

Studies on the role of zinc in maintaining antioxidant status in the heart.

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**STUDIES ON THE ROLE OF ZINC IN MAINTAINING ANTIOXIDANT
STATUS IN THE HEART**

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1418943

A thesis submitted in partial fulfilment of the
requirements of the
Robert Gordon University
for the degree of Masters of Research

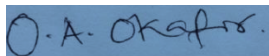
April 2020

Declaration

The work carried out in this thesis for a Masters in research was done by me. All data and results obtained was written and generated by myself. Other sources of information contained that did not come from the results generated have been acknowledged accordingly.

Signed

Date

A blue rectangular stamp containing the handwritten signature "O. A. Okafor." in black ink.

31.07.2020

Oronne A G Okafor

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Finally, I thank God for the journey thus far.

Abstract

Zinc (Zn^{2+}) is an essential micronutrient which plays a pivotal role as a signalling, catalytic and structural component in cells and functions in several physiological processes such as immune function, wound healing, cognition, reproduction and as co-factor for various enzymes involved in antioxidant system of the body. It functions as a component of the antioxidant defence system within tissues and cells and has been identified as a factor of great significance in cardiovascular health. Obesity is a major risk factor for cardiovascular disease. It is thought that an abnormality in adipocyte Zn^{2+} transport may contribute to obesity development. Hypozincaemia (low Zn^{2+} level in blood) in an obese person is followed by a decrease in the systemic antioxidant status resulting in a reduced response to oxidative stress, while Zn^{2+} supplementation reduces inflammation caused by obesity. The connection between obesity and the outcome from an acute myocardial infarction (AMI) is many-sided. Both obesity and AMI have been associated with production of inflammatory mediators and reactive oxygen species (ROS), which cause cell injury and eventually cell death. The pro-inflammatory and pro-oxidant status associated with obesity in the presence of an AMI may therefore present an increased risk of morbidity and mortality. The present study aimed to investigate the role of Zn^{2+} in maintaining antioxidant status in the heart of both obese and non-obese settings.

Results from biochemical assays showed that, chronic exposure of mice to a suboptimal level (5mg/kg) of dietary Zn^{2+} in combination with a high fat diet did not significantly alter cardiac levels of glutathione (GSH) compared with the Zn^{2+} adequate group (Zn^{2+} 35 mg/kg) at 15 and 25 weeks. However, the level of total GSH in the heart was significantly reduced by approximately 50% in the Zn^{2+} adequate group at 25 compared with 15 weeks. A suboptimal level of Zn^{2+} (5 mg/kg) did not significantly affect Copper Zinc superoxide dismutase (Cu/Zn-SOD) activity after either 15 or 25 weeks of dietary intervention. However, heart Cu/Zn-SOD activity was higher in all the groups at 25 compared to 15 weeks. In a second study in rats subjected to *in vitro* myocardial I/R injury, 2 weeks of severe dietary Zn^{2+} depletion (<1 mg Zn/kg diet) was associated with reduced cardiac GSH levels, while Cu/Zn-SOD activity was not affected by dietary Zn^{2+} intake. In contrast, *in vitro* acute Zn^{2+} depletion with TPEN reduced Cu/Zn-SOD activity, but not GSH content. Neither method of Zn^{2+} depletion had any effect on either myocardial Zn^{2+}

content or Caspase 3 activity but plasma levels of Zn^{2+} were reduced following dietary Zn^{2+} depletion.

These results demonstrate that Zn^{2+} depletion results in a worse outcome from I/R via different mechanisms, depending upon the method used to deplete Zn^{2+} . and shows the protective effect of Zn^{2+} as a co-factor of antioxidant enzymes in preventing injuries caused by I/R and propose its use as a possible treatment against cardiovascular disease. Further studies are required to better understand the effect of acute or chronic Zn^{2+} depletion on biomarkers of antioxidant status in the heart.

Abbreviations

AF	Atrial fibrillation
AMI	Acute myocardial infarction
ATP	Adenosine Triphosphate
BAT	Brown adipose tissue
BMI	Body Mass Index
BW	Body weight
CAD	Coronary artery disease
CAT	Catalase
CK	Creatine kinase
CK-MB	Creatine kinase myocardial band
CO ₂	Carbon dioxide
COX-2	Cyclooxygenase-2
Cu/Zn-SOD	Copper Zinc superoxide dismutase
CVD	Cardiovascular disease
EAR	Estimated average requirement
ECG	Electrocardiographic
ED	Endothelial Dysfunction
eNOS	Endothelial nitric oxide synthase
FA	Fatty acid
GCL	Glutamate cysteine ligase
GSH	Glutathione
GPx	Glutathione peroxidase
HFD	High fat diet
H	Hour

H ₂ O	Water
IDC	Idiopathic dilated cardiomyopathy
I/R	Ischaemia/reperfusion
IL-6	Interleukin-6
LDH	Lactate dehydrogenase
MnSOD	Manganese dependent-superoxide dismutase
mPTP	Mitochondrial permeability transition pore
MT	Metallothionein
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric Oxide
OH	Hydroxyl
OS	Oxidative stress
PPAR α	Peroxisome proliferator activated receptor alpha
RDR	Recommended dietary intake
RISK	Reperfusion injury salvage kinase pathway
RNI	Reference nutrient intake
ROS	Reactive oxygen species
SH	Sulfhydryl
SOD	Superoxide Dismutase
STEMI	ST-Segment elevation myocardial infarction
TCA	Tricarboxylic acid cycle
TNF- α	Tumour necrosis factor-alpha
TPEN	N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine
WAT	White adipose tissue
WHO	World Health Organisation

WT	Wild type
Zn ²⁺	Zinc
ZIP	Zinc/Iron Permease
ZNT	Zinc transporter

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1: Literature review

1. The physiological role of zinc (Zn^{2+})

1.1 Zinc (Zn^{2+}) as an essential trace element

In the human body, Zn^{2+} is the most abundant intracellular trace element and second most widely distributed after iron (King et al. 2006). It participates in many (>300) key enzyme reactions that are important in cell proliferation and differentiation, apoptosis, and metabolic activation (Jansen et al. 2009; Li et al. 2017). Zn^{2+} regulates gene expression and protects proteins from oxidation by binding to their sulfhydryl (SH) groups (Wiernsperger and Rapin 2010). A large number of proteins possess Zn^{2+} in their structure and it is the only trace element that is associated with all six enzyme classes in the body, the transferases, hydrolases, oxidoreductases, lyases, isomerases, and ligases (Valee and Falchuk 1993).

1.1.1 Zn^{2+} storage and homeostasis

Normal cellular activity necessitates the mobilization of Zn^{2+} and its transfer from one Zn^{2+} -binding site to another (Feng et al. 2005), and its homeostasis is controlled through the Zn^{2+} transporters, metallothionein (MT), and permeable membranes (Fukada et al. 2011). Free Zn^{2+} does not exist in the cell at an appreciable concentration as the vast majority is always bound to MT, which is a well preserved low molecular weight, cysteine-rich protein that maintains the cellular homeostasis of Zn^{2+} (Miles et al. 2000). A number of proteins (Zn^{2+} transport proteins) including MT, regulate Zn^{2+} homeostasis at different levels (tissue, cellular, and subcellular) in the human body (Baltaci and Yuce 2018). Zn^{2+} is stored in discrete compartments inside or outside intracellular organelles and vesicles by Zn^{2+} transporters (ZnT and ZIP) as shown in Figure 1.1. In mammals, there are 14 ZIP and 9 ZnT transporter genes (Baltaci and Yuce 2018) (Figure 1.1) each of which, when absent or dysfunctional, is associated with different physiological roles and/or health problems (Table 1.1) (Baltaci and Yuce 2018). Both types of transporters display specific changes in protein stability and cellular localization in response to various stimuli including Zn^{2+} deficiency (Kimura and Kambe 2016). ZIP (Zinc/Iron Permease) members facilitate the influx of Zn^{2+} into the cytosol from the outside of cells and the lumen of intracellular compartments, while ZNT (Zinc Transporter) members facilitate its efflux from the cytosol (Kambe et al. 2004) as shown in Figure 1.1.

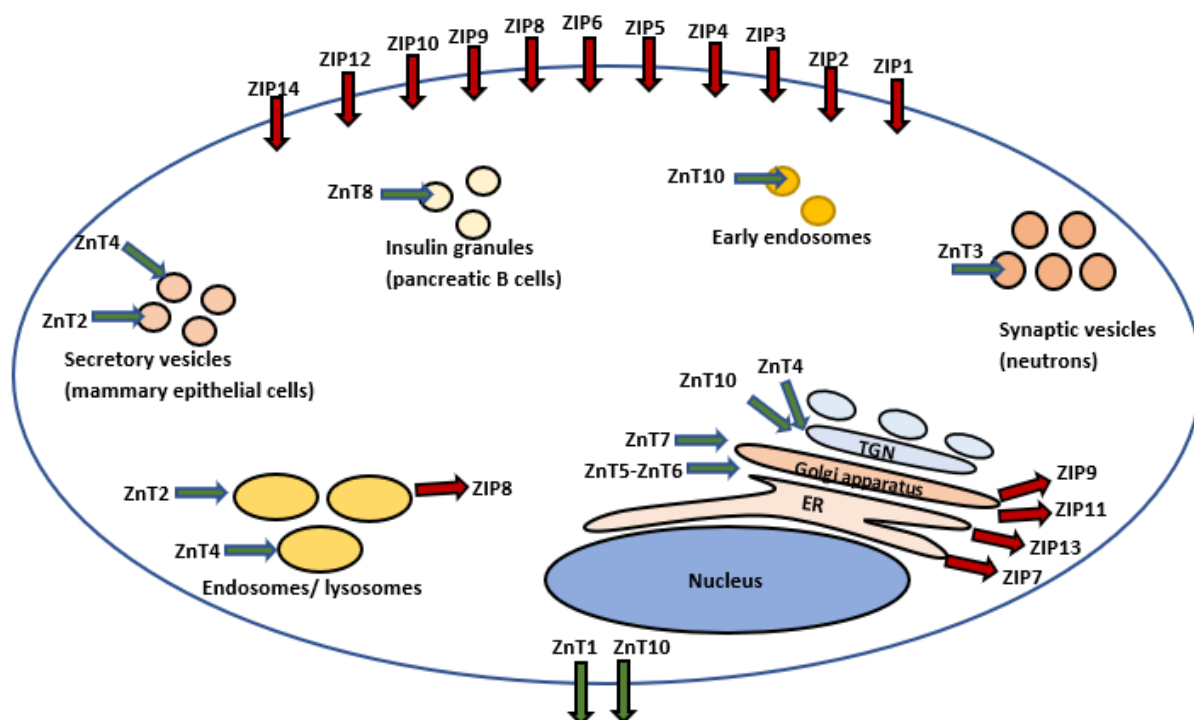


Figure 1.1: Localization of ZnT and ZIP transporters at the subcellular level. TGN - transgolgi network; ER- endoplasmic reticulum; (adapted from Kimura and Kambe 2016).

Table 1.1: Physiological occurrences and health problems associated with ZnT and ZIP proteins (Kambe et al. 2014; Baltaci and Yuce 2018)

ZnT Proteins	Physiological events and health problems
ZnT5	Involved in cardiovascular development, the maturation of osteoblasts and delayed allergic reactions mediated by mast cells
ZnT7	Involved in Insulin signalling pathway (Znt7-null mice displayed diet-induced glucose intolerance and insulin resistance) Huang et al. 2012.
ZnT9	Found to be significantly lower in obesity
ZnT10	Dysregulation contributed to disease progression in Alzheimer's patients (Bosomworth et al. 2013).
ZIP 4	Mutations in this gene causes the inherited disease Acrodermatitis enteropathica (AE)

ZIP13	This gene mutation causes Spondylocheiro dysplastic Ehlers-Danlos syndrome (SCD-EDS)
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1.1.2 Role of Zn²⁺ as an antioxidant

Amongst its several functions, such as a membrane stabilizer and anti-inflammatory entity, Zn²⁺ has been shown to function as an antioxidant by protecting SH groups against oxidation and hindering ROS production in cultured porcine endothelial cells and 3T3 cell models, respectively (Hennig et al. 1996; Oteiza et al. 2000; Zhang et al. 2018). Zn²⁺ inhibits NADPH oxidase, resulting in decreased production of ROS, and induces the generation of MT, which acts as a free radical scavenger (Prasad 2014). Zn²⁺ also causes a rise in the activation of antioxidant enzymes such as superoxide dismutase (SOD) and catalase, prevents lipid peroxidation, and decreases the actions of the oxidant-promoting enzymes inducible nitric oxide synthase (iNOS) and NADPH oxidase (Wiernsperger and Rapin 2010; Prasad 2014). Moreover, Zn²⁺ exerts additional antioxidant effects by protecting thiol groups from oxidation via complexation; a result of this is upregulated expression of GLC (Glutamate Cysteine Ligase), which controls the rate-limiting step in glutathione (GSH) synthesis via activation of the transcription factor Nrf2 (Cortese et al. 2008).

1.1.3 Dietary Zn²⁺ deficiency

In the developing countries, nutritional Zn²⁺ deficiency may affect more than 2 billion people (Prasad 2014) while in developed nations, the aged are a high-risk group due to poor dietary intake and reduced Zn²⁺ uptake with age (Haase et al. 2008). Two to three grams of Zn²⁺ is contained in the adult human body of which 0.1% is replaced every day (Maret and Sandstead 2006). Some food sources include oysters, meat, dairy products and cereals (Kaur et al. 2014). Across the world, there are varying estimated average requirement (EAR) for nutrients, including Zn²⁺. EARs are the recommended average daily level of intake estimated to meet the needs of half of the healthy population (Lawrence and Robertson, 2007). In the UK, the recommended daily intake of Zn²⁺ for males and females aged 19-75+ year is 7.0mg/day (PHE 2016). In the US and Canada, the Recommended Dietary Allowance (RDA; the key reference value adopted from the

EAR) is set to meet the dietary demands of the population of a sex group or in a given life stage; for Zn^{2+} it is 15 mg/day for males and 12 mg/day for females. In Australia and New Zealand, the Recommended Dietary Intake (RDI) is the official guidance, while in the UK the Reference Nutrient Intake (RNI) is the preferred reference value (Lawrence and Robertson 2007). Across the Canada, New Zealand and Australia, the EAR for zinc ranges from 5.5 to 12 mg/day (Karen et al. 2013), whereas the Committee on Medical Aspects of Food and Nutrition Policy (COMA) set the UK Zn^{2+} RNI ranges at 5.5-9.5 mg/day for males and 4.0-7.0 mg/day for females (COMA 1991).

However, approximately 30 % of the global elderly population are deficient in Zn^{2+} , thus supplementation of this important nutrient in this high-risk group has been proposed to support homeostasis, reduce susceptibility to infections, decrease OS and lower inflammatory cytokine production (Prasad, 2014; Haase et al. 2007). Zinc supplementation studies in the elderly have been found to decrease oxidative stress, and generation of inflammatory cytokines suggesting that it may have a protective effect in atherosclerosis and cardiovascular disease (Bao et al. 2010, Prasad, 2014).

Zn^{2+} cannot be stored therefore there is a need for regular intake, inadequate intake of Zn^{2+} resulting in deficiency is a risk factor for cardiovascular diseases (CVD) (Jansen et al. 2009). For example, Salehifar et al. (2008) reported a trend of lower Zn^{2+} level of 0.97 ± 0.25 mg/l vs. 1.12 ± 0.42 mg/l in idiopathic dilated cardiomyopathy (IDC) patients compared to healthy volunteers, respectively with a risk ratio of 0.2. This finding therefore suggests that Zn^{2+} imbalance plays a significant role in development of IDC. Similarly, a study has reported that in patients with congestive heart failure, both with or without atrial fibrillation (AF), there is a significant hypozincemia ($23.2 \pm 16.8 \mu\text{g/dl}$ (AF); $24.7 \pm 27.6 \mu\text{g/dl}$ (non-AF)) when compared to the normal control group who were volunteers with normal ejection fraction ($70.9 \pm 21.6 \mu\text{g/dl}$; Ghaemian et al, 2011).

1.2 Cardiovascular diseases (CVD).

CVDs are conditions which affect the heart and blood vessels and they include heart conditions a person is born with, inherited disorders and conditions that develop later in life, examples of which are atherosclerotic heart disease and

coronary heart disease. People who are at risk of CVDs are likely to be overweight/obese, hypertensive, diabetic, or have abnormal lipid levels (dyslipidaemia) (Sjöström et al. 2007; D'agostino et al. 2008). According to the World Health Organisation (WHO), the number one cause of death worldwide is cardiovascular disease (CVD) and is estimated to be about 7.4 million (Jefferson and Topol 2005; WHO 2019). Indeed, Roth et al (2017) revealed that CVD accounts for 31.80% of all global deaths. Of these, 85% of deaths are caused by heart attack (acute myocardial infarction; AMI) and stroke). Moreover, CVD accounts for 82% of deaths in low- and middle-income countries and, by 2030, 23.6 million people are estimated to die from CVDs (WHO 2019). In the UK, the British Heart Foundation (BHF) UK Factsheet for January 2020 states that 460 people will die daily from CVDs, and AMI has been identified as the leading cause of hospital admission and mortality (Moran et al. 2014; Asaria et al. 2017). More than 7 million people in the UK live with CVD (BHF 2020), and around 80% of these will have at least one risk factor (BHF 2020). Indeed multiple risk factors for CVD are more prevalent in the Western world (Jefferson and Topol 2005).

1.2.1 Acute myocardial infarction (AMI)

Myocardial infarction (MI) is a term used when there are signs suggestive of myocardial necrosis in the clinic alongside myocardial ischaemia (Thygesen et al. 2007). Acute MI (AMI) is a significant cause of morbidity and mortality globally with a yearly ratio of 1:6 for men and 1:7 for women in Europe (Keller et al. 2018) and this usually results from reduced blood supply to the heart muscle as a consequence of either a decrease or complete obstruction of blood flow in the coronary arteries. This subsequently leads to ischaemia and an accumulation of toxic metabolites within the tissue causing loss of function and ultimately death of the cardiomyocytes (Jefferson and Topol 2005). AMI can be confirmed clinically by grouping 2 or 3 features which include chest pain, electrocardiographic (ECG) abnormalities (\pm Q wave development, \pm ST segment changes) and a rise in cardiac enzymes (cardiac troponin and the MB fraction of creatine kinase; CK-MB) (Antman et al. 2000; Thygesen et al. 2012). An AMI may lead to sudden death due to ventricular tachyarrhythmia/fibrillation activated by ischaemia due to arterial occlusion resulting from the rupturing of atheromatous plaque (Davies and Thomas 1985; Huikuri et al. 2001; Solomon et al. 2005).

1.2.2 Energy metabolism in the myocardium (aerobic and anaerobic)

1.2.2.1 Aerobic metabolism

Ordinarily, the function of the heart is dependent on a continuous production of energy in the form of ATP by oxidative phosphorylation in the mitochondria (Stanley and Chandler 2002). The heart requires a high energy supply to function because of its constant function of pumping blood around the body to maintain circulation. This energy is derived from free fatty acids (FAs), ketones, carbohydrate (glucose and lactate) and amino acids (reviewed in Sambandam and Lopaschuk 2003). About 60–90% of this energy is from FA oxidation (a process that is highly dependent on oxygen for ATP generation) with the remainder being derived from glucose and lactate metabolism (Stanley and Chandler 2002). FA oxidation is more energy efficient with an ATP production of 42 Joules (J) compared to glucose with 38 J, however glucose utilisation is more oxygen efficient with 6.3 moles of ATP consumed per O₂ molecule compared to 5.5 moles of ATP per O₂ molecule for FA utilisation (Wolff et al. 2002). Under conditions of normal (aerobic) metabolism, FAs are converted to acyl-CoA upon entry into the cardiomyocyte. This is then transported into the mitochondria where it undergoes β -oxidation to release acetyl-CoA, which in turn enters the tricarboxylic acid cycle (TCA) cycle to produce ATP, CO₂ and H₂O (Figure 1.2). In addition, under aerobic conditions glucose undergoes glycolysis to give rise to pyruvate which is also oxidised in the mitochondria via the tricarboxylic acid (TCA) cycle, or converted to lactate. Excess glucose is stored as glycogen in the myocardium as an additional energy source for anaerobic metabolism if the demands of the heart exceed the supply of oxygen to the cardiomyocyte (Wolff et al. 2002; Sambandam and Lopaschuk 2003).

The uptake of glucose from outside the cell is controlled by the concentration of glucose transporters (GLUT 1 and 4) and the glucose gradient across the membrane (Stanley and Chandler 2002). GLUT 1 and GLUT 4 are the predominant isoforms of the glucose transporter family identified in the heart and they are located in the intracellular vesicles and sarcolemmal membrane (Stanley and Chandler 2002). Therefore, the first step of glucose metabolism in the heart is its transport across the sarcolemma and the cardiomyocyte is able to take up glucose depending on the quantity of transporters in the plasma membrane (Young et al. 1997; Russell et al. 1998). Insulin and ischaemia have been found to have an additive effect on causing a shift of these transporters from the intracellular site

to the plasma membrane and this leads to a faster entrance of glucose into the cell (Slot et al. 1991; Sun et al. 1994; Stanley and Chandler 2002). In AMI, insulin enhances myocardial glucose uptake bringing about the translocation of glucose transporters thereby leading to an increase in glucose metabolism (Sun et al. 1994). Apart from the heart muscle, GLUT 4 can also be found in adipocytes and skeletal muscle (Slot et al. 1991). Once glucose enters the cell, it is quickly phosphorylated to form glucose 6 phosphate and then converted either to glycogen or goes through the glycolytic pathway (Stanley and Chandler 2002).

1.2.2.2 Anaerobic metabolism

Myocardial ischaemia causes a striking change in metabolism leading to an enhanced rate of conversion of glucose to lactate as well as a switch from lactate being taken up by the myocardium to it being produced, causing an interference in cell homeostasis (Wolff et al. 2002). As a consequence of the lack of O₂ supply, oxidation of FA and glucose in the mitochondria is impaired. The anaerobic glycolytic pathway is therefore activated to meet the critical energy demand of the heart, the main characteristic of which is the generation of ATP through the conversion of stored glycogen to pyruvate and H⁺ ions (hydrolysis). However, since pyruvate cannot be oxidised in the mitochondria, there is an excess accumulation of both lactate and H⁺ ions in the cytosol leading to Na⁺ influx via Na⁺/H⁺ exchanger, which subsequently leads to Ca²⁺ accumulation via the Na⁺/Ca²⁺ exchanger (Kloner and Jennings 2001; Figure 1.3).

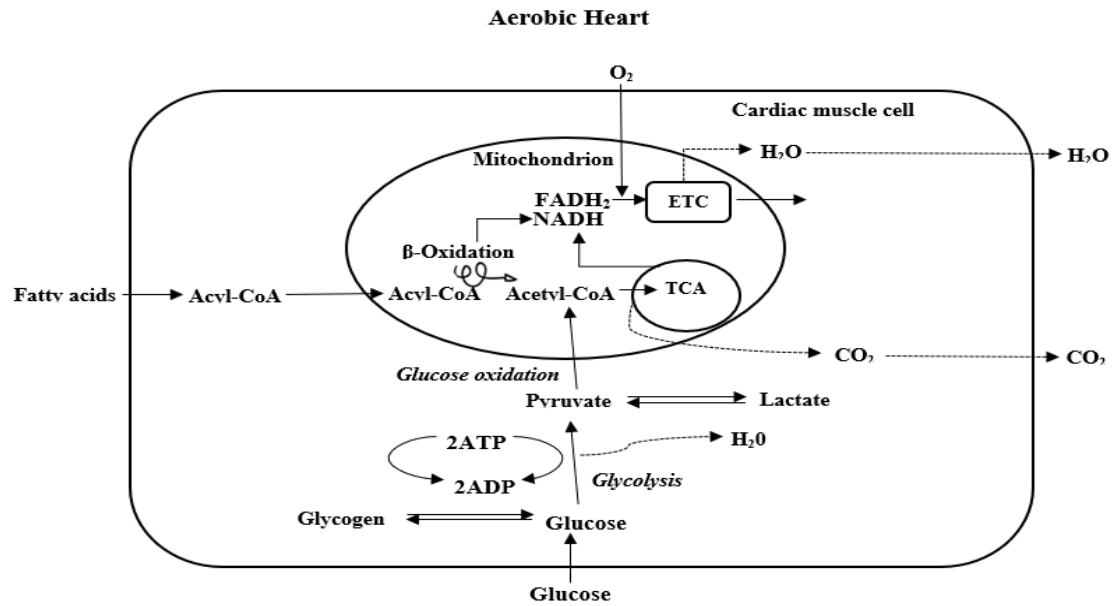


Figure 1.2: Schematic representation of energy utilisation of the cardiomyocyte under aerobic conditions, the main pathways of which are fatty acid and glucose oxidation. (TCA - Tricarboxylic acid pathway) (adapted from Sambandam and Lopaschuk 2003).

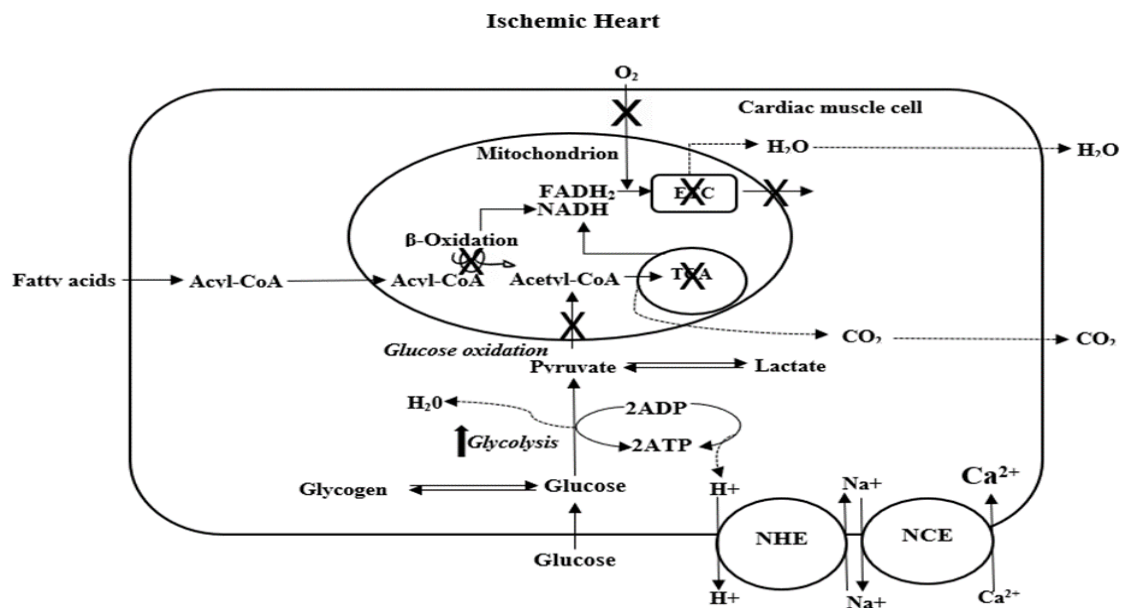


Figure 1.3: A schematic representation of energy metabolism in the ischaemic heart. (adapted from Sambandam and Lopaschuk 2003) (Kloner and Jennings 2001).

1.2.3 Reperfusion injury (RI)

Reperfusion is a term used to describe the restoration of the flow of blood to the heart. Principally, restricted blood flow to the heart (ischaemia) causes an imbalance between demand and supply of O₂ causing injury and an impaired function of the heart (Frank et al. 2012). Reperfusion is achieved clinically using thrombolytic treatments or the use of percutaneous coronary intervention (PCI) (Yellon and Hausenloy 2007). Reperfusion is the utmost effective method to restore ischaemic cells that are not yet apoptotic or necrotic, leading to restoration of function and thus causing the final infarct size sustained to be reduced (Maroko et al. 1972; Hausenloy and Yellon 2004). However, sudden reintroduction of oxygen after a period of ischaemia leads to an increased production of free radicals in the heart muscle (Garlick et al. 1987) and immediate cell death; this response to reperfusion is known as the 'oxygen paradox' (Maxwell and Lip 1997; Hausenloy and Yellon 2004; Yellon and Hausenloy 2007). This leads to the death of cardiomyocytes that were viable prior to reperfusion, thereby increasing the infarct size (Liu et al. 1996; Yellon and Hausenloy 2007). The predominant mechanisms involved in reperfusion injury include the O₂ (oxygen) paradox, the Ca²⁺ (calcium) paradox, the pH paradox and inflammation (Yellon and Hausenloy 2007). Studies have shown that reperfusion-induced oxidative stress (OS) also reduces the amount of intracellular signalling molecules such as the cardioprotective nitric oxide (NO), a known antioxidant. Furthermore, sarcolemmal membrane damage induced by OS leads to increases in intracellular Ca²⁺ which results in Ca²⁺ overload and ultimately cardiac cell death (Liu et al. 1996; Yellon and Hausenloy 2007). As the heart continues to be reperfused, there is clearance of the accumulated lactic acid, the ion exchangers return to normal and the intracellular pH also rapidly returns to normal (Liu et al. 1996; Kloner and Jennings 2001; Yellon and Hausenloy 2007). In terms of cardiac energy, the re-introduction of oxygen allows FA oxidation to recommence, restoring ATP levels within the heart (Belke and Lopaschuk 1997; Sambandam and Lopaschuk 2003). Sometimes, there is a delayed recovery of metabolism in a previously ischaemic heart and which can cause a loss of function of the myocardium after recovering (Schwaiger et al. 1985).

Delayed RI also occurs as a result of activation and build-up of neutrophils (inflammatory cytokines) (Vinten-Johansen 2004) which cause direct damage to

cells via production of more ROS, endothelial dysfunction (ED) and plugging of capillaries (Hoffman et al. 2004). ED is characterized by diminished bioavailability of vasodilators, predominantly NO, and increased contractile factors derived from the endothelium supporting the development of cardiovascular disease and atherosclerosis (Couillard et al. 2005; Hadi et al. 2005; Fernández-Sánchez et al. 2011) which is a consequence of both increased ROS production and decreased antioxidant capacity. ED can also be caused by stimulating inflammation and cytokines (Couillard et al. 2005). Reperfusion of the heart can therefore contribute to a worse outcome after a myocardial ischaemic injury accounting for up to 50% of the infarct's final size (described as lethal reperfusion injury) (Frank et al. 2012).

1.2.4 The reperfusion injury salvage kinase (RISK) pathway in I/R

ROS also activate glycogen synthase kinase-3 β (GSK-3 β), a serine/threonine kinase that phosphorylates and downregulates glycogen synthase, the rate-limiting enzyme of glycogen metabolism. In the basal state, GSK-3 β keeps the activities of the antiapoptotic BH4 and the "derepressor" Bcl-2 protein family members in check. However, under conditions of oxidative stress this GSK-3 β subdomain pool is inactivated, causing a shift in interaction within the mPTP-regulatory complex in favour of the proapoptotic elements (Juhászivá et al.

2008) leading to mitochondrial permeability transition pore (mPTP) opening (a mechanism involved in I/R injury and cell death) (Hausenloy et al. 2002). Opening the mPTP permits the entrance of water and solutes into the mitochondria which causes an increase in the matrix volume, and ruptures the outer mitochondrial membrane eventually initiating apoptosis by leading to the release of intermembrane cytochrome C (Hausenloy et al. 2002). In contrast ischaemic pre-conditioning (i.e. exposure of heart tissue to a short period of ischaemia prior to a more prolonged ischaemic period followed by reperfusion) have been linked to the activation of a protective sequence of events (the reperfusion injury salvage kinase; RISK) pathway), the activation of which results in activation of two key pro-survival kinases; extracellular-signal regulated kinase 1/2 (ERK; also referred to as p42/p44 MAPK) and Akt (Protein Kinase B; PKB) (Hausenloy and Yellon 2004; Hausenloy and Yellon 2007; Figure 1.4). PI3K/Akt activation, and subsequent inhibition of mPTP opening, leads to preservation of tissue (Hausenloy et al. 2002),

while ERK activation results in tissue preservation via P70s5K. Both pathways have been demonstrated to have cardioprotective effects when activated at the time of reperfusion (Hausenloy and Yellon 2004).

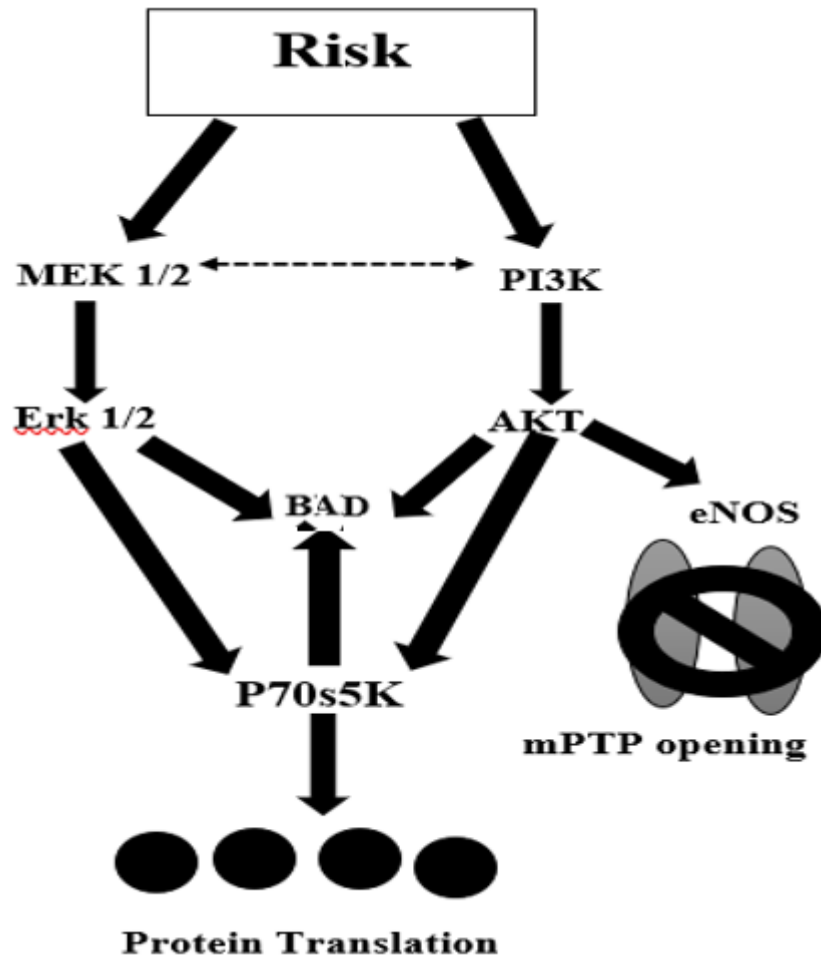


Figure 1.4: Schematic structure showing the 2 key pro-survival kinase pathways (1/2 ERK and Akt). Upregulation of the RISK pathway by means of activating these pathways phosphorylates downstream targets like eNOS-producing NO, which inhibits mPTP opening. (adapted from Tsang et al. 2004). MEK1/2: mitogen-activated protein kinase, P13: prosurvival kinase, Erk1/2: extracellular signal-regulated kinases. AKT/PK B: Protein kinase B, BAD: Bcl2 antagonist of cell death, Enos: Endothelial nitric oxide synthase.

1.2.5 Antioxidant defences within the heart

The rapid rise in ROS and oxidative damage that occurs during reperfusion is caused by an imbalance in antioxidant levels and ROS production (Yamashita et al. 1998). This burst of ROS upon reperfusion of the ischaemic heart can lead to molecular damage to vital structures and functions (protein, DNA) of the heart (Ambrosio et al. 1993; Møller et al. 1996; Becker et al. 1999; Couillard et al. 2005). Glutathione (GSH) works alongside superoxide dismutases (SODs) to remove ROS, however during OS the amount of ROS exceeds the capacity of the antioxidant enzymes, leading to tissue damage (Chan et al. 1998). GSH is a tripeptide (L-gamma--glutamyl-L- cysteinyl-glycine) antioxidant that constitutes the highest number of non-protein thiols in the human body (Cheung et al. 2000). It is oxidized to glutathione disulphide by GSH peroxidase (Anderson 1998) which is then regenerated from glutathione disulphide by GSH reductase. This redox cycle is important for the cell to combat oxidant stress (Cheung et al. 2000).

Antioxidants (GSH and SOD) generally have two ways in which they carry out their actions - the immediate (acute) and the later (chronic). The immediate effect involves different mechanisms, amongst which are decreasing the formation of $\cdot\text{OH}$ (the hydroxyl radical) group and protecting sulfhydryl proteins from hydrogen peroxide (H_2O_2) through the antagonism of redox-active transition metals (iron and copper) (Powell et al. 1994). The chronic effect of GSH and SOD's action can be achieved by the binding of Zn^{2+} directly to sulfhydryl groups of GSH and SOD, thereby reducing the reactivity these sulfhydryl group proteins (Powell et al. 1994).

SOD is an endogenous antioxidant that scavenges ROS by converting superoxide radicals to H_2O_2 and oxygen (Förstermann et al. 2017). The SOD enzymes are important in the detoxification of ROS by scavenging them, thus protecting against OS and preventing the degradation of NO by superoxide in the vascular wall (Neves et al. 2012). There are 3 isoforms of mammalian SOD namely (1) SOD1 (copper/zinc-SOD; Cu/Zn-SOD) usually seen in the cytoplasm and mitochondrial intermembrane space; (2) SOD2 (manganese-SOD; Mn-SOD) found in the mitochondrial matrix, and (3) SOD3 (extracellular SOD) expressed in the extracellular matrix, on the cell surface, and in extracellular fluids and these 3 isozymes have crucial antioxidant roles viz catalyst to the dismutation of superoxide to H_2O_2 and oxygen (Förstermann et al. 2017).

1.2.6 Role of Zn²⁺ in controlling apoptosis-induced myocardial cell death

Apoptosis (programmed cell death) also contributes to myocardial I/R (Hausenloy et al. 2002), the hallmark of which is activation of caspases. Cysteine aspartate proteases (caspases) are a family of proteases that cleave a variety of critical cellular proteins containing the caspase recognition site. In the case of caspase 3 and 7 this is essential to the apoptotic process, making all models of apoptosis to be characterized by increased caspase activity (Jankowski-Hennig et al. 2000). The caspase family of enzymes are among the major proapoptotic proteins and have been implicated in many biochemical pathways leading to apoptosis (Chai et al. 2000). Three major pathways involve (1) membrane receptor-ligand interactions (e.g., Fas-FasL binding) that result in activation of the upstream caspase, caspase-8; (2) triggering by chemical stimuli, which causes changes in mitochondrial membrane permeability, release of cytochrome c into the cytoplasm, and subsequent activation of the upstream caspase, caspase-9; and (3) activation of caspase-12 triggered in response to stress-induced changes in the endoplasmic reticulum (Chai et al. 2000). Caspase-8 and caspase-9, triggered either directly or indirectly, activate the major effector caspase, caspase-3/DEVD-caspase (Chai et al. 2000).

Zinc deficiency has been associated with the induction of apoptosis, and in order to aid our understanding of mechanisms by which zinc deficiency induces apoptotic cell death different studies have been carried out. *In vitro* studies have shown that depletion of intracellular Zn by culturing chronic lymphatic leukaemia (CLL) cells in Zn-depleted medium, or by treatment of cells with N,N,N',N'-tetrakis-2-pyridylmethylethylenediamine (TPEN), results in apoptosis (Zalewski et al. 1991, 1993). Zn deficiency induced apoptosis is dependent on caspase-3 activation, since cytosolic caspase-3 activity is increased in Zn deficient cells while the specific caspase-3 inhibitor z-DEVD-fmk can partially suppress apoptosis (Truong-Tran et al. 2001). Moreover, a study carried out by Martin et al. (1991), using three human cell lines of lymphoid (Molt-3 and Raji) or myeloid (HL-60) origin maintained *in vitro* under zinc-sufficient or zinc-deficient conditions, assessed cell proliferation, viability and mode of death. Their data showed that apoptosis occurred in zinc-deficient HL-60 and Raji cells, and was characterized by a slow decline in cell viability, since cells exhibited condensed and fragmented nuclear DNA and these HL-60 cells responded and maintained viability to Zn²⁺ levels in a dose dependant

manner (Martin et al. 1991). In another study, Zalewski et al (1991) demonstrated, in chronic lymphatic leukaemia (CLL) cells, that the intracellular pool of chelatable Zn^{2+} blocks apoptosis and that this pool is increased by uptake from the medium (Zalewski et al. 1991; Zalewski, and Forbes 1993). Finally, a study carried out by Chai et al (2000) using three human malignant epithelial cell lines (LIM1215 (colonic); NCI-H292 (bronchial); and A549 (alveolar type II)) demonstrated that, in TPEN-treated cells, depletion of Zn^{2+} was followed by activation of caspase-3, DNA fragmentation, and morphologic changes and these effects were completely prevented by exogenous $ZnSO_4$ (Chai et al. 2000).

Caspase 3 can also be inhibited *in vivo* by physiologically relevant concentrations of Zn^{2+} , since a study has shown that rat embryos collected mid-gestation from dams fed a Zn-deficient (0.5mgZn/g) displayed increased caspase activity compared to embryos from pair-fed dams, or dams fed a Zn-adequate (25mg Zn/g) diet ((Jankowski-Hennig et al. 2000)). The authors of this study proposed that Zn-deficiency-induced apoptosis may be mediated via increased ROS concentrations since they also observed increased concentrations of ROS and apoptosis in 3T3 cells cultured in Zn-deficient medium (Jankowski-Hennig et al. 2000).

1.2.7 Role of Zn^{2+} in ischaemia and CVD

The protective potential of Zn^{2+} in CVD has been widely reported in the literature (Little et al. 2010; Lee et al. 2015). With ROS causing a marked increase in free radicals, there is release of Zn^{2+} by MT from both the mitochondria and cytosol (Feng et al. 2005; Lin et al. 2011). This release of Zn^{2+} affects Ca^{2+} regulation in cardiomyocytes because electrical stimulation induces its entry through voltage gated Ca^{2+} channels and this affects intracellular Zn^{2+} stores (Beyersmann and Haase 2001). Intracellular Zn^{2+} , when either increased or decreased, has been linked with apoptotic cell death (Truong-Tran et al. 2001; Viswanath et al. 2011), while *in vitro* I/R following a state of acute depletion of Zn^{2+} with TPEN (a Zn^{2+} chelator) has been shown to result in a greater degree of infarct size (Skene et al. 2019). In addition, studies in an isolated heart model have demonstrated that Zn^{2+} supplementation enhances post-arrest cardiac function, which is suggestive of its protective potential if used as an additive to cardioplegic solutions (Powell et al.

1995). In the clinical setting, indeed, circulating Zn^{2+} levels have been shown to decrease (group mean-76-83 $\mu\text{g}/100\text{ ml}$) within the first 3 days following an AMI and then begin to increase towards normal (90-118 $\mu\text{g}/100\text{ ml}$) levels within 7-10 (Handjani et al. 1974) and 10-14 days (Low and Ikram 1976; Huang et al. 2018).

1.2.8 The role of Zn^{2+} in cardioprotection

Since patients who suffer from myocardial infarction have been found to have depleted plasma Zn^{2+} levels as well as high levels of enzyme markers (creatine kinase [CK] and lactate dehydrogenase [LDH]) of myocardial injury (Low and Ikram 1976), supplementation with Zn^{2+} may be a useful way of reducing the extent of myocardial injury. Zinc pyrithione is a well-known Zn^{2+} ionophore that has been shown to reduce cell susceptibility to oxidative stress induced by H_2O_2 in rats (Oyama et al. 2012). Chanoit et al (2008) demonstrated that in isolated hearts, administration of Zn^{2+} pyrithione reduces injury caused by reperfusion via activation of the RISK pathway. Zn^{2+} pyrithione also decreased the release of LDH and CK-MB, reduced collagen content and decreased myocardial infarct size in hyperlipidemic rat hearts as well as enhancing the cardioprotective effect of ischaemic preconditioning in this setting (Kansal et al. 2015).

1.3 Obesity

According to the World Health Organisation, obesity is defined as accumulation of excess fat (adipose tissue) that presents a risk to health (WHO 2019). Obesity is characterised by a build-up of lipids in mature adipocytes of white adipose tissue (WAT) leading to an increased number and size of these cells as a consequence of energy intake constantly outweighing energy expenditure (Otto and Lane 2005). Obesity was once considered a problem only in developed countries however, the incidence in low- and middle-income countries is now increasing (WHO 2019). It is regarded as a major risk factor for the development of cardiovascular disease and has been associated with reduced life expectancy (Wilson et al. 2002; Olshansky et al. 2005; Yusuf et al. 2005). The mechanisms connecting obesity with cardiovascular disease involves adipokine signalling and changes in circulating glucose and lipids levels (Lopaschuk et al. 2007).

In obesity, adipose tissue is invaded by macrophages that contribute to insulin resistance as well as other disorders related to obesity and these macrophages produce tumour necrosis factor- α (TNF- α), interleukin 6 (IL-6), and other pro-inflammatory cytokines that interfere with the insulin signalling pathway (Cinti 2012). Thus, WAT dysfunction is an important factor in the pathogenesis of metabolic and cardiovascular diseases.

In the United States of America, approximately 70% of adults are grouped as either overweight or obese in comparison to 40 years ago when it was less than 25% (Lavie et al. 2009). Body mass index (BMI) is the most commonly used anthropometric tool to classify obesity (Table 1.2). However, BMI cannot distinguish an elevated body weight due to high levels of lean versus fat body mass therefore other anthropometric measures are used to evaluate the degree of obesity includes waist circumference (WC) and waist-to-hip ratio (WHR) (Table 1.2) (Vecchie et al. 2017).

Obesity and being overweight are associated with chronic and low-grade inflammation with irregular production of some pro-inflammatory signalling pathways (IL-6, TNF- α , and C-reactive protein (CRP)) (Bulló et al. 2003; Costarelli et al. 2010). Chronic, subclinical inflammation is one pathophysiological status contributing to the increased risk of atherosclerotic disease associated with adiposity (Festa et al. 2001).

Table 1.2 Definition of obesity by anthropometric measurements (adopted from Vecchie et al. 2017).

BMI WHO classification			
BMI < 18.5 kg/m ²	Underweight	BMI 30–34.9 kg/m ²	Class I obesity
BMI 18.5–24.9 kg/m ²	Normal weight	BMI 35–39.9 kg/m ²	Class II obesity
BMI 25–29.9 kg/m ²	Overweight	BMI > 39.9 kg/m ²	Class III obesity (morbid)
Waist circumference			
Men > 102 cm		Women > 88 cm	
Waist to hip ratio			
Men > 0.9		Women > 0.85	

1.3.1 Adipose tissue

The adipose tissue is a large organ with a distinct structure and vascular and nerve supplies. It is a vital, yet complex and highly active, endocrine and metabolic organ that contains adipocytes, immune cells, stromovascular cells, nerve tissue and connective tissue matrix that act in concert as an integrated unit (Kershaw and Flier 2004). It is vital for energy homeostasis and is distributed throughout the body in two morphologically distinct white (WAT) and brown (BAT) adipose tissue depots as described in Table 1.4 (Lefterova and Lazar 2009).

Adipocytes are specialized cells that play a key role in vertebrate energy homeostasis (White and Stephens 2010) and increases in adipocyte number or size are a hallmark of obesity. Hence, understanding adipocyte differentiation is appropriate for an in-depth knowledge into the pathogenesis of metabolic diseases including obesity.

1.3.2 Adipogenesis

Adipogenesis is the process by which mature adipocytes develop from fibroblast-like preadipocytes which then differentiate into insulin-responsive and lipid-laden adipocytes (Lefterova and Lazar 2009). This process occurs through numerous stages (Figure 1.5; Table 1.3) involving a cascade of transcription factors, including peroxisome proliferator-activated receptor gamma (PPAR γ), CCAAT/enhancer-binding proteins (C/EBPs) and SREBP-1 (Gregoire et al. 1998; Lefterova and Lazar 2009; White and Stephens 2010). Interactions between individual cells, or between cells and the extracellular environment, control growth and differentiation of preadipocytes with extracellular matrix proteins and preadipocyte factor-1 involved (Gregoire et al. 1998). The key steps in adipogenesis are summarised in Table 1.3.

1.3.3 The secretory function of white adipose tissue (WAT)

The main cellular components of WAT are mature adipocytes. As the store house for triacylglycerols (TAGs) and the main long-term fuel reserve in mammals, the WAT has a significant impact on plasma lipid levels, glucose uptake rates and energy flux (Otto and Lane 2005). WAT secretes a number of adipokines, including

leptin, adiponectin, and cytokines (TNF- α and IL-6) which control numerous physiological processes in the body including appetite, glucose metabolism, inflammatory responses and blood pressure (Leftorova and Lazar 2009).

Table 1.3: Key steps of adipogenesis and their characteristics (adapted from Leftorova and Lazar 2009).

Key steps of adipogenesis	Characteristics
Mesenchymal precursor	Proliferation
	Differentiate into several lineages
Committed preadipocyte	Proliferation
	Committed to differentiate along adipocyte lineage
	Fibroblast-like morphology
Growth-arrested preadipocyte	Unable proliferation due to contact inhibition
Mitotic clonal expansion	Initiation of C/EBP β and C/EBP δ expression and activity
	Series of cell divisions (i.e. mitotic clonal expansion)
	Re-entry into the cell cycle induced by hormonal stimulation
Terminal differentiation	Cell-cycle arrest
	Induction of C/EBP α and PPAR γ expression
	Transcriptional activation of adipocyte genes
Mature adipocyte	Signet-ring morphology: large lipid droplet occupies majority of cell volume.
	High expression of adipocyte genes
	Transcriptionally active PPAR γ , C/EBP α and C/EBP β

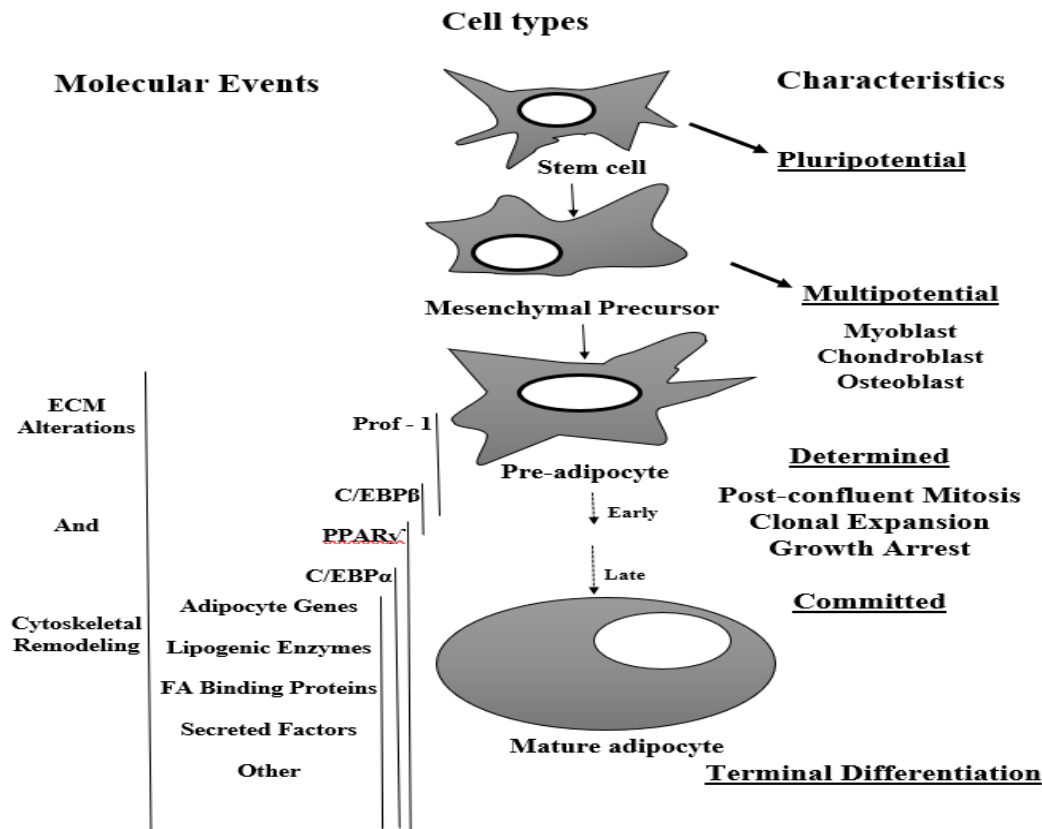


Figure 1.5: An overview of phases in adipocyte differentiation. Pref-1, preadipocyte factor-1; C/EBP (β and α), enhancer binding protein; PPAR-g, peroxisome proliferator-activated receptor-g; ECM, extracellular matrix; FA, fatty acid (adapted from Gregoire et al. 1998).

Table 1.4: Functions of white and brown adipose tissue (adapted from Otto and Lane 2005; Tang et al. 2008; Lefterova and Lazar 2009; Rosen and Spiegelman 2014).

White adipose tissue (WAT)	Brown adipose tissue (BAT)
Unilocular lipid-filled adipocytes that store energy in the form of triacylglycerol during times when input exceeds expenditure.	Multilocular adipocytes that specialize in breakdown of stored lipid into free fatty acids when energy is required.
Predominant type of fat in humans (adults) that store excessive caloric intake in the form of lipid.	Burn lipids through non-shivering thermogenesis and generates heat in new-borns and rodents.

1.3.4.1 Leptin

Leptin, a 16,000 molecular-weight adipocyte-specific hormone, has a variety of biological functions, including the control of appetite and energy balance (crucial hypothalamic signal) and eliciting a proinflammatory immune response (Trayhurn and Beattie 2001; Trayhurn and Wood 2004; Trayhurn 2005). It acts on the limbic system (hypothalamus) bringing about a state of satiety by stimulating dopamine uptake and activating the sympathetic nervous system, thus reducing the desire to eat via the locus coeruleus nucleus leading to an increase in resting energy expenditure (Fernández-Sánchez et al. 2011). It is primarily made and secreted by mature adipocytes. Leptin improves insulin sensitivity by inhibiting lipogenesis and stimulating lipolysis, thereby resulting in reduction of intracellular lipid levels in pancreatic beta cells, liver and skeletal muscle (Ehrhart-Bornstein et al. 2003; Fernández-Sánchez et al. 2011). Elevated levels of leptin are found in animal models of obesity and human obesity (Gregoire et al. 1998). In conditions of weight loss, circulating levels reduce, causing a reduction in the levels of obesity-associated inflammatory markers in plasma (Hukshorn et al. 2004).

Leptin has also been shown to stimulate the release of TNF- α and NO from epididymal fat pads *in vitro*, which suggests important roles in both inflammation and the regulation of blood flow throughout the adipose tissue (Mastronardi et al. 2002). It also promotes oxidative stress and stimulates the proliferation and migration of vascular smooth muscle and endothelial cells, suggesting a role in the development of cardiovascular disease (Fernández-Sánchez et al. 2011).

1.3.4.2 Adiponectin

Adiponectin is expressed and secreted by differentiated adipocytes (Fernández-Sánchez et al. 2011) and assists in regulating energy homeostasis, and glucose and lipid metabolism (Sikaris 2004; Trayhurn 2005). In contrast to other adipokines, and leptin in particular, adiponectin expression and plasma concentrations are decreased in disease conditions presenting with both insulin resistance and obesity, such as Type 2 diabetes (Arita et al. 1999; Trayhurn and Beattie 2001; Fernández-Sánchez et al. 2011). Adiponectin is considered to protect against diabetes and cardiovascular disease (Trayhurn 2005; Skurk et al.

2007) and high levels of this adipokine are associated with weight loss (Sikaris 2004; Fernández-Sánchez et al. 2011).

1.3.4.3 Tumour necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6)

TNF- α was the first inflammatory cytokine shown to be expressed in and secreted by white adipocytes and is a key regulator of the synthesis of IL-6 (Chiellini et al. 2002). It is primarily made by lymphocytes, monocytes and skeletal muscle, when TNF- α is produced in large amounts, it contributes to the pathogenesis of obesity-related metabolic syndrome (Hotamisligil et al. 1995; Fernández-Sánchez et al. 2011). Mature white adipocyte size is regulated by TNF- α , therefore the larger the adipocytes the more TNF- α produced, which in turn initiates changes to limit adipocyte size or to induce apoptosis (Stephens and Pekala 1991; Hotamisligil et al. 1995).

IL-6 is produced by adipocytes, endothelial cells, macrophages, immune cells, fibroblasts and skeletal muscle cells and has many functions which includes defence, tissue damage and proinflammatory properties (Fonseca-Alaniz et al. 2007; Fernández-Sánchez et al. 2011). In adult humans, the amount of IL-6 in circulation correlates with the BMI, carbohydrate intolerance and insulin resistance, while in experimental animals IL-6 promotes gluconeogenesis and glycogenolysis thereby increasing triglycerides (TG) levels while it inhibits glycogenesis (Fernández-Sánchez et al. 2011).

1.3.5 Cardiac energy metabolism in obesity

Although FAs and glucose are the main energy source for the normal heart, in obesity there is an increase in FA oxidation due to the inhibition of glycolysis, which suppresses glucose oxidation and leads to high levels of circulating free FAs (Lopaschuk et al. 2007); this creates an imbalance between FA uptake and oxidation, leading to lipid accumulation in the heart (cardiac lipotoxicity) (Chiu et al. 2001). With this state of impaired glucose oxidation, even if there is normal glucose uptake there is a higher inflow of glucose via the pentose phosphate shunt (resulting in ROS formation and lipid peroxidation) (Gupte et al. 2006) and the hexosamine biosynthesis pathway (Fülöp et al. 2007) and the production of

advanced end products of glycosylation (AGEs) (Cooper 2004). Together these negatively affect myocardial energy metabolism, diastolic function and contractility (Lopaschuk et al. 2007). Mitochondrial dysfunction also occurs as a result of the energy switch, causing accumulation of ROS (through the inability of the mitochondria to minimise the superoxide generated) which dysregulates Ca^{2+} homeostasis in cardiomyocytes (Harmancey et al. 2008). One main mechanism whereby the hearts responds to its high fat environment is through FA-mediated activation of the transcription factor PPAR α (fatty acid oxidation regulator) (Harmancey et al. 2008).

1.3.6 Adipokine levels in obesity and cardiac energy metabolism

Obesity causes an increase in leptin and a decrease in adiponectin alongside its effects on the secretion of other adipokines like resistin, which affects multiple signalling pathways as well as FA metabolism in the heart (Hug et al. 2004; Yildiz et al. 2004; Lopaschuk et al. 2007; Blüher 2012). Leptin and adiponectin act on the heart to influence various cellular functions, including alterations in fat metabolism and cell growth (Ahima and Flier 2000; Friedman 2004; Hug et al. 2004; Yildiz et al. 2004; Blüher 2012). Adiponectin knockout mice have been shown to develop greater infarct sizes following ischaemia/reperfusion and the latter is associated with increased TNF- α expression and myocyte apoptosis, suggestive of a cardioprotective role for adiponectin (Shibata et al. 2005). Furthermore, the high levels of leptin seen in obesity are thought to offer protection via oxidation of FAs and interrupting the de novo ceramide synthesis pathway preventing apoptosis in cardiomyocytes (Unger 2005). The protective effect of adiponectin is thought to be via activation of cyclooxygenase-2 (COX-2) in cardiac myocytes and its anti-ischemic effect is exerted via angiogenesis stimulation and high quantity of endothelial NO production (Ouchi et al. 2004).

1.3.7 Obesity, obesity paradox and cardiovascular disease (CVD)

Some overweight and obese patients have shown an improved cardiovascular (CV) outcome in comparison to leaner ones; this phenomenon is known as the “obesity paradox” (OP) (Vecchié et al. 2017; Keller et al. 2018). Ordinarily, high BMI is

connected to high risk of CVD (Kenchiah et al. 2002) but surprisingly obesity paradox studies have also shown that overweight and obese individuals with CVD experience a better outcome than their leaner counterparts (Lavie et al. 2003; Kalantar-Zadeh et al. 2004; Oreopoulos et al. 2010). A study carried out by Horwich et al (2001) showed that 4 groups (n= 1,203) of patients categorised according to their BMI (underweight :n=164, recommended weight :n=692, overweight :n=169 and obese :n=179) who had CVD but did not display any difference in their survival rates and the high rate of death amongst patients in the study was not related to obesity. Indeed, rather surprisingly, the patients who had higher BMI's displayed a trend of improved survival, giving the impression that obesity may be associated with a more favourable outcome (Horwich et al. 2001). Normally CVD has been shown to be a consequence of obesity not related to blood pressure (BP), age, glucose and lipid levels and these effects increase the risk of coronary heart disease (CHD) (Lavie et al. 2014). However, a study carried out by Keller et al (2018) showed that obese patients with MI aged 70 years or older had a decreased death rate following hospital admission compared to their non-obese counterparts, although the obese patients with MI were more intensively treated suggesting that as part of the reason for better survival. Iglesias et al (2009) recorded larger infarct sizes after AMI in obese subjects (compared to lean counterparts) contradicting the obesity paradox. The inconsistencies amongst studies could be due to: 1) the anthropometric parameter used to classify obesity as BMI is not considered the best predictor of CVD though the most common method used to define obesity, 2) the levels of visceral adipose tissue causing different levels of chronic inflammation, or 3) different nutritional status and micronutrient levels of the individual.

1.4 Oxidative stress (OS) in obesity

Obesity can induce oxidative stress by the generation of ROS via different mechanisms. The first of these is via mitochondrial and peroxisomal oxidation of FAs (Fernández-Sánchez et al. 2011; Habib et al. 2015) and nitrotyrosine in the coronary endothelium (Galili et al. 2007). Another being the over-consumption of oxygen, generation of free radicals in the mitochondria during mitochondrial respiration coupled with oxidative phosphorylation (Habib et al. 2015). Many

factors influence the susceptibility to OS and this affects the antioxidant status or free oxygen radical generation. For example, increases in adipose tissue (obesity), cause a dramatic decrease in the activities of the antioxidant enzymes such as glutathione peroxidase (GPx), SOD, and catalase (CAT) in the body (Amirkhizi et al. 2010; Fernández-Sánchez et al. 2011). Lipoprotein oxidation and plasma lipid peroxidation are also markers of OS as free radicals also target lipids (Couillard et al. 2005). It has been reported that obesity may induce systemic OS leading to an irregular production of adipokines, thus contributing to the development of metabolic syndrome (Esposito et al. 2006; Galili et al. 2007). Antioxidant defence markers are negatively correlated with both the amount of body fat and central obesity (Chrysohoou et al. 2007).

1.4.1 Role of Zn^{2+} in oxidative stress

According to Marreiro et al (2017) "Oxidative stress is a metabolic dysfunction that favours the oxidation of biomolecules, contributing to the oxidative damage of cells and tissues" and this leads to the progression of several chronic diseases for example cardiovascular diseases. The function of Zn^{2+} as an antioxidant reduces the effect of oxidative stress and as such it regulates gene expression and gives structural stability to cell membranes (Marreiro et al. 2017). In the cell membrane, Zn^{2+} competes with iron and copper, inhibits the enzyme NADPH-oxidase and decreases hyperglycemia and chronic inflammation (Cruz et al. 2015). Under normal conditions, Zn^{2+} is bound to MT (free radical or ROS scavenger) however, in oxidative stress it is released from metallothionein-zinc complex and redistributed into cells to carry out its antioxidant actions (Özcelik et al. 2012). MT also binds to copper regulating its homeostasis in tissues (Özcelik et al. 2012). In diabetic mice, Zn^{2+} supplementation has been found to induce the expression of cardiac MT preventing diabetic cardiomyopathy (Wang et al. 2006). Increased inflammatory responses (cytokines) have been seen in endothelial cells as a result of Zn^{2+} deficiency and this has been associated with increased cellular oxidative stress whereas Zn^{2+} supplementation gives protection against vascular damage (Marreiro et al. 2017). The relationship between chronic low-grade inflammation and oxidative stress has been explored in the literature. Pro-inflammatory

transcription factors like NF- κ B have been shown to induce the generation of ROS, thus releasing inflammatory cytokines (Marreiro et al. 2017).

Adipokines regulate obesity and adipose tissue secretes adipokines which in turn generate ROS (Habib et al. 2015). The raised inflammatory state and reduced antioxidant capacity in obesity increases the risk of developing atherosclerosis, which subsequently increases the risk of both AMI and stroke (Saper and Rash 2009). Moreover, insufficient dietary intake of Zn^{2+} may increase the risk of inflammation and OS (Bruno et al. 2007) and reduce SOD activity in people who are overweight and obese (García et al. 2009; Costarelli et al. 2010). Zn^{2+} has also been shown to be involved in brain cell apoptosis caused by hypoxia or ischaemia (Wang et al. 2015) and the prevention of apoptosis (Hennig et al. 1996). This difference in Zn^{2+} apoptotic activity could be due to the physiological and pathological conditions of these cells and ultimately the amount of zinc inside in the cell (Franklin and Costello, 2009). Knowing that Zn^{2+} plays a role in protecting against OS, its deficiency may enhance the development of CVD (Beattie and Kwun 2004; Ou et al. 2013).

1.5 Statement of aims and objectives

The overarching aim of this work is to highlight and explore the relationship between dietary Zn^{2+} intake and the development of CVD. To this end, two studies have been carried out:

- A. To understand the impact of a chronic combined suboptimal Zn^{2+} and high fat diet on cardiac antioxidant status (GSH content and Cu/Zn-SOD activity). The effect of the diet on body weight, adipose tissue weight, liver weight, and the ratios of these tissues to body weight was also determined in the study.
- B. To understand the impact of acute severe Zn^{2+} depletion, achieved *in vivo* through dietary intervention or *in vitro* through Zn^{2+} chelation with TPEN, on the extent of injury in the heart following a period of ischaemia/reperfusion on cardiac antioxidant capacity (GSH and Cu/Zn-SOD activity) or induced apoptosis (caspase 3 activation).

2: General methods

2.1 Tissue homogenisation

Heart tissues were homogenised in 10 ml of the appropriate homogenization buffer per gram of tissue using a Teflon homogenizer. Homogenates were centrifuged (4 degrees) at 10,000 g for 15 minutes and the supernatant was removed, divided into aliquots and stored at either -80°C (myocardial Zn²⁺ and Cu/Zn-SOD) or -20°C (GSH and caspase) depending on the assay to be carried out.

Table 2.1: Homogenization buffers used

<i>Myocardial Zn²⁺ and Cu/Zn-SOD assay</i>	<i>Caspase 3 activity assay</i>	<i>GSH assay</i>
Ice cold 20mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer - pH 7.2	Ice cold 25mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer - pH 7.4	Ice cold MES Buffer (0.4M 2-(N-morpholine)ethanesulphonic acid)-pH 6.0
1mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid)	5mM EDTA (Ethylenediaminetetraacetic acid)	2mM EDTA (Ethylenediaminetetraacetic acid)
210mM mannitol	2mM DTT (Dithiothreitol)	0.1M phosphate
70mM sucrose	0.1% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate)	

2.2 Protein determination

The Bradford assay is used to determine the amount of protein in samples tested. In an acidic environment, proteins bind to Coomassie Brilliant Blue G-250 dye causing a rise in the dye's absorbance (470nm to 595nm) and this is read off from spectrophotometer (Bradford 1976).

Tissue homogenates were thawed at room temperature in readiness for protein quantification. Bovine serum albumin (BSA) standards were prepared in PBS over a wide concentrations range (0.01-1.5mg/ml) and mixed thoroughly using a vortex. Ten μL of the buffer (blank), standard or sample was added to an Eppendorf tube and 500 μL of Bradford reagent added and mixed by vortexing. Two hundred μL of each standard and samples were pipetted into duplicate wells of a 96-well plate and incubated for 5 minutes at room temperature. Absorbance was read at 595nm using a BioTek MQX200 UQuant Microplate Reader. A standard curve was created by plotting the concentration of the standard used against the absorbance values corrected by the blank. The concentration of proteins in each sample was calculated by using the equation obtained from the plotted standard curve (linear regression). First of all, absorbance values for the samples were corrected by subtracting the value of the blank

(Average absorbance of blank concentrations (1+2))

After which the equation $y = ax + b$ was used.

Where y is the derived absorbance, a is the slope and x is the concentration.

Therefore, $x = (y - b) / a$.

2.3 Tissue Zinc Assay

Cardiac Zn^{2+} concentrations were determined using a commercial zinc assay kit (Sigma Aldrich – MAK032). The principle behind the assay is that Zn^{2+} ions binds to a ligand resulting in a product which is measured using the BioTek MQX200 UQuant Microplate Reader at 560 nm and this product is proportional to the quantity of Zn^{2+} present. Standards were prepared from assay kit using 0 (blank), 1, 2, 3, 4 and 5 nmole/well of already prepared Zn^{2+} standard. Thawed samples were first subjected to the Bradford assay to quantify protein content and samples were subsequently diluted to a concentration of 0.5mg protein/ml. Samples and standards (0-5 nmol/well). Fifty μL of samples and standards were pipetted into 96-well plates and 200 μL of Zn^{2+} reagent mix (4 parts Zn^{2+} reagent and 1 part Zn^{2+} reagent 2) were added to each well. Samples were mixed well by pipetting, covered with foil to protect from the light and incubated for 10 minutes at room temperature prior to reading absorbance at 560nm.

The amount of Zn^{2+} present in the samples was determined from the standard curve using the following equation:

Concentration of zinc (nmoles/ μL or mM) = Sa/S

Where Sa =Amount of Zn^{2+} in unknown sample (nmole) determined from the standard curve and S =Sample volume (μL) added to reaction well.

2.4 Caspase 3 activity

Myocardial Caspase 3 activity was determined using a commercial kit (Sigma Aldrich – CASP-3-C). Caspase 3 activation is a marker of the induction of apoptosis (programmed cell death). The underlying principle of this assay is to measure the conversion of the Caspase 3 substrate N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-Pna) to p-nitroaniline. Homogenised samples were thawed, protein content quantified using the Bradford assay and sample concentrations subsequently normalised to 0.5mg protein/ml. Caspase 3 substrate (Ac-DEVD-pNA) stock solution (20mM) was prepared by dissolving the already prepared Caspase 3 substrate which is in powder form in 1.2 ml of DMSO to make a stock solution of 2mM. p-Nitroaniline (pNA) standards (10-200 μM) were prepared in assay buffer (50mM HEPES buffer pH 7.4 containing 1.0mM EDTA, 10mM DTT, 0.1% Chaps, 100mM NaCl, 10% glycerol). All standards and samples were added to 96 well plate in duplicate and 10 μL of 2mM Ac-DEVD-pNA subsequently added to wells with samples. Plates were covered in tinfoil and initially incubated for 90 minutes at 37°C however because the signal was low, the plate was left and further incubated for up to 24 hours (as per the assay instructions for samples with low signals), and absorbance read at 405nm (t-value of 1,440). Caspase 3 activity was calculated in μmol pNA released per minute per ml of tissue lysate/supernatant based on the formula:

$$\text{Activity; } \mu\text{mol pNA/minute/ml} = \frac{\mu\text{mol pNA} \times d}{t \times v}$$

Where;

v - Volume of sample in ml, d - Dilution factor, t - Reaction time in minutes (i.e. 1,440)

The activity in $\mu\text{mol/minute/ml}$ converted and expressed as picomoles of the product (pNA) per minute per mg of protein.

2.5 Glutathione (GSH) Assay

Total GSH content was determined using a Cayman chemical Glutathione Assay Kit (catalogue number: 703002). This assay employs an enzymatic recycling technique. In this technique, Glutathione reductase is used for quantifying the GSH. GSH's sulfhydryl group reacts with DTNB (5,5'-dithio-bis-2-(nitrobenzoic acid) producing TNB (5-thio-2-nitrobenzoic acid) which is yellow in colour. GSTNB (mixed disulphide: GSH and TNB) produced is further reduced by glutathione reductase thus recycling the GSH and producing more TNB. TBN production rate is directly proportional to the recycling reaction and also to the sample's GSH concentration. Absorbance is measured using a BioTek MQX200 UQuant Microplate Reader at 405nm.

Homogenised samples were, thawed and protein content quantified (Bradford assay) and normalised to 0.5mg/ml for all samples. Samples were then deproteinated to reduce protein content to a minimum in order to avoid particle interferences as well as sulfhydryl groups interference with proteins present in the assay.

To deproteinate, equal volumes of both samples and MPA (metaphosphoric acid) reagent (5g in 50 ml of water) were added together and mixed by vortexing. The mixture was left to stand at room temperature for 5 minutes, then centrifuged at 2000g for 2 minutes. Thereafter, the pH was adjusted by adding 50µl of TEAM Reagent (4M triethanolamine solution) to 1 ml of the deproteinated samples and vortexed immediately. The Assay Cocktail was prepared by mixing MES Buffer (11.25ml), reconstituted Cofactor Mixture (0.45ml), reconstituted with enzyme Mixture (2.1ml), water (2.3ml) and reconstituted DTNB (0.45ml) in a 20ml vial. GSSG standards (under these assay conditions 1 mole of GSSG is reduced by glutathione reductase to produce 2 moles of GSH) were prepared in the concentration range of 0–8µM (equivalent to 0-16µM of GSH). Using a 96 well plate, 150µl of the prepared assay cocktail was added to each well containing both standards and the samples, the plate covered with tinfoil, and incubated on an orbital shaker for 25 minutes at room temperature before the absorbance was read at 405nm using a microplate reader.

$$\text{Total GSH} = \frac{[\text{Absorbance at 405nm} - (\text{y-intercept})]}{\text{slope}} \times 2 \times \text{Sample dilution}$$

2.6 Cu/Zn-SOD activity

A commercial superoxide dismutase activity kit (Cayman Chemical catalogue number: 706002) was used to carry out this assay. The principle behind this assay is superoxide radicals being detected by xanthine oxidase and hypoxanthine using triazolium salts. A SOD unit is said to be the enzyme amount which is required to exhibit 50% superoxide radical dismutation. Although the assay kit is not specific to Cu/Zn-SOD, to specifically measure the Cu/Zn-SOD activity the samples were homogenized then subjected to three steps of centrifugation (10,000g for 15 minutes at 4°C) to separate mitochondrial SOD from cytosolic SOD, whereby the cytosolic SOD is retained in the supernatant. The Cu/Zn-SOD-containing supernatant was then stored at -20°C for subsequent analysis.

Samples were thawed and protein content quantified and normalised to 0.5mg/ml for all samples. 10µl of samples and standards (0 - 0.05U/ml I SOD Activity) were put into 96 well plates and 200µl of dilute radical detector was added. Thereafter, 20 µl of xanthine oxidase was added to the plate which was then covered with foil to protect from light and incubated for 30 minutes on a shaker at room temperature prior to reading absorbance at 460nm.

$$\text{SOD (U/ml)} = \frac{[(\text{sample LR} - \text{y-intercept}) \times 0.23]}{\text{Slope} \times 0.01} \times \text{sample dilution}$$

LR = Linearized rate

3: Study 1 Impact of high fat diet (HFD) on cardiac antioxidant levels in wildtype (WT) mice

3.1 Introduction:

In an animal experimental setting, low Zn^{2+} status, combined with consumption of a diet high in cholesterol and saturated fat, has been shown to promote the development of atherosclerosis (Beattie et al. 2012). In that study, both wildtype (WT) and apolipoprotein E knockout ($\text{ApoE}^{-/-}$) mice were given a HFD with varying levels of Zn^{2+} for 6 months to determine the effects of suboptimal (3 and 8 mg/kg) or optimal (35 mg/kg) Zn^{2+} levels on the development of vascular inflammation and arterial plaque development (Beattie et al. 2012). The animals were studied over a 6-month period with results showing that suboptimal dietary Zn^{2+} intervention promoted inflammation of the vessels and increased plaque formation and development of the lesion (atherosclerosis) implicating a relationship between high fat diet and Zn^{2+} deficiency in CVD.

The present study looked at the impact of suboptimal Zn^{2+} status alongside a high fat diet on cardiac antioxidant status. The tissues for this work were obtained from a bio-bank generated as part of the study of Beattie et al (2012) which included three sub-optimal dietary zinc levels of 3, 5 and 8mg/kg. All body, liver and white adipose tissue weights used for the present study were provided by Prof. John Beattie's team. For the tissue analyses the 5mg/kg Zn^{2+} group was used as there was sufficient tissue to obtain satisfactory sample sizes, while the other sub-optimal zinc groups had very few tissue samples available.

3.1.1 Aim:

In light of the relationship between dietary Zn^{2+} intake and development of CVD, which can ultimately lead to the development of AMI and subsequent myocardial injury, the primary aim of this study was to understand the impact of a combined suboptimal Zn^{2+} and high fat diet on cardiac antioxidant status. To address this question, heart tissue from WT mice fed high fat diet for 15 and 25 weeks, stored in a biobank generated by the above study by Beattie et al (2012), was tested for Cu/Zn-SOD activity and GSH content. The present study also determined the effect of this dietary intervention on body weight and both adipose tissue and liver weight to body weight ratios.

3.2 Methods:

The tissues used in this project were obtained from an animal study carried out by Beattie et al (2012). In this study, 5 weeks old male C57BL/6 mice were fed custom made semi-synthetic diet by Harlan-Teklad (Indianapolis, IN, USA) containing high saturated fat (21% w/w) and high cholesterol (0.15%) with either 35 mg/kg Zn²⁺ (zinc adequate; n=10-15) or 5mg/kg Zn²⁺ (suboptimal zinc; n=10) for 15 or 25 weeks (chronic dietary intervention). The composition of these diets is detailed in Table 3.1. Animals were euthanised following either 15 or 25 weeks of dietary intervention. Mice were anaesthetised with sodium pentobarbital (1g/kg body weight; Euthatal; Merial Animal Health, Harlow, UK) via intraperitoneal injection and exsanguinated under terminal anaesthesia. Total GSH content and Cu/Zn-SOD activity were measured in heart samples from mice using commercially available assay kits, as detailed in Chapter 2. Details of body weight, WAT and liver weights were also provided to determine WAT and liver to body weight ratios.

Table 3.1: basic composition of the high-fat, high-cholesterol, western-type diet by Harlan-Teklad (Indianapolis, IN, USA) (adapted from Beattie et al. 2012).

Components	Dietary Zn (5 and 35 mg/kg) grams
Egg white solids, spray-dried	212
Corn starch	75
Maltodextrin	75
Cellulose	46.2
Anhydrous milkfat	210
Cholesterol	1.5
Mineral mix, Zn deficient (81264)	25.7
Calcium Phosphate, dibasic	3.0
Chromium Potassium Sulfate, dodecahydrate	0.02
Vitamin mix (40060)	10.0
Biotin	0.004
Ethoxyquin, antioxidant	0.04

Diet was supplemented with 1% ZnSO₄·7H₂O in sucrose and was adjusted to achieve a Zn²⁺ content for the 5 mg/kg and 35 mg/kg. Diets were also supplemented with sucrose which was adjusted to maintain levels at 341.5 mg/kg.'

Statistical analysis

For all datasets, values are expressed as mean ± s.e.m of n animals/tissue samples. Differences between groups were determined by Two way Analysis of Variance with Tukey's post-hoc test to allow comparison between all experimental groups. Statistical significance was accepted when P<0.05.

3.3 Results:

3.3.1 The impact of suboptimal dietary Zn²⁺ on body weight in mice subjected to either 15 or 25 weeks of high fat feeding.

Chronic exposure to suboptimal (5 mg/kg) dietary Zn²⁺ levels and high fat diet in mice did not significantly influence body weight (BW) compared with time-matched mice fed a Zn²⁺ adequate – (35 mg/kg) diet (Figure 3.1).

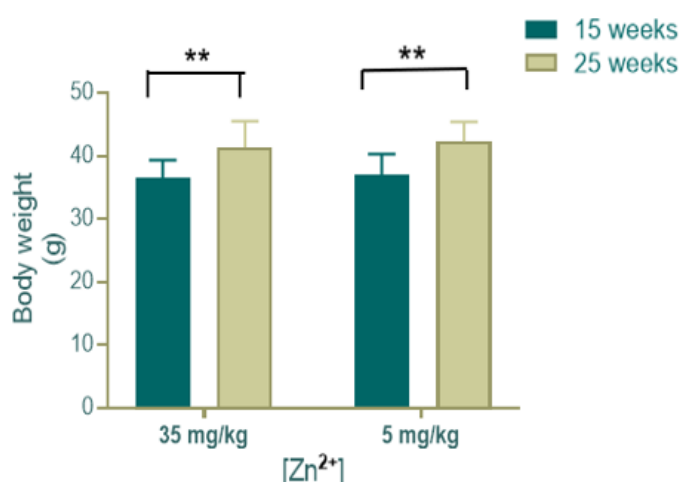


Figure 3.1: The impact of suboptimal Zn²⁺ status on body weight changes in mice subjected to either 15 or 25 weeks of high fat feeding with altered dietary Zn²⁺ i.e. adequate (35 mg/kg) Zn²⁺ and suboptimal (5 mg/kg), respectively. All data are expressed as mean ± SEM. Statistical significance was determined using a two-way ANOVA and Tukey's post-hoc test **indicates P=0.01, n:10-15.

3.3.2 The impact of suboptimal dietary Zn^{2+} status on white adipose tissue (WAT) weight in mice subjected to either 15 or 25 weeks of high fat feeding

Chronic exposure to suboptimal (5mg) dietary Zn^{2+} levels and high fat diet did not significantly influence WAT weights between either the level of Zn^{2+} diet (5 and 35 mg/kg diet) or duration of diet intervention (15 and 25 weeks; Figure 3.2).

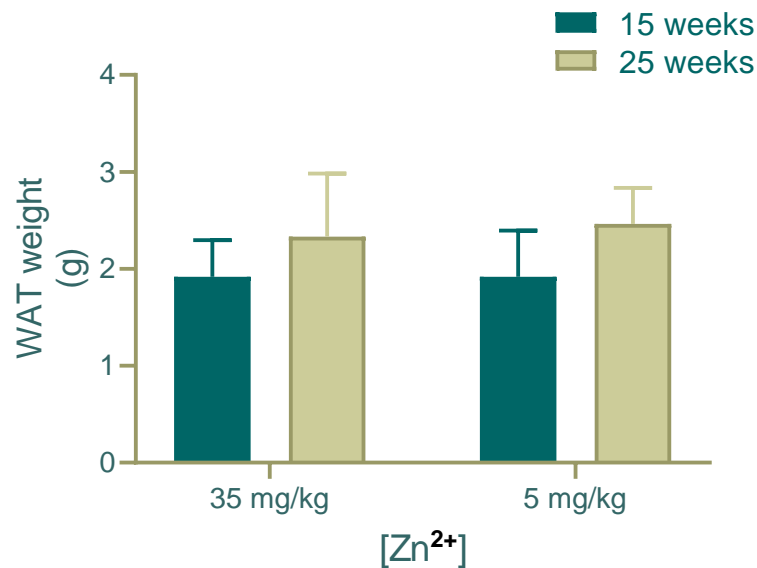


Figure 3.2: The impact of suboptimal Zn^{2+} status on white adipose tissue (WAT) in mice subjected to either 15 or 25 weeks of high fat feeding with altered dietary Zn^{2+} i.e. adequate (35 mg/kg) Zn^{2+} and suboptimal (5 mg/kg), respectively. All data are expressed as mean \pm SEM. Statistical significance was determined using a two-way ANOVA and Tukey's post-hoc test, n:10-15.

3.3.3 The impact of suboptimal dietary Zn^{2+} status on WAT:BW ratio in mice subjected to either 15 or 25 weeks of high fat feeding

Chronic exposure to suboptimal (5 mg/kg) dietary Zn^{2+} levels and high fat diet in mice did not significantly alter the animal white adipose tissue and body weight ratio between either the level of Zn^{2+} diet (5 and 35 mg/kg) or duration of diet intervention (15 and 25 weeks: Figure 3.3).

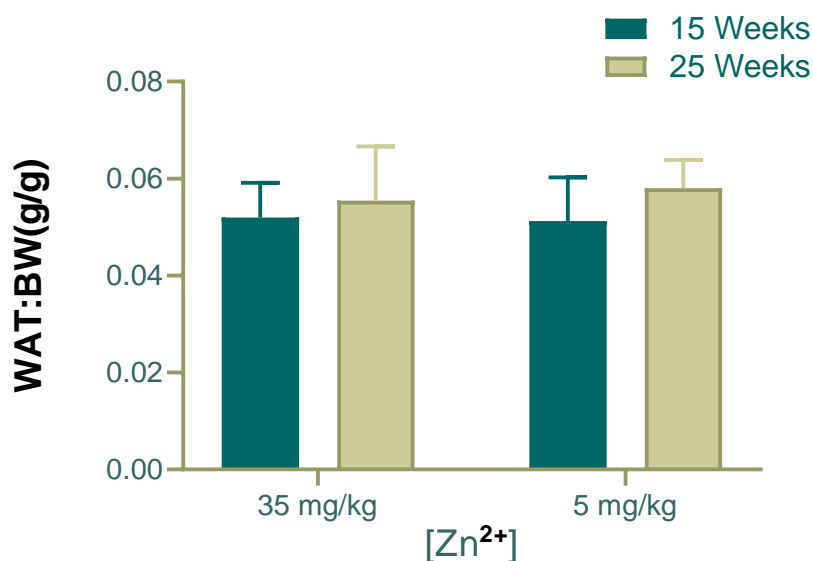


Figure 3.3: The impact of suboptimal Zn²⁺ status on white adipose tissue body weight ratio in mice subjected to either 15 or 25 weeks of high fat feeding with altered dietary Zn²⁺ i.e. adequate (35 mg/kg) Zn²⁺ and suboptimal (5 mg/kg), respectively. All data are expressed as mean \pm SEM. Statistical significance was determined using a two-way ANOVA and Tukey's post-hoc test, n=10-15.

3.3.4 The impact of suboptimal dietary Zn²⁺ status on liver weight (LW) and LW:BW ratio in mice subjected to either 15 or 25 weeks of high fat feeding.

Chronic exposure to suboptimal (5 mg/kg) dietary Zn²⁺ levels and high fat diet for 25 weeks increased liver weights compared with the Zn²⁺ adequate (35mg/kg) fed and time-matched groups ($P < 0.01$; Figure 3.4). Whereas in the 15 weeks animal groups, no difference in LW was observed for either exposure to suboptimal (5 mg/kg) dietary Zn²⁺ or Zn²⁺ adequate (35 mg/kg).

Furthermore, LW:BW ratio (Figure 3.5) showed no significant differences between the suboptimal Zn²⁺ groups and the controls following 15 weeks of dietary intervention (Figure 3.5). However, LW:BW ratio was significantly increased in animals fed a high fat diet with suboptimal dietary Zn²⁺ for 25 weeks compared to mice on an equivalent diet for 15 weeks or control animals on a Zn²⁺ adequate diet for 25 weeks (Figure 3.5).

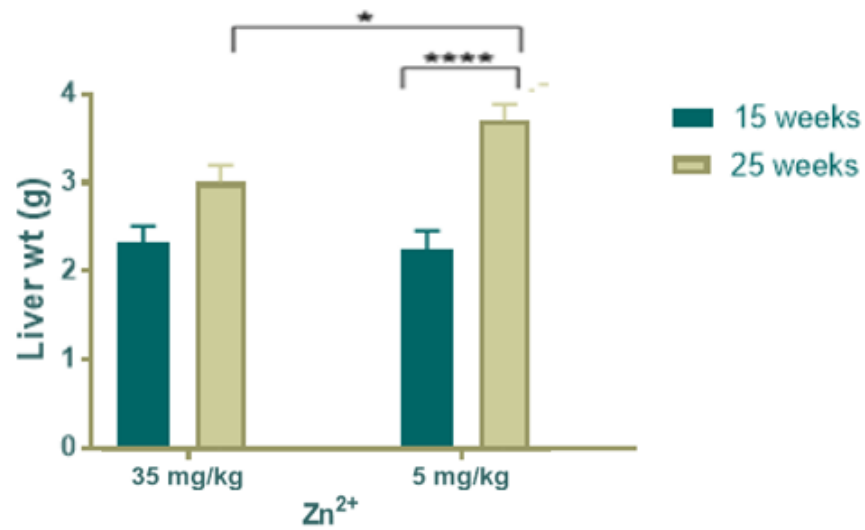


Figure 3.4: The impact of suboptimal Zn²⁺ status on liver weight in mice subjected to either 15 or 25 weeks of high fat feeding with altered dietary Zn²⁺ i.e. adequate (35 mg/kg) Zn²⁺ and suboptimal (5 mg/kg), respectively. All data are expressed as mean \pm SEM. For this study, statistical significance was determined using a two-way ANOVA and Tukey's post-hoc test, $P < 0.01$, $n = 5-15$.

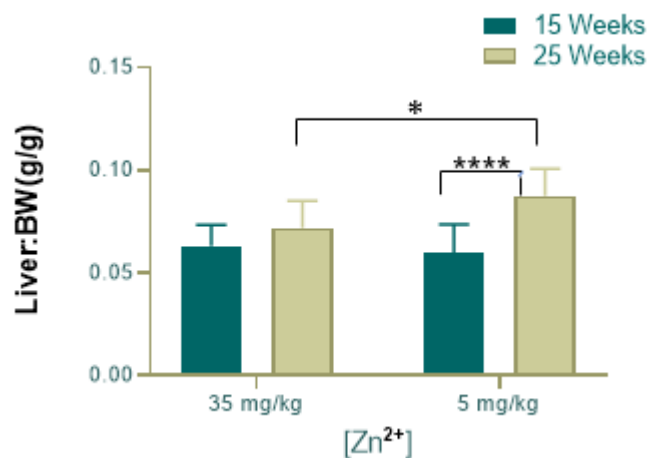


Figure 3.5: The impact of suboptimal Zn²⁺ status on liver/body weight ratio in mice subjected to either 15 or 25 weeks of high fat feeding with altered dietary Zn²⁺ i.e. adequate (35 mg/kg) Zn²⁺ and suboptimal (5 mg/kg), respectively. All data are expressed as mean \pm SEM. For this study, statistical significance was determined using a two-way ANOVA and Tukey's post-hoc test. There was statistical significance comparing 5 mg/kg Zn²⁺ at 15 and 25 weeks ($P < 0.0001$) and at 25 weeks both 5 and 35 mg/kg Zn²⁺ diet ($P < 0.05$) $n = 10-15$.

3.3.5 The impact chronic feeding of a combined suboptimal Zn²⁺ and high fat diet on myocardial GSH content.

Chronic exposure to suboptimal (5 mg/kg) dietary Zn²⁺ levels and high fat diet did not significantly alter cardiac levels of total GSH compared with controls (Zn²⁺ adequate – 35 mg/kg) at either 15 or 25 weeks (Figure 3.6). However, levels of total GSH were significantly reduced ($P < 0.01$) by approximately 50% in the Zn²⁺ adequate group (35 mg/kg) at 25 weeks compared with 15 weeks (Figure 3.6).

GSH levels in the 25 week suboptimal Zn²⁺ group were similarly lower than the matching 15-week group, although this did not reach statistical significance ($P = 0.11$; Figure 3.6).

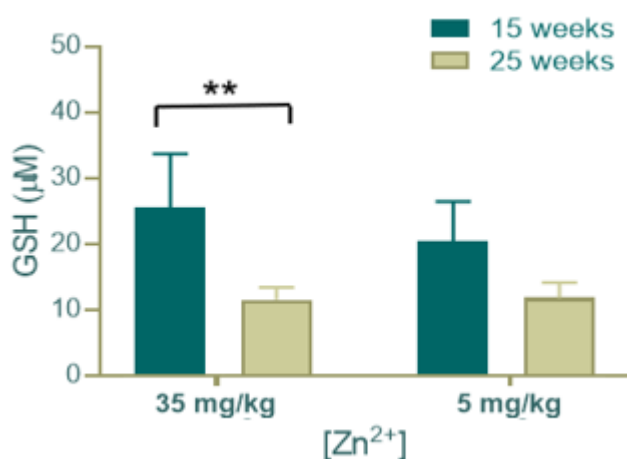


Figure 3.6: The impact of suboptimal Zn²⁺ status on cardiac GSH following high fat feeding with altered dietary Zn²⁺ levels (i.e. adequate (35 mg/kg) and suboptimal (5 mg/kg) Zn²⁺) for either 15 or 25 weeks. All data are expressed as mean \pm SEM. For this study, statistical significance was determined using a two-way ANOVA and Tukey's post-hoc test **indicates $P < 0.01$, $n = 3-6$.

3.3.6 The impact of chronic feeding of a combined suboptimal Zn²⁺ and high fat diet on myocardial Cu/Zn-SOD activity.

Chronic exposure to suboptimal (5 mg/kg) dietary Zn²⁺ levels and high fat diet in mice did not significantly alter cardiac Cu/Zn-SOD activity compared with controls (Zn²⁺ adequate – 35 mg/kg; Figure 3.7). However, when comparing Cu/Zn-SOD activity between the two time points (15 and 25 weeks), an increase in Cu/Zn-SOD activity was observed in the 25 week, compared to the 15 week, groups ($P < 0.01$) in both the zinc-adequate and sub-optimal Zn²⁺ animals (Figure 3.7).

Overall, there was an approximate five-fold increase in Cu/Zn-SOD activity between both groups at 15 and 25 weeks of dietary intake (Figure 3.7).

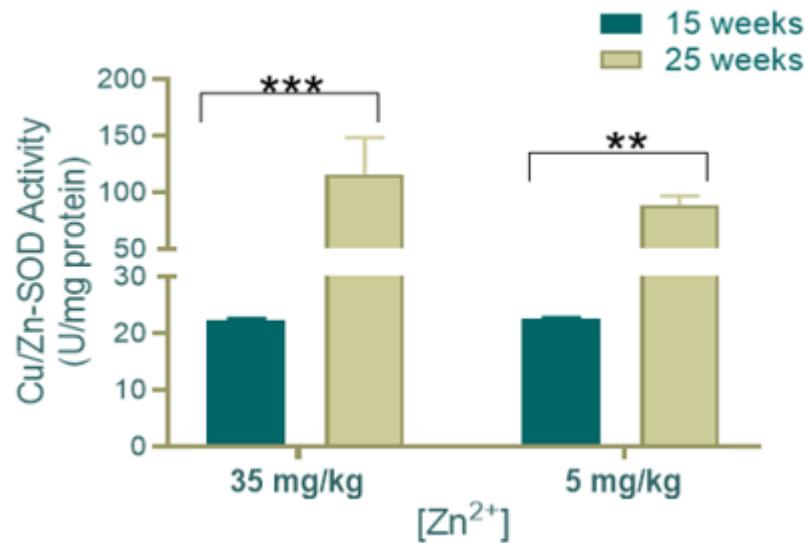


Figure 3.7: The impact of suboptimal Zn²⁺ deficiency status on cardiac SOD following high fat feeding with altered dietary Zn²⁺ levels (adequate (35 mg/kg) and suboptimal (5 mg/kg)) for either 15 or 25 weeks. All data are expressed as mean \pm SEM. Statistical significance was determined using a two-way ANOVA and Tukey's post-hoc test ***indicates $P < 0.01$, ** indicates $P < 0.01$, $n = 3-6$.

3.4 Discussion:

The primary aim of this study was to understand the impact of a combined suboptimal Zn²⁺ and high fat diet on cardiac antioxidant status. The key findings from this study were that suboptimal intake of dietary Zn²⁺ *per se* has no effect on either total GSH content or Cu/Zn-SOD activity in cardiac tissue. However, the duration of high fat feeding does appear to differentially influence both GSH levels and Cu/Zn-SOD activity. Moreover, altered dietary Zn²⁺ levels do not significantly affect body weight or the amount of white adipose tissue of the animals after 25 weeks of high fat feeding, while suboptimal Zn²⁺ was associated with increased liver weights (compared to normal dietary Zn²⁺) after 25 weeks of a high fat diet.

3.4.1 The impact of duration of dietary intervention on antioxidant biomarkers (GSH and Cu/Zn-SOD) in the heart.

While the results of this study have shown that, in the presence of high fat feeding, suboptimal Zn^{2+} does not influence either the amount of GSH or Cu/Zn-SOD activity in the heart, an extended duration of the dietary intervention (25 vs 15 weeks) was observed to induce changes (decreased GSH content and increased Cu/Zn-SOD activity) in both the dietary Zn^{2+} deficient and Zn^{2+} adequate groups. These changes could be due to the duration of the high fat diet, to the age of the animal, or a combination of the two.

Ageing is associated with an increase in free radical generation, which causes oxidative damage that affects the integrity and functions of cells (Kim et al. 2002; Cao et al. 2004). The present study has shown that myocardial levels of GSH in both suboptimal and Zn^{2+} adequate animals (5 mg/kg and 35 mg/kg) at 25 weeks of feeding were found to be lower than that of the 15 weeks dietary intervention groups. This finding is consistent with a study by Kim et al (2002) which measured the levels of serum GSH from male Fischer 344 rats aged 13 (young) and 31 (old) months and demonstrated the presence of low levels of serum GSH in old rats compared to young rats, suggesting that aging increases oxidative stress. Cao et al (2004) carried out a study on 5 and 24-month-old Sprague-Dawley rats to establish a functional link between excess oxidative stress and age-related decline in steroidogenesis. This study showed that among the non-enzymatic antioxidants, the level of GSH was drastically reduced during aging, as well as the catalytic activity of Cu/Zn-SOD in testicular Leydig cells of old rats (Cao et al. 2004). GSH levels have also been shown to reduce with age in the brain of rats (aged 2, 10 and 20-months) (Samarghandian et al. 2015), while Jung and Henke (1996), studied developmental changes in antioxidants biomarkers in the liver and kidney of male Wistar rats of different ages (1, 2, 3, 6, and 12 months) and observed that there was a continuous decline of hepatic GSH content in older rats. Taken together these findings are consistent with the present observation that age negatively affects the GSH content of the heart.

Zn^{2+} is essential for the activity of SOD1 (Cu/Zn-SOD), which is a highly efficient antioxidants that prevents the occurrence of free radical induced injury (Huang et

al. 2018). Similar to GSH, Cu/Zn-SOD activity was not affected by chronic exposure to suboptimal Zn^{2+} for either 15- or 25-weeks. This is in contrast to findings reported in the literature where Zn^{2+} deficiency has been shown to result in significantly reduced Cu/Zn-SOD activity in cardiac tissue (Pucneau et al. 1995; Barandier et al. 1999) and plasma (Huang et al. 2018). Interestingly, and in contrast to the effects on GSH, aging was found to increase Cu/Zn-SOD activity in cardiac tissue in the present study. Again, this is not in alignment with previous reports (albeit in different tissues), since Gomes et al. 2009 showed an increase in the expression of the SOD isoforms 2 and 3, but no change in SOD1 (Cu/Zn-SOD) levels, in the renal cortex of old (52-week-old) versus young (13-week-old) rats.

3.4.2 Impact of high fat diet on antioxidant systems (GSH and Cu/Zn-SOD).

A key promoter of obesity and cardiovascular disease is high fat diet (HFD), which itself has been shown to cause an increase in ROS production and induce alterations in the oxidative stress status of the vascular system (Reil et al. 1999; Sweazea et al. 2010; Mayyas et al. 2017). Moreover, increased ROS production can lead to a decrease in GSH antioxidant levels (Birben et al. 2012). Studies have shown that HFD differentially impacts on antioxidant defences. For example, a short-term (10 weeks) dietary intervention study to determine the effects of different diets, where the control group had 5% fat, 0.5% NaCl, the HFD group were given 25% fat, 0.5% NaCl and the high salt group given 5% fat, 8% NaCl, on myocardial oxidative stress status in young (6–10 weeks old) Sprague Dawley rats showed no changes in the levels of GSH after a 10 week dietary intervention period (Mayyas et al. 2017). Another study in four-month-old female obesity prone mouse strains (C57BL/6J, DBA/2J and AKR/J) fed a HFD similarly showed no changes in GSH levels in the liver after 10 weeks (short term) feeding (Norris et al. 2016). Due to the lack of normal fed controls in the present study, the individual effects of increasing fat content in diet alone on antioxidant status in the heart cannot be assessed. In contrast, in a 16 week study of HFD-induced obesity in white albino rats, examination of oxidative stress markers in tissues (liver, heart and kidney) found that, compared to animals fed normal chow, GSH levels were significantly decreased in both liver and renal tissues, while in heart tissue GSH was significantly increased (Noeman et al. 2011). While these results do not follow

the trend of the current study, none of the studies above looked at prolonged HFD diet intervention, and so it is difficult for a direct comparison with the present study. Although GSH content reduced with time in the present study (high fat fed mice) their comparison to levels in normal chow mice over the same period is unknown. However, the fact that most studies show that diet does not appear to alter GSH levels (compared to normal fed animals) it is perhaps unlikely to that the lower levels seen with a longer diet duration in the present study is due to diet duration, rather most probably an age-related effect.

The present study showed that mice subjected to prolonged (25 weeks) high fat feeding had significantly higher cardiac levels of Cu/Zn-SOD activity than mice fed HFD for 15 weeks. Very few studies have determined the impact of HFD on Cu/Zn-SOD activity, however a study in rats that assessed the impact of a 36-week HFD on skeletal muscle gene expression and mitochondrial function, showed that HFD altered transcript levels of 18 genes: HFD increased the expression of genes involved in stress response but reduced expression of free-radical scavenger enzymes including Cu/Zn-SOD, resulting in reduced DNA repair/metabolism (increased DNA damage) (Sreekumar et al. 2002).

3.4.3 Influence of suboptimal dietary Zn²⁺ status on body, adipose and liver tissues weights in older mice.

In general, obese individuals have high body weights typically because they have higher amounts of body fat, although changes in body weight can correspond to changes in body water, fat, and/or lean tissue content. The data from this animal study showed no correlation between body and WAT weight and dietary Zn²⁺ status. This is consistent with a study conducted by Tallman and Taylor (2003) that investigated the effects of dietary fat and Zn²⁺ on adiposity in C57BL/6J mice and showed that, after 16 weeks of dietary treatment, body weights were similar, irrespective of dietary zinc or dietary fat content. It has been proposed that over a long time (aging animals), the animal becomes better suited to a condition of low Zn²⁺ intake (Tallman and Taylor 2003) which could explain the current finding that there is no difference in between the 5 mg/kg and the 35 mg/kg dietary Zn²⁺ groups at 25 weeks.

In contrast, liver weights in the sub-optimal Zn^{2+} group, at 25 weeks, were higher than the weight of livers from mice fed the a sub-optimal Zn diet for 15 weeks, or a Zn^{2+} adequate diet for either 15 or 25 weeks. This implies that prolonged suboptimal Zn^{2+} results in changes at the level of the liver, possibly through increased fat accumulation in the liver. Although this was not assessed in the present study, this could be measured either by Oil Red-O staining and histological analysis or by measuring lipid content. While few studies have looked at the effects of Zn deficiency on liver metabolism, Reeves and O'Dell, 1983 carried out a study using Male Wistar rats to demonstrate the effect of Zn deficiency on glucose metabolism in meal-fed rats (a low-Zn diet: $>1\text{mg/kg}$ Zn and an adequate-Zn diet; 100mg/kg), and rats fed *ad libitum* on either a low-Zn ($>1\text{mg/kg}$) diet or an adequate-Zn diet (100 mg/kg) for 3 weeks. The results showed that Zn deficiency caused a slight, but significant, decrease in glucose incorporation into liver fatty acids and a significant increase in glucose incorporation into liver glycogen of meal-fed rats compared to meal-fed Zn adequate rats. Moreover, liver size and the fatty acid pool size in the livers of the Zn-deficient, rats was lower than that of the zinc-adequate controls, which the authors proposed resulted from an increased rate of turnover of fatty acids by the liver of the Zn-deficient rats. Although this study showed that the liver was a major site of adaptation to Zn^{2+} deficiency, when expressed per unit tissue the response was not as large as that found in adipose tissue, presumably due differences in the effect of insulin on glucose uptake and metabolism in these tissues.

3.4.4 Limitations of the Study and Further Work

The absence of a group of animals fed a normal fat diet in the present study makes it difficult to conclude whether prolonged feeding with high fat diet causes changes in antioxidant biomarkers' levels in the heart. However, dietary intervention studies which compared normal fed and high fat fed groups have shown that there is no effect of high fat diet on cardiac antioxidants (GSH and Cu/Zn-SOD) following short term (10 weeks) feeding (Norris et al. 2016; Mayyas et al. 2017). Although the present study compared HFD groups of animals on short term (15 weeks) and long term (25 weeks) feeding, the results observed for the short-term dietary

intervention arm of this study are in line with the literature. The trends in the 25 week diet groups are comparable to those in the literature (Sreekumar et al. 2002) hence this is suggestive that high fat feeding induces a gradual decline of antioxidant capacity over time. Although literature implies that the effects seen on anti-oxidant status are due to ageing, to test this in future, studies performed with different groups of age-matched animals receiving normal or high fat diet for either short or long term intervention would allow determination of the impact of both age (within diets) and duration of HFD diet feeding (differences between diets).

3.5 Conclusion

In conclusion, sub-optimal Zn^{2+} had no effect on two cardiac biomarkers of antioxidant status, and there have been no previous studies to this effect. However, the changes seen in the cardiac biomarkers of antioxidant status measured are likely to be due to ageing rather than duration of diet, and again this is the first time this effect has been demonstrated in cardiac tissue.

4: Study 2: Impact of acute severe dietary and invitro (TPEN) Zn²⁺ depletion on cardiac antioxidant levels in rat hearts following myocardial I/R

4.1 Introduction:

In post MI patients, cardiac and plasma Zn^{2+} levels have been reported to decline within the first 3 days (mean fall = 76-83 $\mu\text{g}/100\text{ ml}$) after infarction and then return to normal (normal range=90-118 $\mu\text{g}/100\text{ ml}$.) levels by the tenth day of disease (Low and Ikram, 1976) which may suggest that the loss of Zn^{2+} contributes, at least in part, to cardiac injury. Cardiac GSH levels increased, while malondialdehyde (marker of tissue injury) levels in the heart decreased following Zn^{2+} supplementation before I/R in rats (Ozyildirim et al. 2017). Following acute myocardial I/R, Zn^{2+} plays several protective roles, viz protection following cardiomyocyte acute redox stress; wound healing, avoidance of myocardial damage triggered inflammatory processes and cardiac stem cell maintenance (Lee et al. 2015). *In vivo*, Zn^{2+} deficiency is linked to a more pronounced pro-inflammatory status (Choi et al. 2018). On the contrary, *in vitro* studies show that cellular Zn^{2+} depletion occurs as a result of a decreased function of the endothelial cell barrier (Hennig et al. 1992). However, no work was present in the literature that explored the underlying effects of acute dietary Zn^{2+} deficiency and its impact on an I/R insult in comparison with acute TPEN-induced Zn^{2+} deficiency. As a consequence, a series of experiments was conducted in my supervisor's laboratory to address this gap; this piece of work was a part of that study. The experiments were carried out in a rat animal model and this piece of work was recently published (Skene et al. 2019) alongside other experiments (all done in my supervisor's laboratory).

4.1.1 Aim:

A project in my supervisor's laboratory aimed to determine the impact of severe Zn^{2+} depletion, achieved *in vivo* through dietary intervention or *in vitro* through Zn^{2+} chelation with N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN), on the extent of injury to the heart following a period of I/R. The present study aimed more specifically to determine whether these interventions influence cardiac antioxidant capacity (GSH levels and Cu/Zn-SOD activity) or induced apoptosis (Caspase 3 activation) in the heart.

4.2 Methods:

Male Sprague–Dawley rats (200–250g; approximately 8 weeks old) were purchased from Harlan UK and allocated to one of three groups (n=10 per group) which were

subjected to two-week dietary intervention periods: Zinc adequate (ZA) diet (35 mg/kg Zn^{2+}) fed ad libitum, Zinc deficient (ZD) diet (<1 mg/kg Zn^{2+}), ZA Pair-fed (PF) controls. The PF controls were included to ensure that any changes seen were not simply due to reduced weight gain that results from severe Zn^{2+} depletion. Animals were anaesthetised via intraperitoneal injection of pentobarbital sodium salt (100 mg/kg body weight; Sigma Aldrich), blood collected via cardiac puncture, the heart quickly removed and arrested in ice cold Kerbs Henseleit buffer (KHB; 25 mm NaHCO_3 , 1.18 mm KH_2PO_4 , 2.41 mm MgSO_4 , 2.52 mm CaCl_2 , 119 mm NaCl , 4.7 mm KCl and 10.88 mm glucose; pH 7.4) prior to mounting on a Langendorff-perfusion system. Isolated heart studies were carried out in which regional ischaemia was induced by placing and tightening a ligature around the left coronary artery (LCA) for 30 minutes, followed by a 2h reperfusion period (release of the ligature). A group of sham hearts were also employed, whereby they were subjected to the same period of perfusion (150 minutes) without the induction of ischaemia or reperfusion.

To compare the effects of *in vivo* dietary Zn restriction with *in vitro* Zn depletion, two further groups of hearts from normal-fed rats were used. To induce Zn depletion 10 μM of the Zn^{2+} chelator TPEN was added to the perfusion medium (infusion rate of 100 μl /minutes through the aortic cannula) from 10 minutes before the induction of ischaemia / occlusion and maintained until the end of the experiment.

Upon completion of the ischaemia/reperfusion protocol, ventricular tissue was snap frozen in liquid nitrogen for biochemical analysis of myocardial Zn^{2+} content, caspase activity, total GSH content and Cu/Zn-SOD activity using commercially available assay kits as described in Chapter 2. The isolated heart experiments were conducted by Dr Karen Skene in my supervisor's laboratory.

Statistical analysis

A one-way analysis of variance (ANOVA) with Dunnett's post-hoc test was used to perform comparisons using one group as a control. For the dietary intervention study, the PF group was the control, while for the *in vitro* Zn^{2+} depletion study the vehicle group served as control.

4.3 Results:

4.3.1 The impact of acute dietary Zn²⁺ deficiency and myocardial I/R on both Zn²⁺ content and Caspase 3 activity in rat hearts.

Acute dietary Zn²⁺ deficiency was not associated with any variation in myocardial tissue Zn²⁺ levels across all groups (ZA, PF, ZD) (Figure 4.1A), whereas, the PF rats (control group) in comparison to the ZD group displayed a significant rise in cardiac caspase-3 activity (Figure 4.1B). Therefore, the ZD rats had lower caspase levels than the PF rats. However, the ZA rats also had lower caspase activity levels, suggesting that this is a characteristic of pair feeding.

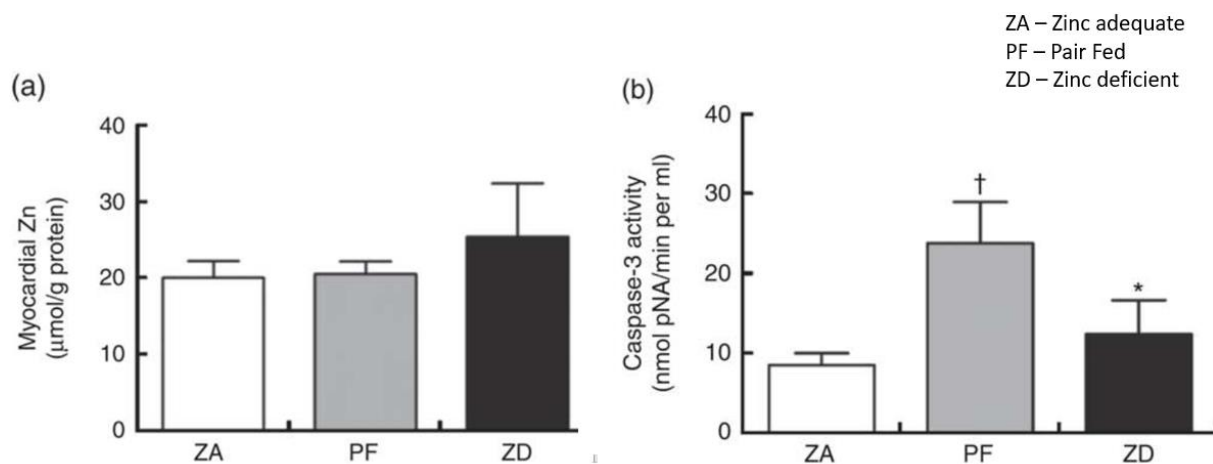


Figure 4.1: The impact of acute Zn²⁺ deficiency and myocardial I/R on myocardial Zn²⁺ content (A) and Caspase 3 activity (B). Data are expressed as mean ± SEM and significance determined as P<0.05 using a one-way ANOVA and Dunnett's post-hoc test; n=3-7. *P<0.05 ZD compared with pair-fed (PF) controls. † P<0.05 PF compared with zinc-adequate (ZA) controls.

4.3.2 The impact of acute dietary Zn²⁺ deficiency on GSH content in hearts subjected to myocardial I/R.

In rat cardiac tissue there was a statistically significant reduction in the GSH content levels in the ZD group compared to the other 2 groups (ZA, PF) which seemed not to be affected by the feeding (Figure 4.2). The difference between the PF and ZD hearts was due to the Zn²⁺ deficiency. Similarly, there is no difference between the PF and ZA hearts suggesting that dietary restriction does not affect GSH levels.

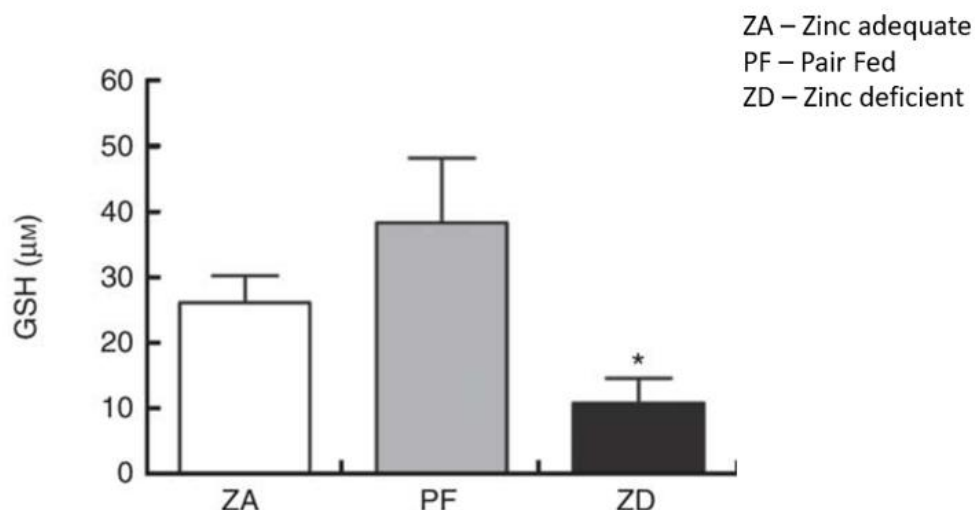


Figure 4.2: The impact of acute Zn^{2+} deficiency on GSH content in rat hearts subjected to 30 minutes regional ischaemia followed by a 2hr reperfusion period. All data are expressed as mean \pm SEM. Statistical significance was determined using one-way ANOVA and Dunnett's post-hoc test (n=5-7). *indicates $P < 0.05$ vs PF.

4.3.3 The impact of acute dietary Zn^{2+} deficiency on Cu/Zn-SOD activity in hearts subjected to myocardial I/R

Cu/Zn-SOD activity in the heart tissue was similar in the 3 groups (Figure 4.3).

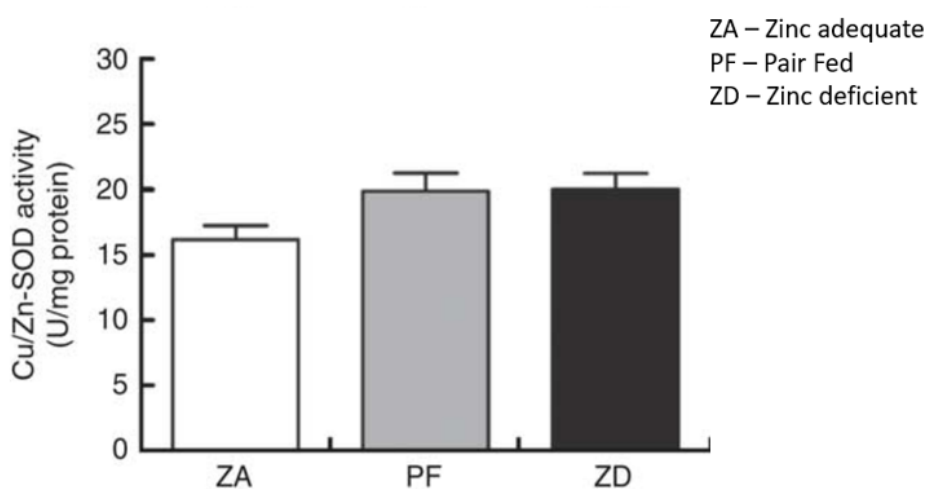


Figure 4.3:

The impact of acute Zn^{2+} deficiency on Cu/Zn-SOD activity in rat hearts subjected to 30 minutes regional ischaemia followed by a 2hr reperfusion period. All data are expressed as mean \pm SEM. Statistical significance was determined using a one-way ANOVA and Dunnett's post-hoc test (n=3-7).

4.3.4 The impact of *in vitro* acute Zn²⁺ depletion using TPEN and myocardial I/R on Zn²⁺ content and Caspase 3 activity in rat hearts.

In vitro acute Zn²⁺ depletion, obtained using TPEN, did not induce any significant difference in either myocardial tissue Zn²⁺ levels or Caspase 3 activity across all groups (Sham, Vehicle, TPEN) (Figure 4.4A and B) following I/R.

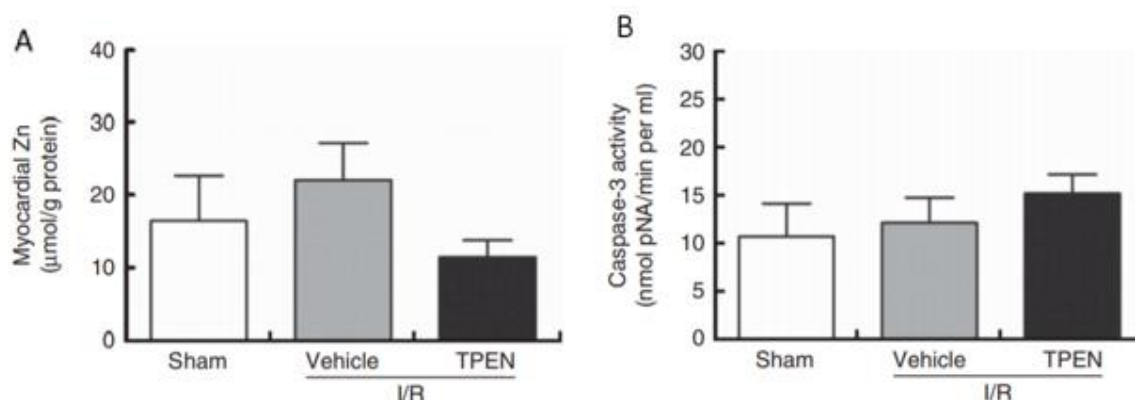


Figure 4.4:

Effect of TPEN (10μM) on tissue Zn²⁺ content (A) and Caspase 3 activity (B) in rat hearts subjected to myocardial I/R. Data are expressed as mean ± SEM and significance determined using one-way ANOVA and Dunnett's post-hoc test; n=10.

4.3.5 The impact of *in vitro* acute Zn²⁺ depletion using TPEN and myocardial I/R on GSH content in rat hearts.

I/R induction displayed a reduced GSH content in both the vehicle and the TPEN groups when compared with the sham hearts (Figure 4.5) although not statically significant.

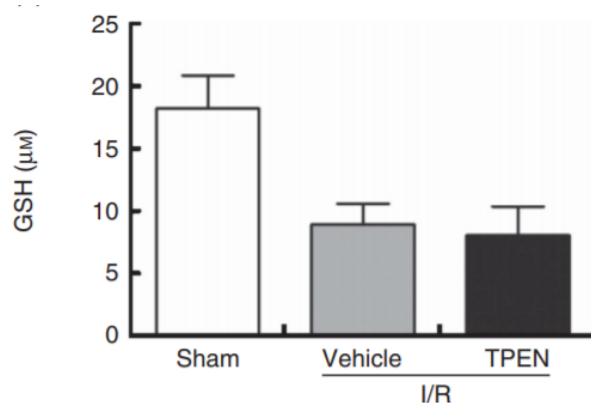


Figure 4.5:

Effect of TPEN (10μM) on GSH content in isolated hearts subjected to 30 minutes acute regional myocardial ischaemia and 2 h reperfusion. All data are expressed as mean ± SEM. Statistical significance was determined using one-way ANOVA and Dunnett's post-hoc test (n=3-8).

4.3.6 The impact of *in vitro* acute Zn²⁺ depletion using TPEN and myocardial I/R on Cu/Zn-SOD activity in rat hearts.

I/R induction resulted in reduced Cu/Zn-SOD activity in both the vehicle and the TPEN groups when compared with the sham hearts (Figure 4.6). However, in hearts given TPEN, there was a significant reduction in Cu/Zn-SOD activity compared with control I/R hearts. Comparing the controls, the vehicle control was also significantly lower than the sham hearts.

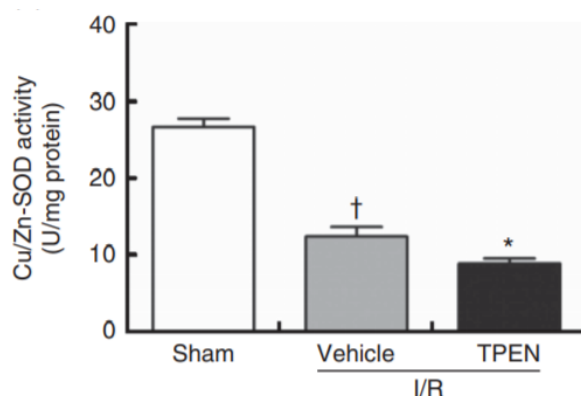


Figure 4.6:

Effect of TPEN (10 μ M) on Cu/Zn-SOD activity in isolated rat hearts subjected to 30 minutes acute regional myocardial ischaemia and 2 h reperfusion. All data are expressed as mean \pm SEM. Statistical significance was determined as $P < 0.05$ using a one-way ANOVA and Dunnett's post-hoc test ($n = 10$). * $P < 0.05$ compared to vehicle controls. † $P < 0.05$ compared to sham controls.

4.4 Discussion:

The primary aim of this study was to understand the impact of both dietary and *in vitro* acute Zn²⁺ depletion on Zn²⁺ levels, Caspase 3 activity, and antioxidants biomarkers in rat hearts subjected to acute regional myocardial ischaemia and reperfusion. The main findings for this study are that dietary Zn²⁺ depletion was associated with reduced cardiac GSH levels, while Cu/Zn-SOD activity was not affected by dietary Zn²⁺ intake. In contrast, *in vitro* acute Zn²⁺ depletion with TPEN reduced Cu/Zn-SOD activity, but not GSH content. Neither method of Zn²⁺ depletion had any effect on myocardial Zn²⁺ content. Caspase 3 activity was observed to be decreased in ZD rats (relative to PF rats) but was not significantly different from controls in the *in vitro* experiments where acute Zn²⁺ depletion was achieved using TPEN.

4.4.1 Tissue Zn²⁺ content and Caspase 3 activity following acute dietary Zn²⁺ deficiency

Assessment of Zn²⁺ levels in the heart showed that severe dietary Zn²⁺ restriction had no significant effect on cardiac Zn²⁺ levels. Although I did not perform plasma Zn²⁺ analysis, the plasma from these rats was analysed by another member of my supervisor's lab (K Skene) and the results showed circulating levels of Zn²⁺ in the ZD group was significantly lower than (<50%) those in both ZA and PF rats (Skenet et al., 2019). Thus there was evidence of severe Zn²⁺ deficiency in these animals. Considering the severity of the extent of dietary Zn²⁺ depletion, one would have expected lower myocardial Zn²⁺ levels compared to Zn²⁺-adequate hearts, but this was not the case. However, the results obtained are in agreement with those obtained from a study conducted by Coudray et al (1993), who also showed that acute dietary Zn²⁺ deficiency (4 ppm Zn²⁺ for the zinc-deficient group and 60 ppm Zn²⁺ for the standard diet group for 3 weeks) caused no notable difference in myocardial tissue Zn²⁺ levels. Similarly, studies in rats with less severe Zn depletion (22 mg/kg vs 98 mg/kg) or Zn²⁺ supplementation (214 mg/kg) showed no alterations in myocardial Zn (Pucheu et al., 1995). The findings that cardiac levels of Zn²⁺ are unaffected by marked alterations in extracellular Zn²⁺ can probably be explained by the tight control of Zn²⁺ in the cardiomyocyte, which is due to a combination of the release of Zn²⁺ from MT stores along with alterations in Zn²⁺ influx/efflux from the cell to maintain levels. Moreover, the accumulation of Zn²⁺ in structures like the sarcoplasmic reticulum of the cardiac cell could help to maintain its level in the myocardial cell. (Lee et al. 2015).

Studies by Karagulova et al (2007); Chanoit et al (2008); Viswanath et al (2011) all agree that Zn²⁺ supplementation improves ischaemic injury and prevents cardiomyocyte death. Although studies have shown that oxidative stress occurring in a dietary Zn²⁺ deficiency state increases apoptosis in vascular tissue (Allen-Redpath et al. 2013), there is no current literature that has examined the impact of Zn²⁺ deficiency on apoptosis in the heart. Being an integral component of proteins, Zn²⁺ is essential for binding DNA (e.g., p53) and can act as an activator or an inhibitor (e.g., caspases) making cell proliferation and cell survival pathways intertwined by the addition or withdrawal of protein growth factors like insulin-like growth factors, IGF-1 and IGF-2. Zn²⁺ binds to an active site cysteine to inhibit caspases and it is essential for protein-protein interactions (e.g., protein kinase c

(PKC) (Clegg et al. 2005). The data from the present study has shown for the first time that acute dietary ZD does not appear to induce caspase-3 activation in the myocardium. However other caspases like caspase 9, although not measured, could have activated apoptosis and this could possibly be the explanation to the greater degree of infarcts in the rat hearts. These caspases would therefore have been of interest to look at these in addition to caspase-3.

Endogenous ROS production initiates DNA damage which activates the tumor suppressor gene: p53 (p53 is a Zn^{2+} finger transcription factor that regulates both G1 and G2 checkpoints, preventing cells with damaged DNA from proceeding with new DNA synthesis or undergoing cell division) (Clegg et al. 2005). This induces the intrinsic pathway of apoptosis by causing cytochrome c into the cytosol release from mitochondria and this recruits the apoptosome complex (caspase-9 zymogen, Apaf-1, and dATP) leading to the auto-catalytic activation of initiator caspase-9 and cleavage of effector procaspase-3 (Clegg et al. 2005). Glutathione depletion by ROS is associated with apoptosis induction and levels have been shown to be reduced in culture mediums with the addition of metal chelators like TPEN inhibiting caspase activation (Carter et al. 2002).

4.4.2 Acute Zn^{2+} deficiency and myocardial I/R alters antioxidant biomarkers in rat hearts

The results from the present study, demonstrating that the GSH content was significantly lower in the dietary Zn^{2+} deficient groups compared to the other groups, is consistent with findings from a study by AL-Rasheed et al(2014) which showed that Zn^{2+} deficiency reduces GSH content due to its consumption at the time of scavenging the high levels of free radicals (AL-Rasheed et al. 2014). AL-Rasheed et al(2014) looked at GSH amongst other antioxidant enzymes in six groups of rats of which one group was Zn-pretreated (30 mg/kg) for 28 days followed by an induction of a MI for 2 days (day 27 day and 28) and tissue homogenates showed decreased levels of GSH (AL-Rasheed et al. 2014). Due to the high myocardial demand of oxygen and the ratio of oxidants to antioxidants, any condition that incapacitates the antioxidant defence system (i.e. ischaemia/reperfusion) may expose the heart to oxidative damage (Barandier et al. 1999).

Zn²⁺ is also essential for the activity of SOD1 (Cu/Zn-SOD) and SODs are highly efficient antioxidants that prevent occurrence of free radical induced injury (Huang et al. 2018). In the present study, Cu/Zn-SOD activity was not affected in animals subjected to acute dietary Zn²⁺ depletion and this is in agreement with the literature as there was no significant change in cardiac SOD levels seen in rats deficient in Zn²⁺ amongst other trace elements (Pucheu et al. 1995; Barandier et al. 1999). Jones et al (2003) have previously reported that SOD does not protect against reperfusion injury in the intact heart, and so it is likely that it is the reduction in GSH that has contributed to the increased infarct size identified in the wider study.. Thus, further studies are required to better understand the effect of acute or chronic Zn²⁺ depletion on biomarkers of SOD activity in the heart.

4.4.3 Outcome of I/R following *in vitro* acute Zn²⁺ depletion with TPEN

In the present study, acute Zn²⁺ depletion with TPEN did not alter the total myocardial tissue Zn²⁺ content. Although myocardial Zn²⁺ levels were not significantly affected by TPEN treatment, the concentration of TPEN used was previously shown to chelate Zn²⁺ at this concentration. Parat et al (2007) conducted a study using the same concentration as used in the present study and found that TPEN caused a dose-dependent reduction cellular Zn²⁺ content in HaCaT keratinocytes (Parat et al. 1997). The difference between the present findings in the whole heart and those in cell preparations could potentially be explained by changes in Zn²⁺ transporter activity in the heart *in situ* causing preservation of cardiac Zn²⁺. Thus, TPEN chelation may block attempts by the cell to adjust Zn²⁺ homeostasis in the face of declining intracellular Zn²⁺ concentrations causing a loss of Zn²⁺ (Clegg et al. 2005). Additionally, since TPEN chelates free Zn²⁺ and the assay used measured total (i.e. bound and free zinc) it is possible that the assay was not sensitive enough to detect the loss of free Zn²⁺.

The wider study conducted with same tissues by my supervisor's team as published in the literature (Skene et al 2019) showed findings of increased ischaemia/reperfusion injury with TPEN. This is in agreement with a study by Ferdinandy et al., who studied the effects of similar concentrations (10 µM) of TPEN ischaemic and reperfusion arrhythmias and observed a reduced incidence of ventricular fibrillation (Ferdinandy et al. 1998).

However, although the concentration of TPEN used in this study gives it a higher affinity for Zn^{2+} than other cations (Ca^{2+}) the effects of TPEN is most likely from the other cations being removed. In I/R endogenous Zn^{2+} has been reported to be harmful and cause cell death by activating extracellular signal-regulated kinase/glycogen synthase kinase β (ERK/ GSK3 β) (Lin et al. 2011). Endogenous Zn^{2+} has been reported to be cardioprotective through its action on signalling process in nitric oxide (NO) via intracellular messenger (Jang et al. (2007). Endogenous Zn^{2+} is also known to play an important role in maintaining redox status within tissues (Oteiza, 2012). Knowing that TPEN is a Zn^{2+} chelator, one would have thought that it would give a significant reduction in myocardial Zn^{2+} level compared to the controls but this was not the case.

In the present study, treatment with TPEN prior to myocardial I/R did not alter Caspase 3 activity compared to the controls. However, results from a study carried out by Pang et al (2013) showed that following treatment with TPEN, Caspase 3 was activated although this was through inhibition of ERK in the hippocampal neurons.

A reduction in Cu/Zn-SOD activity (Figure 4.6) was observed following I/R induction compared with the sham hearts. TPEN reduced Cu/Zn-SOD activity compared to vehicle treated hearts (Figure 4.6). Cu/Zn-SOD is a Zn^{2+} dependent isoform thus post reperfusion, it is not likely to contribute to tissue preservation. Cu/Zn-SOD has been proven not to protect against I/R rather the interstitial levels of the extracellular SOD (Omar and McCord 1991).

The results obtained from the TPEN arm of the study is different to what was seen in the dietary intervention arm. Though in vitro endogenous Zn^{2+} depletion with TPEN demonstrated an increase in infarct size, however, the increase in infarct size due to dietary deficiency was associated with a reduced Cu/Zn- SOD activity since there was preservation of the GSH levels and no evidence of caspase-3 activation. However, it is known that endogenous SOD-1 is not the only Zn-dependent isoform of SOD although has demonstrated to be an unlikely contributor to post-reperfusion tissue preservation. In the vascular wall, between the endothelium and the extra cellular space and the cells of the smooth muscles is where the extracellular SOD (SOD-3) is most available and this plays a crucial role in the regulation of the vascular redox state(Strålin et al. 1995) and it is indicated that the SOD-3 (interstitial levels) confer protection against I/R injury rather than SOD-1 (Omar and McCord, 1991). Moreover, since the Cu/Zn-SOD assay carried out in this study measured the total

Cu/Zn-SOD activity (i.e. SOD-1 and SOD-3), the infarct size increase might likely be due to an inhibition of SOD-3, rather than SOD-1 activity.

Conclusions

The key results from these studies are that both acute dietary Zn^{2+} deficiency and acute *in vitro* Zn^{2+} depletion worsen the extent of myocardial injury resulting from I/R via different mechanisms. Dietary Zn^{2+} deficiency results in a decrease in the content of myocardial GSH which is the likely cause of damage to the cardiac tissue following I/R (as reported in Skene et al., 2019) due to the induced oxidative stress. However, *in vitro* Zn^{2+} depletion is likely to worsen I/R injury because of the reduced availability of SOD to mop up the superoxide radicals, again resulting in increased oxidative stress. These results have demonstrated that severe dietary Zn^{2+} insufficiency poses a problem and may lead to a worse outcome following acute myocardial ischaemia, causing increased morbidity and mortality. However the results from the TPEN studies, where different mechanisms are induced, suggest that further research into the role of Zn^{2+} in cardiovascular physiology and pathophysiology should be carried out using a dietary intervention model to eliminate misleading findings.

5.1 General Discussion:

As mentioned earlier, even though the human body contains about 2–4g of Zn^{2+} , only 12–16 $\mu\text{mol/L}$ exists as a mobile Zn^{2+} pool in plasma. This low plasma concentration of Zn^{2+} is due to its tight binding to proteins (MT) and zinc transport proteins (Jansen et al. 2012). Zn^{2+} is involved in the regulation of glutathione peroxidase and is a cofactor for SOD hence its maintenance in the cell is crucial to adequate antioxidant system function (Marreiro et al. 2017).

Studies pertaining to the role of Zn^{2+} in cardiac protection tend to focus more on the effect of exogenous Zn^{2+} supplementation. However, endogenous Zn^{2+} is mobilized by NO via its opening of mitochondrial ATP channels and this has been shown to offer cardioprotection (Powell et al. 1994; Jang et al. 2007). The importance of the role of endogenous Zn^{2+} in cardiovascular system maintenance cannot be over emphasized. A human study, carried out to assess the relationship between Zn^{2+} intake and the prevalence of coronary artery disease (CAD) in urban and rural areas in India, showed that urban men and women consume lower amounts of Zn^{2+} compared to their rural counterparts and have a significantly higher prevalence of CAD, suggesting that the prevalence of CAD is higher in individuals with low Zn^{2+} intake (Singh et al. 1998). There is growing evidence that Zn^{2+} intake is associated with reduced plasma glucose. For example, Chen et al. (1997) studied a population of obese ($\text{BMI} > 30 \text{ kg/m}^2$) and lean ($\text{BMI} = 21.2 \pm 0.6$) individuals free from endocrine disease hence not on any medication, also not on any supplement (vitamin or minerals), between the ages of eighteen and twenty two and found that the obese individuals had lower fasting plasma Zn^{2+} concentrations ($[13.5 \pm 1.0 \text{ vs. } 18.1 \pm 0.9] \mu\text{mol/L}$). Another study this time in animals also showed that Zn^{2+} supplementation lowered fasting plasma glucose levels in obese mice using eight weeks old male obese C57BL/6J mice and their lean controls given 20 mM of ZnCl_2 in drinking water for eight weeks (totalling 16 weeks of age) and plasma glucose tolerance test (1 g glucose/kg body wt, ip) was measured following more than 12 hr of fasting (Chen et al. 1998). In all, obesity brings about changes in the biochemical parameters of Zn^{2+} and an increase in the production of free radicals contributing to OS which eventually leads to low Zn^{2+} concentrations in the plasma, erythrocytes and an increase in the antioxidant enzyme (GPx and SOD) activities in tissues (heart, liver, kidney and muscle) (Ferro et al. 2011). This increase in antioxidant enzyme activities is caused by an

increased demand to fight against the obesity induced free radical (Ferro et al. 2011).

In the present study, how endogenous Zn^{2+} depletion induced by *in vivo* dietary deficiency and *in vitro* removal of intracellular Zn^{2+} affected the antioxidant capacity of the heart was investigated. The results demonstrated how important it is to maintain adequate levels of Zn^{2+} to protect the heart from developing atherosclerotic plaques associated with western type diet or acute myocardial infarction.

The dietary interventions in the first study looked at dietary Zn^{2+} deficiency in conjunction with high fat diet where animals (mice) were fed HFD containing either 35 or 5 mg/kg Zn^{2+} (adequate and suboptimal Zn^{2+} , respectively) over 15 and 25 weeks (chronic intervention). Whereas, the second study involved rats that were fed standard diets with different Zn^{2+} compositions (ZA – 35 mg/kg Zn^{2+} , ZD- <1 mg/kg Zn^{2+} and PF) for 14 days (acute intervention) as stated in chapter 4. The acute study also looked at Zn^{2+} depletion achieved via *in vitro* Zn^{2+} chelation with TPEN.

Overall, mice subjected to chronic dietary intervention showed more age-related effects in the parameters measured: GSH concentration in the older animals (25 weeks group) showed a significant difference comparing the Zn^{2+} adequate and the suboptimal Zn^{2+} groups but no difference was observed within the 15 week groups (Zn^{2+} adequate: 35 mg/kg vs suboptimal: 5 mg/kg). However, the acute Zn^{2+} deficiency study showed better GSH levels in the ZA group compared with the ZD and TPEN groups. Zn^{2+} deficiency has been shown to reduce GSH levels in other tissues like the brain (Omata et al. 2012) and the liver (Kojima-Yuasa et al. 2005) and a reduction in the levels of GSH can cause a harmful effect on myocardial tissue following I/R as GSH inhibits tissue injury caused by ROS (Chatham et al. 1988; Cheung et al. 2000).

In both studies, results observed in the Cu/Zn-SOD activity might be due to the endogenous production of SOD-1 and this agrees with the literature (Jones et al. 2003). In study one, an extended duration of the dietary intervention (25 vs 15 weeks) was observed to increased Cu/Zn-SOD activity whereas study two (acute

dietary Zn²⁺ deficiency study) showed similar Cu/Zn-SOD activity in the heart tissue in the 3 groups (ZD, PF, and ZA).

Myocardial Zn²⁺ content was measured and there was no significant difference in the Zn²⁺ levels which is probably due to the tight regulation of Zn²⁺ homeostasis intracellularly by metallothionein, ZnT and ZIP (Liuzzi and Cousins 2004; Baltaci et al. 2018). Hearts treated with TPEN also did not show significant differences in Zn²⁺ content.

5.2: Conclusion

Reduced or suboptimal intake of dietary Zn²⁺ differentially affects total GSH content in cardiac tissue. More specifically, exposure to acute Zn²⁺ deficiency reduces levels of total GSH whereas chronic exposure does not. On the contrary, Cu/Zn-SOD activity is not affected by either acute Zn²⁺ depletion or chronic exposure to a suboptimal Zn²⁺ level for either 15 or 25 weeks. The primary findings from these studies is that, in both studies, suboptimal or acute Zn²⁺ deficiency affect the antioxidant status of the cardiovascular system differently.

Chronic Zn²⁺ intake (mouse study) and acute dietary Zn²⁺ deficiency results in a reduction in total GSH content. However, *in vitro* Zn²⁺ depletion is affected by the partial superoxide scavenging capacity. Altogether, dietary insufficiency of Zn²⁺ has been identified as a risk factor for a worse outcome from cardiovascular disease which is linked to an increase morbidity and mortality rates.

Since Zn²⁺ deficiency is linked with obesity, and dietary Zn²⁺ supplementation has been shown to reduce the incidence of diabetic cardiovascular complications, a similar approach may be applicable to protect against obesity-related enhanced risk to cardiovascular disease. Knowing that the percentage of the global population that is currently obese (~500M people according to the 2008 WHO statistics) and therefore at risk of a cardiovascular disease with a worse outcome is on the increase, this makes the morbidity burden huge. Similarly, the global incidence of dietary Zn²⁺ deficiency is high, therefore given the important role of Zn²⁺ in endogenous cardioprotection (SOD1 and the RISK pathway), the potential value of dietary Zn²⁺ supplementation in obesity as a means of providing protection

against both the development of obesity and abrogating against severe cardiac damage needs exploration.

Interestingly, to date this piece of work is the first to explore the role of Zn^{2+} in maintaining antioxidant status in the heart with respect to the impact of high fat diet on cardiac antioxidant status in wild type mice and the impact of acute severe dietary and invitro (TPEN) Zn^{2+} depletion on cardiac antioxidant levels in rats hearts following myocardial I/R and whether Zn^{2+} levels contributes to the extent of myocardial injury.. The differences seen in the two experiments in terms of antioxidant status is that severe deficiency in rats decreased GSH, while sub-optimal Zn^{2+} alongside HFD in mice did not. Therefore, future studies would be recommended to determine the impact Zn^{2+} deficiency would have alongside a HFD following injury in other to tie the two studies together. Furthermore, the potential value of dietary Zn^{2+} supplementation in obese people as a means of abrogating against I/R injury has not been assessed. Hence, further research is needed to explore the molecular mechanisms involved and if Zn^{2+} supplementation may be manipulated and used as a therapeutic tool in obese patients with CVD. A novel approach involving Zn^{2+} supplementation in obese individuals, could significantly reduce long term morbidity in an enormous population and therefore future therapies may include nutritional supplements for people who have been identified as having the genetic or clinical potential to benefit from it.

Animal models have been used extensively in research with some models providing more insight into the pathogenesis of the human disease. The species of animal used is determined by several factors. For these present studies, different species of rodents were used due to impact they have made following this type of study in relation to human health. on the outcome of the experiment. Mice are known to be good animal models for arteriosclerosis and obesity which is very similar in comparison to humans. Thus this model was used to explore whether high fat diet intervention alongside sub-optimal Zn^{2+} levels influence cardiac anti-oxidant status. In contrast, the rat is a well-established model for ischaemia / reperfusion injury studies, hence the reason for undertaking these studies in this species.

In general, the smaller the animal, the more manageable and cheaper the experiment although rodents may not adequately reflect the human situation. In the United Kingdom, animal research is governed by the three Rs—reduction,

refinement and replacement. Effort is made to reduce the number of animals required for any particular study and experimental design is refined so that it causes the least discomfort to the animals and, ultimately, animal models should be replaced altogether.

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