptibility to oxidant damage following exercise in obese subjects (Saiki *et al.,* 2001; Vincent *et al.,* 2004; Vincent *et al.,* 2005a; Vincent *et al.,* 2005b). Saiki *et al* (2001) studied 7 obese and 16 normal-weight subjects and observed changes in serum hypoxanthine following aerobic exercise on a treadmill (15 minutes at 70% heart rate reserve). Baseline hypoxanthine levels were significantly higher in the obese group compared to the normal-weight group [obese vs normal-weight (3.46±3.70 vs 1.23±1.16mmol/L, $P<0.05$)]. Exercise-induced a pronounced increase in serum hypoxanthine level in the obese group compared with the normal-weight group [obese vs normal-weight

(43.86±4.56 vs 10.65±6.81mmol/L, P<0.01)]. Vincent *et al* (2004) assessed LH levels in normal-weight (n=14) and obese (n=14) subjects, pre and post aerobic exercise. At rest LH levels were not significantly different between the normal-weight and obese group, but post-exercise LH levels increased by 70% and 62% in the obese and normal-weight, respectively, which was significantly different to pre-exercise LH levels and significantly different between the normal-weight and obese group. Vincent *et al* (2005a) also found significantly increased post-exercise LH levels following a maximal graded exercise stress test on a treadmill in obese women compared to normal-weight women [obese vs normal-weight (0.13 vs 0.02 (nmol/mL) (O_2/kg/min)]. This occurred despite a 20% shorter exercise time for the obese women than for the normal-weight women. After controlling for body fat percentage and baseline LH, the major contributors of the change in LH levels during exercise were age, peak heart rate and exercise duration. However, when exercise loads were matched by relative intensity, LH responses still remained higher in obese than normal-weight persons [obese vs normal-weight (0.289 vs 0.054 (nmol/mL) (O_2/kg min)] (Vincent *et al.,* 2005b). All studies have shown that compared to normal-weight, obese subjects have a 300-600% greater increase in LH lipid peroxidation markers, post high-intensity exercise. In this study the overweight group had a 133% greater increase in LH post high-intensity exercise when compared to the normal-weight group. The lower increase in LH values may be due to using subjects with lower BMI values compared to the above studies. For example, in this study the mean BMI of the overweight group was 28.40kg/m^2 compared to 33.3kg/m^2 and 30.6kg/m^2 in the obese group of Vincent *et als* (2005) and Saiki *et als* study. In addition other factors which affect differences in LH responses post high-intensity exercise include exercise intensity and exercise duration (Poulsen *et al.*, 1996).

Despite matching for oxygen uptake during exercise in the normal-weight and overweight groups, LH levels post-exercise were still greater in the overweight group.

The exacerbated exercise-induced lipid peroxidation in the overweight group may be due to several factors including an insufficient antioxidant defence. Several studies suggest that obesity is associated with lower plasma antioxidant concentration (Reitman *et al.*, 2002; Strauss *et al.*, 1999; Decsi *et al.*, 1997; Kuno *et al.*, 1998; Moor De Burgos *et al.*, 1992) and decreased activities of erythrocyte cytoprotective enzymes (Olusi, 2002; Ozata *et al.*, 2002; Beltowski *et al.*, 2000). A lowered antioxidant defence leaves the tissues susceptible to free-radical attack during exercise (Ashton *et al.*, 1998). Higher tissue lipid levels in the obese may also provide a larger target for oxidative damage by free-radicals (Vincent *et al.*, 2001). In the obesity exercise-induced study by Vincent *et al* 2004, elevated dietary fat intakes and plasma triglycerides were present in the obese group compared to the non-obese group, which was shown to be positively correlated with exercise-induced change in LH levels. Despite the increase in consumption of dietary fats in the obese group compared to the non-obese group, this did not enhance consumption of antioxidants, which suggests that the obese group may not have taken in antioxidant amounts necessary to prevent oxidant stress. High cell respiration and O_2 consumption may also have been exacerbated in muscle tissue during physical activity in the overweight group due to the additive mechanical load of carrying excessive body weight (Vincent *et al.*, 2004) and mechanical inefficiency (Vincent and Taylor, 2006). For example during the same absolute load-bearing walking activity, obese persons have 38% higher VO_2 (oxygen consumption) values than non-obese persons and these values are correlated with post-exercise LH values (Vincent *et al.*, 2004). Lastly, increased body weight, may cause oxidant stress via endothelial enzymatic sources within tissues (i.e. increased NADH oxidase activity and H_2O_2) (Rajagopalan *et al.*, 1996; Kaminski *et al.*, 2002) or by pro-oxidant complications of increased body weight such as hypertension that increase vascular $O_2^{\cdot}$ and LH levels (Frisbee *et al.*, 2002).

In addition to this study, other research has also found no change in TAS levels pre-

and post-aerobic exercise in and between normal-weight and obese subjects (Vincent *et al* 2004). SOD and GSH also showed no significant difference in and between the normal-weight and obese group, pre- and post-high intensity exercise. Likewise, Vincent *et al* (2005b) observed no difference in antioxidant thiol profiles (total thiols, protein thiols, and nonprotein thiols (glutathione) before and after exercise between normal-weight and obese groups. It was anticipated that in this study the overweight group would have lower antioxidant enzymes pre- and post-exercise compared to their normal-weight counterparts, because of the evidence for reduced antioxidant status at rest in obesity (Vincent *et al.*, 2001). It is possible that the lack of reduction in TAS post high-intensity exercise may be a result of mobilization of secondary sources of antioxidants (Cooper *et al.*, 2002). For example mobilization of vitamin E has been suggested as a mechanism to explain the changes in increased vitamin E levels secondary to oxidative stress (Elsayed *et al.*, 1990). However mobilization of secondary sources of antioxidants might rise antioxidant defences in the blood, but impair defences at the site from which the antioxidants were mobilized (Cooper *et al.*, 2002).

This study aimed to identify the link between oxidant stress and percentage ADP-induced platelet aggregation during acute aerobic high-intensity exercise. Results from chapter five concluded that at rest oxidant stress in obesity was not a source of increased platelet aggregation in obesity. However Davi *et al* (2002) demonstrated a linear relationship between 8-iso $PGF_2\alpha$ and 11-dehydro-TxB_2 ($r=0.61$, $P<0.001$) concluding that the association between lipid peroxidation and platelet activation may be a novel mechanism, through which obesity may affect cardiovascular morbidity and mortality (Grundy *et al.*, 2002; McGill *et al.*, 2002). Furthermore oxidant stress-mediated platelet aggregation has also been found in several settings of risk factors for atherosclerosis and cardiovascular thrombosis, including diabetes mellitus,

hypertension and hypercholesterolemia (Davi *et al.*, 2003; Minuz *et al.*, 2002; Davi *et al.*, 1997). This study is the first to identify whether there is a possibility of 'oxidant stress-mediated platelet aggregation' during and post acute aerobic high-intensity exercise in normal-weight and overweight subjects. Tozzi-Ciancarelli *et al* (2002) demonstrated that oxidant stress induced by strenuous exercise interfered with platelet responsiveness. This was evident by adding LDL-Ox to PRP obtained from blood samples collected from 6 subjects immediately after strenuous exercise as ADP-induced platelet aggregation increased and intra-platelet NO content decreased. However the speculation by Tozzi-Ciancarelli *et al* (2002) that strenuous exercise interfered with platelet responsiveness by promoting LDL-Ox mediated platelet activation did not necessarily prove that the exercise-associated increase in platelet aggregation was mediated by LDL-Ox formation. In this study, following correlation coefficient analysis for platelet aggregation and LH responses, pre and post acute aerobic high-intensity exercise in normal-weight and overweight subjects, it was found that LH demonstrated a significant negative association with platelet aggregation at high ADP-induced platelet aggregation (20μM: r=-0.0.329, P<0.01) and a negative trend towards being associated with platelet aggregation at low ADP-induced platelet aggregation (10μM: r=-0.240, P=0.065). This confirms the suggestion that increased LH may decrease percentage ADP-induced platelet aggregation (which is an expression of enhanced *in vivo* platelet reactivity). However this finding, despite increased LH post-exercise did not cause decreases in ADP-induced platelet aggregation in both normal-weight and overweight subjects following high-intensity acute aerobic exercise. It is proposed that high-intensity exercise was not strenuous enough to induce platelet aggregation because both Tozzi-Ciancarelli *et al* (2002) and Wang *et al* (1994) demonstrated increased platelet aggregation following strenuous exercise.

7.9 CONCLUSION

In conclusion, exercise-induced oxidant stress is enhanced in overweight subjects, compared to normal-weight subjects, despite controlling and matching VO_2 usage during high-intensity exercise. A combination of obesity related factors including elevated body fat and triglyceride levels, decreased antioxidant status, and increased VO_2 during exercise have been implicated in this process. During high-intensity aerobic exercise, LH levels may be associated with decreased platelet aggregation (expression of enhanced *in vivo* platelet reactivity) but decreased platelet aggregation (expression of enhanced *in vivo* platelet reactivity) was not observed in either the normal-weight group and overweight group.

Chapter EIGHT

Study 5

The effect of selenium supplementation on lipid peroxidation, total antioxidant status and platelet responsiveness at rest and post high-intensity acute exercise in normal-weight and overweight subjects

8.0 INTRODUCTION

Given that both obesity and acute high-intensity exercise induces oxidant stress, it is crucial that therapies are developed to reduce this exacerbated response. Oxidant stress is associated with reduced contractile function, arrhythmias and muscle fatigue during acute exercise (Kukreja and Hess, 1992; Vincent *et al.,* 1999; Yu, 1994) and is implicated in pathologies such as diabetes, atherosclerosis and obesity (Bouloumie *et al.,* 1999; Gackowksi *et al.,* 2001; Johnson, 2002; Kennedy and Lyons, 1997; Vincent *et al.,* 1999). Potential treatment therapies to reduce oxidant stress include calorie restriction (Velthuis-te Wierik *et al.,* 1996), increased antioxidant intake (diet composition) (Armstrong and Doll, 1975; Rimm *et al.,* 1996), exercise training (Fukai *et al.,* 2000) and antioxidant supplementation (Skrha *et al.,* 1999; Manning *et al.,* 2004; Anderson *et al.,* 1999).

Several researchers have demonstrated that antioxidant therapy may reduce oxidant stress levels at rest in obesity. For example both Skrha *et al* (1999) and Manning *et al* (2004) observed decreased MDA levels at 3-months and reduced LH levels at 3-months and 6-months following vitamin E supplementation in obese subjects, respectively. In addition, Anderson *et al* (1999) indicated protection against formation of oxidative biomarkers with an 8-week antioxidant treatment [β-carotene (24mg), vitamin C (1000mg) and vitamin E (800IU)]. Antioxidant therapy has also been investigated as a therapy to reduce the exercise-induced oxidant stress during high-intensity exercise. For example, Sumida *et al* (1989) demonstrated that 4-weeks of vitamin E supplementation prevented a rise in plasma MDA levels following maximal-intensity cycle exercise. Ashton *et al* (1999) demonstrated that acute ascorbic acid supplementation prevented exercise-induced oxidant stress in healthy subjects. In contrast to these findings, daily supplementation with an antioxidant mixture (30 mg ß-

carotene, 592 mg vitamin E and 1000 mg vitamin C) did not prevent the exercise-induced rise in plasma MDA after moderate- to high-intensity treadmill running (Kanter et al., 1993). The effects of antioxidant supplementation on oxidant stress levels in overweight and obese subjects has not been studied following acute exercise.

Alternatively, selenium (Se) supplementation may be a suitable candidate to reduce the obesity-associated oxidant stress and exercise-induced oxidant stress. Se is an essential component of the GSH-Px system (Ladenstein *et al.*, 1979), which functions as part of an antioxidant system to protect PUFAs and proteins from the damaging effects of peroxides and LH (Richter, 1987; Del Maestro *et al.*, 1980). In humans, Se deficiency has been implicated in the etiology of CVD and other conditions in which oxidant stress and inflammation are prominent features, but there is still only limited evidence from epidemiological studies for this and the therapeutic benefit of Se administration in the prevention and treatment of CVD remains insufficiently documented (Alissa *et al.*, 2003).

Epidemiological studies have indicated an association between a low Se status and an increased risk for ischaemic heart disease (Huttunen, 1986). However these results are not consistent with the findings of Miettinen *et al* (1983) and Kok *et al* (1987) who found no association between blood Se levels and the risk of CVD. In addition, no differences in tissue Se were apparent in patients who died, with or without myocardial infarction (Ringdal *et al.*, 1986). However Kiem and Feinendegen (1984) did observe diminished activities of GSH-Px and decreased Se levels in platelets from patients with acute myocardial infarction exhibiting a greater tendency to aggregation. Neve (1996) supported the proposed protective effect of Se against CVD by the ability of GSH-Px to combat the oxidative modification of lipids and to reduce platelet aggregation. The prevention of LH build-up promotes the production of vasodilatory prostacylin by the

endothelium which in turn reduces the production of thromboxane, thus reducing vasoconstriction and platelet aggregation (Neve, 1996).

Considering potential consequences of low Se intake, it is no surprise that current UK Se intake is a cause for concern (Rayman, 2002). UK daily intakes of Se have reduced from 60-63 ug/d to the current level of 34-39µg/d (Barclay *et al.,* 1995; Ministry of Agriculture Fisheries and Food, 1997, 1999) which does not meet the reference nutrient intake for males and females (75 and 60µg/d respectively) (Department of Health, 1991). In addition, current plasma or serum Se concentrations may not allow maximal expression of plasma GSH-Px (Duffield *et al.,* 1999). Consequently Se supplementation in all individuals may be of benefit but specific population groups may need to be targeted first. For example obese subjects are prone to both enhanced oxidant stress (Yesilbursa *et al.,* 2005; Mohn *et al.,* 2005; Keany *et al.,* 2003; Ozata *et al.,* 2002; Davi *et al.,* 2002; Dandona *et al.,* 2001; Prázný *et al.,* 1999) and platelet hyperactivity (Anfossi *et al.,* 2004) and furthermore have been shown to have reduced GSH-Px levels (Olusi *et al.,* 2002).

Although the effect of Se supplementation in overweight or obese subjects on oxidant stress levels at rest or following exercise is unknown, it can be speculated that Se supplementation will reduce both the obesity-associated oxidant stress and exercise-induced oxidant stress. Several studies support that Se supplementation has the potential to reduce oxidant stress. For example Sarada *et al* (2002) noted that Se supplementation reduced lipid peroxidation in male albino rats during exposure to hypoxia-induced oxidant stress. Bortoli *et al* (1991) and Wilke *et al* (1992) observed reduced MDA levels following Se supplementation in elderly women and phenylketonuric (PKU) children (at risk of Se deficiencies), respectively. However a

double-blind, cross-over study demonstrated no beneficial effect of Se supplementation on reducing organic hydroperoxide levels in cystic fibrosis children (Portal *et al.*, 1995).

This study investigated the effect of Se supplementation on LH, TAS, GSH, SOD and ADP-induced platelet aggregation in healthy normal-weight and overweight subjects at rest and following acute aerobic high-intensity exercise. The study aimed to evaluate the potential Se supplementation may have on reducing both obesity-associated oxidant stress and exercise-induced oxidant stress and associated impact on platelet aggregation, which may determine whether Se supplementation is a potential therapeutic tool for reducing the risk of CVD.

8.1 METHODOLOGY

Subject Characteristics

The study group included 20 subjects [10 normal-weight (BMI: 23.4±0.46kg/m^2) and 10 overweight (26.6±0.36kg/m^2) healthy males and females (normal-weight (n=4/6), overweight (n=4/6)] (see table 8.0 for subject characteristics). Volunteers were invited to take part in the study by local advertisement. An inclusion criterion was age between 18 and 50 years old and BMI between 20 and 35kg/m^2. Subjects with a history of diabetes, cardiovascular or cerebrovascular disease, hepatic or renal disease, tobacco abuse, or those on hormone replacement therapy were excluded. In addition subjects were excluded if they were hypertensive (with or without treatment), taking treatment for dyslipidaemia, taking any antioxidant supplementations or a smoker. Written informed consent was obtained from all the subjects after they had been given a full explanation of the study. The research was given ethical approval by Bedfordshire Local Research Ethics Committee.

Table 8.0. Subject characteristics

Characteristic	Normal-weight NW (n=10) m/f (5/5)	Overweight OW (n=10) m/f (5/5)	P Value NW vs OW
Age, yr	27.9 ±2.19	31.4±1.98	.251
Body mass, kg	67.72 ± 2.98	82.82 ± 3.73	<.01
Body mass index, kg/m^2	23.55 ± 0.53	28.42±0.73	<.001
Body fat, kg	16.64±1.53	28.01±3.17	<.01
Waist, cm	77.95±1.94	92.58±3.02	<.001
Systolic BP, mmHg	116.30±3.19	128.30±3.37	.08
Diastolic BP, mmHg	73.60±2.00	83.10±1.49	<.001
Fasting glucose, mmol/L	6.51±0.45	5.87±0.44	.327
Fructosamine, mmol/L	188±8.34	201.33±7.30	.38
Total cholesterol, mmo/L	4.60±0.53	4.77±0.18	.856
HDL-cholesterol, mmol/L	1.66±0.11	1.52±0.13	.32
LDL-cholesterol, mmol/L	2.58±0.45	2.72±0.22	<.05
Triglycerides, mmol/L	0.79±0.07	1.14±0.27	.47

Values expressed as the mean±SEM. BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Results were compared using a one-way ANOVA and P values are given in the Table. P values < 0.05 were considered significant. For further details see text.

Experimental design

Subjects visited the Centre for Obesity Research, Luton and Dunstable Hospital on four separate occasions between 9-11am over a 14-week period and were instructed to fast for 10-12 hours and refrain from exercise, caffeine and alcohol for 48 hours before the study visits. Subjects were also asked to maintain their usual dietary pattern.

A placebo-controlled, double-blind, cross-over study was performed, with a wash-out period before crossing over. Each subject received 200µg Se (sodium selenite) for 3-weeks and placebo during another 3-week period. Ten subjects underwent the treatment in the following order: Se/placebo and the other 10 subjects in the reverse order: placebo/Se. The wash-out period was observed for 2-months after the first treatment period. A general scheme of the intervention is presented in figure 8.0.

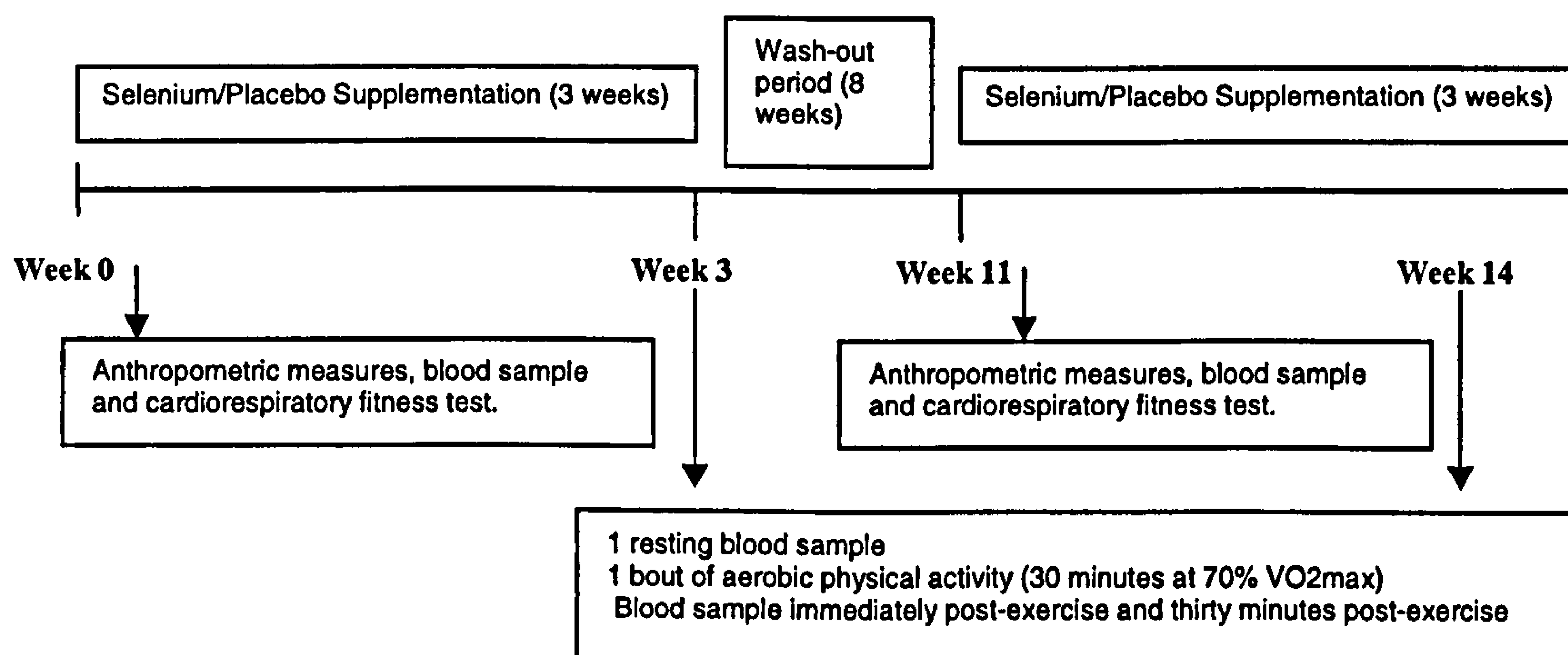


Figure 8.0. General schema of the selenium supplementation intervention

At visit 1 and 3, prior to each treatment, all subjects underwent anthropometric measures, a venous blood sample and an incremental exercise test to exhaustion for assessment of cardio-respiratory fitness.

Following each 3 week treatment, subjects completed an exercise session which adopted the following protocol (visit 2 and 4):

- 5 min warm up (40% VO_{2max})
- The subject was then required to perform a constant workload corresponding to 70% VO_{2max} for 30 minutes.

Heart rate was monitored throughout the exercise sessions. Prior to, immediately post-exercise and 30 minutes post exercise, a venous blood sample was collected.

Anthropometric and cardiovascular measurements and venous blood sampling

Height, weight and waist measurements are described in detail in the methodology chapter (section 3.2.1, 3.2.2, 3.2.3). Body composition was assessed using the BodPod, which is outlined in the methodology chapter (section 3.2.4.2). Cardiovascular measurements are described in the methodology chapter (section 3.3). Venous blood samples were collected as described in the methodology chapter (section 3.1.1 and 3.2.2).

Maximal aerobic exercise test

The maximal exercise test incorporated the Bruce Protocol (Bruce, 1972). O_2 uptake was assessed using the MetaMax 3B®. Maximal aerobic capacity was determined when the subject met two of the following criteria:

- Within 10 beats of maximal age predicted heart rate (220 – age)
- Respiratory quotient greater than 1.1
- Rating of perceived exertion >17

Biochemical measurements

LH, TAS, SOD, GSH and Se were performed as described in methodology chapter (section 3.1.12.1, 3.1.14.1, 3.1.14.2, 3.1.14.3 and 3.1.14.4). Other biochemical measures included, fasting plasma glucose, plasma fructosamine, plasma cholesterol, plasma LDL, plasma HDL and plasma triglycerides as described in methodology chapter (section 3.1.6, 3.1.7, 3.1.8, 3.1.9, 3.1.10 and 3.1.11). ADP-induced percentage platelet aggregation was measured using PlateletWorks®, which is outlined in the methodology chapter (section 3.1.14.2). Final concentrations of ADP used for platelet aggregation were 10 and 20µM. Haematocrit and Haemoglobin was also measured using PlateletWorks® which is also outlined in the methodology chapter (section 3.1.4.1 and 3.1.5.1). Haematocrit and Haemoglobin were measured to calculate the change in plasma volume (Dill and Costill, 1974).

Statistical Analysis

Values reported as mean±SEM. SEM was appropriate because it demonstrates how liable to error the mean is (e.g. the mean value or the mean change or improvement). The Kolmogorov-Smirno test confirmed that the data was normally distributed. The data was analyzed using analysis of variance (ANOVA) with repeated measures, which is appropriate for detecting differences between groups, time effects and interactions. A three-way (AxBxC) mixed ANOVA was used which incorporated two between (groups: normal-weight vs overweight and placebo vs selenium supplementation) and one within (time: rest vs post-exercise vs thirty minutes post-exercise) subject factor. When significant F values were noted paired t-tests were used to detect specific differences within groups at certain time points during the study. To detect differences between groups, one-way ANOVA was incorporated with one within (time: rest vs post-exercise) subject factor. Differences were considered statistically significant at $P<0.05$.

Statistical analysis was carried out using a computer software package (SPSS for Windows, Version 13.0).

8.2 RESULTS

Subject characteristics at week 1 for the normal-weight and overweight groups are shown in table 8.0. Body mass, BMI, body fat and waist circumference, DBP and LDL cholesterol were all significantly higher in the overweight group compared to the normal-weight group (P<0.01, P<0.001, P<0.01, P<0.001, P<0.001 and P<0.05, respectively). All subjects demonstrated similar systolic blood pressure, cholesterol, HDL, triglycerides, fasting glucose and fructosamine levels. At week 12, following the wash-out period, the subject characteristic differences between the normal-weight and overweight group remained the same, indicating there was no change in physiological and biochemical measurements over the duration of the study.

LH, TAS, SOD and GSH values in the normal-weight and overweight groups at week 0 and 12 (following wash-out period) are given in table 8.1. No significant differences were found in LH, TAS, SOD and GSH between week 0 and 12 in the normal-weight and overweight group. However when comparing between the normal-weight and overweight group, at weeks 0 and 12, several differences were noted. For example, at week 0, LH levels in the overweight group were significantly higher than the normal-weight group [overweight vs normal-weight (0.61±0.06 vs 0.74±0.06μmol/L, P<0.05)] and at week 12, LH levels demonstrated a non-significant increased trend in the overweight group compared to the normal-weight group [overweight vs normal-weight (0.60±0.06 vs 0.73±0.06μmol/L, P=0.06)]. No significant differences in TAS, SOD and

GSH levels were observed between the normal-weight and overweight group at week 0 and 12.

Table 8.1. LH, TAS, SOD and GSH values in the normal-weight and overweight group at week 0 and 12 (following wash-out period).

	Normal-weight (n=10)		Overweight (n=10)	
	Wk 0	Wk 12	Wk 0	Wk 12
LH, µmol/L	0.61±0.06	0.60±0.06	0.74±0.06 *	0.73±0.06
TAS, mmol/L	686.10±32.16	755.07±66.61	831.20±46.35	797.60±64.83
SOD, U/g Hb	3735.14±264.16	3947.29±291.86	3265.75±3565.44	3565.44±222.97
GSH, µmol/g Hb	18.03±1.41	18.47±2.31	16.33±1.55	17.69±2.31

Values expressed as the mean±SEM. LH, lipid hydroperoxide; TAS, total antioxidant status; SOD, superoxide dismutase; GSH, reduced glutathione. Results were analyzed using repeated measures, a one-way ANOVA and paired t-test. (*, $P<0.05$, compared to normal-weight group). P values < 0.05 were considered significant. For further details see text.

Plasma Se levels in the normal-weight and overweight group at week 0, 12 (following wash-out period) and immediately following 3 weeks placebo and Se treatment are shown in table 8.2. Plasma Se levels in both the normal-weight and overweight group remained similar at week 0, week 12 and following placebo treatment. However, compared to week 0, plasma Se levels significantly increased following 3-weeks Se supplementation in both the normal-weight and overweight group [wk 0 vs post Se treatment (normal weight, 68.44±6.91 vs 97.38±6.08µg/L, $P=0.028$ and overweight, 46.40±1.55 vs 81.72±6.71µg/L, $P=0.003$). Interestingly, plasma Se levels were

significantly lower in the overweight group when compared to the normal-weight group at wk 0, 12 and following placebo treatment [overweight vs normal-weight (wk 0, 68.44±6.91 vs 46.40±1.55μg/L, P=0.014, Wk 12, 65.46±5.45 vs 46.46±1.68μg/L, P=0.010, post placebo treatment, 71.46±7.06 vs 45.20±2.28μg/L, P=0.008)], but following Se supplementation this significant difference disappeared as plasma Se levels in the overweight group increased to a level similar to the normal-weight group [normal-weight vs overweight (97.38±6.08 vs 81.72±6.71μg/L, P=0.122)

Table 8.2. Plasma Se levels in the normal-weight and overweight group at wk 0, wk 12 (following wash-out period), following 3 weeks placebo treatment and following 3 weeks Se treatment.

Selenium (μg/L)	Wk0	Wk 12 (post wash-out)	Post placebo treatment	Post selenium treatment
Normal-weight	68.44±6.91^	65.46±5.45^^	71.46±7.06^^	97.38±6.08 * ≠ °
Overweight	46.40±1.55	46.46±1.68	45.20±2.28	81.72±6.71** ≠≠ °°

Values expressed as the mean±SEM. Wk0, baseline visit; Wk 12, visit after wash-out period. Results were analyzed using repeated measures, one-way ANOVA and paired t-test (^, P<0.05, compared to overweight, ^^, P<0.01, compared to overweight, *, P<0.05, compared to wk 0, **, P<0.01, compared to wk 0, ≠, P<0.02, compared to wk12, ≠≠, P<0.01, compared to wk12, °, P<0.05, compared to placebo, °°, P<0.01, compared to placebo. P values < 0.05 were considered significant. For further details see text.

Table 8.3 and 8.4 and figure 8.1 and 8.2 show LH levels in the normal-weight and overweight groups, pre- and post-acute aerobic high-intensity exercise following placebo and Se supplementation. During placebo treatment in the normal-weight group, an acute high-intensity exercise session did not significantly increase LH levels post-exercise [rest vs post exercise (0.64±0.06 vs 0.72±0.06μmol/L, P>0.05)]. Se supplementation in the normal-weight group also did not have any beneficial effect on LH levels at rest, post-exercise and 30-minutes post-exercise when compared to the placebo supplementation [Se vs placebo, pre vs post vs 30-minutes post (0.60±0.07 vs 0.64±0.06μmol/L, P>0.05; 0.70±0.05 vs 0.72±0.06μmol/L, P>0.05 and 66±0.05 vs 0.65±0.06μmol/L, P>0.05)]. During placebo treatment in the overweight group, LH levels increased significantly following the high-intensity acute exercise session [rest vs post (0.77±0.09 vs 0.96±0.09μmol/L, P<0.02)] and following 30-minutes recovery LH levels returned to near resting levels (0.78±0.12μmol/L, P<0.05). Following Se supplementation in the overweight group, LH was reduced immediately post acute exercise when compared to the placebo supplementation [Se vs placebo (0.71±0.08 vs 0.96±0.09μmol/L, P<0.02)] and LH levels did not significantly increase following high-intensity acute exercise (as seen following placebo treatment). When comparing the normal-weight and overweight group, LH levels were significantly higher in the overweight group immediately post-exercise and 30-minutes post-exercise in the placebo group [normal-weight vs overweight, post vs 30-minutes post-exercise (0.72±0.06 vs 0.96±0.09μmol/L, P<0.05 and 0.65±0.06 vs 0.78±0.12μmol/L, P<0.05)] but this significance disappeared post-exercise and 30-minutes post-exercise following Se supplementation in the overweight group [normal-weight vs overweight, post vs 30-minutes post-exercise (0.70±0.05 vs 0.71±0.08μmol/L, P>0.05 and 0.66±0.05 vs 0.69±0.07μmol/L, P>0.05)].

Table 8.3. LH, TAS, SOD and GSH values in the normal-weight group, pre and post high-intensity acute exercise, following placebo and Se supplementation.

Placebo			
	Pre	Post	30mins post
LH, µmol/L	0.64±0.06	0.72±0.06	0.65±0.06
TAS, mmol/L	748.84±46.81	724.20±29.68	770.13±31.73
SOD, U/g Hb	3965.90±306.02	4005.28±347.22	3770.49±279.54
GSH, µmol/g Hb	20.86±3.00	15.16±3.10	17.45±3.00
Se supplementation			
	Pre	Post	30mins post
LH, µmol/L	0.60±0.07	0.70±0.05	0.66±0.05
TAS, mmol/L	646.04±10.80≠	779.17±53.61*	778.32±39.36**
SOD, U/g Hb	3473.80±430.77	3727.88±370.77	2931.48±232.12≠
GSH, µmol/g Hb	20.43±3.76	21.65±4.97	19.32±5.03

Values expressed as the mean±SEM. LH, lipid hydroperoxide; TAS, total antioxidant status; SOD, superoxide dismutase; GSH, reduced glutathione. Results were analyzed using repeated measures, a one-way ANOVA and paired t-test. (*, $P<0.05$, compared to pre-values, **, $P<0.02$, compared to pre-values, ≠, $P<0.05$, compared to placebo values). P values < 0.05 were considered significant. For further details see text.

Table 8.4. LH, TAS, SOD and GSH values in the overweight group, pre and post high-intensity acute exercise, following placebo and Se supplementation.

Placebo			
	Pre	Post	30mins post
LH, μmol/L	0.77±0.09	0.96±0.09** °	0.78±0.12 °
TAS, mmol/L	853.88±52.33	836.08±70.27	851.10±50.0
SOD, U/g Hb	4212.07±422.36	3908.85±262.58	3237.41±259.73*^
GSH, μmol/g Hb	16.39±2.39	17.36±2.72	19.12±3.41
Se supplementation			
	Pre	Post	30mins post
LH, μmol/L	0.67±0.08	0.71±0.08≠≠	0.69±0.07
TAS, mmol/L	902.95±77.72 °°	931.51±72.83	895.10±57.99
SOD, U/g Hb	3298.30±236.32≠	3674.96±315.66	3644.49±321.59
GSH, μmol/g Hb	14.61±2.57	13.55±3.00	12.05±3.02

Values expressed as the mean±SEM. LH, lipid hydroperoxide; TAS, total antioxidant status; SOD, superoxide dismutase; GSH, reduced glutathione. Results were analyzed using repeated measures, a one-way ANOVA and paired t-test. (*, P<0.05, compared to pre-values, **, P<0.02, compared to pre-values, ^, P<0.02, compared to post-values, ≠, P<0.05, compared to placebo values, ≠≠, P<0.02, compared to placebo values, °, P<0.05, compared to normal-weight group, °°, P<0.02, compared to normal-weight group. P values < 0.05 were considered significant. For further details see text.

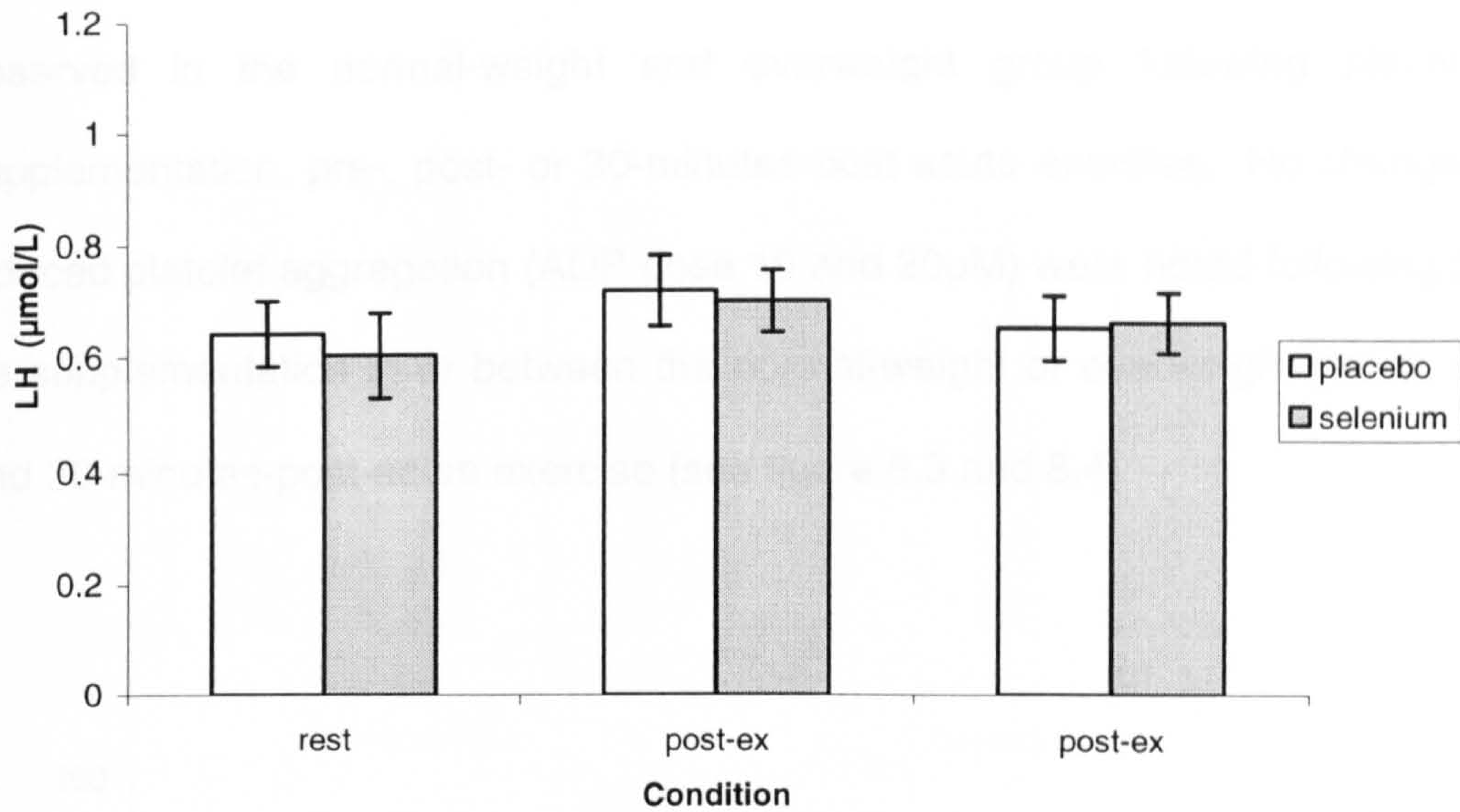


Figure 8.1. LH values (µmol/L) in the normal-weight group, pre and post high-intensity acute exercise, following placebo and Se supplementation. Results (mean±SEM) were analyzed using repeated measures, one-way ANOVA and paired t-tests. For further details see text.

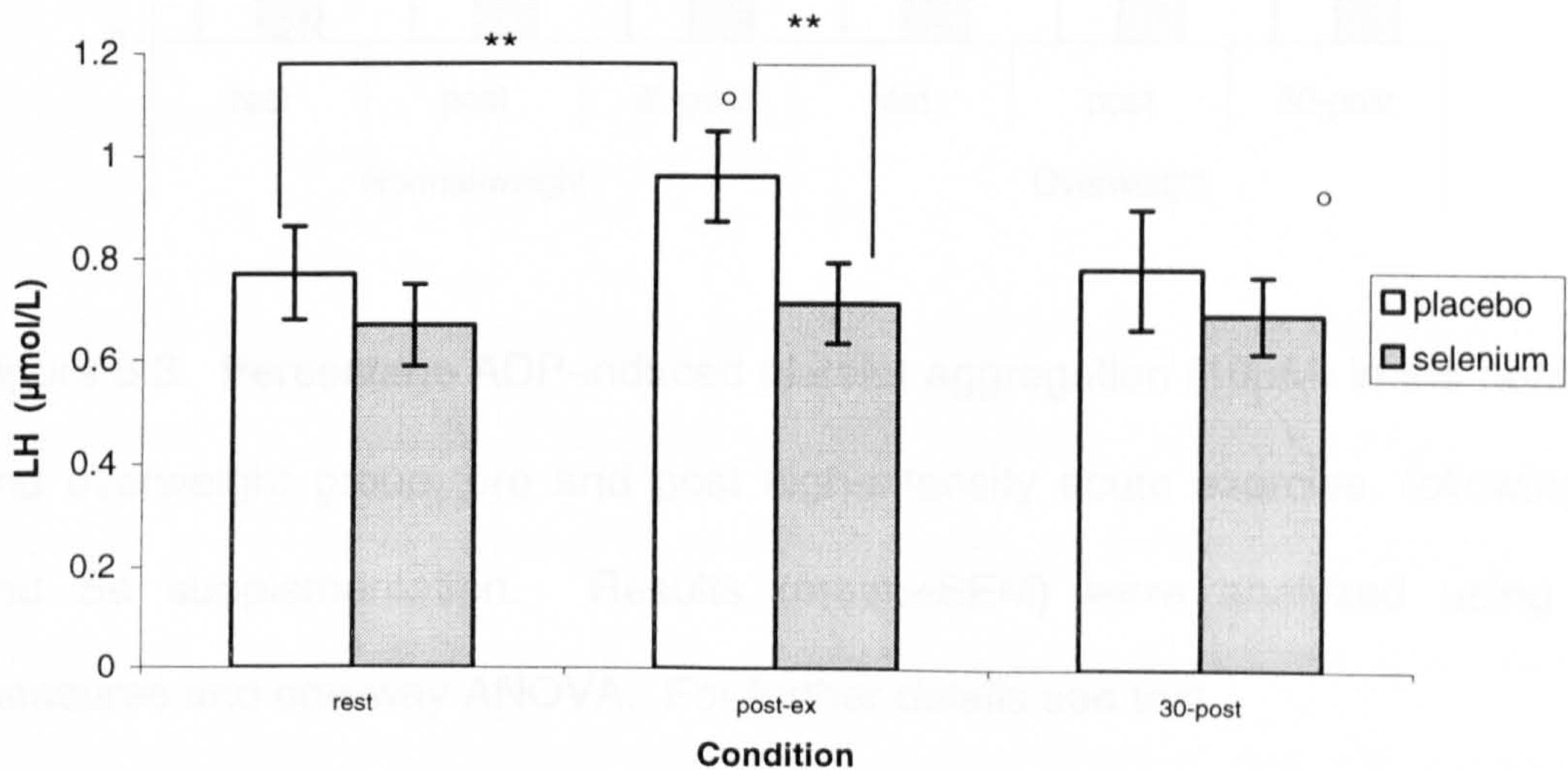


Figure 8.2. LH values (µmol/L) in the overweight group, pre and post high-intensity acute exercise, following placebo and Se supplementation. Results (mean±SEM) were analyzed using repeated measures, one-way ANOVA and paired t-tests (**, P<0.02, °, P<0.05 compared to normal-weight group). P values < 0.05 were considered significant. For further details see text.

No consistent changes in TAS, SOD and GSH levels (see table 8.2 and 8.3) were observed in the normal-weight and overweight group following placebo or Se supplementation, pre-, post- or 30-minutes-post acute exercise. No changes in ADP-induced platelet aggregation (ADP dose 10 and 20µM) were noted following placebo or Se supplementation in or between the normal-weight or overweight group, pre-, post- and 30-minutes-post-acute exercise (see figure 8.3 and 8.4)

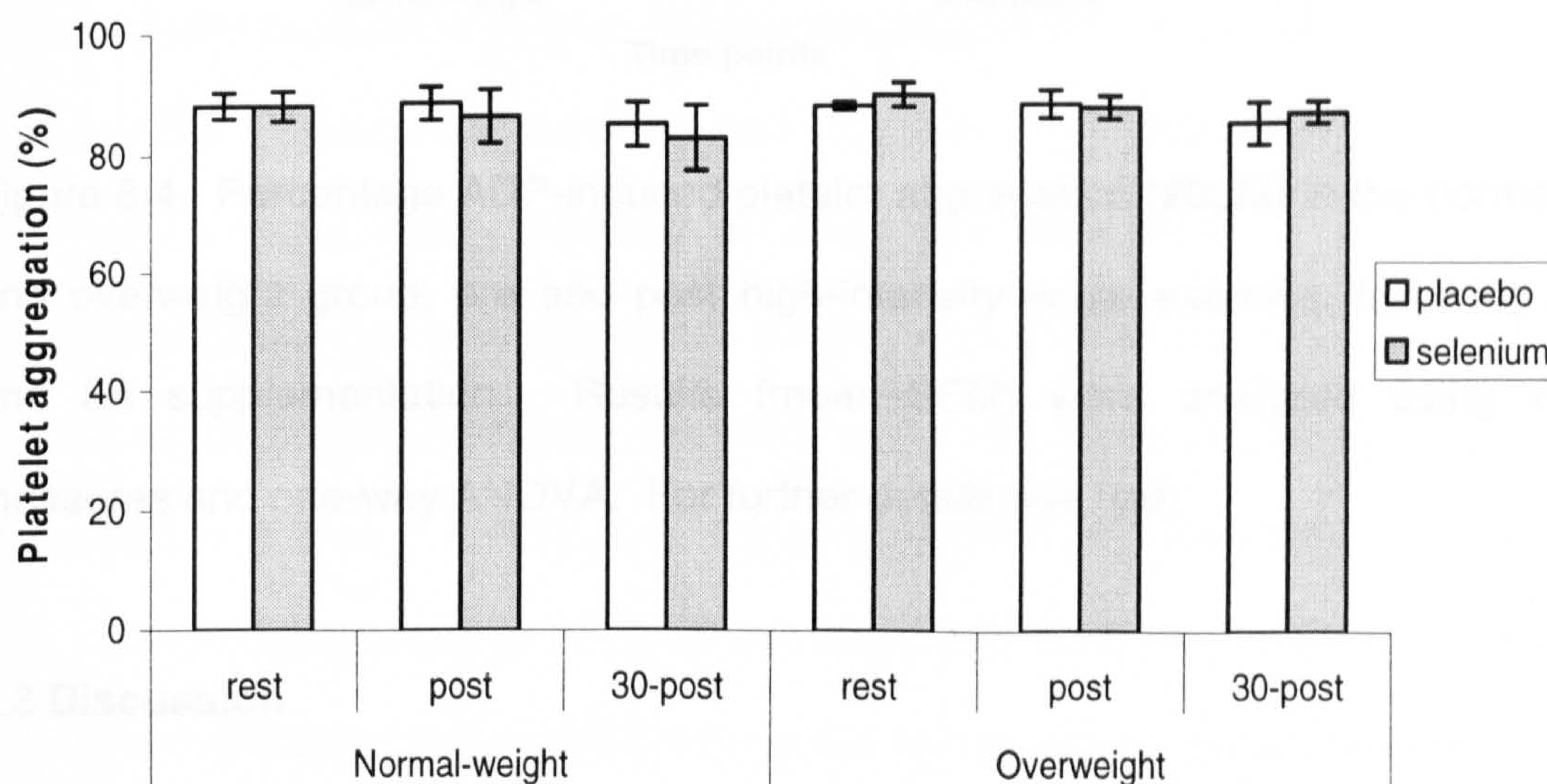


Figure 8.3. Percentage ADP-induced platelet aggregation (10µM) in the normal-weight and overweight group, pre and post high-intensity acute exercise, following placebo and Se supplementation. Results (mean±SEM) were analyzed using repeated measures and one-way ANOVA. For further details see text.

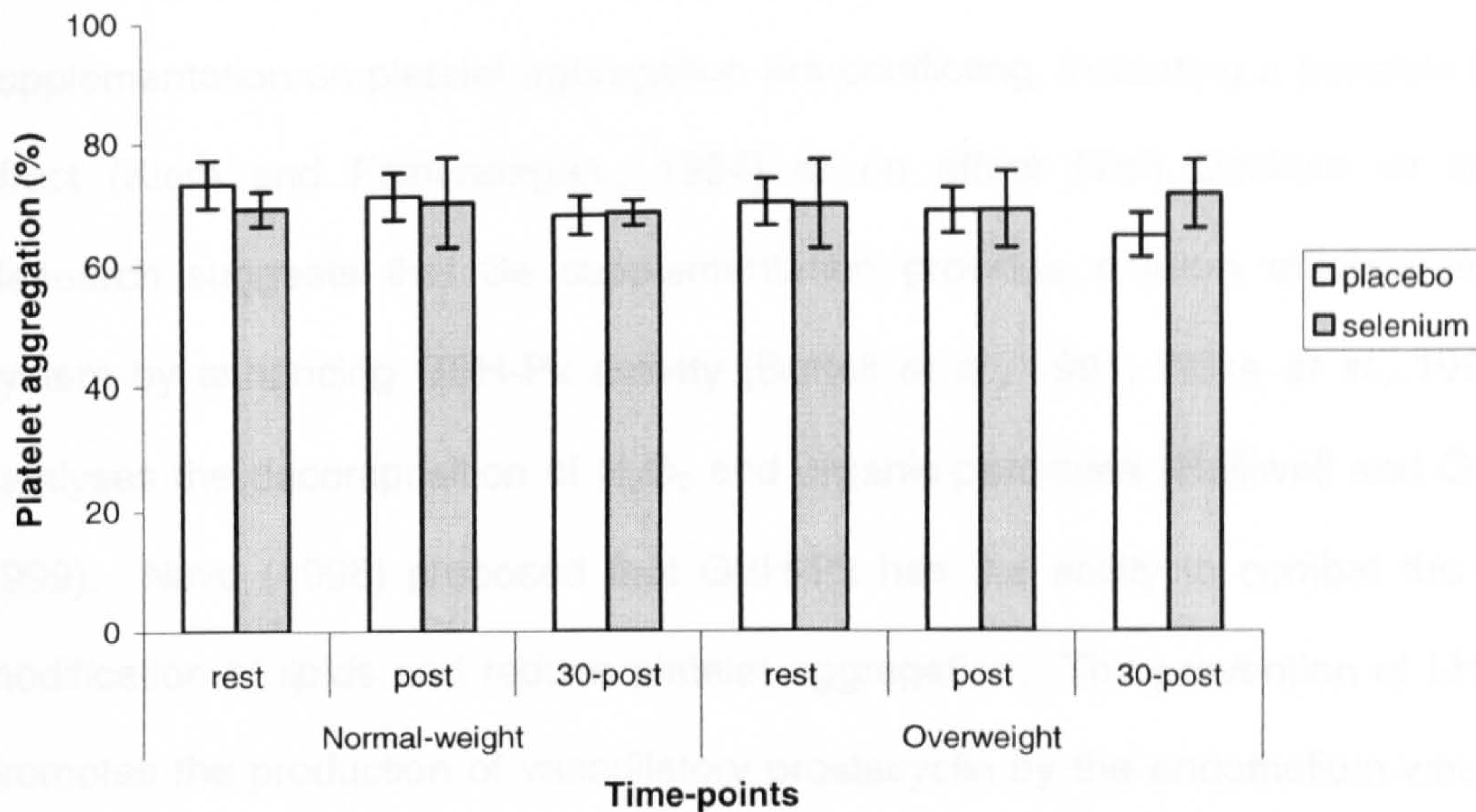


Figure 8.4. Percentage ADP-induced platelet aggregation (20μM) in the normal-weight and overweight group, pre and post high-intensity acute exercise, following placebo and Se supplementation. Results (mean±SEM) were analyzed using repeated measures and one-way ANOVA. For further details see text.

8.3 Discussion

In a double-blind cross-over study, the short term effects of Se supplementation were investigated on LH, TAS, SOD, GSH and ADP-induced platelet aggregation at rest and following an acute high-intensity aerobic exercise in both normal-weight (n=10) and overweight (n=10) subjects. This is the first study to assess the impact of Se supplementation on the obesity-associated oxidant stress at rest, the exercise-induced oxidant stress response and ADP-induced platelet aggregation.

Very few studies have assessed the independent role of Se supplementation on reducing both LH, TAS, SOD, GSH levels and platelet aggregation, but the general consensus is that Se supplementation may reduce oxidant stress levels (Wilke *et al.*,

1992; Bortoli *et al.,* 1991; Sarada *et al.,* 2002). However, the effects of Se supplementation on platelet aggregation are conflicting, indicating a possible beneficial effect (Kiem and Feinendegen., 1984) or no effect (Van Dokkum *et al.,* 1992). Research suggests that Se supplementation provides a more efficient antioxidant system by enhancing GSH-Px activity (Bortoli *et al.,* 1991; Wilke *et al.,* 1992) which catalyses the decomposition of H_2O_2 and organic peroxides (Halliwell and Gutteridge, 1999). Neve (1996) proposed that GSH-Px has the ability to combat the oxidative modification of lipids and reduce platelet aggregation. The prevention of LH build-up promotes the production of vasodilatory prostacyclin by the endothelium which in turn reduces the production of thromboxane, thus reducing vasoconstriction and platelet aggregation (Neve, 1996). Alternatively, reduced oxidant stress levels as a result of Se supplementation may indirectly affect platelet aggregation as research suggests that oxidant stress participates in the regulation of platelet activation (Krotz *et al.,* 2004) (as discussed in chapter 5 and 7). The impact of GSH-Px on reducing both oxidant stress and platelet aggregation is of particular relevance to obese subjects as Olusi *et al* (2002) demonstrated reduced GSH-Px levels in this subject group compared to a non-obese group (84.3±6.7 vs 98.4±3.3U/g Hb, $P<0.001$). Since Se is an essential metal cofactor for the activity of GSH-Px (Rotruck *et al.*, 1973), Se supplementation in the obese group may increase GSH-Px activity (Bortoli *et al.,* 1991; Wilke *et al.,* 1992).

To date few researchers have investigated the effect of Se supplementation on oxidant stress levels and platelet aggregation which are discussed below. Wilke *et al* (1992) noted improvements in oxidant stress status in PKU children (n=15) (at risk of Se deficiencies) who were given a daily sodium selenite (0.13μmol Se/kg/day) supply for 6 months. Compared to control values, PKU children had significantly lower plasma and erythrocyte Se, significantly lower plasma and erythrocyte GSH-Px activity and significantly higher plasma MDA. Following Se supplementation, compared to control

levels, plasma Se, and GSH-Px values normalised after 1 month, erythrocyte Se after 2 months, and erythrocyte GSH-Px and plasma MDA after 4 months. Bortoli *et al* (1991) studied the effects of 30 days Se supplementation (4 x 16.5μg inorganic Se and 5.0mg Vitamin E) in twenty elderly women. MDA levels showed insignificant changes during the Se supplementation period but 30-days post-Se supplementation, MDA decreased to a level lower than pre-Se supplementation [baseline vs 30-days post Se supplementation (4.3±0.6 vs 3.3±0.3μmol/L, $P<0.05$)]. Sarada *et al* (2002) found a reduction in hypoxia-induced oxidant stress in male Sprague-Dawley rats. Compared to the hypoxia only group, hypoxia plus Se supplementation demonstrated a significant decrease in MDA [hypoxia vs hypoxia + Se (4.0±0.4 vs 1.7±0.7nmol/mL, $P<0.05$)] and subsequent increase in plasma GSH levels [hypoxia vs hypoxia + Se (11.9±1.0 vs 25.8±9.7nmol/mL, $P<0.05$)]. Similarly, blood GSH-Px, plasma protein and plasma Se content also increased ($P<0.05$, $P<0.05$, $P<0.05$, respectively) in the Se supplemented hypoxia group compared with hypoxia alone. However, no beneficial effect of Se supplementation was found by Portal *et al* (1995) who performed a double-blind cross-over Se supplementation study on lipid peroxidation markers in cystic fibrosis patients. Similar to obese patients, cystic fibrosis patients have been shown to have increased lipid peroxidation markers (Wilke *et al.,* 1990), as a result of increased production of ROS mediated by infections or a defect in antioxidant defences (Portal *et al.,* 1995). Portal *et al* (1995) assessed twenty seven cystic fibrosis children who were given 2.8μg of sodium selenite per kg per day for 5-months and 5-months with a placebo control and inversion of treatment periods. Simultaneously, 17 healthy children were also investigated as control subjects. Although Se status was similar in both the control (n=17) and cystic fibrosis children (n=27), cystic fibrosis children had significantly higher lipid peroxidation markers (organic hydroperoxides) (122.6±23.3 vs 171.5±54.4μmol/L, $P<0.05$). However organic hydroperoxides were normalized at 12-months on either Se or placebo treatment. After the initial treatment, Se

supplementation increased plasma Se concentration and increased GSH-Px- activity whilst the placebo group demonstrated a reduction in plasma Se concentration and no significant changes in GSH-Px activity [baseline vs first treatment: Se concentration (Se group, 0.83±0.17 vs 1.11±0.18μmol/L, P<0.05 and placebo group, 0.78±0.14 vs 0.67±0.13μmol/L, P<0.05), GSH-Px concentration (Se group, 269.8±40 vs 340.4±77μmol/L, P<0.05 and placebo group, 260±45 vs 291.4±67μmol/L, P>0.05)]. However compared to month 5, following the second treatment period, Se supplementation increased plasma Se concentration (P<0.05) but decreased GSH-Px activity (P>0.05) whilst in the placebo group plasma Se concentration decreased (P<0.05) and GSH-Px activity decreased (P>0.05). These results indicated that improvement of lipid peroxidation markers in cystic fibrosis was not related to the Se supplementation. The decrease in organic hydroperoxide levels observed following Se treatment may be linked to the improvement of the biological indices of Se status as reported in studies by Bortoli *et al* (1991) and Wilke *et al* (1990). However this relationship is inconsistent with data observed in the placebo group since organic hydroperoxide levels normalized in spite of a reduced plasma selenium concentration. It may be possible that the variable organic hydroperoxide levels in healthy subjects was a result of seasonal variations or the interpretation of the data may have been limited as a result of inferences in the organic hydroperoxide marker, despite complying with the criteria of good analytical practice. Alternatively, the reduced organic hydroperoxide levels in the placebo group may be a placebo effect which may have been influenced by change in patient behaviour due to inclusion in the clinical protocol (Portal *et al.*, 1995).

With regards to the effect of Se supplementation on platelet aggregation, research is limited but Kiem and Feinendegen (1984) did observe diminished activities of GSH-Px and decreased Se levels in platelets from patients with acute myocardial infarction

exhibiting a greater tendency to aggregation. But Van Dokkum *et al* (1992) concluded that dietary Se supplementation (200µg Se as Se-rich bread for six weeks (Se-wheat)) did not improve platelet aggregation in healthy young men (n=6) despite showing significantly increased GSH-Px activity. However in a pilot study (Van der Torre *et al.*, 1989), platelet aggregation was monitored in four subjects after supplementation with Se-rich yeast tablets which observed an increased platelet GSH-Px activity and decreased platelet aggregation. Levander *et al* (1983) suggested that the Se compounds in yeast differed from those in wheat so Van Dokkum *et al* (1992) confirmed that it may be conceivable that another Se compound in yeast could be of importance to platelet aggregation.

In this study the effects of 3 weeks Se supplementation (200µg sodium selenite) versus placebo in a double blind manner (with wash-out period) was investigated on LH. TAS, SOD and GSH and ADP-induced platelet aggregation at rest and following acute high-intensity aerobic exercise in both normal-weight (n=10) and overweight (n=10) subjects. For the duration of the study, no changes in physiological and biochemical measurements were observed which could have had an impact on oxidant stress levels. The effect of Se supplementation in plasma was clearly identified in both the normal-weight and overweight groups. Compared to week 0, Se levels significantly increased following 3-weeks Se supplementation in both the normal-weight and overweight groups [wk 0 vs post Se treatment (normal weight, 6.84±0.69 vs 9.74±0.61µg/L, P=0.028 and overweight, 4.64±0.15 vs 8.17±0.67µg/L, P=0.003)]. Interestingly, plasma Se levels were significantly lower in the overweight group when compared to the normal-weight group at wk 0, 12 and following placebo treatment [overweight vs normal-weight (wk 0, 6.84±0.69 vs 4.64±0.15µg/L, P=0.014, Wk 12, 6.54±0.54 vs 4.64±0.17µg/L, P=0.010, post placebo treatment, 7.15±0.71 vs

4.52±0.23μg/L, P=0.008)], but following Se supplementation this significant difference disappeared as Se levels in the overweight group increased to a level similar to the normal-weight group [normal-weight vs overweight (9.74±0.61 vs 8.17±0.67μg/L, P=0.122). At time-points other than following Se supplementation, such as Wk 0, Wk 12 (following wash-out period) and following placebo treatment, plasma Se levels were all similar in both the normal-weight and overweight groups which highlights that the wash-out period was effective at clearing the body of Se as a result of Se supplementation.

LH levels at rest in week 0, 12 and following placebo treatment demonstrated either a significant or a non-significant increased level in the overweight group compared to the normal-weight group. Following Se supplementation, LH levels decreased in both the normal-weight and overweight groups but this was not significant. In addition, following Se supplementation LH levels were not significantly different between the normal-weight and overweight groups. TAS levels significantly decreased in the normal-weight group and SOD levels significantly decreased in the overweight group compared to placebo treatment. Both of these changes cannot be explained. Se supplementation demonstrated no affect on GSH and percentage ADP-induced platelet aggregation levels compared to placebo treatment in both the normal-weight and overweight groups.

LH responses immediately post and 30 minutes post high-intensity exercise in the normal-weight group following placebo treatment did not demonstrate any significant changes but in the overweight group LH levels significantly increased immediately post-exercise, which returned to resting levels 30-minutes post exercise. This confirms an exercise-induced oxidant stress response following high-intensity exercise in the overweight group. Furthermore compared to the normal-weight group, LH levels were

significantly higher in the overweight group at both immediately post and 30-minutes post high-intensity exercise time-points. Se supplementation in the normal-weight group demonstrated no positive effect on LH levels immediately post-exercise and 30 minutes post-exercise. However in the overweight group, Se supplementation prevented a significant increase in LH levels immediately post-exercise. As a result, following Se supplementation, LH levels between the normal-weight and overweight groups were not significantly different immediately post and 30-minutes post exercise. No significant changes in TAS, SOD, GSH and platelet aggregation levels were observed in the normal-weight and overweight groups following placebo or Se supplementation, immediately post or 30 minutes post high-intensity exercise.

This study found that 3 weeks Se supplementation (200μg/d sodium selnite) was affective at increasing plasma Se levels in both the normal-weight and overweight groups. Furthermore Se supplementation in the overweight group reduced the exercise induced oxidant stress response observed following placebo treatment. This is particularly important for overweight or obese individuals who exercise sporadically because they may be at greater risk of exercise-induced oxidant stress since the lack of regular training would not permit an enhancement of the defence mechanisms. This is perhaps even more relevant in the overweight / obese patient because obese people have been shown to have lower antioxidant concentration (Reitman et al., 2002; Strauss et al., 1999; Decsi et al., 1997; Kuno et al., 1998; Moor De Burgos et al., 1992) and decreased activities of erythrocyte cytoprotective enzymes (Olusi, 2002; Ozata et al., 2002; Beltowski et al., 2000). Furthermore in this study, overweight subjects were shown to have lower plasma Se levels [normal-weight vs overweight (6.84±0.69 vs 4.64±0.15μg/L, $P<0.05$) which may lead to a more ineffective antioxidant system by reducing GSH-Px activity (Bortoli *et al.*, 1991; Wilke *et al.*, 1992).

The findings of this study have been compared to other antioxidant therapy studies in obesity aimed at reducing oxidant stress levels. It is difficult to make comparable conclusions between this study and others with regards to the effectiveness of Se supplementation on reducing oxidant stress levels because studies have all used various oxidant stress markers. At rest, this study demonstrated a reduction in LH levels of 13% in the overweight group and a 6.25% reduction in the normal-weight group following 3-weeks Se supplementation [placebo vs Se (overweight, 0.77±0.09 vs 0.67±0.08μmol/L, P>0.05, normal-weight, 0.64±0.06 vs 0.60±0.07μmol/L, P>0.05)]. In comparison, Skrha *et al* (1999) found that vitamin E (600mg daily) administration in obese diabetic patients for 3 months decreased plasma MDA from 3.13±0.68 to 2.87±0.97μmol/L which is an 8% reduction in MDA levels. Similarly Manning *et al* (2004) found reduced LH levels in obese subjects following 6-months of vitamin E supplementation (3 months 800IU vitamin E/day, 3 months 1200IU vitamin E/day). LH was decreased by 27% at 3-months and by 29% at 6-months following vitamin E supplementation and the decrease in LH was positively correlated with plasma vitamin E concentrations at the 6-month time point (r = 0.40, P = 0.01, n=39). Another study also indicated protection against formation of oxidative biomarkers with antioxidant treatment, for example in overweight type II diabetic versus non diabetic contols (Anderson *et al.*, 1999). Twenty diabetics completed an 8-week control period, 8 week treatment period (β-carotene (24mg), vitamin C (1000mg) and vitamin E (800IU) followed by an 8-week control period (subjects were on a weight maintaining diet). Following the treatment period in the diabetic group, TBARS formation decreased by 30% (pre vs post: 101.5±10.70 vs 70.6±9.75nmol/mg, P<0.001).

The effect of antioxidant therapy on reducing exercise-induced oxidant stress has not been undertaken in the obese setting but research has been completed in normal-weight subjects. In this study, compared to placebo, Se supplementation reduced LH

production immediately post high intensity exercise by 2.7% in the normal-weight group [placebo vs Se (0.72±0.06 vs 0.70±0.05μmol/L, P>0.05)] and by 26% in the overweight group [placebo vs Se (0.96±0.09 vs 0.71±0.08μmol/L, P<0.02)]. In comparison, Sumida *et al* (1989) demonstrated that 4-weeks of vitamin E supplementation prevented a rise in plasma MDA levels following maximal-intensity cycle exercise. Ashton *et al* (1999) demonstrated that acute ascorbic acid supplementation prevented exercise-induced oxidant stress in healthy subjects. For example in the control phase, strenuous exercise caused a significant increase in LH levels [pre-exercise vs post-exercise (1.14±0.06 vs 1.62±0.19μmol/L, P=0.005)] and after acute ascorbic acid supplementation no change in LH levels were found post strenuous exercise [pre-exercise vs post-exercise (1.12±0.21 vs 1.12±0.08μmol/L)]. In contrast to these findings, daily supplementation with an antioxidant mixture (30 mg ß-carotene, 592 mg vitamin E and 1000 mg vitamin C) did not prevent the exercise-induced rise in plasma MDA after moderate- to high-intensity treadmill running (Kanter *et al.*, 1993). This study found that the normal-weight group did not benefit from reduced LH levels following high-intensity exercise, which might be because the normal-weight subjects did not have reduced GSH-Px activity (Olusi *et al.*, 2002) suggesting that Se levels prior to Se supplementation may have been at a level which allowed for maximal expression of GSH-Px activity (Pearson *et al.*, 1990). Alternatively, in all Se supplementation studies, variability in results may be associated with differences in study protocol. For example, the form of Se ingested affects the response of the selenoenzymes (Brown *et al.*, 2000), the concentration of some selenoenzymes is affected more than others by scarce selenium supply owing to the hierarchy of selenoprotein expression (Behne *et al.*, 2000). In addition there is considerable variation between individuals in the extent of the response of the selenoenzymes to Se supplementation so Se requirements between individuals in the same population may

differ (Institute of Medicine, 2000) and adaptation to low Se intake can occur by sparing excretion (Thomson *et al.,* 1993).

This study attempted to elucidate if oxidant stress mediates platelet aggregation by examining the effect of reduced oxidant stress following Se supplementation on platelet aggregation levels. Several research studies suggest that oxidant stress participates in the regulation of platelet activation (Krotz *et al.,* 2004) and oxidant stress-mediated platelet aggregation has been found in several settings of risk factors for atherosclerosis and cardiovascular thrombosis, including diabetes mellitus, hypertension and hypercholesterolemia (Davi *et al.,* 2003; Minuz *et al.,* 2002; Davi *et al.,* 1997). Similarly, Tozzi-Ciancarelli *et al* (2002) demonstrated that oxidant stress induced by strenuous exercise interfered with platelet responsiveness. This was evident by adding LDL-Ox to PRP obtained from blood samples collected from 6 individuals immediately after strenuous exercise as ADP-induced platelet aggregation increased and intra-platelet NO content decreased. Several *in vitro* experimental approaches have also been used to investigate the specific role for distinct ROS on platelets. Briefly, platelets exposed directly to H_2O_2 have enhanced collagen-dependent platelet activation and enhanced arachidonic acid (AA)-dependent platelet activation (Practio *et al.,* 1992). Platelets exposed to $O_2^{\cdot-}$ have shown a reduction in the threshold for platelet activation to thrombin, collagen, ADP or AA and induced spontaneous aggregation (Handin *et al.,* 1977; Krotz *et al.,* 2002; Salvemini *et al.,* 1989; De la Cruz *et al.,* 1992). $O_2^{\cdot-}$ also degrades NO which is a potent inhibitor of platelet activation. In addition to exogenously derived ROS affecting the regulation of platelet activation, recent data also suggests that the platelets themselves generate ROS (Marcus, 1977).

This study does not support the association that oxidant stress mediates platelet aggregation. At rest and post-high intensity exercise, compared to placebo treatment, Se supplementation had no affect on reducing *in vivo* platelet reactivity in both the normal-weight and overweight groups. These findings are similar to that of Van Dokkum *et al* (1992) who concluded that dietary Se supplementation did not improve platelet aggregation in healthy young men despite showing significantly increased GSH-Px activity. However Kiem and Feinendegen (1984) found that platelets that aggregated have significantly lower concentrations of Se (P=0.02) and that platelets with higher Se concentrations were less prone to aggregate. The lack of an association between Se supplementation and reduced platelet aggregation in this study may be because sodium selenite is not important in the role of platelet aggregation. For example Levander *et al* (1983) suggested that the increased platelet GSH-Px activity and decreased platelet aggregation in a pilot study following Se-rich yeast tablets suggests that particular Se compounds may be importance to platelet aggregation because Van Dokkum *et al* (1992) observed no beneficial effect of wheat Se supplementation on platelet aggregation levels. However the increased platelet aggregation and TxB_2 levels in diabetic rates was shown to reverse following sodium selenite supplementation (Ersöz *et al.*, 2003). Another possibility for the lack of association between Se supplementation and reduced platelet aggregation may be because 3 weeks sodium selenite supplementation is too short a term to establish lipid peroxidation-mediated changes in platelet function (Salonen *et al.*, 1991). Stampfer *et al* (1988) observed no significant effects on either *in vitro* platelet aggregation ability or serum thromboxane B_2 concentration in 20 healthy university staff member who were supplemented with 727mg vitamin E/d for 5 weeks. Salonen *et al* (1991) attributed the lack of changes to the short term supplementation period. Salonen *et al* (1991) found that antioxidant supplementation daily for 5 months (600mg ascorbic acid, 300mg α-tocpherol, 27mg ß-carotene, and 75µg selenium in yeast) in men (39 controls and 39

supplemented) with low antioxidant status and high fat intake, reduced lipid peroxidation, the capacity of platelets to aggregate and to produce thromboxane A_2 and in vivo platelet activation. Furthermore, Salonen *et al* (1991) found that the presence of the associations between antioxidant changes and change in lipid peroxides was in accordance with the hypothesis that antioxidants reduce platelet activity through the elimination of circulating lipid peroxides. The discrepancy in the different findings between Stampfer *et al* (1988) and Salonen *et al* (1991) may also be attributed to the difference in baseline antioxidant status and dietary fat intake between the two study populations.

This study provided an insight into the potential role Se could play as an antioxidant therapy to reduce oxidant stress at rest and following high-intensity exercise in 'high risk' population groups. Although the study benefits from being a double-blind cross-over study, questions still remain unanswered regarding the use of Se supplementation in everyday practice. For example, the type of supplement, dosage and length of consumption must be investigated. It is important to be aware of the reduced Se intake in the UK (Rayman *et al.*, 20002) and a Se type and dosage should be selected on the basis of normalizing plasma selenium levels in individuals. Normalization of plasma Se levels may in turn optimize GSH-Px activity (Duffield *et al.*, 1999), and reduce oxidant stress levels.

8.4 CONCLUSION

Three-weeks Se supplementation in overweight subjects has the potential to decrease LH levels at rest and normalize LH levels immediately post high-intensity exercise. However the improvements in LH levels did not extend to reducing *in vivo* platelet reactivity, suggesting that oxidant stress may not be a pivotal determinant of platelet aggregation.

Chapter NINE

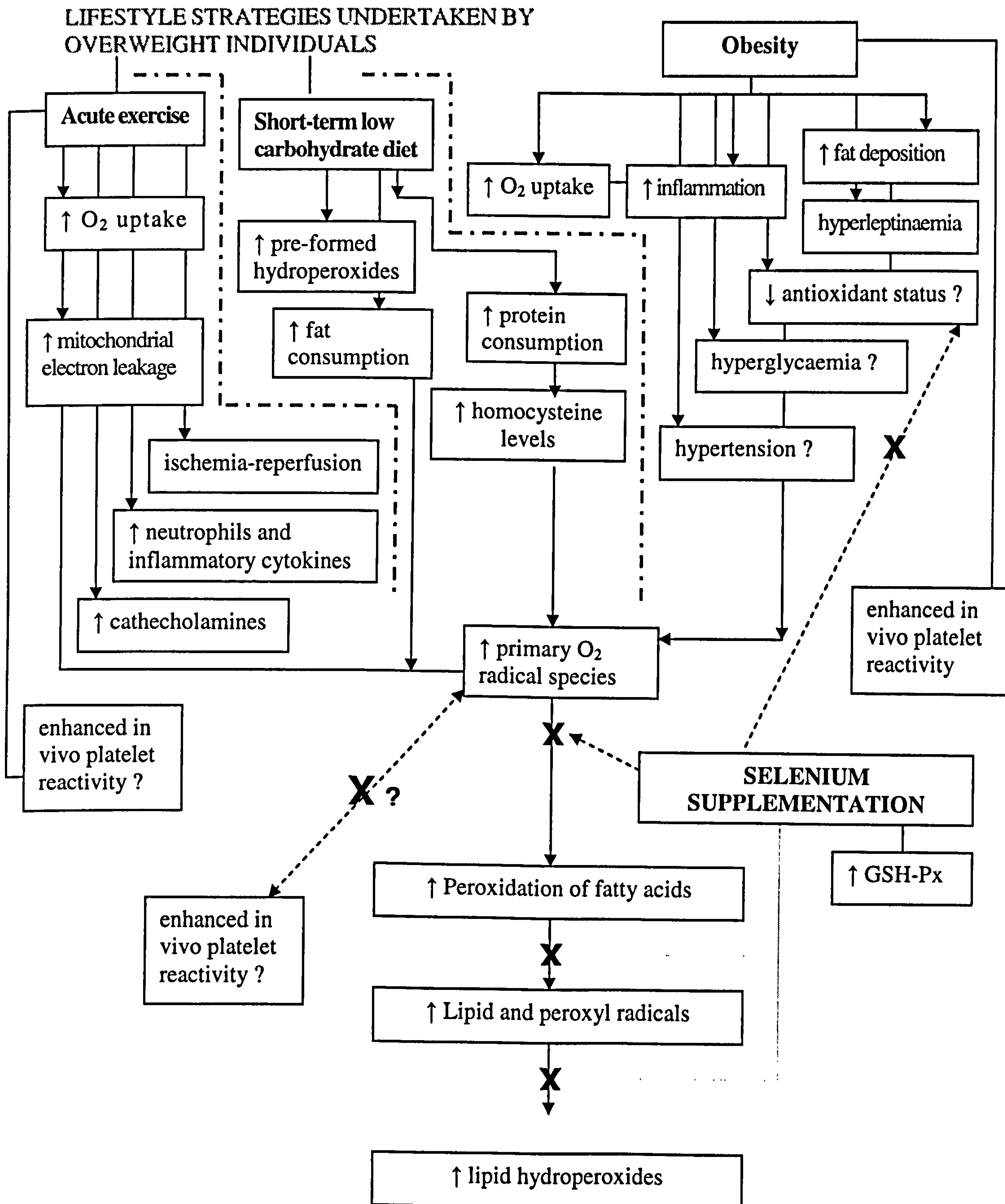
General discussion

9.0 Integration and summary of research findings

Oxidant stress is an imbalance between the formation of reactive oxygen / nitrogen species and antioxidants (Powers *et al.*, 2004). The level of redox signalling is elevated in conditions such as hypertension, hyperinsulinemia, hyperlipidaemia and obesity (Vincent and Taylor, 2006). In addition, high fat (Slim *et al.*, 1996) and high sugar diets (Faure *et al.*, 1997) and moderate unaccustomed exercise all pose an acute oxidant stress (Alessio *et al.*, 2000). Paradoxically though, regular endurance exercise is associated with increased intracellular antioxidants and antioxidant enzymes and decreased ROS production during exercise (Fukai *et al.*, 2000). Similarly alternative strategies such as dietary modifications (Velthuis-te Wierik *et al.*, 1996), weight loss (Davi *et al.*, 2002) and antioxidant therapy (Skrha *et al.*, 1999; Manning *et al.*, 2004) may improve oxidant stress levels, leading to long-term vascular protection, or protection against several diseases associated with oxidant stress such as premature aging, diabetes mellitus, arthritis and cancer (Niki, 2001). The present research was conducted to investigate whether (1) both overweight and obese individuals are susceptible to increased oxidant stress (2) a short-term low carbohydrate diet in overweight subjects may generate oxidant stress (3) overweight individuals are more susceptible to exercise-induced oxidant stress (4) selenium supplementation may attenuate oxidant stress in overweight subjects at rest and following aerobic exercise (5) oxidant stress may play a pivotal role in platelet aggregation.

Figure 9.0 integrates and summarises the research findings of all the studies conducted as part of this thesis. Where a question mark is presented, this indicates that the finding or mechanism is open to discussion since it was not sufficiently elucidated in this work.

Figure 9.0. Chain of events for oxidant stress and platelet aggregation in overweight/obese individuals, following acute high-intensity exercise and short-term low carbohydrate diets and the effect of selenium supplementation.



9.1 BMI and oxidant stress

Study 1 demonstrated significantly elevated levels of oxidant stress levels in obese individuals when compared to normal-weight and overweight individuals as evidenced by increases in LH levels. This may be associated with an increase in oxygen consumption, implicating the mitochondria as a potential source of primary oxygen-centred free radical species. Other possibilities include reduced antioxidant status, cell injury/inflammation and increased fat deposition (Vincent *et al.*, 2001), hyperglycaemia, hypertension and hyperleptinaemia (Vincent and Taylor, 2006). Although this study did not set out to identify potential causes of increased oxidant stress in obesity, lowered antioxidant status, hypertension and hyperglycamia were found not to be factors contributing to increased oxidant stress in obesity because (1) antioxidant status was not shown to be lower in obesity and (2) obese individuals with either hypertension or hyperglycaemia were excluded from the study.

Despite overweight individuals not being predisposed to enhanced oxidant stress, studies 3 and 4 found that lifestyle habits such as short-term low carbohydrate diets and/or acute exercise can increase oxidant stress levels in overweight individuals. It can therefore be speculated that obese individuals would also respond to the above strategies with increased oxidant stress levels. It was appropriate to study the effect of these strategies in overweight subjects because overweight/obese individuals often attempt to lose with weight by exercising and changing dietary habits (Goldbeter, 2006).

9.2 Short-term low carbohydrate diets and oxidant stress

Study 3 investigated the impact of commercial diets for weight loss in a real-life setting on oxidant stress parameters. Compared to a conventional and control diet, a low carbohydrate diet caused an increase in LH levels during the first two weeks which decreased at week 4 whilst total antioxidant status levels increased gradually to week 4. This occurred despite the low carbohydrate diet achieving the greatest weight loss compared to the conventional and control diet (which can be a potential mechanism to reduce oxidant stress). The change in oxidant stress levels are likely to reflect initial increased susceptibility to oxidants possibly caused by changes in macro-nutrient intake (Slim *et al.,* 1996; Velthuis-te Wierik *et al.,* 1996) versus baseline [e.g. increased fat (total or saturated fat content) and or protein intake], followed by compensatory elevations in antioxidant enzyme levels to help protect tissues against further tissue damage. For example SFAs have been shown to be susceptible to oxidation (Mata *et al.*, 1996) and MUFAs lower LDL oxidation (Berry *et al.*, 1991; Reaven *et al.*, 1991) whilst lipoproteins isolated from individuals consuming diets rich in PUFAs and MUFAs demonstrate greater pre-disposition to peroxidation than lipoproteins from individuals given SFAs (Kleinveld *et al.,* 1993). Changes in the consumption of lard and compound cooking fat (such as baking and frying margarines) which contain high levels of pre-formed hydroperoxides (Wolff and Nourooz-Zadeh, 1996) may explain changes in LH levels on the LowCD or explain the diverse results in lipid peroxidation responses in studies investigating the impact of composition of dietary fat on oxidant stress levels (largely dependent upon the intake of food containing hydroperoxides generated by pyrolysis such as fatty fried foods). Increased protein intake on the LowCD may also promote oxidant stress (Fang *et al.*, 2002). High protein diets increase homocysteine levels, which can increase endothelial $O_2^{\cdot-}$ production and induce oxidant stress in the vasculature (Wu

and Meininger, 2002). In addition, increased protein intake has also been shown to stimulate generation of ROS and lipid peroxidation in human polymorphonuclear leukocytes and mononuclear cells (Mohanty *et al.*, 2002) and increase whole-body NO production by constitutive and inducible NOS in rats (Wu *et al.*, 1999). It is important to note that the above changes in oxidant stress may have increased further if subjects adhering to the low carbohydrate diet were not taking a daily multivitamin. It would be interesting to determine the number of individuals in the public who consume low carbohydrate diets without a daily multivitamin. The conventional diet and control diet had no significant effect on oxidant stress levels in overweight women over a 4-week period, which demonstrates that a diet promoting low-fat consumption (consisting of MUFAs and PUFAs) and low-glycaemic index foods are favourable for lower oxidant stress levels. It is important to highlight that although the low-carbohydrate diet achieved the greatest weight loss compared to the conventional and control diet, oxidant stress levels still increased on the low-carbohydrate diet.

9.3 Acute exercise and oxidant stress

In study 4, despite matching oxygen consumption during exercise between the normal-weight and overweight groups, it was shown that overweight individuals are still predisposed to a greater increase in oxidant stress levels post high-intensity exercise compared to normal-weight individuals. Post-high intensity exercise, LH levels significantly increased in the overweight group (+19.8 %) but not in the normal-weight group (+10.3 %). Although differences in resting LH levels between the normal-weight and overweight were not significantly different, they were immediately post-exercise. At 30-minutes post-exercise these levels reduced to pre-exercise levels. No consistent changes were identified within or between the normal-weight

and overweight groups for TAS, GSH and SOD pre-exercise, post-exercise and thirty-minutes post-exercise. The exacerbated exercise-induced oxidant stress in the overweight group is likely to be due to the factors which are associated with the obesity-associated oxidant stress e.g. reduced antioxidant status, cell injury/inflammation and increased fat deposition (Vincent *et al.*, 2001), hyperglycaemia, hypertension and hyperleptinaemia (Vincent and Taylor, 2006). As a result of exercise-induced oxidant stress, overweight/obese individuals who are repeatedly undertaking infrequent bouts of exercise (high-intensity) to lose weight may be frequently enhancing oxidant stress and placing themselves at greater risk of physiological complications such as cardiac arrthymias, fibrinolysis and angina (Alessio, 1994). However this risk may be lessened by exercising at moderate exercise intensities (Tozzi-Ciancarelli *et al.*, 2002). In addition continuous exercise training (Fukai *et al.*, 2000) and antioxidant therapy (Skrha *et al.*, 1999; Manning *et al.*, 2004; Anderson *et al.*, 1999) may be useful strategies to reduce oxidant stress in obesity at rest and during exercise.

Due to obesity and lifestyle habits such as short-term low carbohydrate diets and acute high-intensity exercise increasing oxidant stress, study 5 was designed to determine the effect of antioxidant supplementation on oxidant stress in overweight individuals at rest and following high-intensity exercise. It was speculated that any significant benefits found may extend into the obese population. Selenium supplementation was chosen as the antioxidant of choice because it is an essential component of the GSH-PX system (Ladenstein *et al.*, 1979), which functions as part of an antioxidant system to protect PUFAs and proteins from the damaging effects of peroxides and LH (Richter, 1987; Del Maestro *et al.*, 1980). Also given that current UK daily intakes of Se are so low (Department of Health, 1991) that they do not allow maximal expression of plasma GSH-PX (Duffield *et al.*, 1999) and evidence that

obese individuals have reduced GSH-PX levels (Olusi *et al.*, 2002) selenium supplementation seemed a good antioxidant choice.

9.4 Selenium supplementation and oxidant stress

The results of study 5 provided an insight into the potential role Se could play as an antioxidant therapy to reduce oxidant stress at rest and following high-intensity exercise in normal-weight and overweight individuals. At rest, although not significant, Se supplementation produced a trend for decreased LH levels in both the normal-weight and overweight group but the greatest effect was observed in the overweight group. Immediately following and 30 minutes post-exercise, compared to placebo, Se supplementation also reduced LH levels in the overweight weight but this did not occur in the normal-weight group. Interestingly, although LH levels between the normal-weight and overweight groups were significantly different post and 30 minutes post exercise on placebo, this was not the case following Se supplementation. These results suggest that Se supplementation in overweight individuals has the potential to reduce LH levels at rest and normalize LH levels during acute aerobic high-intensity exercise. The minimal changes in TAS, SOD and GSH do not explain why Se supplementation may have reduced LH levels in overweight subjects. However, it can be speculated that the improvements in LH in the overweight group may be due to a significant increase in plasma selenium levels which in turn maximised GSH-PX activity (since it was shown that overweight individuals compared to normal-weight individuals in the placebo group had significantly lower plasma selenium levels).

9.5 Oxidant stress-mediated platelet aggregation

Since platelets play a central role in the process of thrombus formation (Hoak, 1988), as well as playing an important role in atherogenesis (Rabbani and Loscalzo, 1994) and the progression of atherosclerotic lesions (Kamath *et al.*, 2001) it seemed significant to investigate whether increased oxidant stress (due to obesity or unhealthy lifestyles) contributes to persistent platelet aggregation. It has already been shown that oxidant stress mediates platelet aggregation in several settings such as diabetes mellitus, hypertension and hypercholesterolemia (Davi *et al.*, 2003; Minuz *et al.*, 2002; Davi *et al.*, 1997) which suggests that increased oxidant stress in obesity may contribute to persistent platelet aggregation. Several *in vitro* experimental studies have identified specific effects of ROS on platelet activity. For example (1) H_2O_2 has been shown to enhance collagen-dependent platelet activation and enhance arachidonic acid (AA)-dependent platelet activation (Practio *et al.*, 1992) (2) $O_2^{\cdot -}$ have been shown to reduce the threshold for platelet activation to thrombin, collagen, ADP or AA and induced spontaneous aggregation (Handin *et al.*, 1977; Krotz *et al.*, 2002; Salvemini *et al.*, 1989; De la Cruz *et al.*, 1992) and also reacts with NO to form $OONO^-$, which decreases bioavailability of NO, a potent inhibitor of platelet activation (Krotz *et al.*, 2004). In addition to exogenously derived ROS affecting the regulation of platelet activation, recent data also suggests that the platelets themselves generate ROS (Krotz *et al.*, 2002).

In this study, oxidant stress-mediated platelet aggregation was investigated using correlation analysis between oxidant stress parameters measured as LH and platelet aggregation measured as ADP-induced platelet aggregation using PlateletWorks. Although percentage ADP-induced platelet aggregation levels progressively decreased (expression of enhanced in vivo platelet reactivity) and LH levels progressively increased with increasing BMI levels, no association was found

between LH and ADP-induced platelet aggregation in healthy individuals (including a range of BMIs). In addition no association was found between TAS and ADP-induced platelet aggregation. However correlation coefficient analysis from data obtained prior to and post high-intensity acute exercise demonstrated that LH has a significant negative association with platelet aggregation at high ADP-induced platelet aggregation (20μM) ($r=-.0.329$, $P<0.01$) and a negative trend towards being associated with platelet aggregation at low ADP-induced platelet aggregation (10μM) ($r=-.240$, $P=0.065$). This suggests that increased LH could decrease the sensitivity of platelet activity to ADP (which is an expression of enhanced in vivo platelet reactivity). However this association is not entirely clear because this study failed to observe reduced platelet aggregation post high-intensity exercise despite observing significantly increased LH levels.

To gain a clearer picture of the potential oxidant stress may have on mediating platelet aggregation, the impact of Se supplementation on platelet aggregation (since Se supplementation reduces LH levels) was examined in both normal-weight and overweight individuals, pre and post high-intensity acute exercise. At rest and post-high intensity exercise, compared to placebo treatment, Se supplementation had no effect on reducing in vivo platelet reactivity in both the normal-weight and overweight groups. The lack of an association between Se supplementation and reduced platelet aggregation in this study may be because sodium selenite is not important in the role of platelet aggregation. For example Levander *et al* (1983) suggested that the increased platelet GSH-Px activity and decreased platelet aggregation in a pilot study following Se-rich yeast tablets suggests that particular Se compounds may be importance to platelet aggregation because Van Dokkum *et al* (1992) observed no beneficial effect of wheat Se supplementation on platelet aggregation levels. However the increased platelet aggregation and TxB_2 levels in diabetic rats was

shown to reverse following sodium selenite supplementation (Ersöz *et al.*, 2003). Another possibility for the lack of association between Se supplementation and reduced platelet aggregation may be because 3 weeks sodium selenite supplementation is too short a term to establish lipid peroxidation-mediated changes in platelet function (Salonen *et al.*, 1991).

9.5 Conclusion

It is well established that obesity enhances oxidant stress but whether this is a cause and effect relationship or a result of obesity-related diseases or a combination of the two remains unclear. Overweight individuals were not predisposed to enhanced oxidant stress but lifestyle habits such as short-term low carbohydrate diets and/or acute exercise were shown to increase oxidant stress levels in overweight individuals. Interestingly this exacerbated exercise induced response in overweight individuals was ameliorated by three-weeks selenium supplementation which is particularly important for overweight or obese individuals who exercise sporadically because they may be at greater risk of exercise-induced oxidant stress since the lack of regular training would not permit an enhancement of the defence mechanisms. In addition to oxidant stress, it seemed significant to identify if increased oxidant stress (due to obesity or unhealthy lifestyles) contributes to persistent platelet aggregation (since platelets play a central role in the process of thrombus formation, atherogenesis and atherosclerotic lesions). Overall, it appeared that oxidant stress does not mediate platelet responsiveness. Although a possible association between oxidant stress and platelet responsiveness pre- and post-high-intensity exercise in both normal-weight and overweight subjects was found, the nature of the association between oxidant stress and platelet aggregation remained unresolved as no association was found between lipid hydroperoxide levels and percentage ADP-induced platelet aggregation at rest across normal-weight, overweight and obese

groups and selenium supplementation did not improve platelet responsiveness in overweight individuals, despite ameliorating oxidant stress levels.

As evident from the following section, much work is still required in the domain of obesity, exercise, diet and oxidant stress in both health and disease. It is therefore hoped that the work contained in this thesis provides an induction into the area of free radical research and platelet aggregation in overweight/obesity, exercise, diet and pathology, and helps to generate future research ideas.

9.6 Future Work

The present study provided evidence that obesity may mediate oxidant stress as shown by elevated lipid hydroperoxide levels. It was also shown that oxidant stress is exacerbated in overweight individuals who were undertaking short-term LowCD, and high-intensity acute exercise. However caution should be taken when employing biomarkers for oxidant stress to examine these concepts as their measurement and interpretation are hampered by several factors. For example, oxidant stress assays (such as TBARS, MDA and LH) lack specificity, sensitivity and definition (in defining commonly accepted normal reference intervals). Precise analytical methods for oxidant stress need to be introduced for application in routing clinical laboratories which allow more precise interpretations of laboratory results to be made. Similarly, the impact of oxidant stress on platelet aggregation should be analysed using a wide range of biomarkers which reflect different aspects of platelet activation and may differ in terms of sensitivity.

To investigate further the effects of obesity and obesity-related diseases on oxidant stress, it would be useful to compare various groups of body mass index and existence of co-morbidities on oxidant stress levels. Future obesity studies into

oxidant stress should also include an assessment of GSH-Px which was not included in the present studies. Olusi *et al* (2002) observed that GSH-Px was lower in obese compared to a non-obese individuals, so it would be interesting to further define its role in the pro-oxidant/antioxidant balance.

Having shown that short-term adherence to LowCD in overweight individuals increased susceptibility to oxidant stress, an investigation is needed of longer duration. In addition, assessment of the effect of the LowCD on oxidant stress in obese individuals is needed. Future research should also be performed to identify the independent effects of dietary consumption and calorie restriction on the pro-oxidant / antioxidant balance.

The present studies have clearly demonstrated that exercise-induced oxidant stress was increased in overweight individuals compared to normal-weight individuals, following high-intensity exercise. Therefore exercise-induced oxidant stress response in obesity could also be examined following both moderate- and high-intensity exercise to identify an exercise intensity which is of lowest risk to the obese individual. Future research could study the effects of an incremental graded exercise stress on oxidant stress to identify if there is a 'oxidant stress threshold' (point at which LH levels significantly increase) during exercise. Exercising below the 'threshold' may potentially put individuals at less risk of the exercise-induced oxidant stress.

Short term Se supplementation may be advantageously used in overweight subjects completing a high intensity aerobic exercise session. However long-term intervention studies are needed to evaluate the efficacy of this new therapeutic approach to the prevention and treatment of exercise-induced oxidant stress following high-intensity exercise in overweight individuals. In addition, the type of Se supplement, dosage

and length of consumption should also be investigated. The application of Se supplementation at rest, pre and post exercise should also be studied in the obese population.

Further research should also examine the effects of long term adherence to weight management (including dietary changes and exercise training) on the pro-oxidant/antioxidant balance, which may provide greater insight into the importance of weight loss or cardio-respiratory fitness.

Finally, the impact of obesity-related oxidant stress should be examined in relation to its impact on clinical outcomes measures and physiological complications such as cardiac arrthymias, fibrinolysis and angina in the short-time and premature aging, inflammation, diabetes, CVD and cancer in the long-term (Alessio, 1994). This could further highlight the importance of oxidant stress measures in health and disease.

Chapter TEN

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10.0 References

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