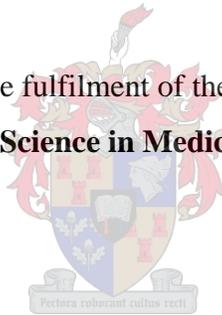


**THE EFFECT OF CPT-1 INHIBITION ON MYOCARDIAL FUNCTION AND
RESISTANCE TO ISCHEMIA/REPERFUSION INJURY IN A RODENT MODEL
OF THE METABOLIC SYNDROME**

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DECLARATION

I, GERALD JEROME MAARMAN, hereby acknowledge that the work contained in this dissertation is my own work and that I have not previously in its entirety or in part submitted it to any other tertiary institution to obtain a degree/qualification.

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ABSTRACT

Background: Obesity is associated with dyslipidemia, insulin resistance and glucose intolerance and together these components characterise the metabolic syndrome (Dandona *et al.* 2005). In the state of obesity, there are high levels of circulating free fatty acids and increased rates of fatty oxidation which inhibit glucose oxidation. This: (i) reduce the heart's contractile ability, (ii) exacerbates ischemic/reperfusion injury and (iii) decreases cardiac mechanical function during reperfusion (Kantor *et al.* 2000; Liu *et al.* 2002; Taegtmeyer, 2000).

Aim: The aim of our study was to investigate the effect of inhibiting fatty acid oxidation, with oxfenicine (4-Hydroxy-L-phenylglycine), on (i) cardiac mechanical function, (ii) mitochondrial respiration, (iii) myocardial tolerance to ischemia/reperfusion injury, (iv) CPT-1 expression, MCAD expression, IRS-1 activation, total GLUT- 4 expression and (v) the RISK pathway (ERK42/44 and PKB/Akt).

Methods: Male Wistar rats were fed a control rat chow diet or a high calorie diet (HCD) for 16 weeks. The HCD caused diet induced obesity (DIO). The animals were randomly divided into 4 groups [Control, DIO, Control + oxfen and DIO + oxfen]. The drug was administered for the last 8 weeks of feeding (200mg/kg/day). Animals were sacrificed and the hearts were perfused on the Langendorff perfusion system. After being subjected to regional ischemia and two hours of reperfusion, infarct size was determined. A separate series of animals were fed and/or treated and hearts were collected after 25 minutes global ischemia followed by 30 min reperfusion for determination of GLUT- 4, CPT-1, IRS -1, MCAD, ERK (42/44) and PKB/Akt expression/phosphorylation using Western blot analysis. A third series of hearts were excised and used for the isolation of mitochondria.

Results: In the DIO rats, chronic oxfenicine treatment improved cardiac mechanical function by improving mitochondrial respiration. Oxfenicine inhibited CPT-1 expression but had no effect on MCAD or GLUT- 4 expression. Oxfenicine decreased IRS-1

expression, but not IRS-1 activation. Oxfenicine also improved myocardial tolerance to ischemia/reperfusion without activation of the RISK pathway (ERK & PKB). In the control rats, chronic oxfenicine treatment worsened cardiac mechanical function by adversely affecting mitochondrial respiration. Oxfenicine also worsened myocardial tolerance to ischemia/reperfusion in the control rats without changes in the RISK pathway (ERK & PKB). Oxfenicine had no effect on CPT-1, MCAD or GLUT- 4 expression. Oxfenicine increased IRS-1 expression, but not IRS-1 activity.

Conclusion: Chronic oxfenicine treatment improved cardiac mechanical function and myocardial resistance to ischemia/reperfusion injury in obese animals, but worsened it in control animals. The improved cardiac mechanical function and tolerance to ischemia/reperfusion injury may be due to improvement in mitochondrial respiration.

UITTREKSEL

Agtergrond: Vetsug word geassosieer met dislipidemie, insulien weerstandigheid en glukose intoleransie, wat saam die metaboliese sindroom karakteriseer (Dandona *et al.* 2005). Met vetsug is daar 'n hoë sirkulasie van vetsure, sowel as verhoogde vetsuur oksidasie wat gevolglik glukose oksidasie onderdruk. Dit: (i) verlaag die hart se vermoë om saam te trek, (ii) vererger isgemiese/herperfusie skade en (iv) verlaag kardiaale effektiwiteit gedurende herperfusie (Kantor *et al.* 2000; Liu *et al.* 2002; Taegtmeier, 2000).

Doel: Die doel van die studie was om die effekte van vetsuur onderdrukking m.b.v. oksfenisien (4-Hidroksie-L-fenielsingien) op (i) meganiese hart funksie, (ii) mitokondriale respirasie, (iii) miokardiale toleransie teen isgemiese/herperfusie skade, (iv) CPT-1 uitdrukking, MCAD uitdrukking, IRS-1 aktiwiteit, totale GLUT-4 uitdrukking en (v) die RISK pad (ERK42/44 en PKB/Akt) te ondersoek.

Metodes: Manlike Wistar rotte was gevoer met 'n kontrole rot dieet of 'n hoë kalorie dieet (HKD) vir 16 weke. Die HKD lei tot dieet-geïnduseerde vetsug (DGV). Die diere was lukraak verdeel in 4 groepe [kontrole, DGV, kontrole + oksfen en DGV + oksfen]. Die behandeling met die middel was toegedien vir die laaste 8 weke van die voeding protokol (200mg/kg/dag). Die diere was geslag en die harte was geperfuseer op die Langendorff perfusie sisteem. Na blootstelling aan streeks- of globale isgemie en 2 ure herperfusie was infark groottes bepaal. 'n Aparte reeks diere was gevoer en/of behandel en die harte was versamel na 25 minute globale isgemie gevolg deur 30 minute herperfusie vir die bepaling van GLUT-4, CPT 1, IRS -1, MCAD, ERK (42/44) en PKB/Akt uitdrukking/aktivering d.m.v. Western blot analise. 'n Derde reeks diere was gebruik vir die isolasie van mitokondria.

Resultate: In die DGV diere, het kroniese oksfenisien behandeling meganiese hart funksie verbeter d.m.v. die verbetering van mitokondriale respirasie. Oksfenisien het CPT-1 uitdrukking verlaag terwyl GLUT- 4 en MCAD uitdrukking nie geaffekteer was

nie. Oksfenisien het IRS-1 uitdrukking verlaag, maar nie IRS-1 aktiwiteit nie. Oksfenisien het ook miokardiale weerstand teen isgemiese/herperfusie verbeter met sonder aktivering van die RISK pad (ERK & PKB). In die kontrole diere, het kroniese oksfenisien behandeling die meganiese hart funksie versleg d.m.v. negatiewe effekte op mitokondriale respirasie. Oksfenisien het die miokardiale weerstand teen isgemiese/herperfusie van die kontrole rotte versleg sonder veranderinge in die RISK pad (ERK & PKB). Oksfenisien het geen effek gehad op CPT-1, MCAD en GLUT-4 uitdrukking nie. Oksfenisien het IRS-1 uitdrukking verhoog, maar nie IRS-1 aktiwiteit nie.

Samevatting: Kroniese oksfenisien behandeling het die meganiese hart funksie en miokardiale weerstand teen isgemiese/herperfusie skade in die vet diere verbeter, maar versleg in die kontrole diere. Hierdie verbetering van meganiese hart funksie en weerstand teen isgemiese/herperfusie skade kon dalk wees a.g.v. 'n verbetering in mitokondriale respirasie.

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LIST OF ABBREVIATIONS

Units of measurement

AU	Arbitrary unit
EC	Energy consumption
FC	Food consumption
g/day	Gram/day
g/mol	Gram/mol
Kbs	Kilo base
kDa	Kilo dalton
Kg	Kilogram
kg/ m ²	Kilogram/square meter
kJ	Kilo joules
kJ/g	Kilo joule/gram
ℓ	Litre
μg	Microgram
μg/ μℓ	Micro gram/micro litre
μIU	Micro international unit
μIU/μℓ	Micro international unit/micro litre
μℓ	Micro litre
μmol/gww	Micromole/gram wet weight
μmol/min/gww	Micromole/minute/gram wet weight
μm	Micro meter
mℓ	Millilitre
mm	Millimetre
mmol	Millimole
mM	Milli-molar
mg/kg/day	Milligrams/kilogram/day
mol/min/gww	Mol/minute/gram wet weight
%	Percentage
RPP	Rate pressure product

rpm	Revolutions per minute
Yrs	Years
m ²	Square meter

Chemical compounds

Ca ²⁺	Calcium ion
CO ₂	Carbon dioxide
Co-A	Coenzyme A
FADH ₂	Flavin adenine dinucleotide
C ₆ H ₁₂ O ₆	Glucose
GTP	Guanosine tri-phosphate
C ₆ H ₁₂ O ₂	Hexanoic acid
H ⁺	Hydrogen ion (proton)
HPG	Hydroxy-L-phenylglycine
NADH	Nicotinamide adenine dinucleotide
Oxfen	Oxfencine
O ₂	Oxygen
K ⁺	Potassium ion
KCl	Potassium chloride
Na ⁺	Sodium ion
Na ₂ S ₂ O ₄	Sodium hydrosulfite
H ₂ O	Water

Enzymes:

ACC	Acetyl-CoA carboxylase
AMPK	Adenosine-monophosphate-activated-protein - kinase

CPT-1	Carnitine palmitoyl transferase-1
G3PDH	Glyceraldehyde-3 phosphate dehydrogenase
KAT	Keto acyl transferase
MCAD	Medium chain acyl-CoA dehydrogenase
MCD	Malonyl CoA Decarboxylase
MTE-1	Mitochondrial thioesterase-1
PI3-kinase	Phosphatidyl-inositol-3-kinase
PFK	Phospho fructose kinase
PKC-θ	Protein kinase C-theta
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PDHK	Pyruvate dehydrogenase kinase
PDHC	Pyruvate dehydrogenase complex

Proteins

CRP	C-reactive protein
ERK (42/44)	Extracellular signal-regulated kinase
FATP	Fatty acid transporter protein
GLUT-4	Glucose transporter- 4
HDL	How density lipoprotein
IRS-1	Insulin receptor substrate 1
LPL	Lipoprotein lipase
LDL	Low density lipoprotein
PKB/Akt	Protein kinase- B
RBP4	Retinol binding protein 4
UCP	Uncoupling protein
VLDL	Very low density lipoproteins

Other abbreviations

ADP: O	ADP: O ratio
ANOVA	Analysis of variance
ATP/O ₂	ATP produced per oxygen used
BMI	Body mass index
CD	Cafeteria diet
CVD	Cardiovascular disease
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
DNA	Deoxyribonucleic acid
DCA	Di-chloro-acetate
ETC	Electron transport chain
FFA	Free fatty acid
GIK	Glucose insulin potassium
HR	Heart rate
IL-6	Interleukin- 6
IUPAC	International union of pure and applied chemistry
I/R	Ischemia/Reperfusion
LAD	Left anterior descending coronary artery
LVDP	Left ventricular developed pressure
mRNA	Messenger RNA
MS	Metabolic syndrome
MVO ₂	Myocardial oxygen consumption
NEFA	Non-esterified fatty acid
PC	Personal computer
PPAR- α	Peroxisome proliferator activated receptor - alpha
PMSF	Phenyl-methyl-sulphonyl-fluoride
PAGE	Poly-acrylamide gel electrophoresis

PVDF	Poly-vinylidene fluoride
P	Probability
QO ₂	Rate of oxygen consumption
ROS	Reactive oxygen species
RCI	Respiratory control index
n	Sample size
Ser	Serine
S.E.M	Standard error of the mean
SRC	Standard rat chow
SDS	Sodium dodecyl sulphate
Thr	Threonine
TCA	Tri carboxylic acid
TG	Triglycerides
TBS	Tris-buffered saline
TTC	1, 2, 3 Tri- phenyl tetrazolium chloride
T2DM	Type 2 diabetes mellitus
Tyr	Tyrosine
TNF- α	Tumour necrosis factor alpha
W.H.O	World health organization

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INTRODUCTION

Over the past couple of decades obesity has increased at an alarming rate and given rise to the observation that it is currently exceeding the boundaries of diseases that threatens human life (Mathieu *et al.* 2008). Since the surpassing of the Palaeolithic era and the progression into the era of western lifestyle-popularity, humans gradually diverted from the diet of the pre-agricultural Hunter-gatherers, to the much indulged western diet of today. A variety of factors that include poor dietary composition (high-trans fatty acids, high saturated fatty acids and high refined sugar content), socio-economic changes, agricultural developments and technological advances appears to be the leading cause of the increased incidence of obesity globally (Hammer *et al.* 2008; Opie, 2009; Poirier *et al.* 2006).

In the United States of America alone, 65% of adults over 20yrs of age are either overweight or obese and deaths ascribable to obesity are 280184/year. South Africa now also has an obesity record, mimicking that of the USA, with approximately 50% of the adult female population \geq 30yrs classified as obese (Cordain *et al.* 2005). Obesity within the South African context appears to be a complicated matter. In 2001 a census was done and 44.8 million people were counted. The culturally diverse South African population consisted of 76 percent blacks, 13 percent whites, 9 percent of mixed ancestry (coloureds) and 2.5% Indians (Puoane *et al.* 2005). The census also revealed that people continuously migrates from rural to urban areas. A recent study concludes that the percentage of the population in urban areas to more than 60 percent. The largest migrating group are black people from the rural areas (Puoane *et al.* 2005).

The emergence of diseases in the previously disadvantaged groups in South Africa initially occurred in the coloured population, which was the first to experience urbanization, industrialization, upward mobility and adoption of the typical western lifestyle (See review by Puoane *et al.* 2005). The black groups are currently in transition of this process and therefore there is now a clear link between urbanization and emergence of diseases in these groups. In the black group the degree of urbanization is

directly linked to the increasing consumption of the typical western diet, smoking cigarettes at an early age in black women, and the development of diabetes and hypertension (Bourne, 1994; Steyn *et al.* 1994; Steyn *et al.* 1996). Other factors contributing to the development of obesity in transitional countries include environmental, socio-economic, behavioural and cultural factors (WHO, 2000). Both research and case studies indicate that environmental and socioeconomic factors contribute to the emergence of obesity in urban black African women (Puoane *et al.* 2005).

These statistics give both researchers and the public a glimpse into the magnitude of the global obesity pandemic. Many continents, including Africa, have adopted a sedentary lifestyle (lifestyle prone to inactivity), which in addition to the above mentioned factors, exacerbates already worrying statistics. This brings health experts and scientists to the conclusion that obesity is single handedly threatening human health and also explains why obesity has received much attention over the past few years (Reaven, 2005; Lopaschuk, Folmes & Stanley, 2007; Lopaschuk *et al.* 2010; Franssen *et al.* 2008).

Obesity is one of a cluster of physical- and metabolic abnormalities that together characterise the metabolic syndrome. Obesity, and particularly visceral/central obesity, is associated with certain metabolic abnormalities/cardiovascular risk factors that include dyslipidemia, hypertension, glucose intolerance, systemic inflammation, obstructive sleep apnoea/hypoventilation and a pro-thrombotic state (Poirier *et al.* 2006). The importance of body fat distribution on the severity of obesity related diseases has only in recent years become better understood. Consensus is that metabolic perturbations, associated with the metabolic syndrome, is worsened by an increased amount of visceral fat which is commonly known as central obesity (Mathieu *et al.* 2008).

There is increasing evidence implicating increased systemic oxidative stress (an imbalance between oxidants & antioxidant systems in the favour of oxidants) in the state of obesity (Diniz *et al.* 2008). Additionally, the accumulation of adipose tissue leads to (i) an altered inflammatory state with lowered adiponectin levels, increased pro-

inflammatory cytokine levels (interleukin-6, C-reactive protein and TNF- α -levels), (ii) altered lipid profiles i.e. dyslipidemia (low High Density Lipoproteins & High Low Density Lipoproteins), (iii) a variety of adaptations/alterations in cardiac structure and function i.e. cells secreting various locally acting molecules, cell dysfunction/death, lipotoxicity, left ventricular diastolic dysfunction and left ventricular hypertrophy as well as (iv) insulin resistance (Poirier *et al.* 2006; Taegtmeyer, 2000).

Recent work has shown that, hearts from obese rats fed an experimental high carbohydrate and -fat diet, were hypertrophied. This was illustrated by the increase in ventricular weight, ventricular weight-to-tibia length, left ventricular posterior wall thickness and an increase in cardiomyocyte size (Du Toit *et al.* 2008). With obesity, ectopic lipid accumulation in the myocardium (as a result of the elevated circulation of free fatty acids and triglycerides) impairs cardiac systolic and diastolic function (Rasouli *et al.* 2007). However, it is almost impossible to adequately discuss the effects of obesity, without touching on the metabolic syndrome.

CHAPTER 1

LITERATURE REVIEW

A marked increase in the number of metabolic syndrome cases has occurred worldwide (Eckel, Grundy & Zimmet, 2005; Miranda *et al.* 2005; Nguyen *et al.* 2008; Lin & Sun). This increase is due to increases in the incidence of obesity and diabetes. There is thus an urgent need for effective therapeutic strategies.

Metabolic syndrome (MS) & Cardiovascular disease

1.1 Metabolic Syndrome

1.1.1 Definition of the Metabolic Syndrome

This syndrome was originally defined as a condition consisting of several classical cardiovascular risk factors that include insulin resistance, hypertension, hyperinsulinemia, dyslipidemia, type II diabetes (T2DM) and glucose intolerance. MS is the consequence of an excessive caloric intake and a sedentary lifestyle (Eckel, Grundy & Zimmet, 2005). An individual is diagnosed with the MS if they present with two or more of these abnormalities. The prevalence of MS increases with age as illustrated by data gathered in the USA: One in three adults between the age 50 and 59 have MS when compared with younger adults (Caglayan *et al.* 2005).

Patients with MS are four times more likely to develop cardiovascular pathologies in comparison with patients without this condition (Bugger & Abel, 2008; Caglayan *et al.* 2005; Klein *et al.* 2002).

Pathologies associated with MS include: coronary artery disease, heart failure and ischemic intolerance. The metabolic syndrome is also associated with renal dysfunction and aorta wall stiffness (Caglayan *et al.* 2005; Klein *et al.* 2002).

1.1.2 The Development of the MS

The general rationale is that certain factors, including a sedentary lifestyle, excessive food intake and genetic predisposition, lead to either visceral- or general obesity. Thus the point of origin of MS is considered to be obesity. This increase in body fat causes dyslipidemia which in turn leads to insulin resistance and ultimately MS (Reaven, 2005). See figure 1 below.

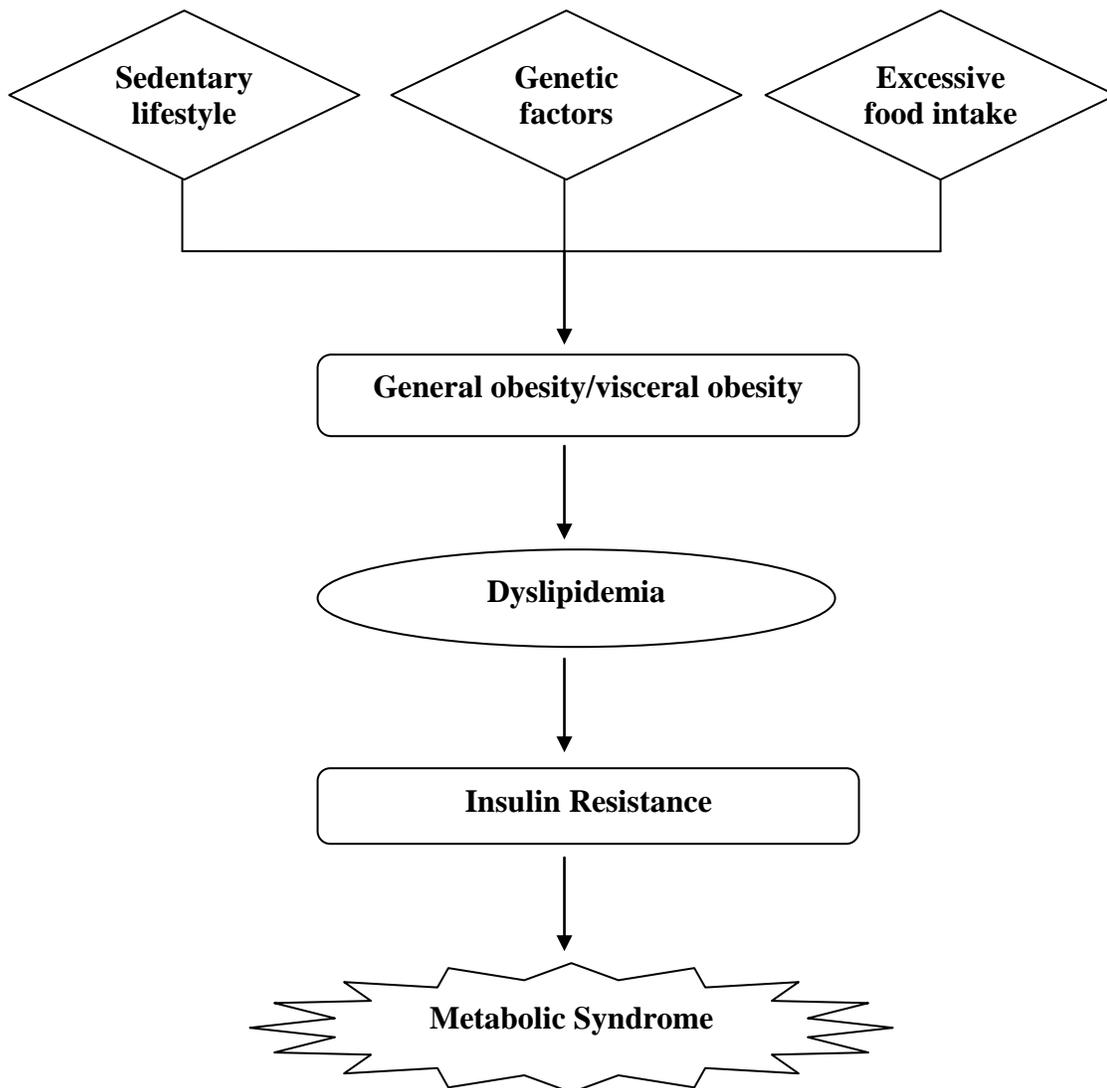


Figure 1: A graphical representation of how MS develops over time (Adapted from Reaven, 2005; Smith 2006)

Type 2 diabetes mellitus (T2DM) is a known consequence of MS (Eckel, Grundy & Zimmet, 2005; Miranda *et al.* 2005; Nguyen *et al.* 2008; Lin & Sun, 2010). Together with obesity, the incidence of T2DM is increasing rapidly and many researchers devote their scientific efforts towards developing treatments to combat T2DM. This condition however remains a major concern.

1.1.3 MS and Type II Diabetes Mellitus (T2DM)

T2DM is the more prevalent form (90%) of diabetes, with a polygenic background, which is acted on by environmental factors in order to enable its distinctive clinical presentation (Thim *et al.* 2006). It is described as a chronic metabolic disorder and a non-insulin-dependent type of diabetes that is the result of obesity, insulin resistance as well as a β -cell secretory defect and can go undiagnosed for many years (Carley & Severson, 2008; Scheuermann-Freestone *et al.* 2003). The same factors that lead to MS is the cause of T2DM. Therefore T2DM is usually associated with the metabolic syndrome (Thim *et al.* 2006). Patients with this form of diabetes have: (i) concomitant hypertension or cardiovascular disease and (ii) limited exercise tolerance, which have been associated with decreased glycemic control and microvascular disease (Scheuermann-Freestone *et al.* 2003).

The pathogenesis of T2DM is described as multifactorial with both genetic and environmental contributions such as diet and physical activity. There is now substantiated scientific evidence suggesting that T2DM is strongly associated with visceral fat accumulation. Furthermore many patients with T2DM have either normal or elevated LDL-cholesterol levels or decreased HDL-cholesterol levels (Scheuermann-Freestone *et al.* 2003).

Statistics show that approximately 15% of all deaths in American patients with T2DM are ascribed to cardiac disease without notable symptoms of coronary artery disease (Christoffersen *et al.* 2007). T2DM affects about 250 million people worldwide and it is

estimated that by the year 2025, the prevalence of this disease will reach 380 million people (Hegarty *et al.* 2009). Epidemiological studies have shown that in diabetic populations, the incidence of myocardial infarction increases with subsequent heart failure. It is reported that diabetes presents as a coexisting condition in 20-35% of heart failure patients (Chandler *et al.* 2007). Furthermore, this heterogeneous disorder accounts for 90-95% of all diabetes cases (Scheuermann-Freestone *et al.* 2003). It has been shown that, T2DM patients with normal cardiac morphology and function, displayed impaired metabolism of high-energy phosphates, in both cardiac- and skeletal muscle (Scheuermann-Freestone *et al.* 2003).

The metabolic syndrome comprises of different components which include obesity, insulin resistance, glucose intolerance, dyslipidemia/hypercholesterolemia and hypertension (Caglayan *et al.* 2005). However, only the components relevant to this thesis will be discussed. The MS has a profound deleterious impact on the incidence and development of cardiovascular disease, through these above mentioned components.

1.2 Components of MS and its Impact on Cardiovascular Disease

1.2.1 Obesity

A person is traditionally classified as obese based on their body mass index (BMI) measurement, as set out by the world health organisation (WHO) standards/guidelines. A person with a BMI measurement ≥ 30 - $40\text{kg}/\text{m}^2$ (Class I-III) is classified as obese (See review by Rasouli & Kern, 2008). Obesity is a storage disorder, which occurs when there is an imbalance between calorie intake and -utilization over a period of time (Mathieu *et al.* 2008; Poirier *et al.* 2006). The ability of obesity to cause damage to the myocardium could be related to the selected metabolic pathway and fuel utilization, whether fatty acid oxidation or glucose oxidation (Diniz *et al.* 2006). Obesity is a heterogeneous condition with a complex etiology which is implicated to decrease life expectancy and is associated

with numerous medical complications such as cardiovascular disease (Mathieu *et al.* 2008; Lima-Leopoldo *et al.* 2008).

Obesity is associated with systemic inflammation during which adipocytes secrete numerous factors known as adipokines. This ultimately led to the classification of the adipose tissue as an endocrine organ and part of the innate immune system. Adipokines include: leptin, tumor necrosis alpha (TNF- α), adiponectin, resistin and visfatin. TNF- α is an adipokine that has been indicated as a possible mediator of insulin resistance with controversial effects on the heart (Xu *et al.* 2002, Torre-Amione *et al.* 1996; Jobe *et al.* 2009). The administration of TNF- α to rats causes insulin resistance and the neutralization of TNF- α *in vivo* reverses both hepatic and skeletal muscle insulin resistance (Lang *et al.* 1992; Borst *et al.* 2004; Borst & Conover, 2005). The above mentioned adipokines are considered important determinants of insulin resistance, via circulating hormonal effects or – local adipocytic effects and contributes to the regulation of cardiac metabolism and inflammatory responses (See review by Rasouli & Kern, 2008).

The adipose tissue derived macrophages also play an important role in obesity mediated systemic inflammation, by secreting cytokines such as IL-6 (Dandona *et al.* 2005). Obesity: (i) affects the cardiovascular system through its influence on certain risk factors (dyslipidemia, hypertension, and glucose intolerance) and (ii) is considered to be an independent risk factor for the development of cardiovascular disease (Poirier *et al.* 2006). Obesity is not just an independent risk factor for cardiovascular disease but is mostly associated with cardiovascular disease and even believed to cause cardiovascular disease (Poirier *et al.* 2006).

The incidence and development of cardiovascular disease (CVD) have been shown to be the result of elevated plasma LDL-cholesterol levels or decreased HDL-cholesterol levels. Under normal conditions the lipid balance is: high HDL and low LDL levels. However, this disturbance in the LDL: HDL ratio is caused by visceral obesity (Scheuermann-Freestone *et al.* 2003). Therefore visceral obesity is of great significance

in the development of cardiovascular disease (CVD). Other possible causes of CVD are (i) consumption of foods which are high in trans-fatty acids and saturated fatty acids as well as (ii) genetic predisposition (Guttmacher & Collins, 2003; Guize *et al.* 2008)

1.2.1.1 Importance of visceral obesity in cardiovascular disease

It has been shown that visceral obesity contributes to the development of coronary artery disease (Kobayashi *et al.* 2001). Visceral obesity is characterized by low high-density lipoprotein (HDL) cholesterol, high LDL cholesterol levels and low plasma adiponectin levels. In visceral adipose tissue there is a greater increase in the lipolytic response to noradrenalin (as indicated by the amount of FFA released) and this could be the reason for the resultant high LDL cholesterol and low HDL cholesterol levels. Furthermore it is believed that in the presence of visceral obesity adipocytes undergo certain changes which cause them to produce less adiponectin. Therefore reduced adiponectin levels are observed in visceral obesity (Kobayashi *et al.* 2001; Mathieu *et al.* 2008). In conclusion, visceral obesity contributes to the development of cardiovascular disease (CVD) by means of high plasma LDL cholesterol, low HDL cholesterol levels and low adiponectin levels. All of these factors promote the progression of arterial plaques, arteriosclerosis and thus CVD (Mathieu *et al.* 2008; Cefalu, 2008; Vague, 1956).

1.2.1.2 Obesity and cardiac remodeling

Obesity leads not only to increased adipose tissue depots but also to significant lipid accumulation in the heart. Thus with the occurrence of obesity, there are adaptations or alterations in the cardiac structure that include: (i) ventricular chamber dilation caused by an obesity induced shift in the Frank-Starling curve and a consequential increase in left ventricular filling pressure and -volume, (ii) left atrial enlargement, (iii) *adipositas cordis* or gradual fat accumulation within in heart muscle fibres, which cause myocyte degeneration and cardiac conduction defects and lastly (iv) left ventricular hypertrophy,

due to an increased demand on the heart to pump blood to systemic circulation because blood dependent tissue (adipose tissue) has increased (Poirier *et al.* 2006; Opie *et al.* 2006).

1.2.1.3 Obesity and cardiac function

With obesity, the deposition of fat can impair cardiac function in two ways: (i) by physical compression or secretion of various locally acting molecules by peri-organ fat cells and (ii) lipid accumulation that occurs in non adipose cells including myocytes which leads to cardiomyocyte dysfunction or cell death. This is a phenomenon of lipids having a toxic effect on the myocardium is known as cardiac lipotoxicity (Wilson *et al.* 2007). Research also showed that lipid accumulation in the myocardium may be due to the activation of genes involved in cardiomyocyte lipid metabolism (Christoffersen *et al.* 2007). The full extent of the effects of obesity on cardiac function has been widely researched and published (Wilson *et al.* 2007; Essop *et al.* 2009; Du Toit *et al.* 2008; Katakam *et al.* 2007; Morel *et al.* 2003; Kenchaiah *et al.* 2002). These research studies have shown evidence of obesity related cardiac dysfunction in experimental animal models of obesity.

In these rat models of obesity, it has been shown that the heart muscle contains more triglycerides compared to heart muscle from control animals and the obese animals display diastolic dysfunction on echocardiographic examination. Obesity is associated with eccentric left ventricular hypertrophy, which is associated with left heart dysfunction (Opie *et al.* 2006).

Wistar rats fed a western diet (which comprises 45% of calories from fats) developed obesity and consequential cardiac dysfunction after 8-12 months on the diet (Wilson *et al.* 2007). However in the same study, cardiac dysfunction was not observed in rats fed on a high fat diet (60% calories from fats) nor on a low fat diet (10% calories from fats) (Wilson *et al.* 2007). In a study done on pre-diabetic/obese rats (after being fed a high

caloric diet), assessment of mitochondrial function showed diminished ADP phosphorylation rates. This damaged mitochondrial function has been shown to be associated with cardiac dysfunction during obesity (Essop *et al.* 2009).

This evidence suggests that the decrease in mitochondrial capacity to produce energy in pre-diabetic rat hearts leads to impaired mitochondrial respiratory capacity. This causes a reduction in cardiac contractile function and increased cardiac susceptibility to ischemia/reperfusion injury in obesity (Essop *et al.* 2009).

1.2.1.4 Obesity and cardiac susceptibility to I/R-injury

Obesity increases myocardial susceptibility to ischemia/reperfusion injury in the isolated heart (Du Toit *et al.* 2007; Katakam *et al.* 2007; Morel *et al.* 2003). A study to determine the myocardial susceptibility to ischemic-reperfusion injury in a pre-diabetic model of diet-induced obesity showed that infarct size was greater in the absence of insulin and smaller in the presence of insulin in obese rats (Du Toit *et al.* 2008). Literature suggests that myocardial susceptibility to ischemia/reperfusion injury is associated with metabolic changes such as decreased myocardial glucose oxidation during ischemia (Gonsolin *et al.* 2007). Obesity thus plays a pivotal role in increased myocardial susceptibility to ischemia/reperfusion injury, by means of metabolic changes which leads to insufficient capacity of the heart to withstand severe oxygen deprivation (Poirier *et al.* 2006; Dandona *et al.* 2005; Du Toit *et al.* 2008).

1.2.2 Insulin resistance

Obesity is associated with MS, a syndrome which has already been shown to impact the cardiovascular system (Mathieu *et al.* 2008). Several studies have shown an association between obesity and insulin resistance and this largely contributes to the free fatty acid supply to the liver. This would then contribute to the development of insulin resistance

according to the portal hypothesis for the pathogenesis of insulin resistance (which suggests that insulin resistance is caused by increased delivery of free fatty acids (FFA) to the liver) (Kobayashi *et al.* 2001; Mathieu *et al.* 2008). The mechanism by which increased central adiposity causes hepatic insulin resistance is unclear. The "portal hypothesis" implicates increased lipolytic activity in the visceral fat and therefore increased delivery of FFA to the liver, ultimately leading to liver insulin resistance (Kabir *et al.* 2005).

Insulin resistance has been described as a state of reduced responsiveness, of insulin sensitive tissues, to the physiological concentration of insulin. It is associated with T2DM and defective pancreatic β -cell functioning that leads to reduced insulin mediated glucose uptake. It has been shown that insulin resistance in aging dog hearts (compared to younger activity-matched controls) is associated with disrupted mitochondrial integrity and diminished electron transport chain enzyme levels (See review by Sack, 2009).

1.2.2.1 Impact of insulin resistance

It is known that the progression from insulin resistance to type II diabetes mellitus is brought about by a whole cascade of molecular- and physiological processes (as illustrated by figure 2). These processes participate in the insulin signalling pathway and involve the phosphorylation and de-phosphorylation of downstream signalling proteins. During the state of insulin resistance, the heart continues to rely on fatty acids as its main source of energy. The reason is the up-regulation of enzymes involved in fatty acid oxidation and down-regulation of glycolytic enzymes, which is promoted by FFA stimulation of PPAR alpha (peroxisome proliferator activated receptor- alpha). This increased FFA stimulation/uptake/oxidation is only present with concomitant high plasma concentrations of free fatty acids which are associated with obesity (Thim *et al.* 2006; Hegarty *et al.* 2009). During ischemia there is a change in energy substrate utilization and energy metabolism i.e. a downregulation of fatty acid oxidation and an increase in glucose uptake and oxidation (See review by Beadle & Frenneaux, 2010).

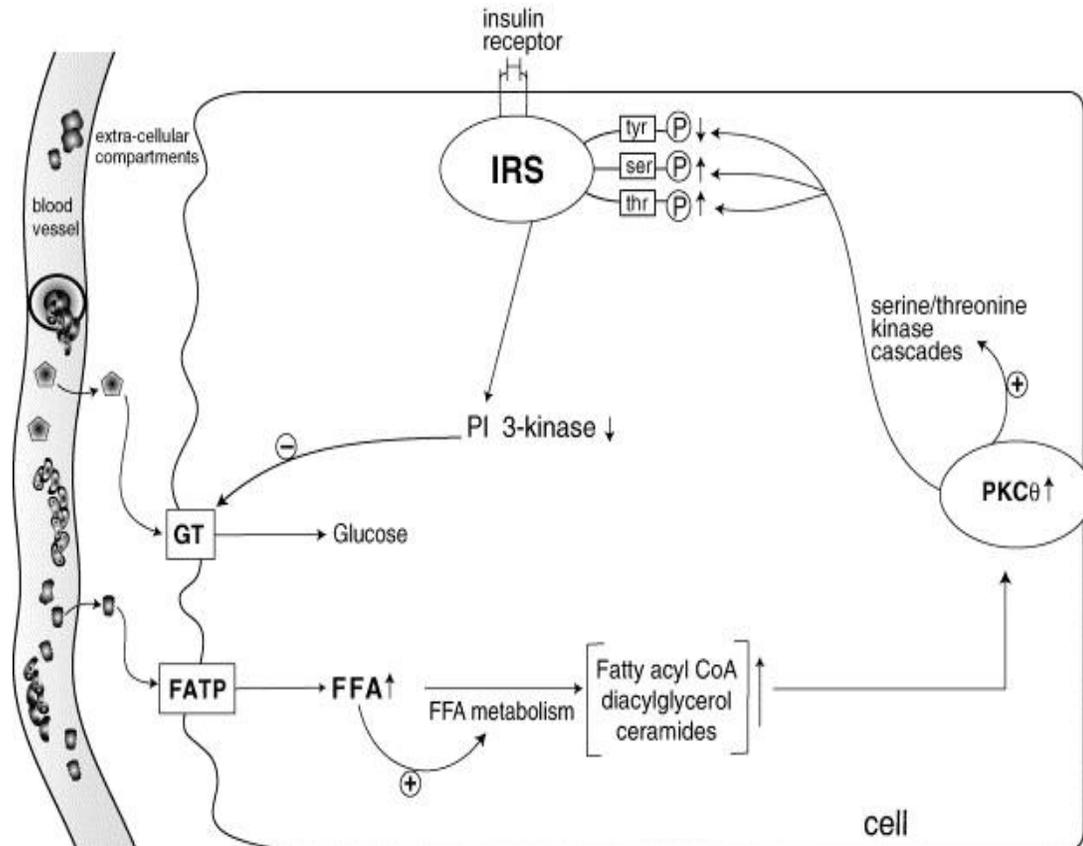


Fig.2: Etiology of Insulin Resistance and/or Pre-Diabetes. This figure is reproduced from Guo & Tabrizchi (2006)

Literature suggests that increased fatty acid availability causes increased fatty acid uptake and oxidation in the mitochondria and increased expression of mitochondrial uncoupling proteins (UCPs). These UCPs are transporters present in the mitochondrial inner membrane, which mediate a regulated discharge of the proton gradient generated by the electron transport chain (Ledesma, de Lacoba & Rial, 2002). The increased fatty acid oxidation and UCP expression decrease the amount of ATP produced per molecule of oxygen consumed in the mitochondrial electron transport chain. Therefore the insulin resistant heart has an increased oxygen requirement to produce equivalent amounts of ATP, a phenomenon known as oxygen wastage (Scheuermann-Freestone *et al.* 2003; Essop & Opie, 2004).

Free fatty acid uptake is determined by serum FFA levels. Increased FFA uptake increases the FFA concentration inside the mitochondrial matrix and promotes mitochondrial fatty acid oxidation (β -oxidation). (See review by Carley & Severson, 2005). There is a consequent accumulation of certain β -oxidation pathway intermediates (Such as ceramides, fatty acyl-CoA and diacyl-glycerol) due to disease induced suppression of the catalytic activity of specific enzymes in the β -oxidation pathway (See review by Opie & Knuuti, 2009). This is usually present together with increased levels of pro-inflammatory cytokines/proteins such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and C-reactive protein (CRP) (See review by Guo & Tabrizchi, 2006).

As a result, the expression and activity of protein kinase C- θ (PKC θ) increase which positively regulates the serine/threonine kinase cascades. Thus the phosphorylation state of the insulin receptor substrate (IRS) is changed. The threonine and serine putative binding sites are phosphorylated and as a result tyrosine phosphorylation decreases. This change in the IRS-phosphorylation state reduces the ability of the IRS to stimulate PI3-kinase (Phosphatidyl-Inositol-3-phosphate-kinase). The reduced PI3-kinase activity, ultimately suppresses the glucose uptake by preventing glucose transporter-4 (GLUT- 4) translocation to the cell membrane (myocytes, adipocytes). The end result is type II diabetes mellitus or pre-diabetes (Guo & Tabrizchi, 2006; McCarthy *et al.* 2005). The rationale is that this proposed mechanism of insulin resistance might also be true for the cardiomyocyte.

1.2.2.2 Insulin resistance and cardiac function

Insulin resistance is associated with increased cardiac fatty acid oxidation and a decrease in glucose utilization/oxidation (Lee *et al.* 2005; Aasum *et al.* 2008; Belke *et al.* 2000). During increased fatty acid oxidation, oxidative phosphorylation is uncoupled from the electron transport and the glucose-oxidation is suppressed via inhibition of the glycolytic pathway (Hue & Taegtmeyer, 2009). The inhibition of glucose-oxidation leads to the

accumulation of lactate and protons within cells. The result is a decrease in the intracellular pH (acidosis) which results in: (i) reduced contractile function, (ii) exacerbated ischemic injury and, (iii) decreased cardiac mechanical function during reperfusion (Dyck *et al.* 2004; Liu *et al.* 2002; Kantor *et al.* 2000; Lee *et al.* 2005; Hue & Taegtmeier, 2009). Thus it is clear that insulin resistance leads to reduced normoxic and reperfusion cardiac mechanical function.

A study that investigated the link between elevated circulating fatty acid concentration, the cardiac structure, and cardiac function in obese-insulin resistant rat models found that obesity/insulin resistance caused ectopic lipid accumulation in the myocardium, elevated circulating fatty acid levels and an increase in triglyceride content of the heart and these changes impaired cardiac systolic and diastolic function (Atkinson *et al.* 2003). The myocardial accumulation of fatty acids and metabolites is also associated with cell damage, suppression of the sarcoplasmic reticulum (SR) calcium pump function, suppression of myofibrillar ATPase activities and decrease in expression of myosin heavy chain isoforms (Stanley, Lopaschuk & McCormack, 1997).

In the heart, the two isoforms of the motor protein myosin heavy chain (MyHC) have been shown to be affected by a wide variety of pathological and physiological stimuli. Hearts that express the faster MyHC motor protein, α , produce more force than those expressing the slower MyHC motor protein, β , leading to the hypothesis that MyHC isoforms play a major role in the determination of cardiac contractility. Therefore MyHC isoform expression may therefore be important to maintain normal cardiac contractile function (Miyata *et al.* 2000).

During severe ischemia, the protons originating from hydrolysis of glycolytically derived ATP is the major contributor to acidosis (Calvani *et al.* 2000). Within the sarcomere, calcium-ions and protons compete for the binding sites on the troponin components of the sarcomeric contractile apparatus. This leads to perturbed contraction and thus a reduction in the myocardial contractile function (Stanley, Lopaschuk & McCormack, 1997). Another adverse effect of the fall in intra-cellular pH (acidosis), especially during

ischemia, is an increase in sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchange. A large pH gradient is created across the cell membrane. $\text{Na}^+/\text{Ca}^{2+}$ exchange is activated and increase intracellular Na^+ levels leads to intra-cellular calcium overload and eventually cell death (Liu *et al.* 2002). Data suggests that $\text{Na}^+/\text{Ca}^{2+}$ exchange is not completely inhibited during ischemia/hypoxia but rather functions in a "reverse mode" to exchange intracellular Na^+ for extracellular Ca^{2+} leading to increased $[\text{Ca}]_i^{2+}$ (Haigney *et al.* 1992; Liu *et al.* 1996a; Liu *et al.* 1996b)

1.2.2.3 Insulin resistance, metabolism and ischemic injury

Many mechanisms contribute to ischemia/reperfusion injury. However, scientific evidence suggests that with insulin resistance, contractile dysfunction during and after myocardial ischemia is partially mediated by changes in cardiac metabolism. This is reflected by severely increased rates of fatty acid oxidation (Lochner *et al.* 2004). Therefore insulin resistance affects cardiac metabolism by impairing glucose uptake and oxidation together with a resultant increase in fatty acid oxidation during ischemia (Guo & Tabrizchi, 2006). The impaired cardiac metabolism in insulin resistance increases the heart's susceptibility to ischemia/reperfusion injury (Taegtmeyer, 2000).

Cellular fatty acid concentrations increase 20-30 minutes after induction of ischemia. During ischemia and insulin resistance, this increase in cellular fatty acid concentrations, elevates fatty acid oxidation and consequently leads to ischemic injury due to enzymatic breakdown of membrane phospholipids and accumulation of toxic metabolites from increased fatty acid oxidation (Calvani *et al.* 2000; Opie 2004; Stanley, Lopaschuk, McCormack, 1997; Stanley, Recchia, Lopaschuk, 2005; Stanley, 2004). Furthermore, indirect evidence has suggested that increased cardiac insulin sensitivity was accompanied by increased resistance to ischemia/reperfusion injury (Yue *et al.* 2005). It is important to remember that myocardial ischemia/reperfusion injury, leads to structural changes in the myocardium, which is later followed by functional decline due to progressive fibrous replacement (See review by Modriansky & Gabrielova, 2009).

1.2.3 Dyslipidemia

1.2.3.1 Hypercholesterolemia, coronary artery disease and the myocardium

The typical lipid profile in MS displays dyslipidemia which includes hypertriglyceridemia (high levels of triglycerides), hypercholesterolemia or low HDL, high LDL and increased plasma fatty acid levels, which is due to increased free fatty acid release from the adipose tissue, secondary to insulin resistance (See review by Mooradian, 2009). All of the above characteristics are independent risk factors for coronary artery disease (CAD) and atherosclerosis. This lipid profile is further aggravated by high dietary intakes of saturated- and trans-fatty acids, which also tend to elevate blood plasma LDL-levels (Ginsberg *et al.* 2006; Fortino *et al.* 2007).

Dyslipidemia plays an important role in the development of atherosclerotic cardiovascular disease (ACVD) associated with the metabolic syndrome. There is a strong association between elevated LDL levels and the initiation- and progression of arterial plaques. Most of the cardiovascular risk associated with the metabolic syndrome is mediated by dyslipidemia (See reviews by Cefalu, 2008; Vague, 1956).

A high cholesterol diet has been associated with intracellular lipid accumulation in cardiomyocytes and several alterations in the structural and functional properties of the myocardium (Puskás *et al.* 2004). Hypercholesterolemia appears to attenuate the cardioprotective effect of ischemic preconditioning via an atherosclerosis independent mechanism (Ferdinandy *et al.* 1998). Recently it was shown that moderate hypercholesterolemia combined with a marked hypertriglyceridemia causes moderate contractile dysfunction in isolated rat hearts (O' nody *et al.* 2003). It also causes marked alterations in the expression of genes in functional gene clusters in the myocardium (Puskás *et al.* 2004). These results indicate that hyperlipidemia exerts complex effects on the myocardium and negatively affects cardiac function (Csont *et al.* 2007; O' nody *et al.* 2003).

1.2.3.2 Hypercholesterolemia and cardiac metabolism

The lipid accumulation caused by hypercholesterolemia might impair cardiac metabolism by promoting the uptake and oxidation of fatty acids and inhibiting glucose oxidation. This has been proven by studies which showed that animals fed a high cholesterol diet have increased fatty acid oxidation and decreased/inhibited glucose oxidation (Puskás *et al.* 2004; Lopaschuk *et al.* 2010). Therefore it is clear that hypercholesterolemia indirectly affects cardiac metabolism by increasing the circulation/uptake of free fatty acids, increasing beta oxidation and inhibiting glucose oxidation (Lopaschuk, Folmes & Stanley, 2007; Guo & Tabrizchi, 2006; Lopaschuk *et al.* 2010).

1.2.3.3 Hypercholesterolemia and myocardial susceptibility to I/R-injury

It has now become clear that hypercholesterolemia increases the risk of coronary artery disease, which has been associated with reduced tolerance to ischemia/reperfusion injury (Puskás *et al.* 2004, Lopaschuk, Folmes & Stanley, 2007; Lopaschuk *et al.* 2010). With hypercholesterolemia, small LDL particles enter the arterial wall proteoglycans more avidly and are extremely susceptible to oxidative modification (Carr & Brunzell, 2004; Noh *et al.* 2006; Franssen *et al.* 2008). Hyperlipidemia, which includes hypercholesterolemia, is often associated with oxidative or nitrosative stress in the myocardium and vasculature. Research has shown that when hypercholesterolemia is induced with a high cholesterol diet, there is an increase in the formation of reactive oxygen species (ROS) as well as peroxynitrite in the rat myocardium. For example, peroxynitrite is a product of a reaction between superoxide and nitric oxide (Csont *et al.* 2007; Franssen *et al.* 2008; Mozaffari & Schaffer, 2008). Peroxynitrite has been reported to induce DNA damage, increase lipid peroxidation, and to cause post-translational modification on proteins (e.g. nitration, oxidation of thiol groups), thereby activating (e.g. poly-ADP-ribose polymerase, matrix metalloproteinases) or inhibiting (e.g. aconitase, superoxide dismutase) certain enzymes. These cellular effects of peroxynitrite may contribute to the development of cardiac contractile dysfunction seen in hyperlipidemic

rats, however, the precise mechanisms leading to increased peroxynitrite remain to be investigated (Csont *et al.* 2007).

Therefore hypercholesterolemia is associated with increased production of reactive oxygen species (ROS), which is known to be a major contributor to reperfusion induced injury. Hypercholesterolemia thus increases myocardial susceptibility to ischemia/reperfusion injury, by promoting the formation/production of reactive oxygen species (ROS) and decreasing cardiac contractile function (Csont *et al.* 2007; Franssen *et al.* 2008; Mozaffari & Schaffer, 2008).

1.2.4. The impact of MS on mitochondria and mitochondrial function

The mitochondria's primary function is the production of energy in the form of ATP. This occurs during a process called respiration or oxidative phosphorylation which is an oxygen dependent process during which the energy substrate (pyruvate) is oxidized to acetyl-CoA. Acetyl-CoA enters the citric acid cycle (Krebs cycle), is oxidized to CO₂ and in the process reducing equivalents (NADH and FADH₂) are produced, which are a source of electrons for the electron transport chain (ETC) and guanosine tri-phosphate or GTP (which is readily converted to ATP) (See review by Gustafsson & Gottlieb, 2008).

It is thus clear that the mitochondria play a very important role in the cardiac energy metabolism, also in cardiomyocytes where its importance is displayed by their primary function, which is to facilitate oxidative phosphorylation for the generation of ATP (Sack, 2009). This is also reflected by the fact that: (i) the heart cell consists of at least 20% mitochondria by dry weight and furthermore (ii) cardiac muscle tissue has a very high mitochondrial content of 23% - 40% of the total volume (Lindenmeyer *et al.* 1968; Bugger & Abel, 2008; Sack, 2009).

Mitochondria do not only produce ATP, but also perform homeostatic functions i.e. oxidative metabolism, reactive oxygen species-(ROS) generation, utilization/breakdown

and intracellular calcium homeostasis (Sack, 2009). In MS there are profound abnormalities in heart mitochondria that lead to mitochondrial dysfunction (Bugger & Abel, 2008; Murray *et al.* 2006; Sharov *et al.* 1998).

1.2.4.1 Mitochondrial dysfunction in obesity

Mitochondrial dysfunction is brought about by uncoupling of mitochondrial respiration because of elevated plasma fatty acid levels (as substantiated by evidence pertaining to measurements of mitochondrial proton leak) (Sack, 2009). Two paradoxical hypotheses exist. The first hypothesis suggests that MS (specifically insulin resistance) causes mitochondrial dysfunction and the second hypothesis suggests that mitochondrial dysfunction leads to insulin resistance and the metabolic syndrome (Irving & Nair, 2007). However, the underlying mechanisms for mitochondrial dysfunction and insulin resistance have yet to be fully elucidated.

1.2.4.2 Impact of mitochondrial dysfunction

Obese (*ob/ob*) mice are insulin resistant, leptin deficient and euglycemic (with concomitant disruption of circulating glucose, fatty acid concentrations). They exhibit diminished glucose oxidation, increased mitochondrial fatty acid oxidation and increased mitochondrial oxygen consumption rates (QO_2). All these metabolic perturbations lead to diminished mitochondrial efficiency (uncoupling) and ultimately result in a reduced capacity of the heart to respond to increased cardiac load (Sack, 2009). MS-associated mitochondrial dysfunction causes a reduced capacity for energy production. This is believed to lead to secondary dysregulation of cellular processes which are important for cardiac pump function (including calcium handling and contractile function) and it results in an increased energy demand, diminished ATP production and impaired cardiac function (Huss & Kelly, 2005). Therefore it has been suggested that mitochondrial dysfunction may contribute to the impaired myocardial contractile ability in obese

animals. Furthermore it is known that damage to mitochondria can also be as a result of ischemia/reperfusion injury i.e. restoration of oxygen flow after a period of ischemia, which is known to be associated with an increase in ROS and intracellular calcium levels (Modriansky & Gabrielova, 2009; Essop, 2007; Yamada *et al.* 1994)

Increased plasma levels of non-esterified-fatty acids (NEFAs) in the MS might contribute to decreased phosphocreatine/ATP ratios by increasing expression/activity of uncoupling proteins (UCPs) (Taegtmeyer *et al.* 2008; Essop & Opie, 2004). This causes uncoupling of the mitochondria and thus mitochondrial dysfunction, which reduces ATP production (referring to membrane protective glycolytic ATP) and ultimately reduced cardiac contractility (Bugger & Abel, 2008; Sharov *et al.* 1998, Essop & Opie, 2004). It should be noted that mitochondrial UCPs are believed to be expressed as an adaptive mechanism/inherent protective mechanism and could have paradoxical functions. These functions include the induction of mitochondrial uncoupling and the export of fatty acids out of the mitochondria which might reduce cardiac lipotoxicity (Bugger & Abel, 2008; Opie & Knuuti, 2009). Another effect of mitochondrial uncoupling is “oxygen wastage”, since more oxygen is now needed to produce equivalent amounts of ATP (Essop & Opie, 2004). Furthermore the disruption in the mitochondrial function, associated with obesity, exacerbates and accentuates the pathophysiology of diabetes (Sack 2009; Yamada *et al.* 1994)

1.3 Ischemia and Reperfusion

1.3.1 Definition of ischemia and reperfusion

Ischemia, whether it is due to a pathological state or not, describes an inadequate blood flow/oxygen deprivation which is seen when arterial blood flow through a damaged blood vessel is reduced to a volume that does not meet the heart's requirements for adequate function (Jennings, 1970). Oxygen deprivation leads to certain metabolic changes in heart cells such as decreased adenosine triphosphate (ATP) levels, which causes a switch from aerobic- to anaerobic metabolism, with a simultaneous

accumulation of protons/decrease in intracellular pH (acidosis) (Jennings & Yellon, 1992).

It is generally agreed that the best treatment for the ischemic myocardium is the re-establishment of oxygen supply to the affected area as soon as possible. This is achieved by reperfusion. However, in addition to the beneficial effects of reperfusion, it can also cause further damage such as cell death/necrosis (Piper & Garcia-Dorado, 1999; Jennings & Reimer, 1983; Park & Lucchesi, 1999). Even though reperfusion is a paradoxical treatment for ischemia associated damage, this remains the only way to salvage reversibly damaged tissue. Thus ischemia and reperfusion is linked and is often referred to in the literature as ischemia/reperfusion.

During ischemia there is a decrease in blood supply which prohibits the removal of possible toxic metabolites. The ATP is insufficient to maintain ion-pump activity which causes disturbances in ion homeostasis (Opie, 2004). Damage during ischemia is caused by factors such as calcium overload, free radical formation and inflammatory processes (Piper & Garcia-Dorado, 1999; Park & Lucchesi, 1999; Opie, 2004; Van Vuuren, 2008).

1.3.2 Changes in the heart during ischemia and reperfusion

There is a range of changes that occur in the myocardium during ischemia and reperfusion. Below is a list of the well documented changes (Ganote & Humphrey, 1985; Jennings *et al.* 1978).

Ischemia and reperfusion can lead to:

- Depletion of energy stores (Jennings *et al.* 1978)
- Intracellular acidosis (Neely *et al.* 1984; Dyck *et al.* 2004; Liu *et al.* 2002)
- Accumulation of metabolic by-products (Guo & Tabrizchi, 2006; Corr, Gross & Sobel, 1984)

- Accumulation of intracellular calcium (Clusin *et al.* 1983; Nayler *et al.* 1988)
- Increased cytosolic sodium (Shen & Jennings, 1972; Tani & Neely, 1989)
- Loss of intracellular potassium (Opie *et al.* 1969)
- Mitochondrial damage (swelling, decreased matrix density, partial loss of cristae) (Jennings & Ganote, 1976; Schaper *et al.* 1979)
- Myofibril relaxation (Jennings & Ganote, 1976; Schaper *et al.* 1979)
- Swelling of the sarcoplasmic reticulum (Jennings & Ganote, 1976; Schaper *et al.* 1979)

The above mentioned changes lead to decreased cardiac function, decreased susceptibility to ischemia/reperfusion injury and decreased cardiac efficiency.

1.3.2.1 Overview of cardiac metabolism

In the heart there are two main metabolic pathways for the production of energy in the form of ATP, i.e. glycolysis and beta oxidation (Taegtmeyer, 2004). The heart has very high energy demands, which is related to the maintenance of cellular processes. These processes include: ion-transport, sarcomere function and intra-cellular calcium homeostasis (Wilson *et al.* 2007). Metabolism and the ability of the heart to contract are linked. The ATP producing pathways must therefore respond appropriately to the heart's physiological demands and the delivery of fuel (Taegtmeyer, 2000; Beadle & Frenneaux, 2010; Macfarlane, Forbes & Walker, 2008).

Glucose is stored in insulin sensitive organs such as the liver in the form of glycogen, by a process called glycogenesis. Glucose is transported into cells by glucose transporters known as GLUT 4 and GLUT 1 (Guo & Tabrizchi, 2006; Abel *et al.* 1999). When blood glucose is high, the pancreas secretes insulin which promotes GLUT 4 translocation to the cell membrane. Glucose is converted to glucose-6-phosphate by an enzyme called Hexokinase. From here glucose-6-phosphate is further metabolically modified by a host of enzymes in order to form pyruvate, (the end product) and energy in the form of ATP.

Pyruvate is oxidized by pyruvate dehydrogenase complex (PDHC) inside the mitochondrial matrix. This oxidative mechanism is set in place to degrade carbohydrates as a fuel in order to synthesize ATP and this is referred to as glucose oxidation. This process is completed in a complex series of steps involving the breakdown/formation of ATP, ADP, NAD^+ and NADPH. All these steps are catalysed by a number of enzymes i.e. hexokinase, glucokinase, phosphohexose-isomerase, phospho-fructokinase I, aldolase, triphosphate-isomerase, glyceraldehyde-3-P-hydrogenase (G3PDH), phospho-glyceride-kinase, enolase and pyruvate kinase. See figure 3.

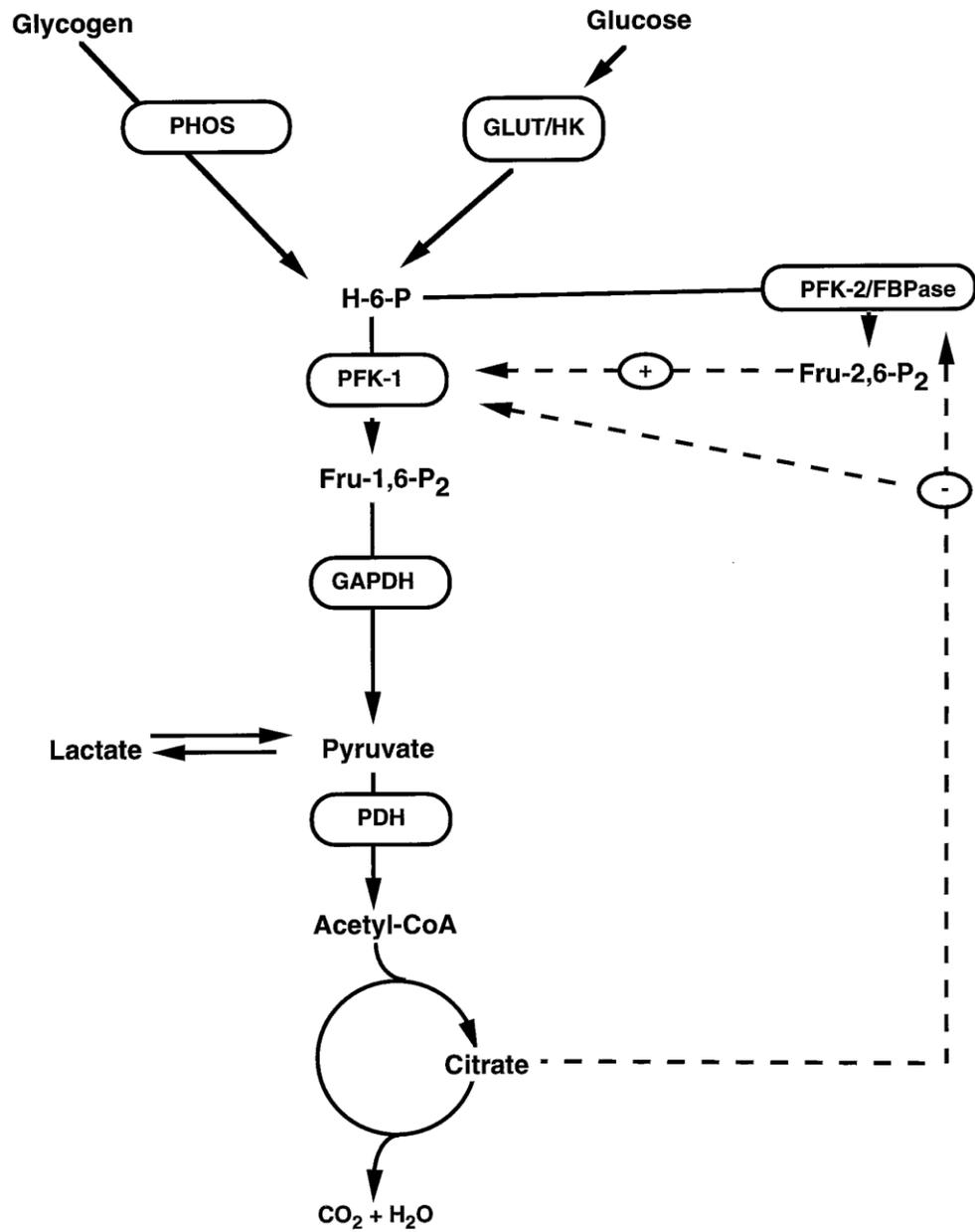


Fig. 3: A schematic representation of glycolysis (Depré, Rider & Hue, 1998)

Of β -oxidation and glycolysis, an important end product is acetyl-CoA which enters the tri-carboxylic acid cycle (TCA-cycle). Here the acetyl-CoA is reduced to reducing equivalents NADH and FADH_2 which function as carriers/donors of electrons to the electron transport chain (ETC). This produces an electro-chemical gradient across the mitochondrial membrane that drives ATP synthesis. The electro-chemical gradient is produced by pumping protons from the mitochondrial matrix to the intermembranal space. The bulk of the protons re-enter the matrix via the F_0F_1 -ATPase (not shown in figure 4). However, a proportion of the protons bypass the F_0F_1 -ATPase via the adenine nucleotide translocator (ANT) and this is known as the proton leak (ANT is not shown in the figure, only the proton leak). The end result of the ETC is the production of ATP in the presence of oxygen. See figure 4.

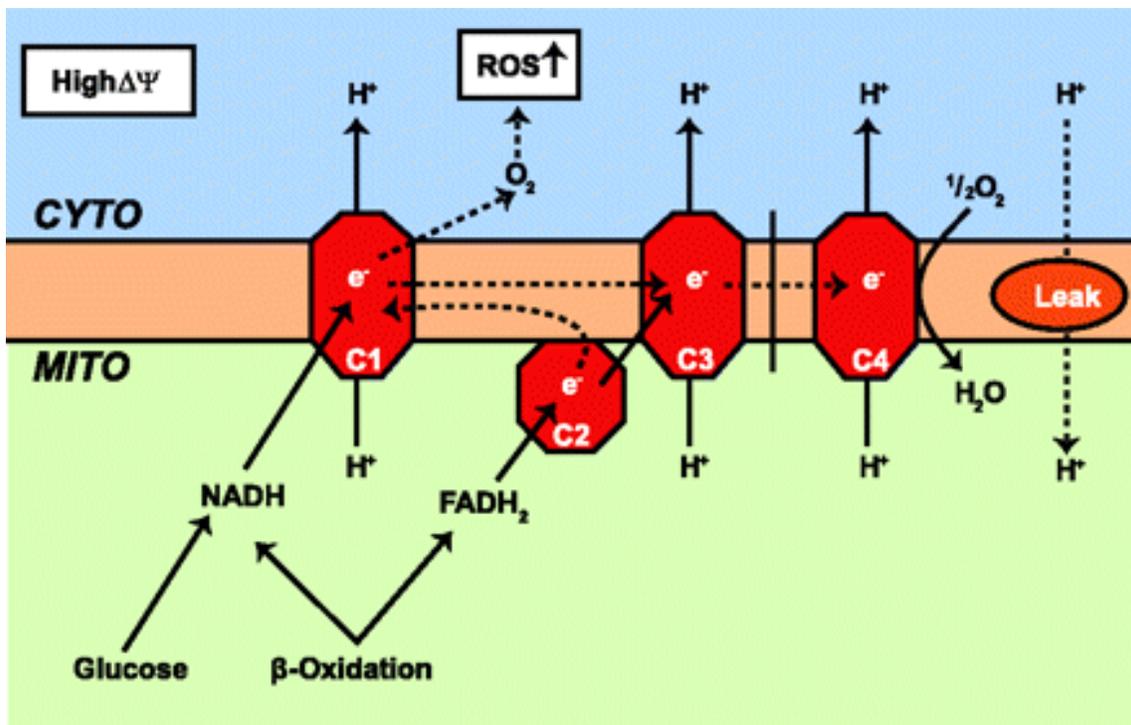


Fig.4: The Electron Transport Chain within the mitochondrion. Illustrated are the different complexes and intermediates (Hue & Taegtmeier, 2009, Essop & Opie, 2004).

These metabolic regulatory processes are time dependent. This involves regulation at various levels: (i) allosteric control of enzyme activity via metabolic intermediates, (ii) signal transduction events and (iii) regulation of genes encoding rate limiting enzymes and proteins.

The majority of the body's lipids are present in the form of triglycerides, free fatty acids, phospholipids and cholesterol. *In vivo*, there are two known sources of fatty acids for metabolism: (i) circulating fatty acids bound to plasma albumin which is derived from adipose tissue-lipolysis and (ii) fatty acids from the hydrolysis of triglyceride rich lipoproteins by an enzyme called lipoprotein-lipase, which is located on the surface of endothelial cells in the vasculature (Carley & Severson., 2005).

Triglycerides are broken down into glycerol and fatty acids by the process of lipolysis, which is activated during periods of fuel deprivation. The fatty acids are taken up into the cardiomyocytes by two transport processes i.e. simple diffusion and protein-mediated-transport (which accounts for 80% of total fatty acid uptake). The predominant sarcolemmal transport proteins are the 88-kDa fatty acid translocase/CD36 (FAT/CD36) and a 43-kDa plasma membrane fatty acid binding protein (FABPpm) (Carley & Severson, 2005).

Co-enzyme-A (CoA) esters are formed with the fatty acids in the cytosol in order to form a compound that is known as fatty acyl-CoA. There are three metabolic pathways that contribute to the production of acyl-CoA: (i) oxidation of fatty acids (derived from plasma) and the breakdown of intra cellular triacylglycerol, (ii) pyruvate oxidation (that originates from lactate dehydrogenase) and (iii) glycolytic activities (Calvani *et al.* 2000).

Fatty acyl-CoA is transported over the mitochondrial inner membrane after its conversion to fatty acyl-carnitine in the cytosol. This chemical reaction is catalysed by carnitine palmitoyl transferase-1 (CPT-1). Before the fatty acyl-carnitine is oxidized in the mitochondrial matrix, it is converted back to the fatty acid (without the carnitine), by carnitine palmitoyl transferase-2 (CPT-2). This enzyme catalyzes the reverse of the

reaction CPT- 1 catalyzes. Once the fatty acid is inside the mitochondrial matrix, β -oxidation occurs and it is further broken down through a number of steps, catalyzed by various enzymes. The end product of β -oxidation is acetyl-CoA and ATP (see figure 5).

When a long chain fatty acid such as palmitic acid (which contains 16-carbon atoms) is degraded by the β -oxidation pathway, it yields approximately 8 molecules of acetyl-CoA or \pm 117 molecules of ATP.

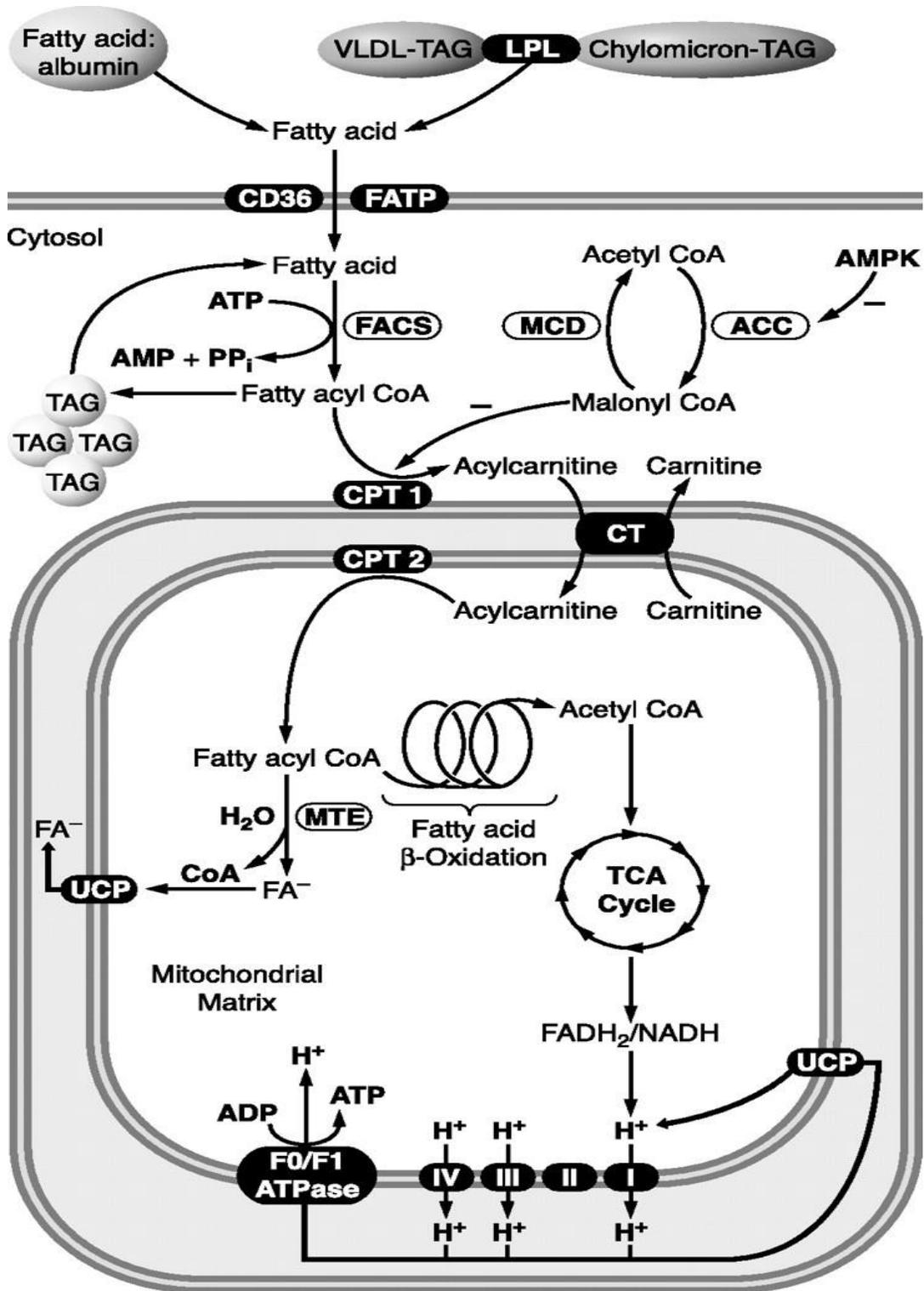


Fig.5: Overview of the β -oxidation pathway reactions. This figure illustrates the range of reactions in the beta oxidation pathway (Lopaschuk *et al.* 2010).

Intracellular control of fatty acid oxidation:

Under normal circumstances, the balance between the utilization/oxidation of the different fuels, for the purpose of energy production, is tightly regulated in the heart. The selection of fuels solely depends on the heart energy requirements, which is most probably determined by the expression of protein coding genes, involved in cardiac energy metabolism (Wang *et al.* 2005). The consumption of fatty acids depends on the availability of fatty acids to the myocardium and the intracellular control of fatty acid uptake/metabolism (Lopaschuk, Folmes & Stanley, 2007). Intracellular control of fatty acid oxidation takes place at the level of mitochondrial uptake of fatty acids by carnitine palmitoyl transferase-1 (CPT-1). When CPT-1 activity decreases (as would be the case during inhibition by malonyl-CoA), then long chain acyl-CoA can be redirected towards complex lipid synthesis e.g. triacylglycerol, phospholipids diacylglycerol etc. Furthermore, malonyl-CoA is another important inhibitor of fatty acid oxidation in the heart (Lopaschuk, Folmes & Stanley, 2007; Beadle & Frenneaux, 2010).

Oxidation of fatty acids is subjected to complex control mechanisms and dependent on a range of factors, including: (i) supply of fatty acids to the heart, (ii) presence of competing energy substrates such as glucose, lactate, ketones and amino acids, (iii) energy demand of the heart, (iv) supply of oxygen to the heart, (v) allosteric control of fatty acid uptake, esterification, and mitochondrial transport; and ultimately, (vi) the control of mitochondrial function, including direct control of fatty acid oxidation, TCA cycle activity, and electron transport chain (ETC) activity (Lopaschuk *et al.* 2010; Beadle & Frenneaux, 2010).

Another important fact to note about fatty acids is that they are ligands for peroxisome proliferators-activated receptors (PPARs) and are thus regulators of gene expression. Genes that encode for proteins which control myocardial fatty acid uptake and metabolism includes PPAR- α , which is regulator of the expression of proteins involved in fatty acid uptake and –oxidation (Lopaschuk, Folmes & Stanley, 2007; Guo & Tabrizchi, 2006).

PPAR- α , has been extensively studied, and its target genes are those which encode proteins that are involved in: (i) the uptake of fatty acids (FAT/CD36, FATP1), (ii) cytosolic fatty acid binding and -esterification (FABPm), (iii) glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase, (iv) malonyl CoA metabolism (MCD), (v) fatty acid uptake (CPT 1), (vi) fatty acid oxidation [very-long-chain acyl CoA dehydrogenase, long-chain acyl CoA dehydrogenase, medium-chain acyl CoA dehydrogenase (MCAD), 3-KAT], (vii) mitochondrial uncoupling [including mitochondrial thi-esterases (MTE-1) and uncoupling proteins (UCP2, UCP3)], and (viii) glucose oxidation [PDH kinase (PDK) 4] (Lopaschuk *et al.* 2010).

1.3.2.2 Metabolism of the obese heart/insulin resistant heart

As previously mentioned, the state of obesity/insulin resistance, is characterized by elevated plasma FFA (free fatty acid) levels which increases FFA uptake, increase cellular FFA concentrations and increase beta oxidation (Guo & Tabrizchi, 2006; Aasum *et al.* 2008, Lopaschuk, Folmes & Stanley, 2007). Data suggest that an increased supply of free fatty acids negatively influences myocardial function in hearts subjected to ischemia/reperfusion (Aasum & Larson, 1997; Lopaschuk *et al.* 2010; see review by Allard, 2004). The negative influence is due to the obesity induced impairment of cardiac metabolism by i) increased beta oxidation and ii) intracellular accumulation of potentially toxic fatty acid intermediates such as fatty acyl-CoA, fatty acyl carnitine as well as iii) the oxygen wasting effect of free fatty acids (Vik-Mo & Mjøs, 1981; Corr, Gross & Sobel, 1984). According to Liedtke *et al.* (1988) excess free fatty acid lead impair membrane integrity, organelle sub-performance and myocardial contractility.

In addition, elevated fatty acid levels during obesity/insulin resistance impair glucose metabolism, specifically glucose oxidation via inhibition of the pyruvate dehydrogenase (PDH) complex. This leads to uncoupling of glycolysis from glucose oxidation (Lopaschuk, Wambolt & Barr, 1993; Aasum & Larson, 1997). High fatty acid levels and the resultant increased fatty acid-oxidation: (i) strongly inhibits the mitochondrial enzyme, pyruvate dehydrogenase, the oxidation of the glycolytic pathway-intermediate,

pyruvate, and thus the general inhibition of the whole glycolytic pathway, (ii) uncouples oxidative phosphorylation from electron transport (this causes the inefficient use of energy stores, especially fatty acids) (Modriansky & Gabrielova, 2009) and (iii) consumes more oxygen than glucose metabolism. These adverse effects may result in impaired cardiac contractile function and arrhythmias during ischemia (Stanley, Lopaschuk & McCormack, 1997). Therefore, there is a definite link between the increased rates of β -oxidation and the deleterious effects on the myocardium as a whole, in the state of obesity/insulin resistance and during ischemia/reperfusion (Bugger & Abel, 2008; Lopaschuk, Wambolt & Barr, 1993; Stanley, 2004; Stanley, 2002; Stanley, Lopaschuk, McCormack, 1997; Aasum *et al.* 2008).

From the literature it is clear that the negative effects of elevated free fatty acids are not necessarily correlated with their increasing effects the inhibition of potentially toxic fatty acid intermediate accumulation. Instead, the negative effects may be related to myocardial glucose oxidation/utilization. Therefore it is suggested that an imbalance between glycolysis and glucose oxidation could be the possible mechanism of the detrimental effects of excess free fatty acid levels (Lopaschuk, Wambolt & Barr, 1993).

Cardiac energy metabolism is regulated by (i) availability of exogenous substrates, (ii) hormones such as insulin, (iii) cardiac work/energy demand and (iv) the supply of oxygen (Carley & Severson, 2005). Irrespective of whether glucose or fatty acids are used to produce ATP, there is a difference in the amount of ATP produced as well as in the amount of oxygen consumed.

Fatty acids are less oxygen efficient fuels that consume more oxygen for each ATP molecule produced, in comparison to glucose. There is a theoretical 12%-14% increase in efficiency of ATP production in shifting from 100% palmitate oxidation to 100% glucose oxidation (Wang *et al.* 2005). Fatty acids may be a more energy efficient fuel (7.3 ATP=CO₂ produced vs. 6.3 ATP=CO₂ produced) but glucose is a more oxygen efficient fuel (5.5 ATP/O₂ consumed vs. 6.3 ATP=O₂ consumed) (Wolff *et al.* 2002). See table 1 below. Glycolysis can generate ATP in the absence of oxygen, a situation of reduced

oxygen availability which resembles ischemia. Thus, theoretically the obese/insulin resistant heart should increase glucose oxidation, especially during ischemia. If the heart uses glucose as major fuel during ischemia, it will function better. However, the heart preferentially oxidizes fatty acids during insulin resistance, which is one of the earliest changes that occur after the onset of experimentally induced diabetes/insulin resistance (Wang *et al.* 2005). This choice of fuel by the heart has deleterious effects on the myocardium (Lopaschuk, Folmes & Stanley, 2007). The following table displays the exact ratios of ATP produced in comparison with oxygen consumed. It clearly highlights the fact that the oxidation of glucose uses less oxygen when compared to fatty acids and is therefore a better choice of fuel, particularly under anoxic/ischemic conditions.

Table 1: The comparison of complete oxidation of glucose and a fatty acid of equivalent carbon chain length (hexanoic acid). The theoretical ATP yields assume perfect coupling of substrate oxidation to oxidative phosphorylation of ADP. In this table it is important to note the differences in ATP production and oxygen efficiency of glucose and fatty acids (hexanoic acid) (Wolff *et al.* 2002).

	Glucose (C ₆ H ₁₂ O ₆)	Hexanoic Acid (C ₆ H ₁₂ O ₂)
Oxidation Equation	C ₆ H ₁₂ O ₆ → 6 O ₂ 6 CO ₂ → 6H ₂ O	C ₆ H ₁₂ O ₂ → 8 O ₂ 6 CO ₂ → 6H ₂ O
ATP Yield	38 ATP	44 ATP
Energy Efficiency	6.3 ATP=CO ₂ produced	7.3 ATP=CO ₂ produced
Oxygen Efficiency	5.5 ATP=O ₂ consumed	6.3 ATP=O ₂ consumed

1.3.2.3 Metabolism of the normal/ischemic heart

Carbohydrate metabolism in ischemia: In normal hearts

Ischemia has a biphasic effect on carbohydrate metabolism. Initially glycolysis is stimulated and then the glycolytic rate decreases as ischemia becomes more severe and then inhibitory metabolites accumulate. During severe ischemia there is an accumulation of the products of glycolysis (lactate and protons). This accumulation leads to a decrease in the pH (acidosis) which increases acetyl-CoA and NADH levels, inhibits pyruvate dehydrogenase (PDH) and ultimately inhibits the glycolytic flux. Therefore, during severe ischemia, the lack of washout of the inhibitory metabolites suppresses glycolysis (Opie, 1991; Opie & Knuuti, 2009).

Fatty acid metabolism during ischemia: In normal hearts

Ischemia inhibits fatty acid metabolism and leads to the accumulation of lipid metabolites such as fatty acyl-CoA, diacylglycerol and ceramides (Guo & Tabrichi, 2006; Beadle & Frenneaux, 2010). These metabolites inhibit the mitochondrial fatty acid translocase, sodium pump and phospholipid cycles. In addition to the inhibited metabolism, membrane phospholipids are broken down by the action of phospholipases, which are activated by an accumulation of calcium (Opie, 1991; Opie & Knuuti, 2009).

1.3.3.4 Metabolism of the obese/ischemic heart

In the obese state, the circulation and uptake of free fatty acids are elevated. However there is an imbalance between fatty acid uptake and the rate of fatty acid oxidation which is believed to cause accumulation of lipids in the myocardium. In other words, more fatty acids are taken up than what is oxidized (Lopaschuk, Wambolt & Barr, 1993; Lopaschuk & Barr, 1997; Lopaschuk, 1997; Lopaschuk & Kelly, 2008; Lopaschuk *et al.* 2010).

Lipid accumulation causes myocyte degeneration and cardiac conduction defects (Poirier *et al.* 2006). Thus the lipids that accumulate exert toxic effects on the myocardium; known as cardiac lipotoxicity (Opie & Knuuti, 2009). The high rates of fatty acid oxidation and the resultant lipid accumulation inhibit glucose oxidation. This results in mitochondrial uncoupling and diminished ATP production (Bugger & Abel, 2008). When the heart from an obese animal is exposed to ischemic circumstances; the increase in fatty acid metabolism predisposes the heart to ischemic damage. The elevation of fatty acid oxidation and the inhibition of glucose oxidation have deleterious effects on the obese heart during ischemia i.e. reduced contractile function, exacerbated ischemic injury and decreased cardiac efficiency during reperfusion (Lopaschuk, Folmes & Stanley, 2007; Opie & Knuuti, 2009; Shipp, Opie 1991).

1.3.2.5 Signaling pathways and proteins/enzymes involved in metabolism

Overview of key enzymes and proteins

Increased oxidation of fatty acids in obesity/insulin resistance, especially during ischemic conditions has harmful effects on the heart. From the literature it is evident that inhibition of fatty acid oxidation can be employed to protect the obese/insulin resistant heart during ischemia (Opie & Knuuti, 2009). When an inhibitor of fatty acid oxidation is administered, the ultimate goal is to inhibit or suppress β -oxidation by inhibiting carnitine palmitoyl transferase-1 (CPT- 1), thus preventing acyl moiety uptake and promoting the use of glucose as a substrate to produce energy.

The inhibition of β -oxidation of free fatty acids exposes the roles of a variety of key enzymes that participate in the β -oxidation pathway i.e. CPT- 1 and medium chain acyl-CoA-dehydrogenase (MCAD). In addition I will also discuss important proteins i.e. insulin receptor substrate-1 (IRS-1) and glucose transporter-4 (GLUT- 4).

CPT 1 and MCAD

The transport of long chain fatty acids from the cytosol to the mitochondrial matrix destined for β -oxidation is initiated by the conversion of fatty acids to acyl-carnitines by CPT-1. This enzyme (i) forms an integral part of the outer mitochondrial membrane, (ii) is known to catalyze the rate limiting step in fatty acid oxidation (catalyzes the synthesis of long chain acyl-carnitine from length corresponding long chain acyl-CoA) (Calvani *et al.* 2000) and (iii) is furthermore tightly regulated by its physiological inhibitor, malonyl-CoA (first intermediate in fatty acid synthesis). There are two isoforms of CPT-1 expressed in mammalian tissue i.e. liver isoform (LCPT-1) and heart/muscle isoform (MCPT-1) (Woldegiogis *et al.* 2000).

Under normal circumstances, ACC (acetyl-CoA carboxylase) carboxylizes acetyl-CoA (from energy substrate oxidation) to form malonyl-CoA, which is a natural inhibitor of CPT-1. It is known that CPT-1 is a rate limiting enzyme in the β -oxidation pathway. Oxfenicine inhibits CPT-1 and thus the β -oxidation pathway. MCAD (Medium Chain Acyl-CoA Dehydrogenase) is also a key enzyme in the β -oxidation pathway and therefore the inhibition of CPT-1 will also lead to the inhibition of MCAD (Barger & Kelly, 1999). A change in the expression of these proteins would provide valuable information regarding the effects of oxfenicine on cardiac energy metabolism in control and obese animals, subjected to chronic oxfenicine treatment. See figure 6.

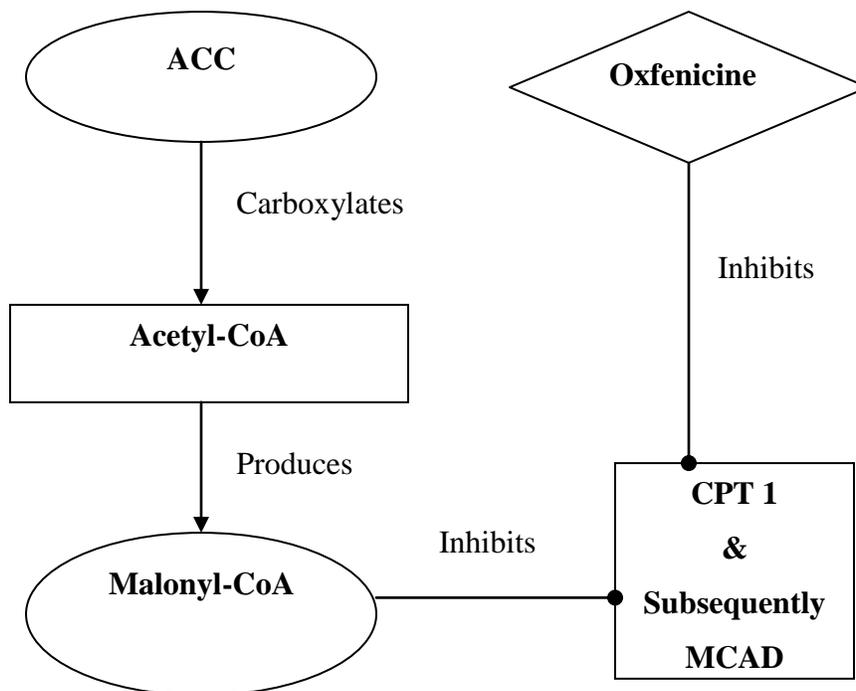


Fig.6: CPT-1 portrayed as a rate limiting enzyme and this figures illustrates how it is affected by the natural inhibitor malonyl-CoA.

IRS -1:

Insulin receptor substrates (IRS-1, -2, -3, and -4) form part of family of docking proteins that function as an interface between the insulin receptor and the rest of the insulin signalling pathway. IRS-1 is of interest due to the fact that it is the major protein involved in the binding and activation of PI3-K (Phospho-Inositol-3-Kinase) (Guo & Tabrizchi, 2006). IRS-1 is the main site for the development of free fatty acid induced insulin resistance (pre-diabetes) due to its inactivation caused by a change in its phosphorylation state. In insulin resistant individuals with high circulating levels of free fatty acids, IRS-1 activity is inhibited due to inappropriate phosphorylation of its phosphorylation sites i.e. increase in the phosphorylation of Threonine (Thr) and Serine (Ser) sites and a decrease in the phosphorylation of Tyrosine (Tyr) sites (Guo & Tabrizchi, 2006).

IRS-1 has three putative binding sites (Ser 270, Ser 374 and Ser 641). IRS-1(Ser 641) being is known to be highly phosphorylated in a state of obesity/insulin resistance and with elevated levels of circulating free fatty acids. This suppresses activation of PI3-Kinase and consequential GLUT- 4 translocation is lost, which leads to insulin resistance and pre-diabetes. Inhibition of β -oxidation (by pharmacological intervention) and the resultant increase in glucose oxidation should theoretically promote the IRS-1 (Ser 641) phosphorylation (Guo & Tabrizchi, 2006).

GLUT- 4:

This protein is the major mechanism of glucose uptake in the mammalian cell (skeletal muscle cells & adipocytes). It is directly involved in glucose homeostasis and glucose uptake in the heart, in response to the stimulation of the insulin receptor (Guo & Tabrizchi, 2006). Under normal conditions, GLUT- 4 resides in intracellular vesicles and is translocated to the plasma membrane in response to an insulin signal, exercise and ischemia (Murray *et al.* 2006).

During a state of free fatty acid induced insulin resistance, circulating insulin levels may very well be high, but due to erroneous phosphorylation of IRS-1, PI3-K is not activated and thus GLUT- 4 translocation to the plasma membrane is perturbed and ultimately decreased. The suppression of GLUT- 4 translocation decreases glucose uptake and thus elevates blood glucose levels (known as a state of pre-diabetes).

With the GLUT- 4 translocation assessment, a decrease in GLUT- 4 translocation reflects a decrease in glucose uptake, as in the case of insulin resistance/obesity (Guo & Tabrizchi, 2006).

1.3.3 Interventions used to protect the heart against I/R-injury

Interventions that alter cardiac energy metabolism and improve cardiac mechanical function should improve myocardial tolerance to ischemia/reperfusion and therefore protect against ischemia/reperfusion injury. This is based on previous observations which showed that elevated rates of fatty acid oxidation are associated with decreased cardiac mechanical function in insulin resistant rats during ischemia/reperfusion. The decrease in mechanical function is especially observed during periods of anoxia/ischemia (Hafstad *et al.* 2007). This leads us to believe that elevated rates of fatty acid oxidation during ischemia cause this whole cluster of deleterious effects on the heart in obesity. This concept is what guided scientists to focus their efforts on compounds that are able to “switch” energy substrate utilization away from fatty acid oxidation towards glucose-oxidation (Hafstad *et al.* 2007; Taegtmeyer *et al.* 2005).

Table 2: Methods used to “switch” cardiac metabolism away from fatty acid oxidation towards glucose oxidation (Rupp, Zarain-Herzberg & Maisch, 2002).

Direct Approaches	Indirect Approaches	Alternative Approaches
<u>Aim:</u> To decrease circulating levels of fatty acids	<u>Aim:</u> To increase glucose - oxidation	<u>Aim:</u> To directly inhibit fatty acid oxidation
By Administering (Rx): <ul style="list-style-type: none"> - Glucose-Insulin-Solutions - Nicotinic Acid - Beta-Adrenergic blocking agents 	By Administering (Rx): <ul style="list-style-type: none"> - Sodium-Dichloro Acetate 	By Administering (Rx): <p><u>CPT-1 Inhibitors</u></p> <ul style="list-style-type: none"> - Oxfenicine - Etomoxir - Perhexiline - ACCβ - Malonyl-CoA <p><u>3-KAT Inhibitors</u></p> <ul style="list-style-type: none"> - Trimetazidine - Ranolazine

The aims of these interventions (in Table 2) are to decrease circulating free fatty acid levels, increase glucose oxidation and inhibit fatty acid oxidation, respectively. With the direct approach, a compound like nicotinic acid has an anti lipolytic effect and suppresses circulating fatty acid levels. With the indirect approach, sodium-dichloro acetate inhibits pyruvate dehydrogenase kinase (PDHK), activates pyruvate dehydrogenase complex (PDHC) and thus increases glucose oxidation. With the alternative approach, compounds are used to inhibit different enzymes in the β -oxidation pathway. Enzymes manipulated include CPT-1 and 3-KAT or CAT (Keto-Acyl Transferase/Ceto-Acyl Transferase).

1.3.3.1 Reperfusion injury salvage kinases (RISK)

Myocardial cells possess prosurvival signaling pathways, which protect the myocardium against ischemia/reperfusion-induced injury. These pathways (including the RISK pathway) are activated at reperfusion and include proteins such as PI3-K, PKB/Akt, P70/S6K and ERK (42/44) (Hausenloy & Yellon, 2004). It would be useful to investigate how oxfenicine treatment before an ischemic event, influences the RISK pathway activity or whether the RISK pathway is at all involved in the oxfenicine induced cardiac effects.

The MAPK kinase family (or mitogen-activated serine/threonine kinases) is comprised of extracellular activated signal-regulated kinase (ERK), p38 MAP kinase, and c-Jun N-terminal kinase (JNK) (Kennedy *et al.* 2000). Also, various trophic factors (i.e., insulin-like growth factor-1) activate protein kinase-B (PKB) (Cicconi *et al.* 2003). The RISK pathway, especially ERK and PKB, is activated during reperfusion. This is an anti-apoptotic pathway and thus confers cardioprotection against ischemia/reperfusion injury when activated (Hausenloy & Yellon, 2004).

1.3.3.2 Glucose-Insulin-Potassium solutions (GIK)

The benefits of enhanced glucose provision by means of GIK administration include increased energy production, reduced loss of K^+ ions, inhibition of changes in the membrane action potential, altered extracellular volume and decreased circulating levels of free fatty acids. The above mentioned benefits cause a reduced incidence of arrhythmias, reduced ischemic contracture and improved recovery of cardiac function (King & Opie, 1998). It has been shown that glucose and/or insulin protects the isolated rat heart from fatty acid-induced ischemic damage, when it is present from the start of coronary ligation (Apstein & Opie, 2005; Lopaschuk *et al.* 2010).

1.3.3.3 Dichloro Acetate (DCA)

DCA treatment is one approach to prevent lactate accumulation in the myocardium from obese animals (Wolff *et al.* 2002; Clarke, Wyatt & McCormack, 1996). DCA promotes myocardial glucose oxidation at the expense of myocardial fatty acid oxidation and appears to be relevant in the therapeutic management of angina pectoris (Lopaschuk *et al.* 2010).

1.3.3.4 Interventions aimed at decreasing/inhibiting FFA metabolism

The aim of this approach is to promote the increase of glucose-oxidation by directly inhibiting or suppressing fatty acid oxidation. Recent studies have indicated that the manipulation of myocardial metabolism may be a valuable tool in the improvement of functional outcomes of patho-physiological/stressful conditions such as an ischemic insult (Aasum, Hafstad & Larson, 2003).

The increase in glucose oxidation and lactate uptake/metabolism is achieved by (i) decreasing citrate levels and the inhibition of phospho-fructo-kinase (PFK) and lastly

also by (ii) lowering acetyl-CoA and/or NADH levels in the mitochondrial matrix, thereby lowering the inhibition of pyruvate dehydrogenase complex (PDHC) (Aasum, Hafstad & Larson, 2003; Lopaschuk *et al.* 2010).

In the laboratory and clinical setting the inhibition of fatty acid oxidation can be achieved by a number of chemical compounds, which achieve their effect by acting on various enzymes within the β -oxidation pathway. These include: carnitine palmitoyl transferase-I (CPT-1), which is partially inhibited by oxfenicine (4-hydroxy-L-phenylglycine) (Rupp, Zarain-Herzberg & Maisch, 2002).

Research interest in oxfenicine started in the early 1980's when it was found to be effective in decreasing chest pain in patients with obstructive coronary artery disease and stable angina. Oxfenicine also protected hearts from necrotic tissue damage during ischemia (Stephens, Higgins & Harris, 1985).

4-Hydroxy-L-phenylglycine is transaminated in the heart and liver to 4-hydroxy-phenylglyoxylate (HPG), which acts at the level of CPT-1. It therefore inhibits palmitoyl carnitine formation from palmitate (Stephens, Higgins & Harris, 1985). It achieves its inhibitory effect by its intermediate 4-hydroxy-phenyl-glyoxylate (HPG) and does this in a pH dependent manner, analogous to malonyl-CoA (Korb *et al.* 1984; Stephens, Higgins & Harris, 1985). This leads to a decrease in NADH/NAD⁺ and Acyl-CoA/CoA ratios, causing secondary activation of pyruvate dehydrogenase with resultant stimulation of glucose utilization (Higgins, Morville & Burges, 1981).

There are, to date, only two mechanisms that have been identified by which oxfenicine induced inhibition of fatty acid oxidation is achieved i.e. (i) by greater sensitivity of heart CPT-1, than liver CPT-1 to HPG inhibition and (ii) by greater oxfenicine aminotransferase activity in heart than in the liver (Stephens, Higgins & Harris, 1985).

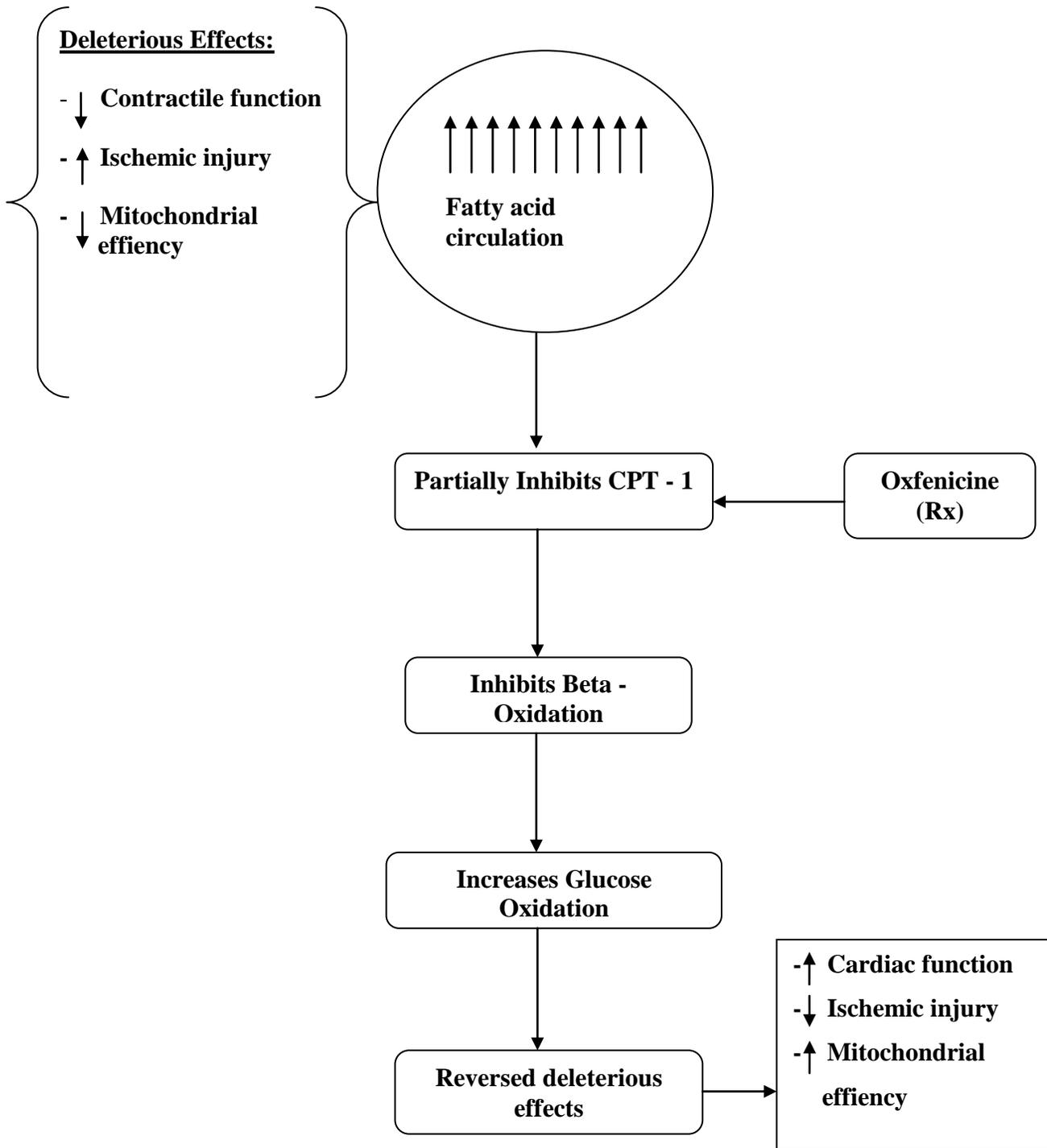


Fig.7: Schematic representation of the proposed mode of action of CPT-1 in the heart of obese animals

1.4 Hypothesis & Aims

We hypothesize that chronic oxfenicine (4-Hydroxy-L-phenylglycine) treatment of obese animals may improve cardiac mechanical function and myocardial tolerance to ischemia/reperfusion injury.

To test this hypothesis we aim to investigate the effects of chronic oxfenicine treatment on:

1. Basal cardiac mechanical function
2. Myocardial tolerance to ischemia/reperfusion as assessed by documenting reperfusion function and myocardial infarct size
3. Mitochondrial respiration in isolated mitochondria
4. The RISK pathway function and activity (ERK42/44 and PKB/Akt)
5. CPT-I, MCAD and GLUT- 4 expression and IRS-1 activation.

CHAPTER 2

MATERIAL AND METHODS

2.1 Experimental groups

For this study, male Wistar rats were used. Rats were housed in the University of Stellenbosch Central Research Facility, under optimal conditions i.e. constant temperature (22°C) and humidity (40%). Throughout the study, the South African Medical Research Council's Guide for Humane Use of Laboratory Animals was followed. Ethical clearance was obtained from the Committee for Experimental Animal Research (CEAR) of the Faculty of Health Sciences, University of Stellenbosch. Ethical clearance number P08/02/002. When the rats reached a weight of 200±5 grams they were randomly divided into a control- and an experimental diet fed (DIO) groups.

Groups for the different experiments were:

Heart perfusion experiments:

For this series of experiments we had four experimental groups (10 rats per group): Control group, DIO group, control + oxfenicine, DIO + oxfenicine.

Infarct size determination experiments:

For this series of experiments we had four experimental groups (10 rats per group): Control group, DIO group, control + oxfenicine, DIO + oxfenicine.

Western blot analysis experiments:

For this series of experiments we had four experimental groups (6 rats per group): Control group, DIO group, control + oxfenicine, DIO + oxfenicine.

Mitochondrial function experiments:

This division yielded four experimental groups (6 rats per group): Control group, DIO group, control + oxfenicine, DIO + oxfenicine. Glutamate experiments were done in

duplicate. Palmitate experiments were performed using 3 rats per group and not in duplicate.

2.2 Rat diets used

The standard rat chow diet (SRC- diet) consisted of: 60% carbohydrate, 30% protein and 10% fat. The high caloric diet (HCD) consisted of: 65% carbohydrate, 19% protein, 16% fat. The control rats were fed a standard rat chow diet, while the DIO group received a high caloric diet (Pickavance *et al.* 1999). The feeding program continued for a period of 16 weeks. The food consumption was measured daily but animals were allowed to feed *ad libitum*. The average daily energy consumption for the SRC and HCD fed animals were as follows:

<u>Energy consumption:</u>	<u>SRC- diet</u>	<u>HC- diet</u>
Food consumption (FC)	29g/day	57g/day
Energy consumption (EC)	13.1kJ/g	10.1kJ/g
Energy consumption/day	379.9kJ	575.7kJ

2.3 Drug administration:

Oxfenicine (Aldrich Sigma Chemicals) was administered in the drinking water at a dosage of 200mg/kg/day (Okere *et al.* 2007). This drug supplementation was initiated from the beginning of week 9 of the feeding program. Rats were allowed to drink/eat *ad libitum*. Therefore, in order to calculate the correct dose of drug in the water in relation to the animals' increased bodyweights and water consumption, we calculated the average bodyweights and water consumption per group weekly. The drug concentration in the water was then determined for the average bodyweight for the animals, taking into consideration the amount of water they drank.

Calculation of oxfenicine dose in 2 litres to obtain 200mg oxfenicine/kg/day:

$$\text{Dose (g/2L)} = \left[(200\text{mg (dose)} \times \text{Ave}_{\text{weight}} \text{ (kg)}) \times 2000 \text{ (ml)} / \text{Ave}_{\text{water consumption}} \text{ (ml)} \right] / 1000$$

2.4. Experimental protocols

2.4.1 Experimental protocol - Part 1

The rats were on the diet for 16 weeks and oxfenicine given to SRC and HCD fed animals for the last 8 weeks of the feeding regime. After that, the animals were sacrificed and a range of experiments were performed. The first series of animals were used to determine body weights and to collect retro peritoneal (RP) fat which was weighed to get the RP weights. This same series of rat hearts were excised and used for the assessment of mitochondrial respiration with the oxygraph. A second series of rats were perfused on the Langendorff perfusion system and used to determine cardiac mechanical function after 40 minutes of regional ischemia and infarct size were determined after two hours of reperfusion. The heart parameters (heart rate and left ventricular developing pressure) were measured during stabilization and during reperfusion. The average of these measurements was used to calculate the percentage cardiac functional recovery during reperfusion, after which infarct size was determined using TTC staining (this procedure is explained in the section 2.5.2. of *experimental procedures*). For further reference, see figure 1 below.

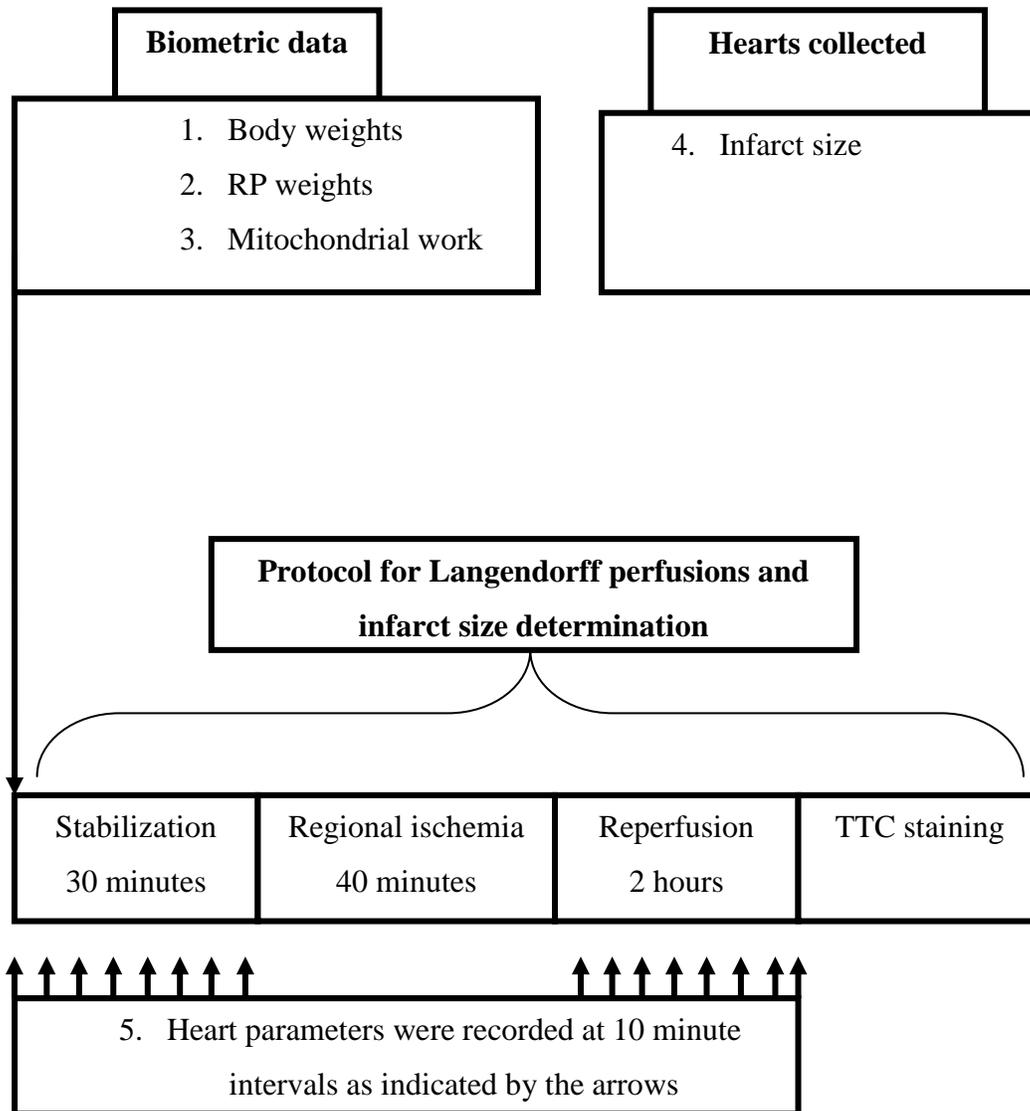


Fig. 1: Outline of the steps followed for the measurement of body weights, retro-peritoneal fat weights, assessment of mitochondrial respiration and measurement of mechanical function as well as the determination of infarct size.

2.4.2 Experimental protocol - Part 2

A third series of rats were used to determine cardiac mechanical function on the Langendorff perfusion system, after 25 minutes of global ischemia and these hearts were

freeze clamped after 30 minutes of reperfusion for Western blot analysis (this procedure is explained in the section 2.5.4. of *experimental procedures*). The heart parameters (heart rate and left ventricular developing pressure) were measured during stabilization and during reperfusion. These measurements were used to calculate the percentage cardiac functional recovery. For a detailed outline, see figure 2.

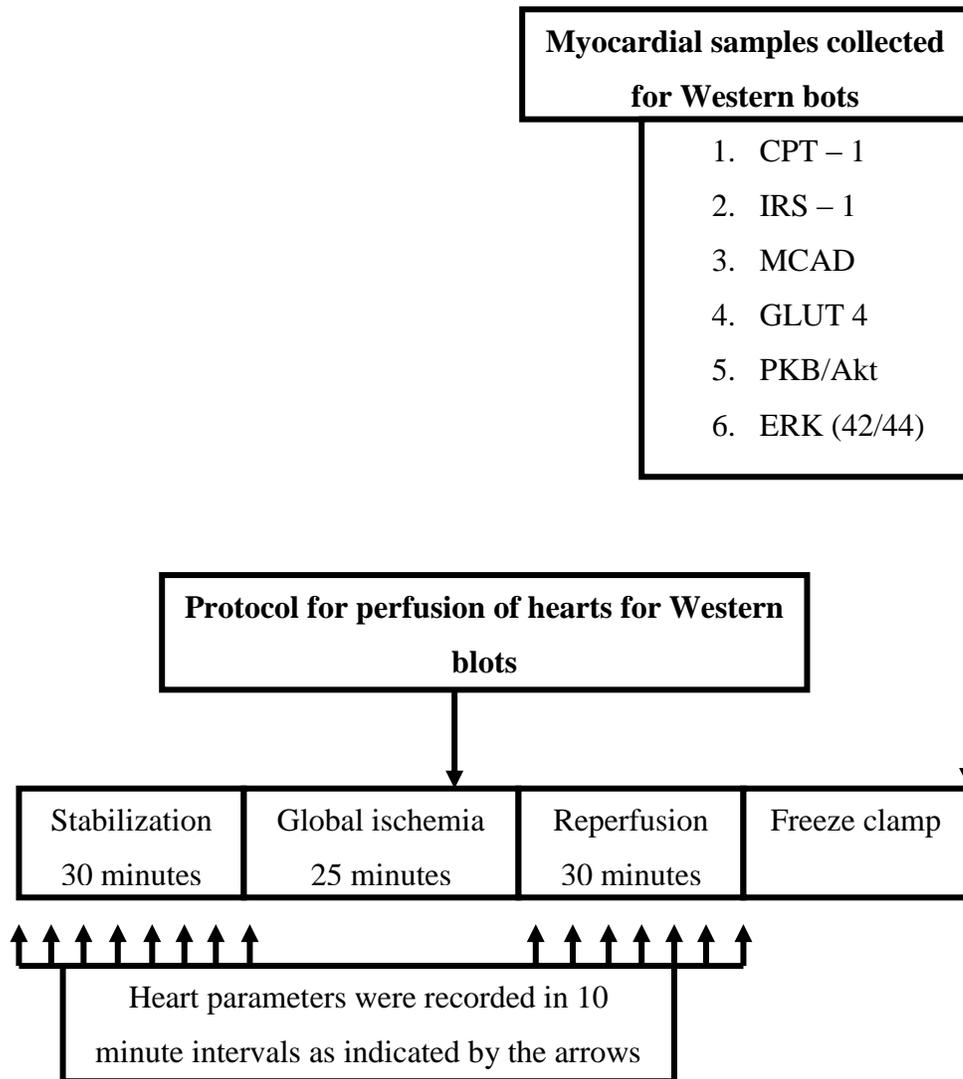


Fig. 2: Outline of the steps followed for the measurement mechanical function and myocardial sample collection for western blot analysis.

2.5 Experimental procedures

2.5.1 Langendorff rat heart perfusion

The rats were euthanized with an intraperitoneal injection of sodium pentobarbitone (injected 0.4ml/0.5kg ~ 160mg/kg). A thoractomy was performed and the hearts were excised. The hearts were then placed in ice cold Krebs Henseleit buffer. Composition of the KH buffer solution: 118.46mM NaCl, 24.995mM NaHCO₃⁻, 4.748mM KCL, 1.185mM KH₂PO₄⁻, 1.19mM MgSO₄.7H₂O, 1.25mM CaCl₂.2H₂O (11mmol glucose, 1.2mmol palmitate and 30µIU/ml insulin). The rat hearts were mounted on the Langendorff perfusion system and perfused for a stabilization period of 30 minutes. A balloon was inserted into the left ventricle. This balloon was connected to a computerized data acquisition system [Gentronics chart recorder, version 1.00, Tygerberg, South Africa] which recorded the left ventricular systolic, left ventricular diastolic pressure, left ventricular developing pressure (LVDP) and heart rate (HR). These functional parameters were used to determine the rate pressure product (RPP = HR x LVDP) which is an index of mechanical heart function. In order to obtain the cardiac functional recovery, the RPP after ischemia was divided by the RPP before ischemia times by 100%.

2.5.2 Infarct size determination

The infarct size determination was only done on the hearts that were subjected to 40 minutes of regional ischemia and 2 hours reperfusion.

After the heart was excised and mounted on the Langendorff perfusion apparatus. All hearts were then allowed to stabilize for at least 30 minutes. The proximal portion of the left anterior descending (LAD) coronary artery was first localized between the left atrial appendage and the right ventricular outflow tract. The coronary artery was ligated/occluded using a 6-0 polypropylene suture around the major trunk of the LAD coronary artery or its prominent branches. The ends of the thread were passed through a

small piece of PE50 tube to form a snare. Ischemia was induced by pulling the snare tight and then fixing it by clamping the tubing with a small hemostat. This achieved coronary artery occlusion. Ischemia was confirmed by regional cyanosis, a substantial decrease in left ventricular developed pressure (LVDP) and a 40% fall in coronary flow. Ischemia was induced for 40 minutes. Reperfusion was initiated by releasing the snare and thus opening the occluded coronary artery. Hearts developing ventricular fibrillation (VF) usually spontaneously reverted to sinus rhythm. VF lasting more than 45 sec was treated with finger flick cardioversion until a normal sinus rhythm was obtained. No pharmacological agents were used for defibrillation. The hearts were then reperfused for two hours after which the silk suture was securely tied around the coronary artery and 0.5% Evans blue solution (5mℓ) was slowly infused via the aorta in order to stain the hearts. The hearts were then placed in a freezer and frozen overnight.

Hearts were then cut into \pm 2mm slices. One heart yields about 5 slices that were then stained by incubation in 1% w/v 2, 3, 5 – triphenyl tetrazolium chloride salt (TTC). Principle of this procedure: The damaged myocardial cells in the infarcted area release an enzyme. TTC binds to this enzyme and causes a red/pink colour to develop. Finally, formaldehyde is added to stop all chemical reactions. The viable area has a blue colour, the infarcted area a white colour and the area at risk has a red/pink colour. The infarct size were determined by planimetry and expressed as a % of the area at risk. During planimetry, the different areas are traced with an ink marker pen onto a transparent sheet, supported by a perspex board. The transparent sheet was then scanned in and a computer program (UTHSCSA Image Tool) was used to determine the infarct size, expressed as a % of the area at risk.

2.5.3 Isolation of mitochondria

Hearts were excised from respective experimental groups (Controls, DIO, Control + oxfen and DIO + oxfen). The atria were cut off and the hearts were placed in tubes containing 5°C KE isolation medium which consisted of: 0.18M KCL, 0.001M EDTA

and 2M Tris to achieve pH-7.4. While in the KE isolation medium the hearts were cut in small pieces and washed 4-5 times with isolation medium to remove all traces of blood. The sample was homogenized using a polytron tissue homogenizer for 2 times 4 seconds at a speed of 4. This was done on ice. The sample was then centrifuged for 10 minutes at 755 x g (2500rpm). The pellet (containing fat and cellular debris) was separated from the supernatant and the supernatant was centrifuged for 10 minutes at 18800 x g (12500rpm). The supernatant was now decanted and this time the pellet (containing the mitochondria) was loosened from the bottom of the tube and resuspended in 500µl of KE isolation medium. The resuspended pellet was pipetted into a glass teflon homogenizer (Potter Elvehjem) and partly homogenized. The refined pellet is pipetted into an Eppendorff; this contained the isolated mitochondria and was placed on ice. From this suspension of isolated mitochondria, 50µl was pipetted in 1ml of 1% TCA for later mitochondrial protein determination using the Lowry method (Lowry *et al.* 1951). The rest of the isolated mitochondria were used for the oxygraph experiments (Lanza & Nair, 2009; Lindenmeyer, Sordahl & Schwartz, 1968).

2.5.3.1 Assessment of mitochondrial function: Isolated mitochondria

For the purpose of oxygraph experiments the mitochondria were isolated from fresh rat heart tissue. 600µl Incubation medium (which contained 1.25M Sucrose, 100mM Tris-HCl, 85mM K₂HPO₄, 50mM Glutamate or Palmitoyl-L-Carnitine, 20mM Malate) was added in the cell of the oxygraph (Hansatech Instruments (2006) version 2.1). Prior to experiments the oxygraph was calibrated. The calibration procedure was as follows: The electrode of the oxygraph was cleaned with rapid electrode disc polish and a cotton bud. A drop of 50% KCl was placed on the electrode and a small piece of each of the membrane and cigarette paper (Rizla quality papers) was placed on the drop of KCl. The membrane and cigarette paper was tightly set in place by using an O-ring and a plunger. The excess membrane and cigarette paper was cut off and the larger black rubber ring was set in place around the electrode. The oxygraph cell chamber was closed by turning the outside cover of the cell. Water was placed in the cell chamber with a pipette (600µl).

This chamber was connected to a water bath with the temperature set to 25°C. The water (600µl) in the chamber was removed and 600µl of incubation medium was pipetted into the cell. The computer features are activated (stirrer speed, graph zoom and axes & temperature). A signal was recorded on the computer screen and once a signal plateau was reached, Na₂S₂O₄ (oxygen scavenger) was added. Once the oxygen signal was zero the calibration was completed and saved. The cell chamber was washed with distilled water. This washing step was repeated 4-5 times. After this, 600µl of incubation medium was pipetted into the cell and allowed to reach 25°C. Once the desired temperature was reached, the experiment was started by pressing “go” and a straight line (signal) was observed and allowed to run for 1 minute.

The mitochondria (100µl) were pipetted into the cell of the oxygraph and once an active mitochondrial respiring state was confirmed, 50µl of 1mM ADP is added and state 3 respiration (ADP converted to ATP) was recorded on a connected computer (PC). The last state, referred to as state 4 respiration (substrate being used up and about to reach plateau), was then recorded after a few seconds. Thereafter, 100µl of 25mM ADP was added and the cell of the oxygraph is closed in order to induce a state of anoxia (25 minutes). After anoxia, the mitochondria were re-oxygenated and the recovery of state 3 was recorded and calculated. During the progress of the experiment, different endpoints were investigated i.e. ADP/O (ADP to oxygen ratio), RCI (respiratory control index), QO₂ (rate of oxygen consumption) as well as the percentage recovery of state 3 (Lindenmeyer, Sordahl & Schwartz, 1968).

2.5.3.2. Calculation of mitochondrial respiratory parameters:

1. **ADP: O ratio** = nmoles ADP / total O₂ uptake (state 3)
2. **RCI** = Rate of oxygen uptake (state 3) / Rate of oxygen uptake (state 4)
3. **QO₂** = Oxygen uptake/mg mitochondrial protein
4. **ADP phosphorylation rate** = nmoles ADP/1 minute/mg mitochondrial protein
5. **% Recovery of state 3** = state 3 (after re-oxygenation) / state 3 (before anoxia) x

100

2.5.3.3. Lowry protein determination protocol: to determine mitochondrial proteins

Preparation of the Lowry:

We took 50µl of the mitochondria suspension and added 1ml 10% TCA. This was allowed to stand on ice for at least 3 minutes or over night in the fridge/cold room. Then the sample was centrifuged at 250rpm for 15 minutes. The supernatant was decanted and 500µl NaOH (1N) was added. This mixture was heated in a water bath at 70°C in order to denature the proteins. The sample was vortexed and 500µl H₂O was added and thoroughly vortexed. After these steps the sample was ready for protein determination. 50µl of the sample and appropriate standards were placed in Lucham tubes and 50µl 0.5N NaOH was added in another tube (blank) [experiment was done in triplicate]. Thereafter 1ml NaK-tartrate-CuSO₄ was added in every tube with time intervals of 10 seconds. Each tube was vortexed after every addition. After ten minutes (measured from the first addition) 100µl Folin-C was added with the same time intervals of 10 seconds. After the addition of Folin-C, the contents of the tubes changed colour. After 30 minutes the optical density was read at 750nm against the blank.

2.5.4 Western blot analysis

Hearts used for western blot protein analysis

A separate series of animals were prepared for tissue sample collection. The hearts were excised and placed in a container with ~ 4°C of Krebs Henseleit solution. Within ~ 1 minute the heart was mounted on the perfusion apparatus via the aortic cannula. The heart was retrogradely perfused for 30 minutes then subjected to 25 minutes of global ischemia and 30 minutes of reperfusion. On completion of these perfusions, the hearts were freeze clamped with Wollenburger tongs, pre-cooled in liquid nitrogen and stored at -80°C.

A general Western blot protocol

Myocardial tissue was collected as described above. Lysates were made from the hearts and added to tubes containing 700 μ l of the lysis buffer. The hearts were then homogenized (using a Polytron homogenizer) in lysis buffer. The contents of this buffer were: 20mM Tris-HCL (pH- 7.5), 1mM EGTA, 1mM EDTA, 150mM sodium chloride (NaCl), 1mM β -glycerophosphate, 2.5mM tetrasodiumpyrophosphate, 10mM sodium vanadate (NaVO₃), 100mM phenylmethylsulphonylfluoride (PMSF), 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1% Triton X-100. After homogenization, the samples were centrifuged at 1000g for 10 minutes. The supernatant was then separated from the pellet, the pellet was decanted and the supernatant was used for further experimentation.

Protein extraction:

A Bradford protein determination assay (Bradford, 1976) was done. Microsoft Office Excel was used to draw a standard curve. From this curve the protein content (μ g) of each of the samples was determined. The samples were diluted with lysis buffer into a final protein concentration that was equal in amount and volume. A final mixture was made of sample and Laemmli sample buffer in a 2:1 ratio.

Protein separation:

The sample (consisting of the heart in lysis buffer and Laemmli sample buffer) was boiled for 5 minutes and stored at -20°C. The same amount (μ g) of protein (depending on the protein of interest) in the same volume (μ l) of lysate was loaded onto a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS – PAGE) gel (the % gel differs from experiment to experiment).

The samples were loaded in a 4% stacking polyacrylamide gel and separated on gels ranging from 7.5%-12% (depending on the protein of interest). The gels were allowed to run (using the standard Bio-Rad Mini-Protean II System) and proteins were transferred to

a polyvinylidene fluoride (PVDF) membrane (Immobilon™ P, Millipore). This transfer process was done in a tank electro transfer set up. In this tank, the membrane-gel stack is immersed in transfer buffer, through which an electrical current is applied. This buffer contained: 25mM Tris, 192mM Glycine and 20% Methanol. Equal loading was verified by Ponceau's red staining and Beta-tubulin.

Table 3: Details for proteins that were investigated by western blot analysis: protein name, type, size, casting gel, staining gel and loading volume.

Protein	Size	Type	Casting gel	Stacking gel	Load (µl)
CPT-1	75 kDa	H-20-rabbit polyclonal antibody	10%	4%	9µl (60µg/prot)
IRS-1	180 kDa	Ser 641-rabbit polyclonal antibody	8%	4%	9µl (80µg/prot)
MCAD	45 kDa	F-15-goat polyclonal antibody	12%	4%	9µl (60µg/prot)
ERK 42/44	42/44 kDa	Thr202/Tyr204-rabbit polyclonal antibody	12%	4%	9µl (60µg/prot)
PKB/Akt	60 kDa	Ser473-rabbit polyclonal antibody	10%	4%	9µl (50µg/prot)
GLUT-4	55 kDa	H-61-rabbit polyclonal antibody	10%	4%	9µl (50µg/prot)
Beta Tubulin	55kDa	rabbit polyclonal antibody	10%	4%	9µl (50-80µg/prot)

Blocking of membranes/secondary antibody:

The membranes were blocked with 5% fat free milk for 2 hours and washed with 0.1 % TBS–Tween. The primary antibody was diluted (1:1000) in TBS – Tween/milk and allowed to bind to the membrane over night (or at least 5 hours). The following morning, the membranes were washed with 0.1 % TBS–Tween and the secondary antibody was added (at room temperature) for an hour. Secondary antibody was an ECL anti-rabbit (or anti-goat depending on the primary antibody) immunoglobulin G, Horseradish peroxidase-linked (from donkey) secondary antibody (Amersham), diluted in TBS-Tween (milk) solution (1:4000). After an hour, the excess/unbound secondary antibody was washed off and the membrane was covered with ECL™ detection reagents for 1 minute, then exposed to an autoradiography film (Hyper film ECL, RPN 2103). Specific signals were detected by Chemiluminescence and captured on the radiography film. Finally the reaction was quantified by laser densitometry (UNSCAN – IT™ version 5.1, Silk science). For comparison between groups, samples from negative control hearts were included in each blot and used for normalization of the unknown samples (i.e. calculation of the ratio between the sample and negative control). Data is expressed in arbitrary units (AU).

2.6 Statistical Analysis

A two way ANOVA was done, followed by a Bonferroni post hoc test. A *t*-test or a one way ANOVA was done where applicable. A $p < 0.05$ was seen as statistically significantly different. All the results are expressed as the mean \pm SEM.

CHAPTER 3 RESULTS

3.1 Biometric-, functional- and infarct size data after 16 weeks on the feeding program

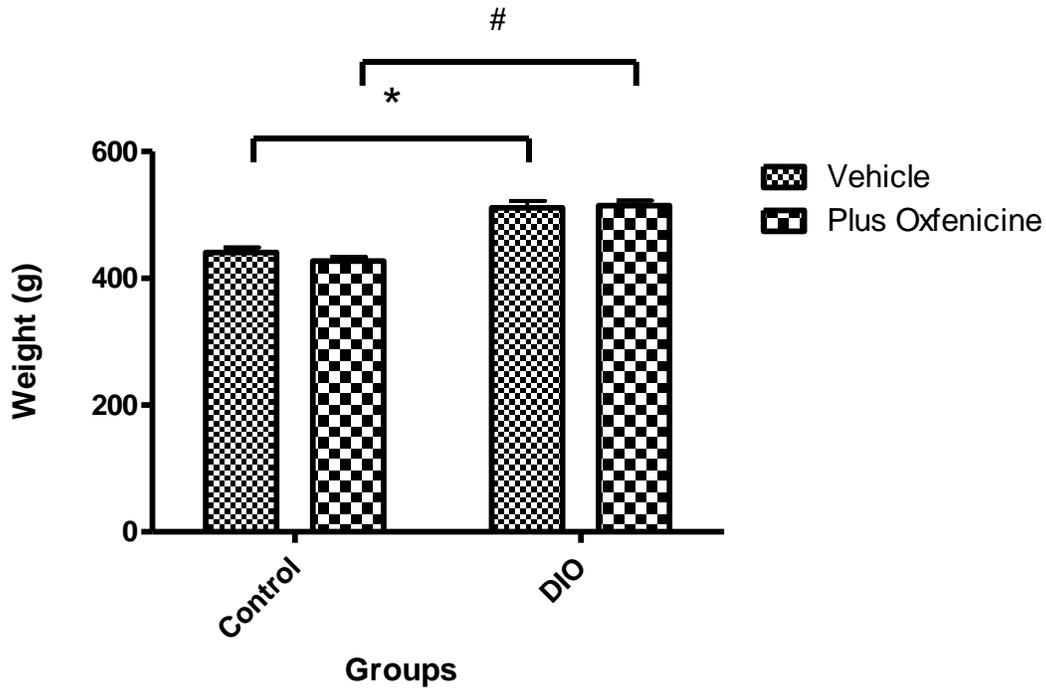


Fig. 3.1.1: The body weights of 16 week control- and high caloric diet fed (DIO) rats, with and without oxfenicine treatment. All values are expressed as the mean \pm SEM. Sample size (n) = 10. *P < 0.0004 and #P < 0.0001.

This data showed that there were significantly higher body weights in DIO group vs. control group ($511.30 \pm 10.86\text{g}$ vs. $440.50 \pm 8.07\text{g}$). This is a clear indication that the high caloric diet did have an effect on the body weights. The DIO + oxfen group also had significantly higher body weights than control + oxfen group ($514.80 \pm 7.73\text{g}$ vs. $427.30 \pm 6.61\text{g}$).

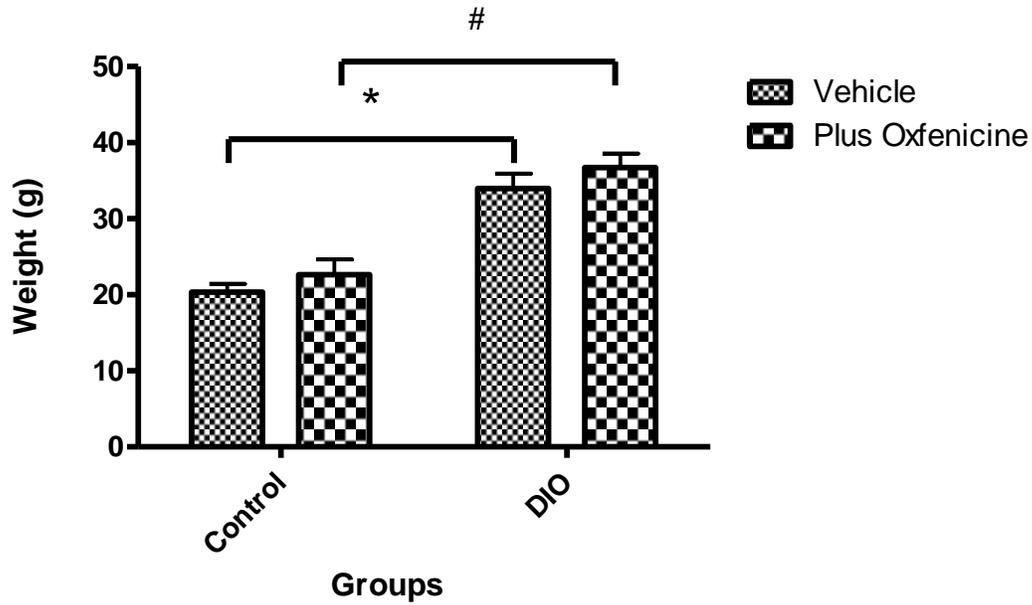


Fig. 3.1.2: The retro peritoneal fat weights of 16 week control- and high caloric diet fed (DIO) rats, with and without oxfenicine treatment. All values are expressed as the mean \pm SEM. Sample size (n) = 10. *P < 0.0001, #P < 0.0004.

The RP fat weights without treatment were significantly higher in the DIO vs. Controls ($33.98 \pm 1.93\text{g}$ vs. $20.37 \pm 1.06\text{g}$). It is clear that the diet did have an effect on the IP fat weights of the animals. The RP weights of the DIO + oxfen are also higher than that of the controls + oxfen ($36.73 \pm 1.84\text{g}$ vs. $22.67 \pm 1.96\text{g}$).

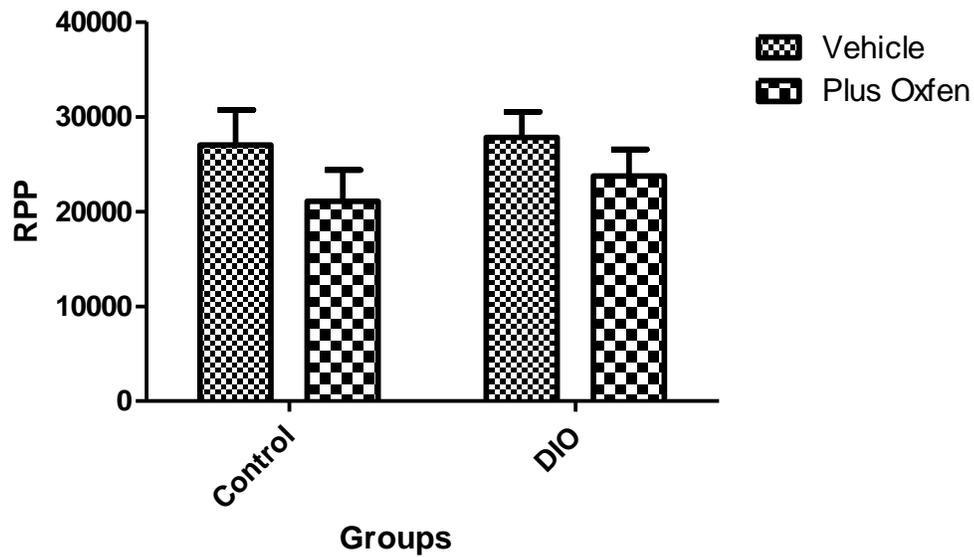


Fig. 3.1.3: Basal cardiac function (RPP) of the different groups, with and without oxfenicine treatment. All values are expressed as the mean \pm SEM. Sample size (n) = 10. P > 0.05

There were no differences in the basal cardiac mechanical function (before regional ischemia) between the control group vs. DIO group (27839 \pm 3417 RPP vs. 27811 \pm 2715 RPP). Comparing for differences within groups that were treated with oxfenicine i.e. control vs. control + oxfen (27839 \pm 3417 RPP vs. 21100 \pm 3277 RPP) and diet vs. DIO + oxfen (27811 \pm 2715 RPP vs. 23754 \pm 2807 RPP) data showed no significant differences in function. Thus neither the drug nor the diet had an effect on either group.

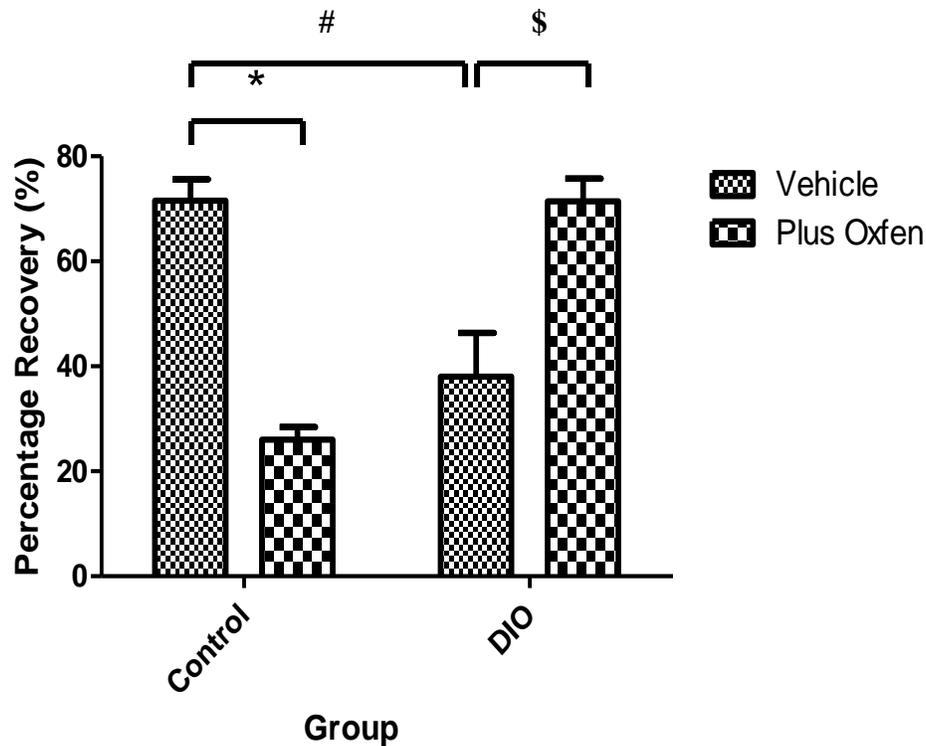


Fig.3.1.4: Cardiac functional recovery, after 40 minutes of regional ischemia and 10 minutes of reperfusion, with and without oxfenicine treatment in the respective groups. All values are expressed as the mean \pm SEM. Sample size (n) = 10. *P< 0.001, #P< 0.001, \$P< 0.0019.

The functional recovery was higher in the control group vs. control + oxfen ($71.57 \pm 4.03\%$ vs. $26.05 \pm 2.36\%$). There was a significantly better functional recovery in the control rats vs. DIO groups ($71.57 \pm 4.03\%$ vs. $38.01 \pm 8.33\%$). Recovery was improved in the DIO + oxfen vs. DIO groups ($71.46 \pm 4.27\%$ vs. $38.01 \pm 8.33\%$). There was a marked decrease in the mechanical function, after ten minutes reperfusion in the control groups treated with oxfenicine. These results suggest that chronic oxfenicine treatment improves cardiac mechanical function in the DIO rats and controversially, decrease functional recovery of the control rats.

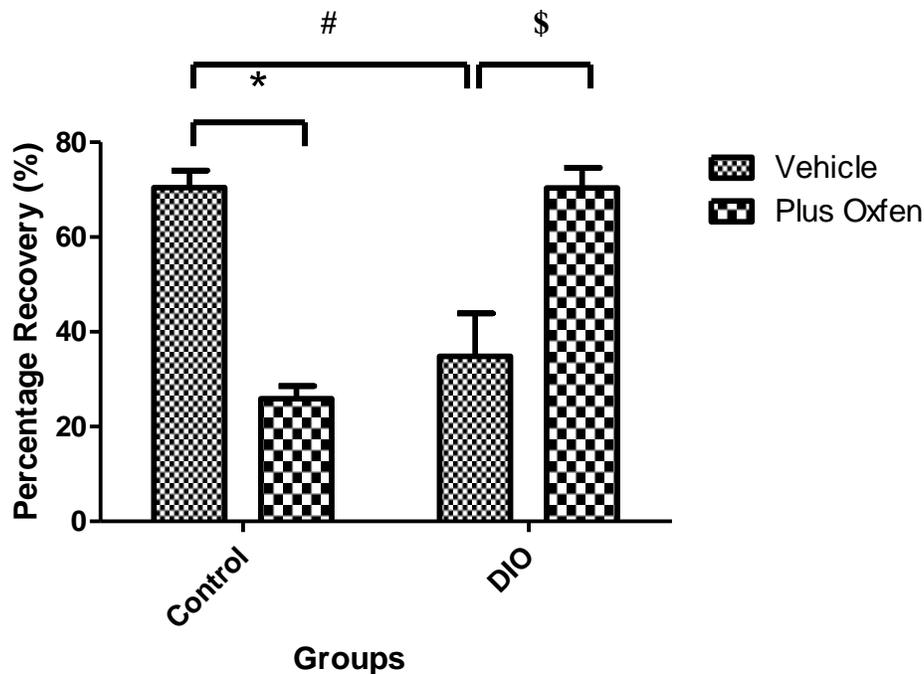


Fig.3.1.5: Cardiac functional recovery, after 40 minutes of regional ischemia and 20 minutes of reperfusion, with and without oxfenicine treatment in the respective groups. All values are expressed as the mean \pm SEM. Sample size (n) = 10. *P< 0.001, #P< 0.001, \$P< 0.001.

The functional recovery was increases in the control vs. control + oxfen ($70.43 \pm 3.58\%$ vs. $25.93 \pm 2.61\%$). There was a significantly better functional recovery seen in the control rats vs. DIO groups ($70.43 \pm 3.58\%$ vs. $34.83 \pm 9.07\%$). Recovery was improved in the DIO + oxfen vs. DIO groups ($70.40 \pm 4.20\%$ vs. $34.83 \pm 9.07\%$). The overall effect of chronic oxfenicine treatment on cardiac mechanical function indicates that the drug has deleterious effects on control hearts and protective effects on DIO hearts.

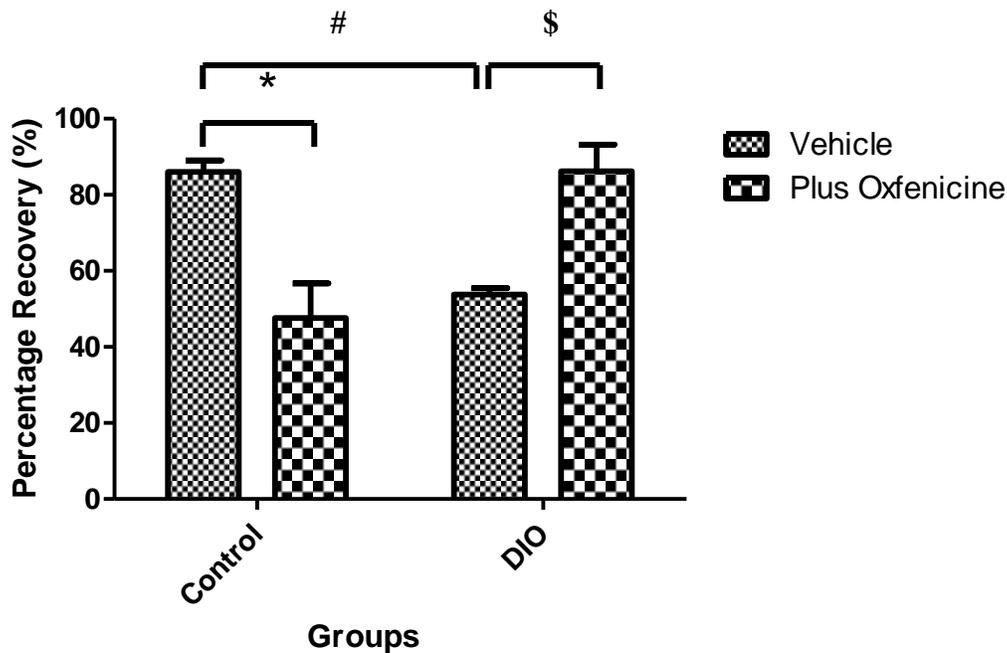


Fig.3.1.6: Cardiac functional recovery, after 25 minutes of global ischemia and 10 minutes of reperfusion, with and without oxfenicine treatment in the respective groups. All values are expressed as the mean \pm SEM. Sample size (n) = 10. *P< 0.0025, #P< 0.0001, \$P< 0.0011.

The functional recovery was increased in the control vs. control + oxfen (86.02 \pm 3.01% vs. 47.63 \pm 9.07%). There was a significantly improved functional recovery seen in the control rats vs. DIO groups (86.02 \pm 3.01% vs. 53.79 \pm 1.67%). Recovery was increased in the DIO + oxfen vs. DIO groups (86.23 \pm 7.01% vs. 53.79 \pm 1.67%). The overall effect of chronic oxfenicine treatment on cardiac mechanical function indicates that the drug has deleterious effects on control hearts and protective effects on DIO hearts.

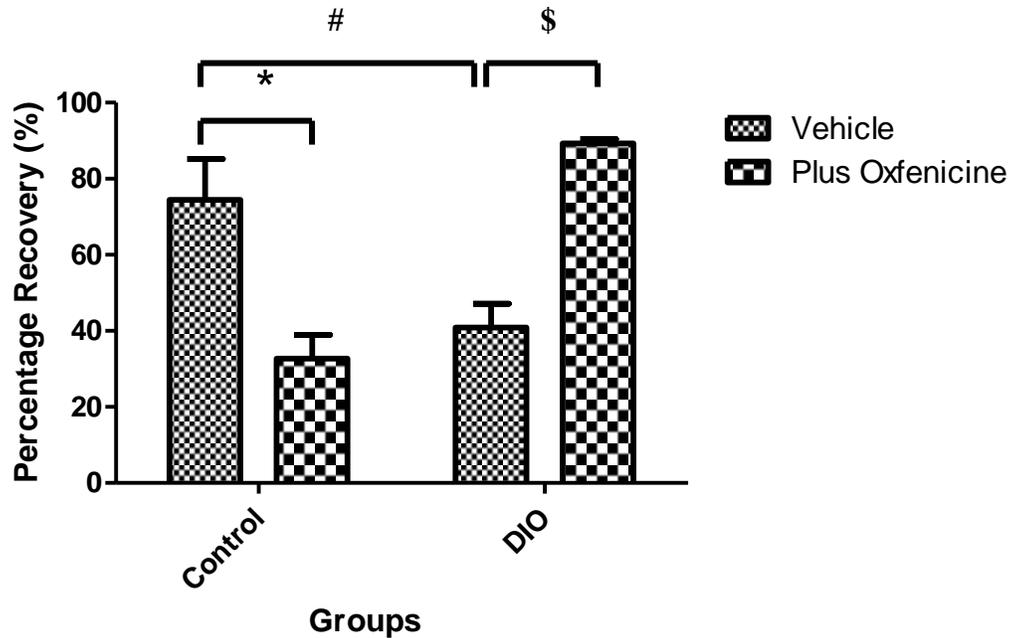


Fig.3.1.7: Cardiac functional recovery, after 25 minutes of global ischemia and 20 minutes of reperfusion, with and without oxfenicine treatment in the respective groups. All values are expressed as the mean \pm SEM. Sample size (n) = 10. *P< 0.0074, #P< 0.0226, \$P< 0.0001.

The functional recovery was increased in the control vs. control + oxfen ($74.44 \pm 10.80\%$ vs. $32.75 \pm 6.17\%$). There was a significantly improved functional recovery seen in the control rats vs. DIO groups ($74.44 \pm 10.80\%$ vs. $40.90 \pm 6.21\%$). Recovery was higher in the DIO + oxfen vs. DIO groups ($89.31 \pm 1.18\%$ vs. $40.90 \pm 6.21\%$). The overall effect of chronic oxfenicine treatment on cardiac mechanical function indicates that the drug has deleterious effects on control hearts and protective effects on DIO hearts.

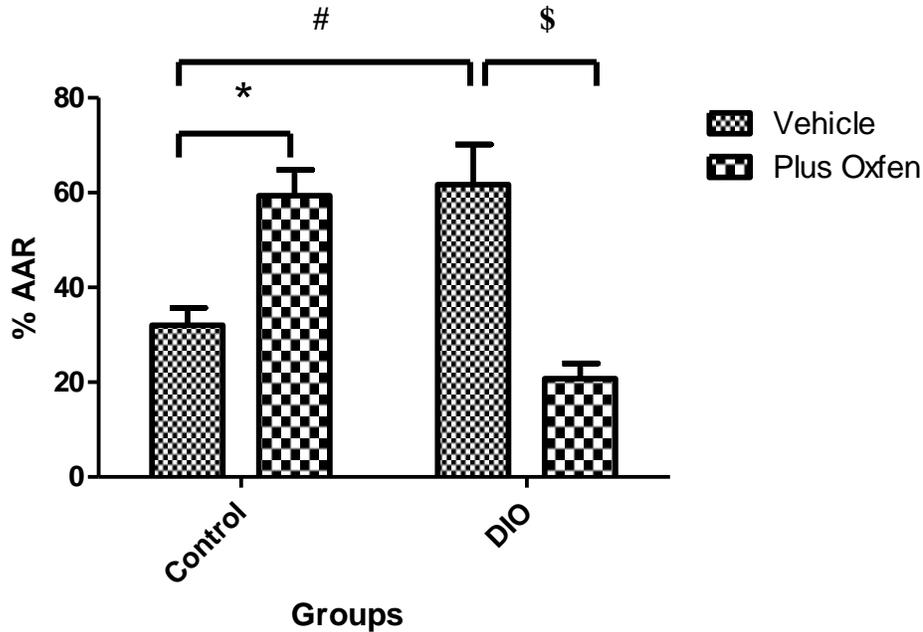


Fig.3.1.8: Infarct size for the four experimental groups as determined using TTC staining, after 40 minutes regional ischemia and 2 hours reperfusion. The infarct size is expressed as a percentage (%) of the area at risk. Mean \pm SEM are given. Sample size (n) = 10. *P< 0.01, #P< 0.0047, \$P< 0.001.

The infarct size was significantly larger in the control + oxfen rats vs. control rats (59.37 ± 5.49 % of AAR vs. 32.05 ± 3.62 % of AAR). With treatment, there was also a significant decrease in the infarct size of DIO + oxfen ($20.79 \pm 3.19\%$ of AAR) vs. DIO rats ($61.70 \pm 8.45\%$ of AAR). The infarct size of the control hearts was significantly smaller vs. that of the DIO hearts (32.05 ± 3.62 % of AAR vs. $61.70 \pm 8.45\%$ of AAR). Chronic oxfenicine treatment appears to improve the myocardial tolerance to ischemia/reperfusion injury, in DIO group and decreases tolerance of the control hearts. There were inherent differences in the myocardial tolerance to ischemia/reperfusion injury in the control and DIO groups, as expressed by the infarct size.

3.2 Isolated mitochondria data

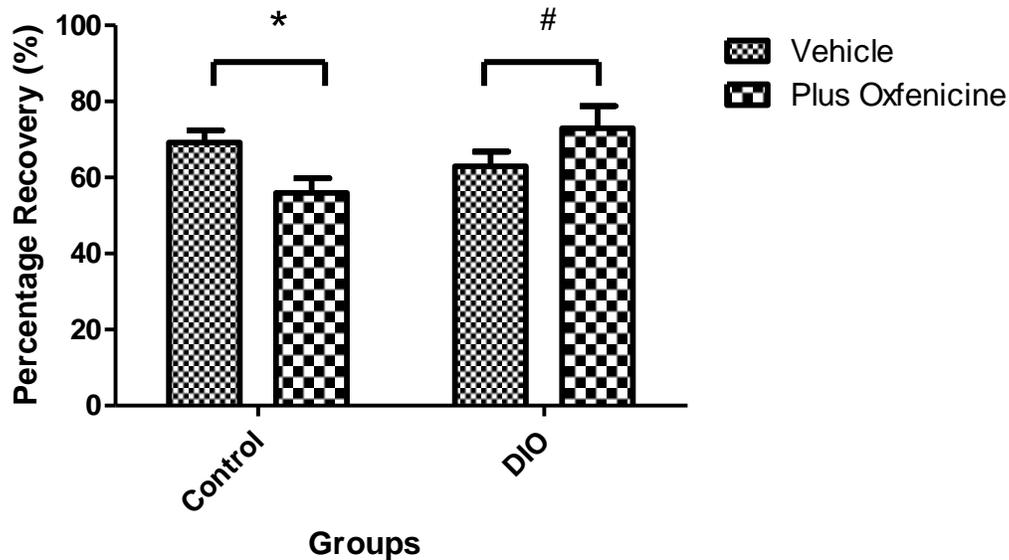


Fig.3.2.1: Mitochondrial state 3 respiration recovery for the four experimental groups as determined by oxygraph mitochondrial assessment. The substrate used was glutamate. Mean \pm SEM are given. Sample size (n) = 6. *P< 0.0253, #P<0.0060.

There were no significant differences between the Control rats vs. DIO rats ($69.17 \pm 3.22\%$ vs. $62.99 \pm 3.83\%$). The effect of the drug was seen as it decreased the percentage recovery of state 3 respiration of the control + oxfenicine group vs. control group ($55.97 \pm 3.87\%$ vs. $69.17 \pm 3.22\%$). In contrast to this, there was a significant increase in the percentage recovery of state 3 respiration of the DIO + oxfenicine group vs. DIO group ($72.93 \pm 5.83\%$ vs. $62.99 \pm 3.83\%$). It appears that after chronic oxfenicine treatment, control rat mitochondrial state 3 respiration recoveries were much lower than that of the control rats without treatment and recoveries of the DIO rats + oxfenicine were much higher than that of the DIO rats without oxfenicine.

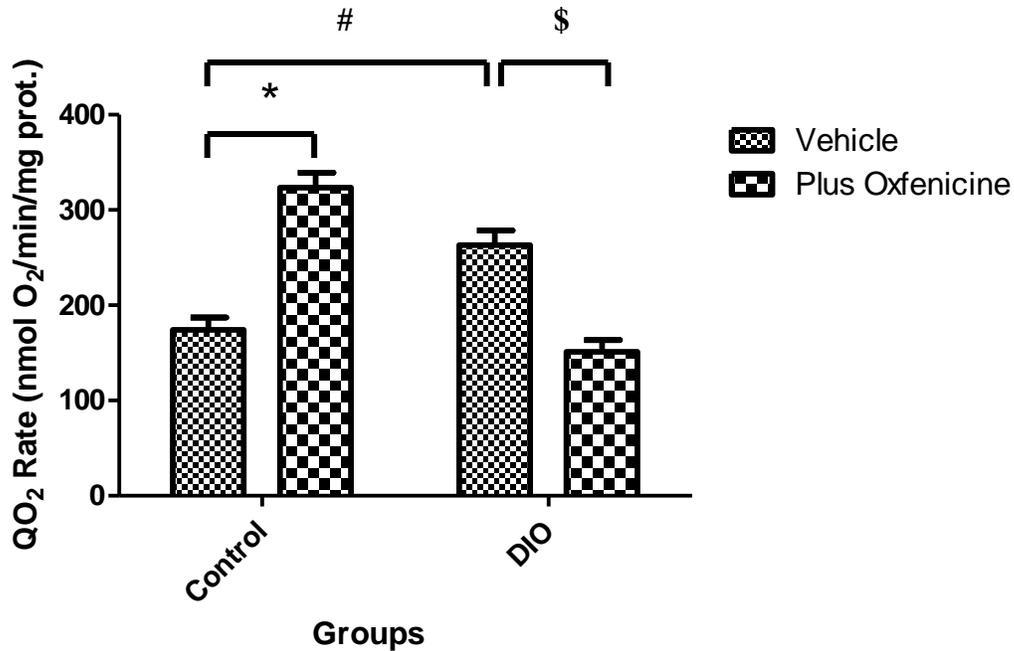


Fig.3.2.2: Myocardial oxygen consumption (QO₂) (nmol O₂/min/mg protein) for the four experimental groups as determined by oxygraph mitochondrial assessment. The substrate used was glutamate. Mean ± SEM are given. Sample size (n) = 6, *P<0.0001, #P<0.0013, \$P<0.0002.

Myocardial oxygen consumption was significantly lower in the Control vs. the Control + oxfenicine group (174.1± 13.08 nmol O₂/min/mg protein vs. 323.5±15.81 nmol O₂/min/mg protein). It was higher in the DIO group vs. Control group (263.0± 15.43 nmol O₂/min/mg protein vs. 174.1± 13.08 nmol O₂/min/mg protein). The oxygen consumption was significantly decreased in the DIO + oxfenicine vs. DIO group (151.1± 12.25 nmol O₂/min/mg protein vs. 263.0± 15.43 nmol O₂/min/mg protein). It appears that the diet did affect the oxygen consumption as shown by the increase in the oxygen consumption of the DIO group. Furthermore, the drug also affected the oxygen consumption in both the Control- and DIO groups.

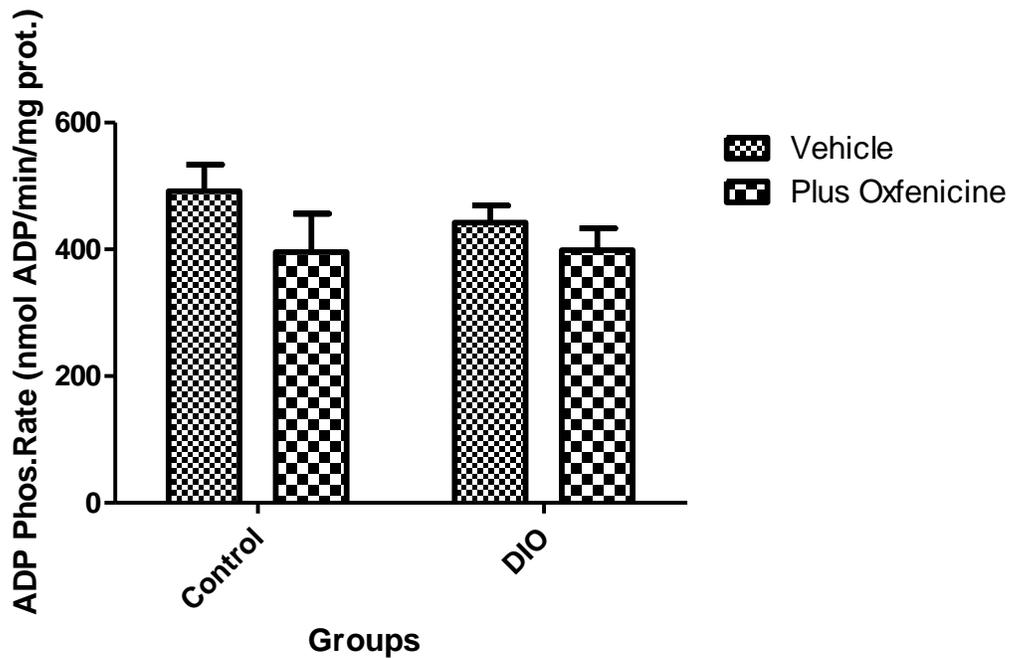


Fig.3.2.3: ADP phosphorylation rate (nmol ADP/min/mg protein) for the four experimental groups as determined by oxygraph mitochondrial assessment. The substrate used was glutamate. Mean \pm SEM are given. Sample size (n) = 6, $P > 0.05$.

There were no significant differences in any of the four groups. Control vs. DIO (491.7 \pm 41.87 nmol ADP/min/mg protein vs. 442.3 \pm 26.89 nmol ADP/min/mg protein). Control vs. Control + oxfenicine (491.7 \pm 41.87 nmol ADP/min/mg protein vs. 395.6 \pm 60.71 nmol ADP/min/mg protein) and DIO vs. DIO + oxfenicine (442.3 \pm 26.89 nmol ADP/min/mg protein vs. 398.9 \pm 34.22 nmol ADP/min/mg protein).

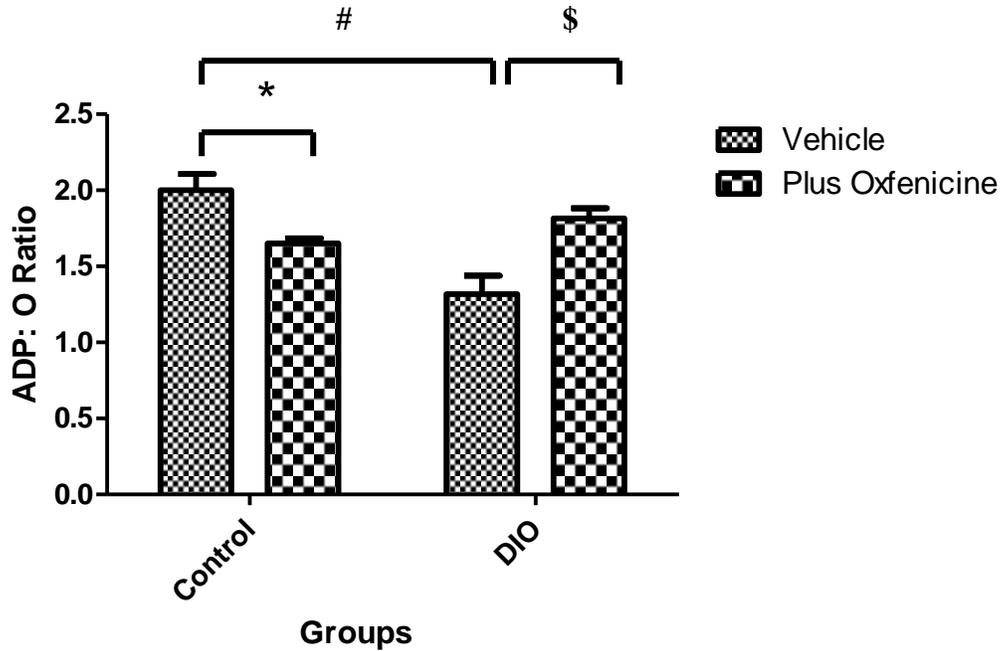


Fig.3.2.4: Mitochondrial ADP: O ratio for the four experimental groups as determined by oxygraph mitochondrial assessment. The substrate used was glutamate. Mean \pm SEM are given. Sample size (n) = 6. *P < 0.0107, #P < 0.00018, \$P < 0.0048

The ADP: O ratio was significantly higher in the control vs. the DIO group (2.00 \pm 0.11 vs. 1.32 \pm 0.12). It was decreased in the control + oxfenicine vs. control group (1.65 \pm 0.03 vs. 2.00 \pm 0.11). The ratio was significantly increased in the DIO + oxfenicine vs. DIO group (1.81 \pm 0.01 vs. 1.31 \pm 0.12). It appears that the diet had an effect on the ADP: O ratio as shown by the decrease in this ratio in the DIO group. Furthermore, the drug also affected the ADP: O ratios of the control + oxfenicine vs. the control group (1.65 \pm 0.03 vs. 2.00 \pm 0.11)

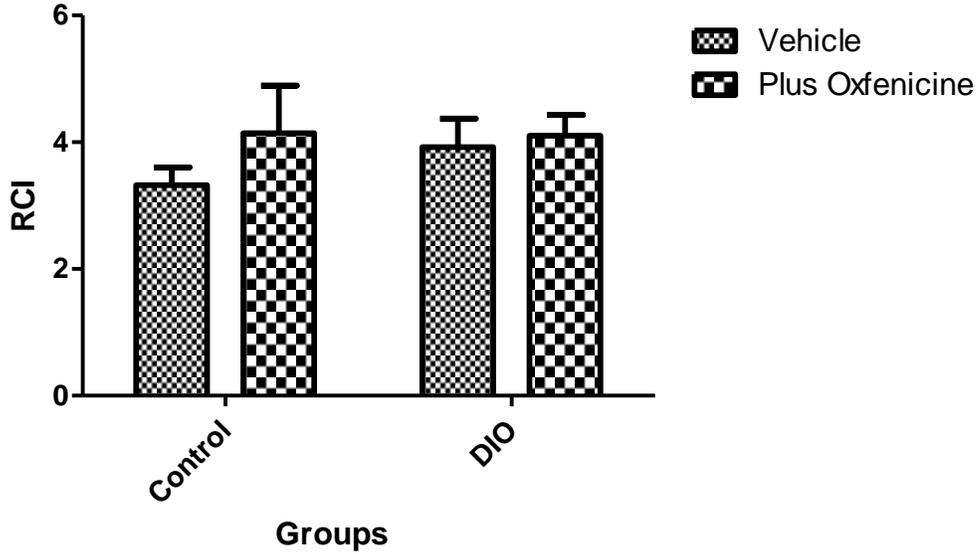


Fig.3.2.5: Respiratory control index (RCI) for the four experimental groups as determined by oxygraph mitochondrial assessment. The substrate used was glutamate. Mean \pm SEM are given. Sample size (n) = 6, P> 0.05.

There were no significant differences in any of the groups. Respiratory control index (RCI) of Control vs. DIO (3.32 ± 0.28 vs. 3.92 ± 0.45), Control vs. Control + oxfenicine (3.32 ± 0.28 vs. 4.15 ± 0.75) and DIO vs. DIO + oxfenicine (3.92 ± 0.45 vs. 4.11 ± 0.33).

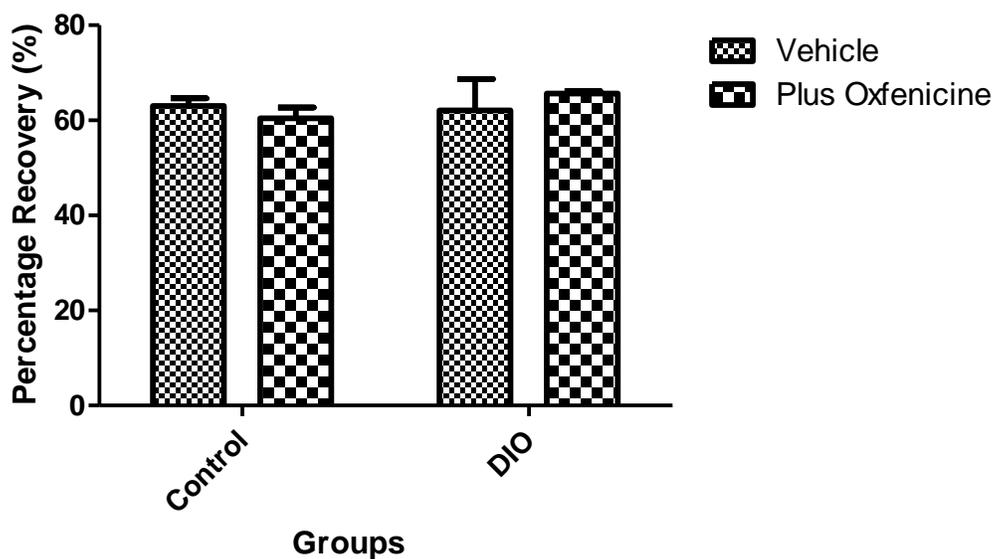


Fig.3.2.6: Mitochondrial state 3 respiration recovery for the four experimental groups as determined by oxygraph mitochondrial assessment. The substrate used was palmitate. Mean \pm SEM are given. Sample size (n) = 3, $P > 0.05$.

There were no significant differences in any of the four groups. Control vs. DIO ($63.03 \pm 1.59\%$ vs. $62.10 \pm 6.57\%$). Control vs. Control + oxfenicine ($63.03 \pm 1.59\%$ vs. $60.43 \pm 2.25\%$) and DIO vs. DIO + oxfenicine ($62.10 \pm 6.57\%$ vs. $65.67 \pm 0.50\%$).

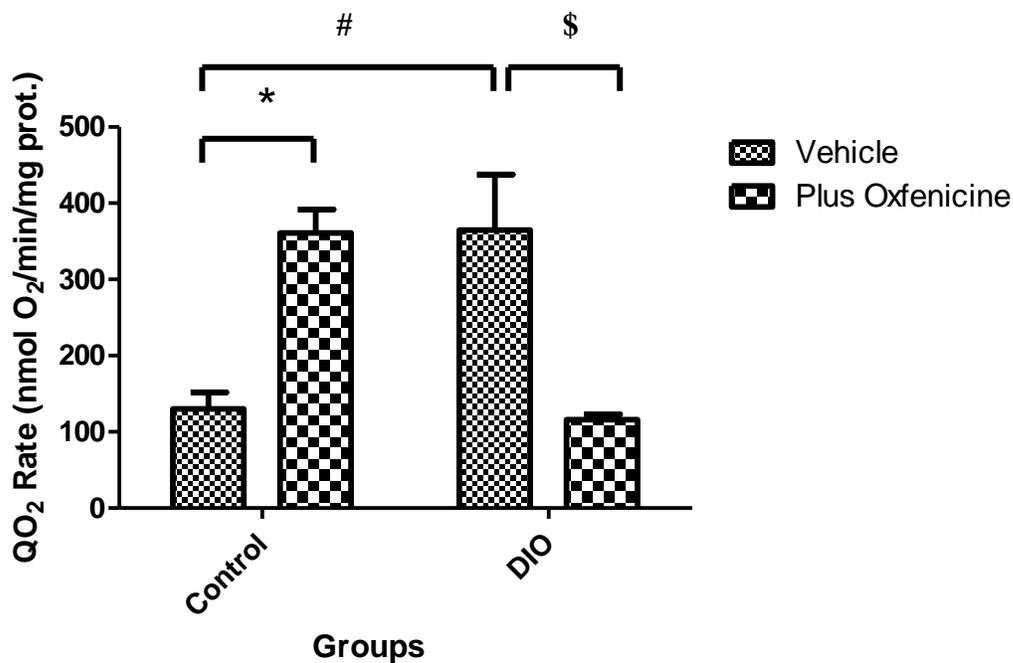


Fig.3.2.7: Myocardial oxygen consumption (QO₂) (nmol O₂/min/mg protein) for the four experimental groups as determined by oxygraph mitochondrial assessment. The substrate used was palmitate. Mean ± SEM are given. Sample size (n) = 3, *P<0.0036, #P<0.037, \$P<0.028.

Myocardial oxygen consumption was significantly lower in the Control vs. the Control + oxfenicine group (130.0± 21.70 nmol O₂/min/mg protein vs. 361.0± 30.99 nmol O₂/min/mg protein). It was higher in the DIO group vs. Control group (364.7± 73.11 nmol O₂/min/mg protein vs. 130.0± 21.70 nmol O₂/min/mg protein). The consumption was significantly decreased in the DIO + oxfenicine vs. DIO group (116.0± 7.21 nmol O₂/min/mg protein vs. 364.7± 73.11 nmol O₂/min/mg protein). The diet did have an effect on the oxygen consumption as shown by the increase in the oxygen consumption of the DIO group. Furthermore, the drug also affected the oxygen consumption in both the Control- and DIO groups.

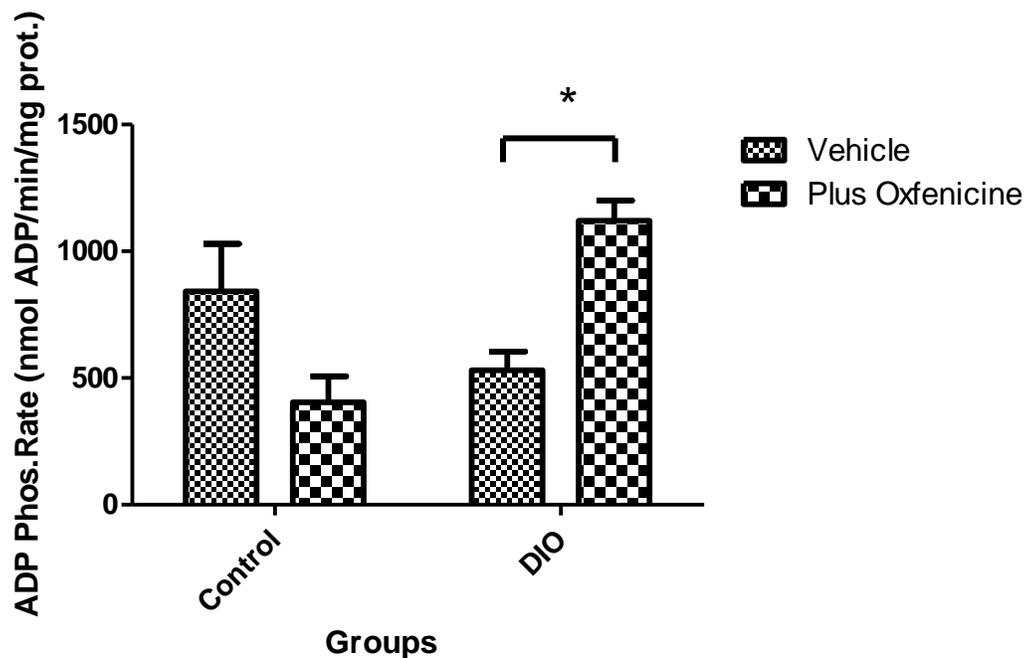


Fig.3.2.8: ADP phosphorylation rate (nmol ADP/min/mg protein) for the four experimental groups as determined by oxygraph mitochondrial assessment. The substrate used was palmitate. Mean \pm SEM are given. Sample size (n) = 3, *P<0.0055

There were no significant differences in the Control vs. DIO groups (842.10 \pm 187.60 nmol ADP/min/mg protein vs. 531.10 \pm 73.06 nmol ADP/min/mg protein). Control vs. Control + oxfenicine (842.10 \pm 187.60 nmol ADP/min/mg protein vs. 404.10 \pm 103.30 nmol ADP/min/mg protein) were statistically similar. The ADP phosphorylation rate was significantly lower in the DIO group vs. DIO + oxfenicine group (531.10 \pm 73.06 nmol ADP/min/mg protein vs. 1121.10 \pm 79.54 nmol ADP/min/mg protein).

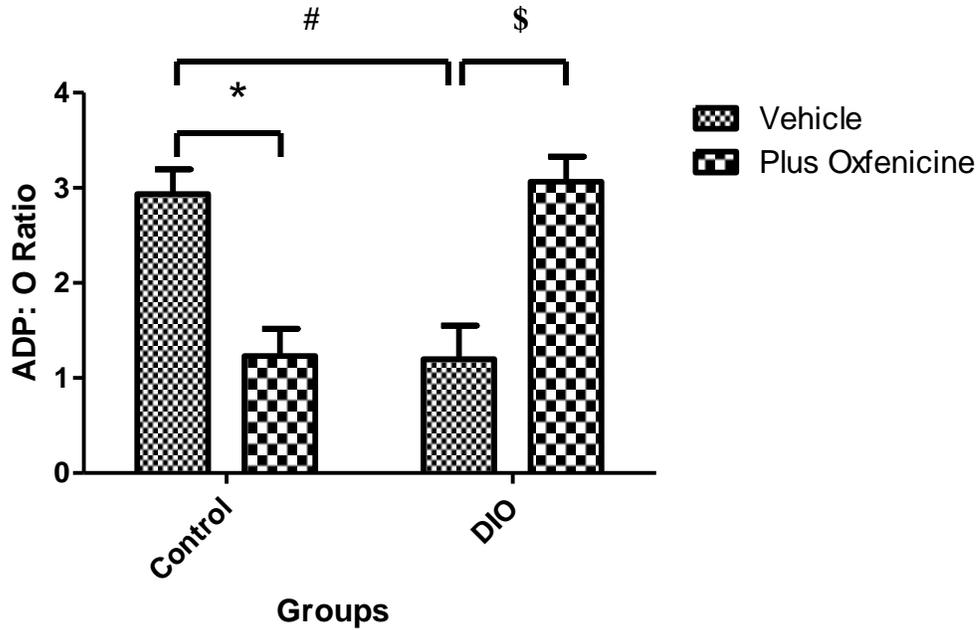


Fig.3.2.9: Mitochondrial ADP: O ratio for the four experimental groups as determined by oxygraph mitochondrial assessment. The substrate used was palmitate. Mean \pm SEM are given. Sample size (n) = 3, *P<0.0166, #P<0.0116, \$P<0.0130

The ADP: O ratio was significantly higher in the Control vs. DIO (2.93 ± 0.26 vs. 1.20 ± 0.35). Control vs. Control + oxfenicine showed higher ADP: O ratios (2.93 ± 0.26 vs. 1.23 ± 0.28) and lower ratios were seen in the DIO group vs. DIO + oxfenicine group (1.20 ± 0.35 vs. 3.1 ± 0.26).

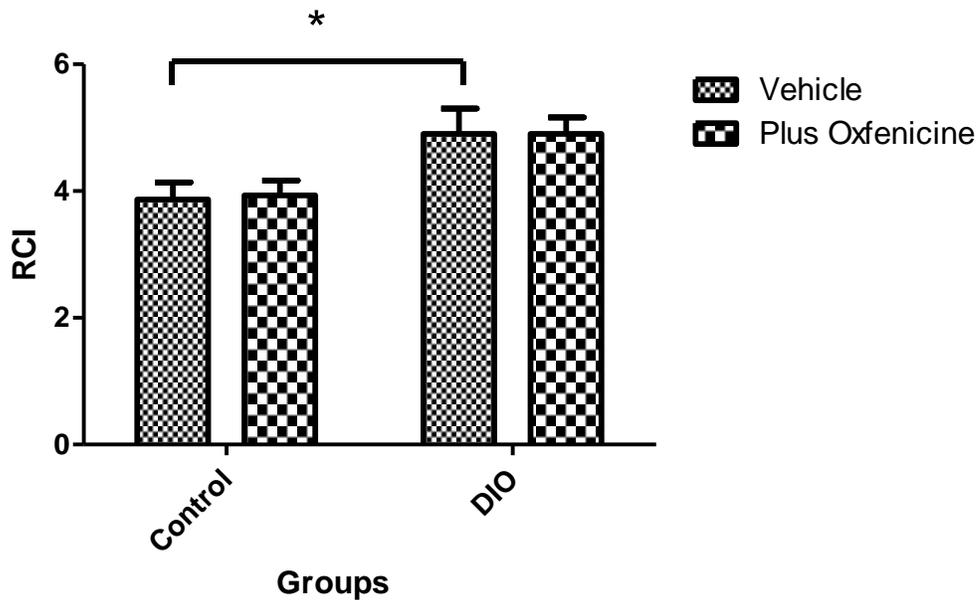


Fig.3.2.10: Respiratory control index (RCI) for the four experimental groups as determined by oxygraph mitochondrial assessment. The substrate used was palmitate. Mean \pm SEM are given. Sample size (n) = 3, *P < 0.1014

Respiratory control index was significantly lower in the Control vs. DIO groups (3.87 ± 0.27 vs. 4.90 ± 0.40). There were no significant differences in any of the other groups. Control vs. Control + oxfenicine (3.87 ± 0.27 vs. 3.93 ± 0.23) and DIO vs. DIO + oxfenicine (4.90 ± 0.40 vs. 4.90 ± 0.26).

3.3 Western Blot Data

The representative blot in each group shows three hearts per group. Beta tubulin was used to indicate equal loading.

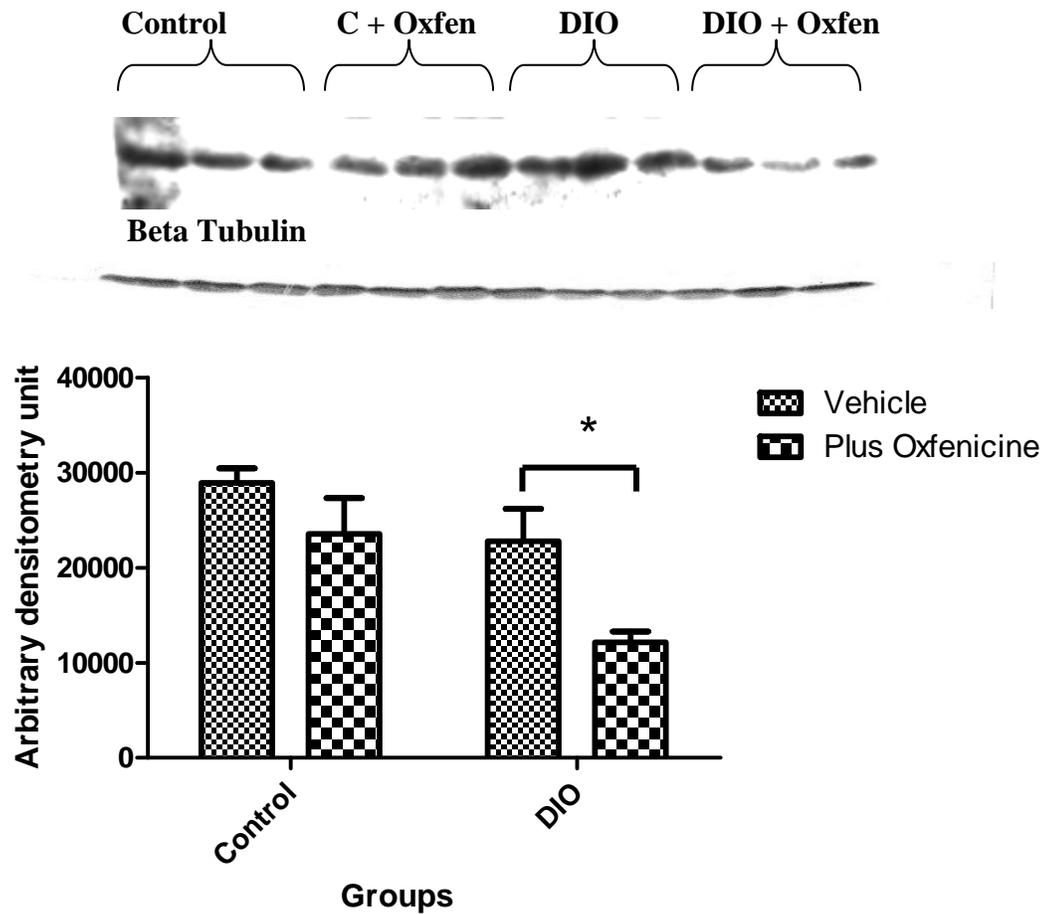


Fig.3.3.1: Total CPT-1 expression with and without oxfenicine treatment. Expression of hearts freeze clamped after 30 minutes reperfusion. Mean \pm SEM are given. Sample size (n) = 6, *P<0.0137

There were no significant differences between the dietary groups. Control vs. DIO (28904 \pm 1530 AU vs. 22792 \pm 3383 AU). Control vs. Control + oxfenicine (28904 \pm 1530 AU vs. 23550 \pm 3777 AU) were not significantly different. However the CPT 1 expression was significantly decreased in the DIO + oxfenicine vs. the DIO group treated group (12159 \pm 1119 AU vs. 22792 \pm 3383 AU).

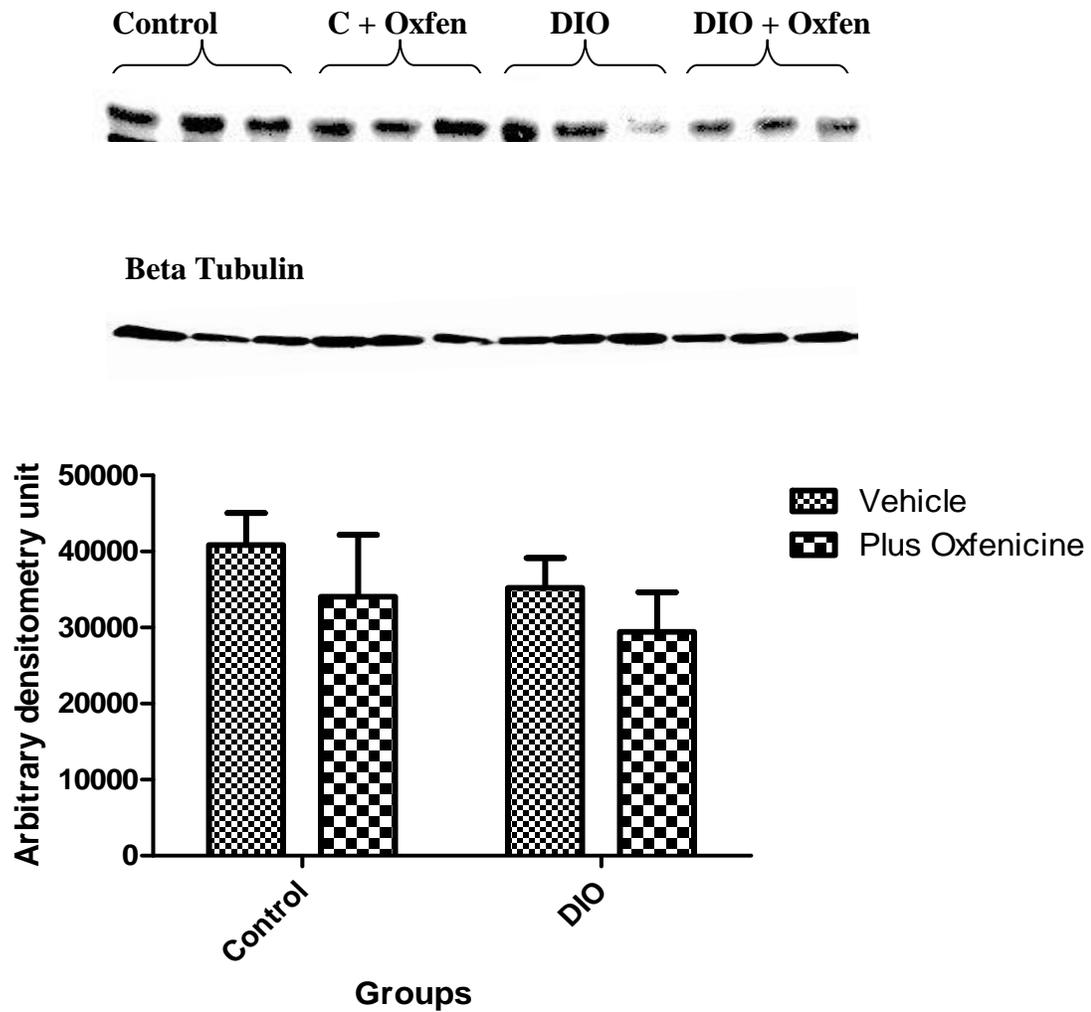


Fig.3.3.2: Total MCAD expression with and without oxfenicine treatment. Expression of hearts freeze clamped after 30 minutes of reperfusion. Mean \pm SEM are given. Sample size (n) = 6, P >0.05.

There were no significant differences in total MCAD expression between any of the groups. Control vs. DIO (40842 \pm 4212 AU vs. 35247 \pm 3899 AU). Control vs. Control + oxfenicine (40842 \pm 4212 AU vs. 34053 \pm 8118 AU) and DIO vs. DIO + oxfenicine (35247 \pm 3899 AU vs. 29453 \pm 5209 AU).

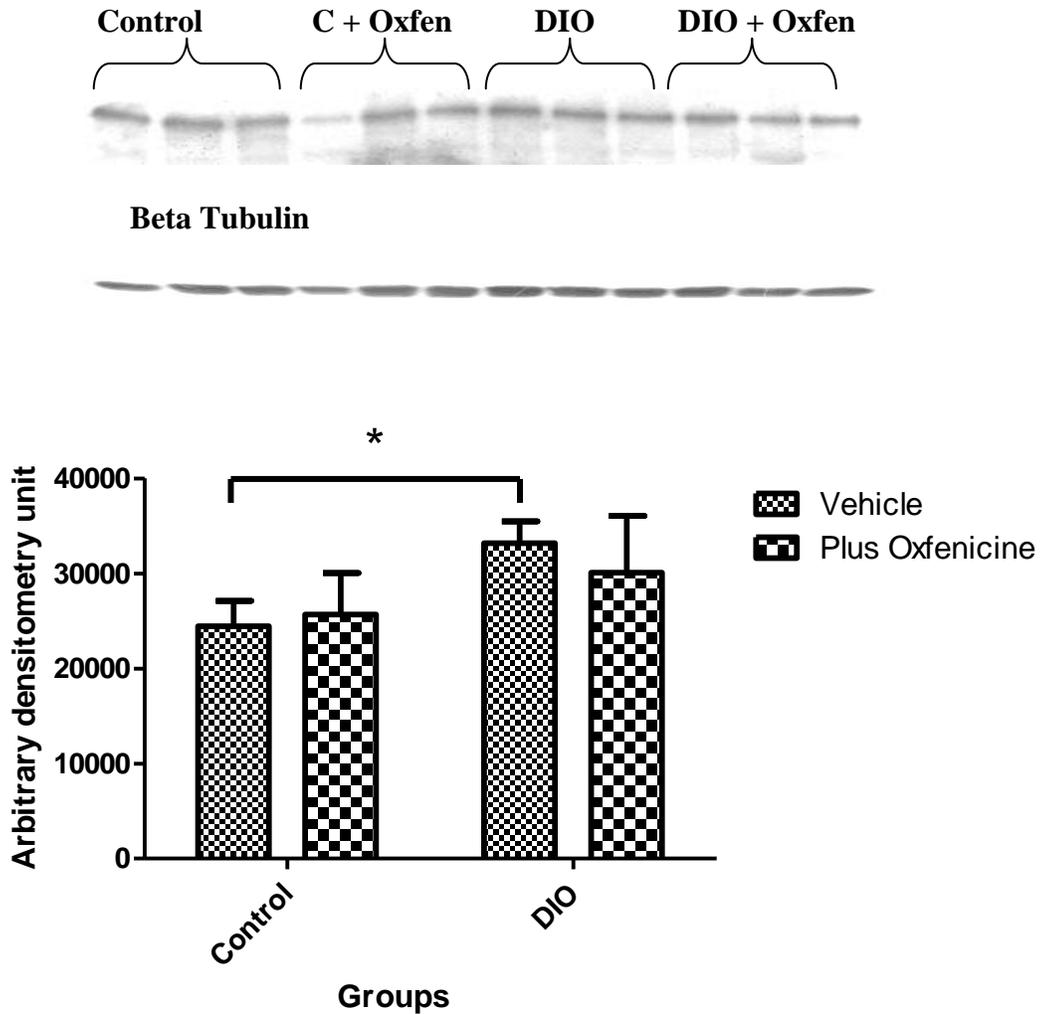


Fig.3.3.3: Phosphorylated IRS-1 (Serine 641) with and without oxfenicine treatment. Expression of hearts freeze clamped after 30 minutes of reperfusion. Mean \pm SEM are given. Sample size (n) = 6, *P <0.03.

Phosphorylated IRS-1 was significantly higher in the DIO group vs. Control group (33170 \pm 2316 AU vs. 24463 \pm 2654 AU). There were no significant differences between the following groups: Control vs. Control + oxfenicine (24463 \pm 2654 AU vs. 25691 \pm 4346 AU) and DIO vs. DIO + oxfenicine (33170 \pm 2316 AU vs. 30089 \pm 5973 AU).

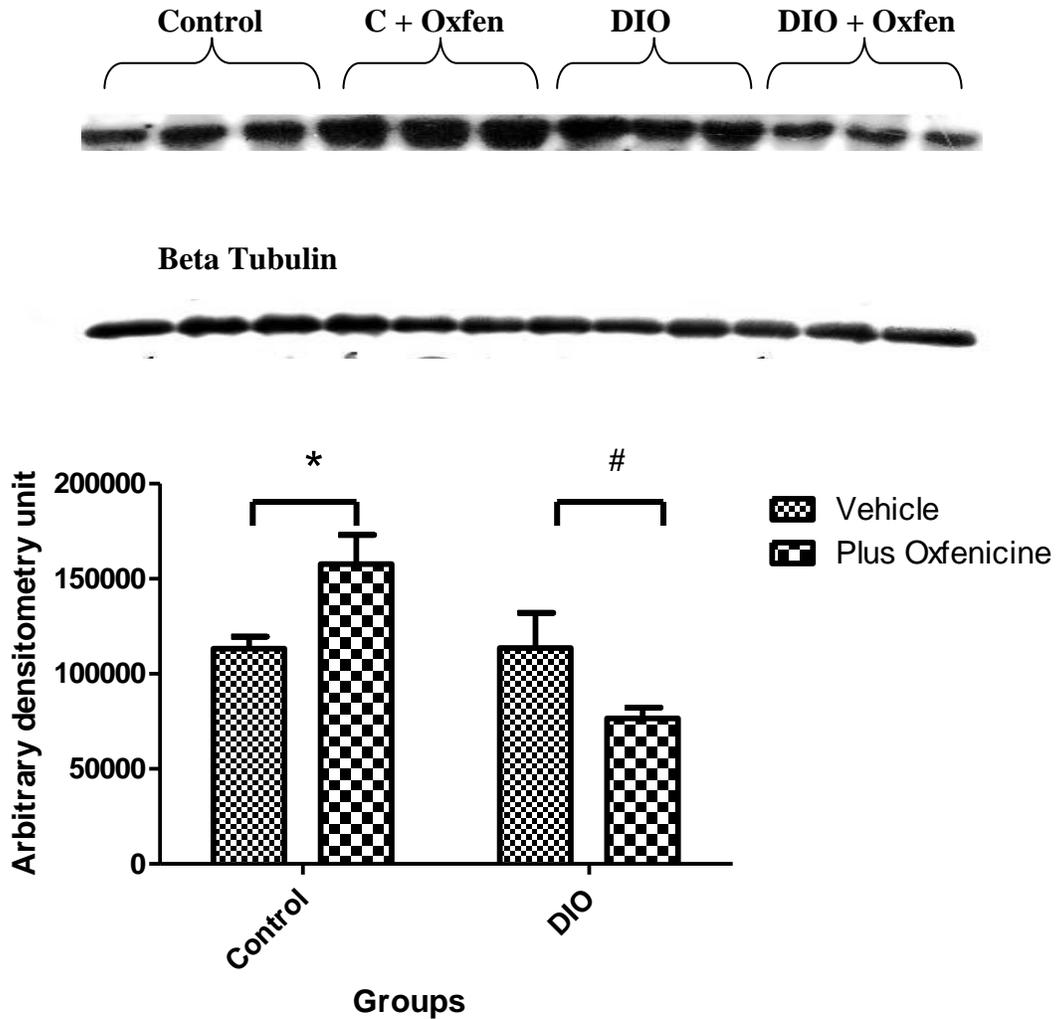


Fig.3.3.4: Total IRS-1 expression with and without oxfenicine treatment. Expression of hearts freeze clamped after 30 minutes of reperfusion. Mean \pm SEM are given. Sample size (n) = 6, *P < 0.022, #P < 0.0024

Total IRS-1 expression was significantly increased in the control + oxfen group vs. control group (157813 ± 15284 AU vs. 113219 ± 6373 AU). The expression was not different between the control group vs. DIO group (113219 ± 6373 AU vs. 113601 ± 18504 AU). Total IRS-1 expression was significantly decreased in the DIO + oxfen group vs. DIO group (76610 ± 5673 AU vs. 113601 ± 18504 AU).

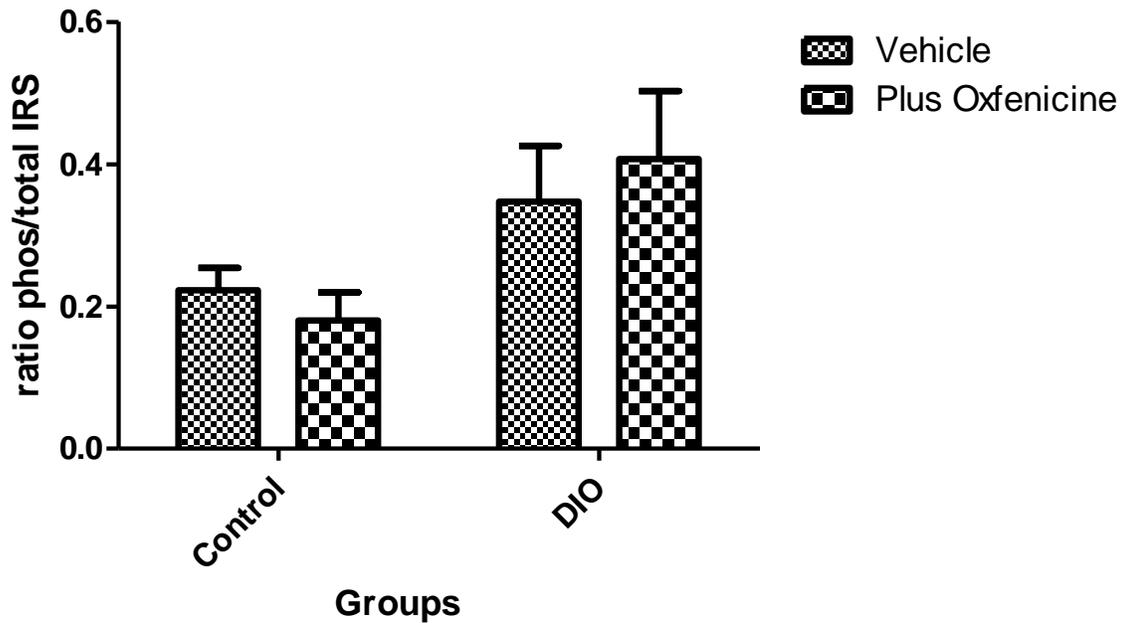


Fig.3.3.5: Ratio of Phospho/total IRS-1 with and without oxfenicine treatment. Hearts were freeze clamped after 30 minutes of reperfusion. Mean \pm SEM are given. Sample size (n) = 6, $P > 0.05$

There were no significant differences between any of the groups. Control vs. DIO (0.22 ± 0.03 vs. 0.35 ± 0.08). Control vs. Control + oxfenicine (0.22 ± 0.03 vs. 0.18 ± 0.03) and DIO vs. DIO + oxfenicine (0.34 ± 0.08 vs. 0.41 ± 0.20).

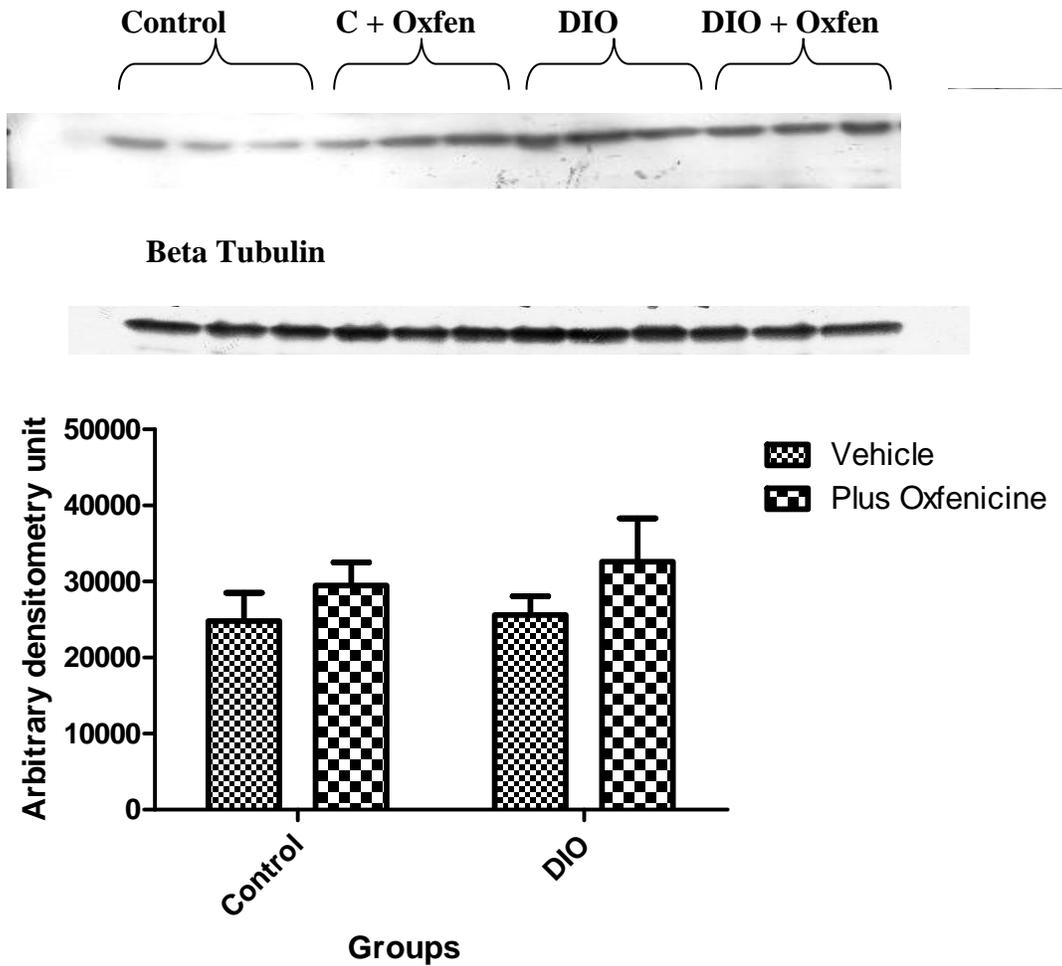


Fig.3.3.6: Total GLUT-4 expression with and without oxfenicine treatment. Expression of hearts freeze clamped after 30 minutes of reperfusion. Mean \pm SEM are given. Sample size (n) = 6, P >0.05.

There were no significant differences between any of the groups. Control vs. DIO (24815 \pm 3686 AU vs. 25597 \pm 2450 AU). Control vs. Control + oxfenicine (24815 \pm 3686 AU vs. 29468 \pm 3067 AU) and DIO vs. DIO + oxfenicine (25597 \pm 2450 AU vs. 32618 \pm 5703 AU).

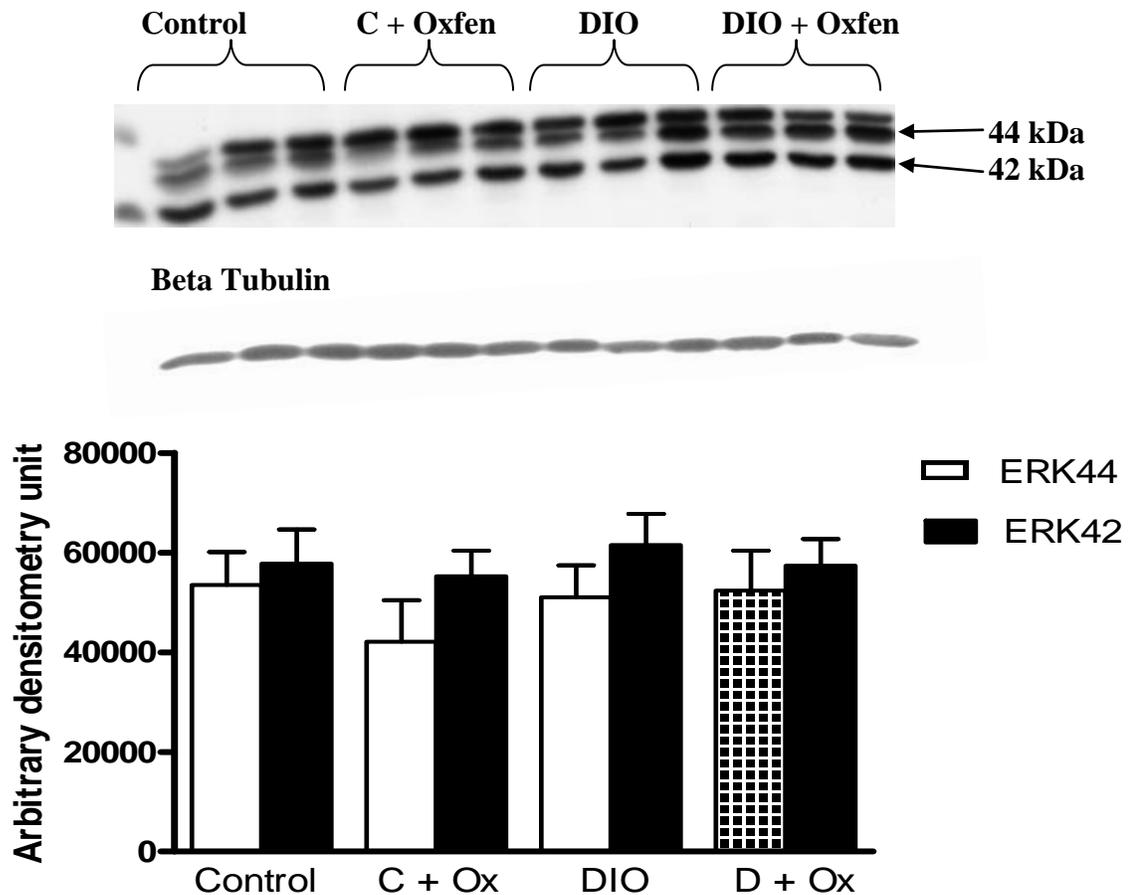


Fig.3.3.7: Levels of phosphorylated ERK- 44 and ERK- 42 with and without oxfenicine treatment. Expression of hearts freeze clamped after 30 minutes of reperfusion. Mean \pm SEM are given. Sample size (n) = 6, P >0.05.

There were no significant differences between any of the groups when pERK 44 was compared between groups and pERK 42 was compared between groups. For pERK 44: Control vs. DIO (53510 \pm 6561 vs. 51025 \pm 6391 AU). Control vs. Control + oxfenicine (53510 \pm 6561 AU vs. 42101 \pm 8314 AU) and DIO vs. DIO + oxfenicine (51025 \pm 6391 AU vs. 52388 \pm 7994 AU). For pERK 42: Control vs. DIO (51293 \pm 3044 AU vs. 61514 \pm 7705 AU). Control vs. Control + oxfenicine (51293 \pm 3044 AU vs. 56194 \pm 6205 AU) and DIO vs. DIO + oxfenicine (61514 \pm 7705 AU vs. 60921 \pm 4820 AU).

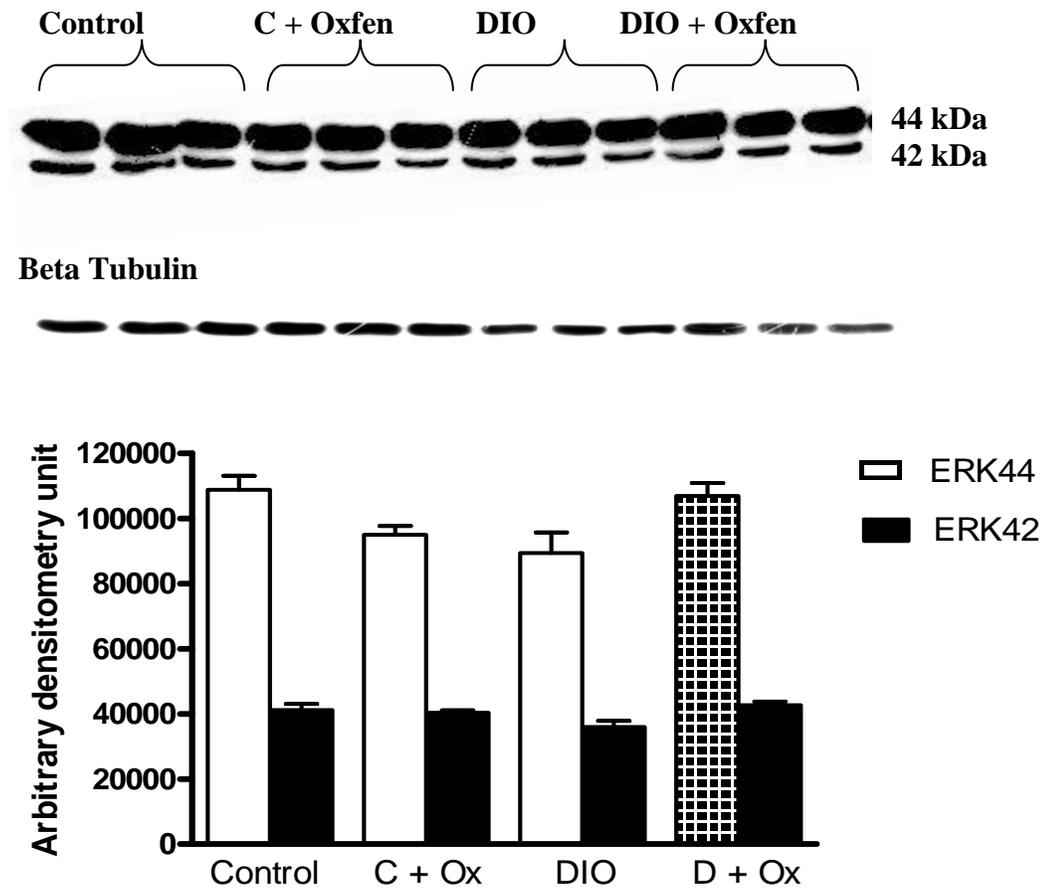


Fig.3.3.8: Levels of total ERK- 44 and ERK- 42 with and without oxfenicine treatment. Expression of hearts freeze clamped after 30 minutes of reperfusion. Mean \pm SEM are given. Sample size (n) = 6, P >0.05.

There were no significant differences between any of the groups when tERK 44 was compared between groups and tERK 42 was compared between groups. For tERK 44: Control vs. DIO (108701 ± 4374 AU vs. 89377 ± 6248 AU). Control vs. Control + oxfenicine (108701 ± 4374 AU vs. 94984 ± 2652 AU) and DIO vs. DIO + oxfenicine (89377 ± 6248 AU vs. 106880 ± 3945 AU). For tERK 42: Control vs. DIO (41152 ± 1931 AU vs. 35952 ± 1917 AU). Control vs. Control + oxfenicine (41152 ± 1931 AU vs. 40392 ± 612.6 AU) and DIO vs. DIO + oxfenicine (35952 ± 1917 AU vs. 42679 ± 1075 AU).

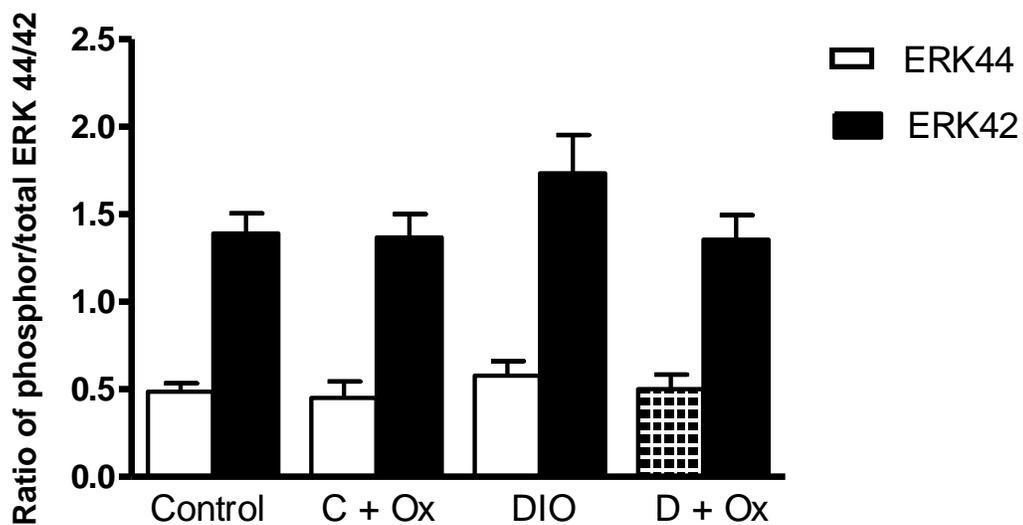


Fig.3.3.9: Ratio of Phosphorylated/total ERK (44/42) with and without oxfenicine treatment. Hearts were freeze clamped after 30 minutes of reperfusion. Mean \pm SEM are given. Sample size (n) = 6, $P > 0.05$

There were no significant differences between any of the groups. For ERK 44: Control vs. DIO (0.49 ± 0.05 vs. 0.58 ± 0.08). Control vs. Control + oxfenicine (0.49 ± 0.05 vs. 0.45 ± 0.09) and DIO vs. DIO + oxfenicine (0.58 ± 0.08 vs. 0.50 ± 0.08). For ERK 42: Control vs. DIO (1.39 ± 0.12 vs. 1.733 ± 0.22). Control vs. Control + oxfenicine (1.39 ± 0.12 vs. 1.37 ± 0.13) and DIO vs. DIO + oxfenicine (1.73 ± 0.22 vs. 1.355 ± 0.14).

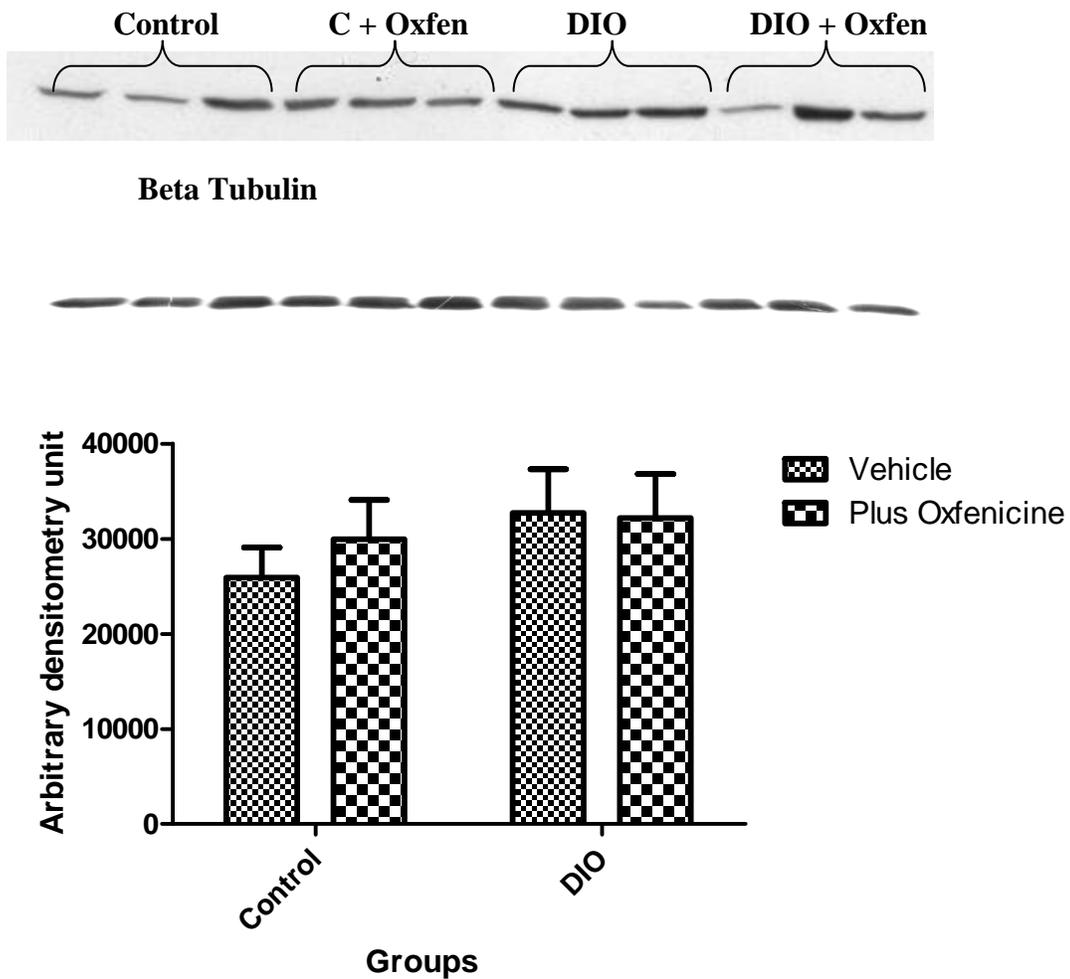


Fig.3.3.10: Phosphorylated PKB expression with and without oxfenicine treatment. Expression of hearts freeze clamped after 30 minutes of reperfusion. Mean \pm SEM are given. Sample size (n) = 6, P >0.05.

There were no significant differences between any of the groups. Control vs. DIO (25909 \pm 3185 AU vs. 32716 \pm 4600 AU). Control vs. Control + oxfenicine (25909 \pm 3185 AU vs. 29953 \pm 4136 AU) and DIO vs. DIO + oxfenicine (32716 \pm 4600 AU vs. 32180 \pm 4646 AU).

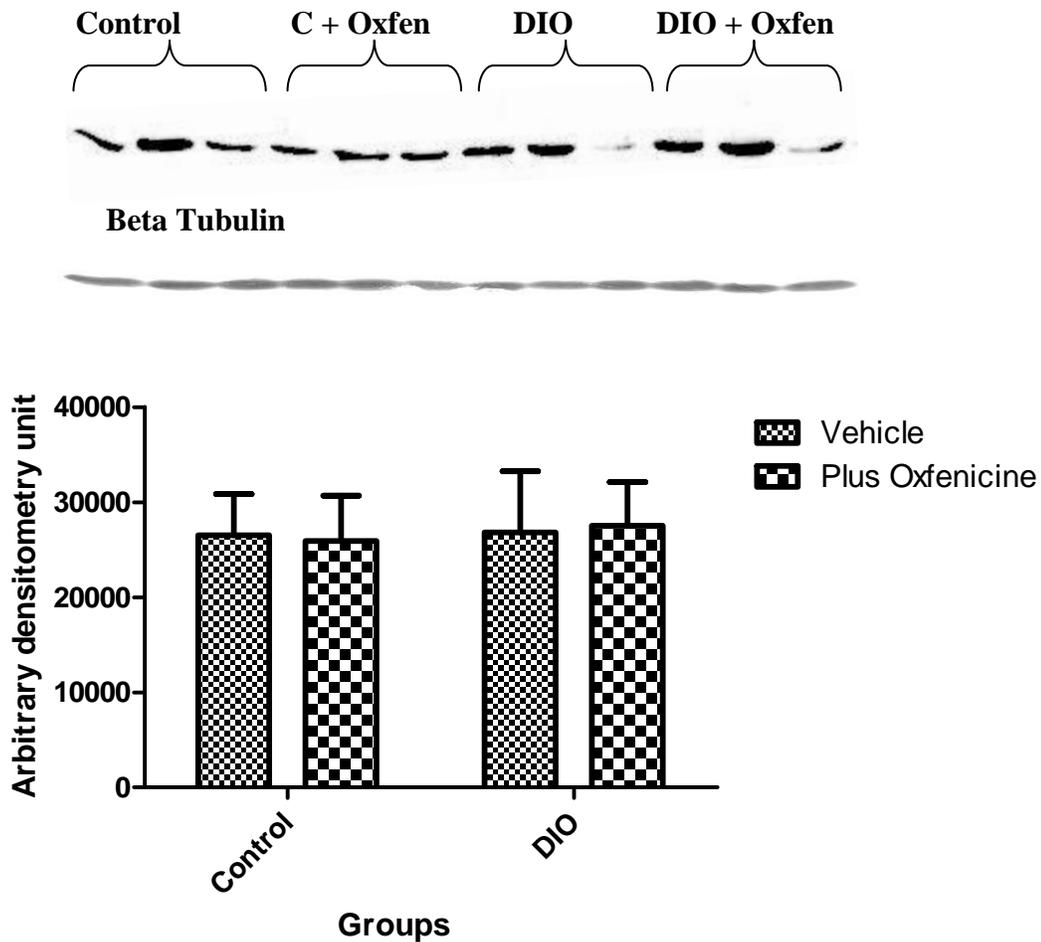


Fig.3.3.11: Total PKB expression with and without oxfenicine treatment. Expression of hearts freeze clamped after 30 minutes of reperfusion. Mean \pm SEM are given. Sample size (n) = 6, P >0.05.

There were no significant differences between any of the groups. Control vs. DIO (26518 \pm 4348 AU vs. 26821 \pm 6446 AU). Control vs. Control + oxfenicine (26518 \pm 4348 AU vs. 25910 \pm 4769 AU) and DIO vs. DIO + oxfenicine (26821 \pm 6446 AU vs. 27527 \pm 4605 AU).

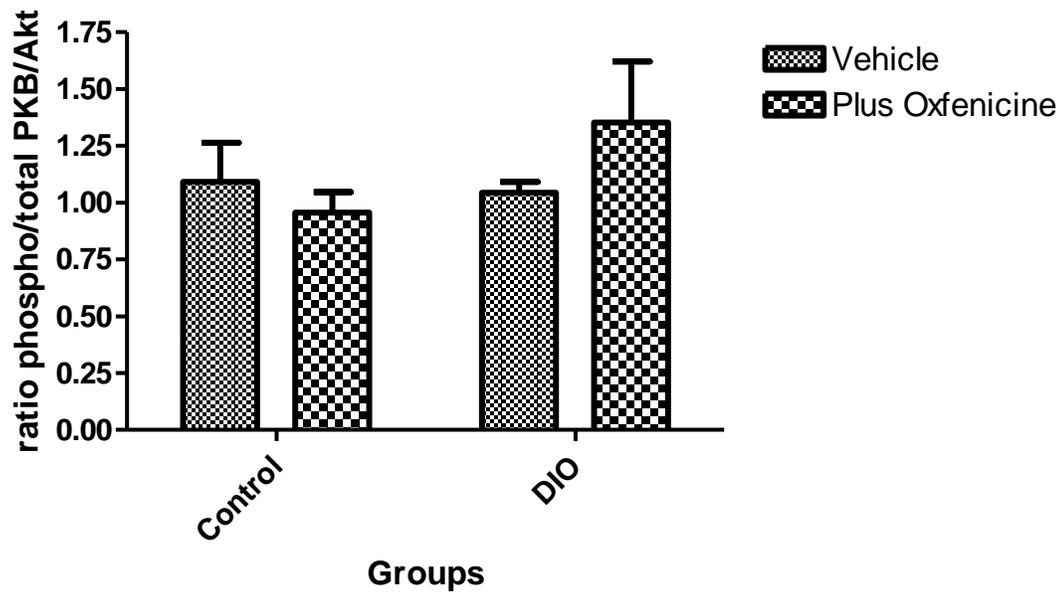


Fig.3.3.12: Ratio of Phosphorylated/total PKB with and without oxfenicine treatment. Expression of hearts freeze clamped after 30 minutes of reperfusion. Mean \pm SEM are given. Sample size (n) = 6, P >0.05.

There were no significant differences between any of the groups. Control vs. DIO (1.09 \pm 0.17 vs. 1.04 \pm 0.05). Control vs. Control + oxfenicine (1.09 \pm 0.17 vs. 0.96 \pm 0.09) and DIO vs. DIO + oxfenicine (1.04 \pm 0.05 vs. 1.35 \pm 0.27).

CHAPTER 4: DISCUSSION OF RESULTS

Effects of chronic oxfenicine on bodyweights

In our study it was important to investigate the efficacy of the switch from fatty acid oxidation to glucose oxidation, in a high calorie induced model of obesity and metabolic syndrome. This is an established model and has been in use in our department for a number of years (Du Toit *et al.* 2008; Essop *et al.* 2009). The body weights were significantly higher in both the DIO groups when compared to the control groups (Fig. 3.1.1). The drug, oxfenicine is an inhibitor of fatty acid oxidation and therefore it is highly unlikely to influence body weights.

The increased retro-peritoneal fat weights also gave an indication that the DIO group had indeed gained weight over the 16 week period (Fig. 3.1.2). The drug had no effect on these fat depots. There is no evidence in literature of any study which indicated that oxfenicine had an effect on bodyweight or intra peritoneal fat weight.

Impact of chronic oxfenicine treatment on:

Myocardial function before ischemia

Our basal mechanical function data show no significant differences in any of the groups (Fig. 3.1.3). It has been shown that the obese rat heart has reduced cardiac mechanical function compared to controls. The reduced function is observed in both basal conditions as well as after an ischemic event (Du Toit *et al.* 2008; Essop *et al.* 2009). Reduced basal function in the DIO groups compared to the control groups was not obtained in our study. Essop *et al.* (2009) represented data that showed reduced basal function in the DIO rats when compared to the controls. These authors perfused the hearts on a working heart perfusion apparatus while we used a Langendorff perfusion system. The heart is not in an

active contractile state on the Langendorff system. This could explain why we did not see any functional differences between the control and obese hearts. The work heart perfusion system is much more sensitive to functional differences between control and obese hearts, than the Langendorff perfusion system. The work done by Essop *et al.* (2009) was in the same animal model as in our study, but hearts were perfused with a buffer containing glucose and no palmitate or insulin. This could be another reason for the fact that they observed worsened function in the DIO rats while we could not. This is in line with findings by Taegtmeyer (2000) who state that the normal heart function is better when both fatty acids and glucose is present in the perfusate. In another study done by Du Toit *et al.* (2008) the addition of insulin (30 μ IU/ml or 50 μ IU/ml) before ischemia appears to improve the mechanical function of the DIO rat hearts and may have played a role. In addition, it has been shown that oxfenicine does not affect heart rate, coronary flow or arterial blood pressure (Stephens & Higgins, 1985).

Myocardial function after ischemia

After regional ischemia (40 minutes), global ischemia (25 minutes) and reperfusion (10- and 20 minutes), we found that the myocardial function of the DIO groups was significantly reduced compared to controls (Fig. 3.1.4 to Fig. 3.1.7). The reason for the different ischemic times used in the two protocols is one of a practical nature i.e. during global ischemia the whole heart is made ischemic as opposed to regional ischemia, where just a region of the heart is made ischemic. Therefore the time of ischemia cannot be the same for both types of ischemia, as global ischemia for 40 minutes (as with regional ischemia) would be too severe on the heart and leads to total mechanical failure during reperfusion. With global ischemia of 25 minutes, the heart can evoke pressure and therefore mechanical function can still be recorded. Furthermore, 40 minutes of regional ischemia yield an average infarct size of ~40% and thus creating an opportunity to either increase or decrease the infarct size. Du Toit *et al.* (2008) show data of decreased contractile function in DIO rats, which was possibly due to what they refer to as a *hypertrophic response*. This hypertrophic response is characterized by increased wall

thickness followed by dilation and contractile dysfunction. We found similar results after subjecting the hearts to ischemia/reperfusion which would suggest they were more prone to ischemia/reperfusion injury.

Oxfenicine is known to improve cardiac contractile function in control animals before and after ischemic insults (Burges *et al.* 1981; Molaparast-Sales, Liedtke & Nellis, 1987; Chavez *et al.* 2003; Chandler *et al.* 2003; Broderick & Glick, 2004). This effect is attributed to the stimulation of glucose oxidation and decrease in lactate production (Stephens & Higgins, 1985; Higgins *et al.* 1981). However, to our knowledge, there is no current data on the effects of chronic oxfenicine treatment in a model of diet induced obesity. Our results show that the drug significantly improved the cardiac mechanical function of the DIO group treated with oxfenicine compared to the DIO group without the drug. The improved function in the DIO rats has not been shown before and could be due to the stimulation of glucose/pyruvate metabolism (Stephens & Higgins, 1985; Higgins, Morville & Burges, 1981; Burges, Gardiner & Higgins, 1981; Molaparast-Sales, Liedtke & Nellis, 1987). Similar results have been found in studies in which animals were treated with oxfenicine and where improvement in cardiac mechanical function was observed. These findings of the effects of oxfenicine on cardiac function were investigated in different experimental models. In a domestic pig model, oxfenicine improved contractile function and cardiac efficiency during demand ischemia (Chavez *et al.* 2003; Chandler *et al.* 2003). Broderick and Glick (2004) found that acute oxfenicine treatment (2mM oxfenicine in perfusate) in age matched male and female Sprague-Dawley rats improved the recovery of systolic pressure, cardiac output and ventricular contraction.

The control + oxfenicine groups had reduced function when compared to the untreated animals. The worsened function could be linked to oxfenicine induced cardiotoxicity. Oxfenicine has been shown to cause (1) cardiac-, liver- and kidney hypertrophy, (2) uncoupling of oxidative phosphorylation, (3) phospholipid-, triglyceride- and fatty acid accumulation as well as (4) inhibition of myocyte membrane components, in control rats (Bachmann & Weber, 1988). In the above mentioned study the administered dose of

oxfenicine was 150mg/kg/day for a period of 8 weeks in control lean animals. In 1988, it was shown that oxfenicine caused lipid accumulation with an intraperitoneal injection of 60mg/kg (Jodalen *et al.* 1988). Oxfenicine treatment has also been associated with cardiac hypertrophy at a dosage of 900mg/kg/day (for up to 2 years, only after three months) (Higgins, Morville & Burges, 1981; Higgins, Morville & Burges, 1980; Greaves *et al.* 1984). All of the above mentioned factors could explain the marked decrease in cardiac function in control rats. However, Okere *et al.* (2007) recently found that at a dosage of 200mg/kg/day, oxfenicine showed none of these negative effects on the rat myocardium. In the same study oxfenicine caused increased cardiac triglyceride levels in control rats fed a high saturated fatty acid diet.

There seems to be a few factors that play a role in the vast range of effects reported for oxfenicine. These include dosage, duration of treatment, mode of administration (injection or ingestion), animal model (control, obese, heart failure/hypertrophy), and the type of diet as well as additional effects of other interventions such as ischemia and/or reperfusion. The influence of these factors may explain why reason oxfenicine treatment yields different effects in the published studies.

Studies of Bachmann and Weber in (1984), Stephens and Higgins (1985), Higgins, Morville & Burges, 1981 and Greaves *et al.* (1984) only briefly discuss the effects of oxfenicine on the liver. It should be kept in mind that in our study, this drug was administered over a period of 8 weeks and was supplemented in the drinking water. Results from the study of Bachmann and Weber in (1984), Stephens and Higgins (1985) and Finck *et al.* (2005) are an indication that oxfenicine possibly has uncharacterised effects on the liver and possibly other organs like the kidney and skeletal muscle (Finck *et al.* 2005; Higgins, Morville & Burges, 1981). It is therefore possible that the inconsistency in oxfenicine effects seen in our data and the literature is not only due to oxfenicine's influence on the heart but also on other organs (Bachmann & Weber, 1984; Jodalen *et al.* 1988; Higgins, Morville & Burges, 1981; Greaves *et al.* 1984; Okere *et al.* 2007; Chavez *et al.* 2003; Chandler *et al.* 2003; Stephens & Higgins, 1985; Higgins *et al.* 1981; Burges, Gardiner & Higgins, 1981; Molaparast-Sales, Liedtke & Nellis, 1987).

Infarct size

When the susceptibility to ischemia/reperfusion was determined in our study, the infarct size was significantly larger in the DIO group compared to the control group (Fig. 3.1.8). This has been previously described by other investigators (Essop *et al.* 2009; Boudina & Abel, 2005). The mechanism for this phenomenon could be related to the mitochondrial permeability transition pore (mPTP) which is thought to play a role in myocardial cell death during ischemia/reperfusion (Hausenloy & Yellon, 2003). Multiple factors may play a role and these include altered lipoprotein levels, ectopic cardiac triglyceride lipid deposition, activation of inflammatory pathways, metabolic derangements, perturbed mitochondrial respiratory capacity and mitochondrial dysfunction (for details see reviews by Boudina & Abel 2005; Bugger & Abel, 2008; Morin *et al.* 2001). We could not find differences in the basal cardiac mechanical function of the DIO group when compared to that of the control group. The differences in the DIO group compared to the control group were only evident after ischemia/reperfusion. This indicates that obesity predisposes the heart to ischemia/reperfusion injury but does not necessarily compromise cardiac function under normoxic conditions.

Control rats treated with oxfenicine had significantly larger infarcts in comparison to untreated controls, which could also be related to the oxfenicine induced cardiotoxicity reported by Bachmann and Weber (1988). The DIO rats treated with oxfenicine displayed smaller infarct size than the DIO rats without treatment. Infarct size was even smaller than those of the controls. This correlates with data from other groups who showed that oxfenicine treatment protects the heart against necrotic damage during ischemia and it reduced infarct size (Higgins, Morville & Burges, 1980; Korb *et al.* 1984; Stephens *et al.* 1985; Vik-Mo *et al.* 1986; Kong *et al.* 2002). Data on trimetazidine, another inhibitor of fatty acid oxidation, suggests that this drug might decrease mitochondrial uncoupling and increase the production of membrane protective glycolytic ATP (Essop & Opie, 2004). This could also apply to oxfenicine. Another proposal is that the experimental stimulation of glucose oxidation, prior to and during ischemia, might cause a rise in intracellular pH (basic) and decrease $\text{Na}^+/\text{Ca}^{2+}$ exchange. This might lead to reduced intra-cellular

calcium overload and eradication of cell death/necrosis (Haigney *et al.* 1992; Liu *et al.* 1996a; Liu *et al.* 1996b; Liu *et al.* 2002). Furthermore it has been shown that oxfenicine efficiently prevents cell death (Kong *et al.* 2002). These are all possible mechanisms for the reduction in the infarct size in the DIO rats treated with oxfenicine compared to the DIO rats without oxfenicine treatment.

For MS there are existing mechanisms for reduced contractile function and susceptibility to ischemia/reperfusion. These mechanisms include: (1) increased rates of fatty acid oxidation and (2) uncoupling of mitochondrial respiration (Bugger & Abel, 2008). Thus, in the case of our study, it was important to investigate these mechanisms (whether the diet had an effect) as well as to investigate the effects of the drug. Therefore we set out to assess mitochondrial respiration and the different proteins within the energy metabolism pathways and the RISK pathway.

Mitochondrial function

State 3 respiration recoveries: glutamate or palmitate as substrate

When glutamate was used as substrate there was no difference in the state 3 respiration recovery (after 25 minutes anoxia followed by 6 minutes re-oxygenation) in the DIO group when compared with the control group (Fig. 3.2.1 and Fig. 3.2.6). This is in line with the findings of Essop *et al.* (2009) who showed that the DIO animals have impaired contractile function and impaired mitochondrial respiratory capacity. We could not find any uncoupling of mitochondrial respiration in the DIO groups, in contradiction to the concept that type 2 diabetes mellitus (T2DM) and the metabolic syndrome (specifically obesity) lead to mitochondrial dysfunction/uncoupling of mitochondrial respiration (See review by Bugger & Abel 2008).

This further supports our existing knowledge that our animal model is not one of diabetic but rather a model of pre-diabetes and insulin resistance (and/or transient insulin

resistance) (Du Toit *et al.* 2008). This would still qualify the model as one of the metabolic syndrome. The state 3 respiration recoveries were significantly reduced in the control groups + oxfenicine when compared to the control groups without treatment. This is in agreement with the results in a study by Bachmann and Weber (1988). Oxfenicine treatment improved the state 3 respiration percentage recoveries of the DIO rats compared to the DIO rats without treatment. Although there were no differences between the recoveries of the DIO group vs. the control group, it should be noted that the recovery of the DIO + oxfenicine rats was increased to control levels.

We assessed mitochondrial respiration using palmitoyl L-carnitine (palmitate) as substrate, but with an *n* of 3. (Numbers were restricted due to funding and animal availability). State 3 respiration percentage recovery data showed no differences in any of the groups, when palmitate was used as substrate. The fact that we could not detect differences could be due to the small sample size or that there simply were no differences.

Myocardial oxygen consumption (QO₂) with glutamate or palmitate as substrate

Oxygen consumption was significantly higher in the DIO groups in comparison with the controls, with glutamate as substrate (Fig. 3.2.2 and Fig. 3.2.7). This could be due to oxygen wastage with increased fatty acid oxidation, since more oxygen is required to produce equivalent amounts of ATP during fatty acid oxidation (Essop & Opie, 2004).

Taking this into consideration, the question might arise as to why there would be increased oxygen consumption in the DIO rats when our results show no mitochondrial uncoupling. This could be due to the fact that our model is not fully diabetic but rather pre-diabetic and/or transiently insulin resistant and thus complete uncoupling is not *yet* evident, mitochondria might still be in transition into uncoupling. Furthermore, even though the oxygen consumption is significantly increased, it might not be severe enough

to impair mitochondrial respiratory capacity/respiratory recovery ability or cause mitochondrial uncoupling.

Oxfenicine increased the oxygen consumption in the control rats when compared to controls without oxfenicine. This negative effect might also relate to oxfenicine induced cardiotoxicity (Bachmann & Weber, 1988). Oxfenicine causes an increase in mitochondrial oxygen consumption in the control rats. This indicates oxygen wastage and may be a mechanism by which the negative effects of the drug are caused. Therefore, in retrospect, it appears that the same drug that decreases oxygen consumption in the DIO rats causes its increase in the control rats. The possible factor that signals the absence of oxygen wastage in the one group but not in the other group again raises the issue of elevated levels of *fatty acids*, which are known to cause oxygen wastage (Essop & Opie, 2004). Thus the oxfenicine induced lipid accumulation could exacerbate oxygen wastage in the control rats treated with oxfenicine.

DIO rats treated with oxfenicine displayed decreased oxygen consumption. The exact mechanism for this improvement remains unclear. This finding correlates with the improved state 3 respiration recoveries, infarct size and function during reperfusion.

Myocardial oxygen consumption when palmitate was used as substrate followed the same pattern as when glutamate was used as a substrate. Therefore the same rationale regarding possible mechanisms are applicable as described earlier. With regards to the oxygen consumption during the experiments with palmitate as fuel substrate, it should be noted that the pattern of changes between groups is similar but more pronounced. These changes are 1) an increase in oxygen consumption of controls + oxfenicine compared to the controls, 2) higher oxygen consumption in DIO rats compared to controls and 3) decreased oxygen consumption in DIO rats + oxfenicine when compared to DIO rats without oxfenicine. The more pronounced differences seen between the groups, when palmitate is used, is in line with the notion that palmitate is more representative and a better reflection of mitochondrial uncoupling since fatty acids are the main fuel for the adult mammalian heart *in vivo* or *ex vivo* (Essop *et al.* 2009).

ADP phosphorylation rates with glutamate or palmitate as substrate

Our data show no differences in the ADP phosphorylation rates in any of the groups when glutamate was used as substrate (Fig. 3.2.3 and Fig. 3.2.8). Similar results were obtained in the study done by Essop *et al.* (2009). From their study, the conclusion can be made that differences in ADP phosphorylation rates between control hearts and DIO hearts are dependent on the type of substrate used. When glutamate was used as substrate, there were no differences in the ADP phosphorylation rates of the DIO rats compared to the control rats. When palmitate was used, they observed higher ADP phosphorylation rates in the control rats when compared to the DIO rats.

However in our study, the ADP phosphorylation rates when palmitate was used as substrate showed no differences between the control rats compared to the DIO rats. As discussed previously this could possibly be because this obese model is not fully diabetic but rather pre-diabetic and transiently insulin resistance. No differences were observed between the ADP phosphorylation rates of control rats and control rats + oxfenicine. Another explanation could also be the small sample size in our study (n=3). The ADP phosphorylation rates were significantly increased in the DIO + oxfenicine group when compared to the DIO group without oxfenicine. When glutamate was used as substrate we observed no differences in any of the groups. This could suggest that for each type of substrate the ADP phosphorylation rates are unique and differ.

ADP: O ratios with glutamate or palmitate as substrate

When glutamate was used as substrate we found that the ADP: O ratios were lower in the DIO rats in comparison to the control rats (Fig. 3.2.4 and Fig. 3.2.9). This is just a representation of our data that showed an increase in oxygen consumption and no change in the ADP phosphorylation rates in these groups. This suggests that the level of obesity or the effect of the high caloric diet might be sufficient to cause oxygen wastage but not

sufficient to influence ADP phosphorylation rates (and thus not sufficient to diminish ATP production).

The ADP: O ratios were increased in the DIO rats treated with oxfenicine compared to the DIO rats without treatment. It should be noted that even though the statistical software expressed the ADP: O ratios of the oxfenicine treated DIO rats as significantly increased; the value of this ratio is raised to that of the control rats. This makes the ADP: O ratios similar to those of the control rats. The ADP: O ratios when palmitate was used as substrate, followed the same pattern as when glutamate was used as a substrate. Therefore the same rationale is applicable. With the ADP: O ratios during the use of palmitate as fuel substrate, it should be noted that the pattern of changes between groups are similar but more pronounced. These changes are: i) increase in ADP: O ratios of controls + oxfenicine compared to controls without oxfenicine, ii) higher ADP: O ratios in DIO rats compared to controls without oxfenicine and iii) decreased ADP: O ratios in DIO rats + oxfenicine when compared to DIO rats without oxfenicine. The more pronounced differences seen between groups, when palmitate is used, is in line the concept that palmitate is more representative of mitochondrial uncoupling/energy substrate oxidation as fatty acids are the main fuel for the adult mammalian heart both *in vivo* and *ex vivo* (Essop *et al.* 2009).

Respiratory control indexes with glutamate or palmitate as substrate

There were no differences in the RCI values between any of the groups when glutamate was used as a substrate (Fig. 3.2.5). However when palmitate was used as a substrate, the RCI was higher in the DIO group when compared to the control group (Fig. 3.2.10). The RCI gives an indication of the tightness of coupling of ATP production to energy substrate oxidation (Lindenmeyer, Sordahl & Schwartz, 1968; Lanza & Nair, 2009). These data suggest that there was no mitochondrial uncoupling in any of the groups. The same results were obtained when palmitate was used as substrate.

Western blot data

Obesity is known to be responsible for a whole range of molecular and metabolic perturbations, which ultimately influence cardiac function and susceptibility to ischemia/reperfusion injury (Dandona *et al.* 2005; Poirier *et al.* 2006). In light of this we also explored for possible changes in the beta oxidation pathway enzyme/protein expression and the involvement of the RISK pathway (namely ERK (44/42) and PBK/Akt) in the effects of oxfenicine in our study. All the Western blots were done on hearts that were subjected to 25 minutes of global ischemia and 30 minutes of reperfusion. Our western blot data highlighted some differences in protein expression and activation between study groups.

Total CPT-1 expression

The diet did not have an effect on the total CPT-1 expression (Fig. 3.3.1). Oxfenicine however decreased the expression in the DIO rat hearts when compared to the DIO rats without treatment (Fig. 3.3.1). The fact that the CPT-1 protein expression was decreased in the DIO rats treated with oxfenicine, may partly explain the dramatic positive effects seen in these DIO rats after treatment. The general understanding is that when a receptor is inhibited, the cell could be expected to respond by overriding the inhibitor's effect and increase receptor expression.

This was not the case in our study, regarding the effect of oxfenicine on the CPT-1 expression in the DIO rats. In an attempt to try and explain this finding, we reverted back to the basic pharmacology of the drug. We could not find information regarding the specific way in which oxfenicine inhibits CPT-1 in the heart. However, the consensus is that oxfenicine inhibits CPT-1 in a pH dependent manner, analogous with the mechanism of malonyl-CoA. Oxfenicine is also structurally similar to malonyl-CoA and could therefore inhibit CPT-1 in a similar manner (Stephens & Higgins, 1985). Lopez-Vinas *et*

al. (2007) proposed an *in silico* model of CPT-1 binding sites for malonyl-CoA (or oxfenicine).

According to Lopez-Vinas *et al.* (2007), CPT-1 has two binding sites for inhibitors (malonyl-CoA and therefore also oxfenicine): one binding site of a high- and one- of a low affinity. These binding sites are said to be at the N- and C terminals of the receptor and the site Met⁵⁹³ amongst others, plays a major role in the inhibition of CPT-1. Under normal circumstances the cell would sense the availability of substrates (like lipids and carbohydrates) by means of AMPK such as after a low fat/high carbohydrate meal. The low availability of fatty acids activates malonyl-CoA which inhibits CPT-1, so that fatty acids are not broken down any further, but rather carbohydrates (Dyck *et al.* 2004). When fatty acid levels return to normal, the sensor (AMPK) signals the cell that fatty acid levels are normal. The inhibition by malonyl-CoA is abolished by an enzyme called malonyl-CoA-decarboxylase (MCD), which decarboxylates and inactivates malonyl-CoA and thus the cell can increase CPT-1 protein expression. If the fatty acid levels increase or are continuously elevated, the cell would inactivate malonyl-CoA related CPT-1 inhibition and further increase CPT-1 protein expression, to correct for the extremely high levels of fatty acids. Therefore, with regards to fuel metabolism, the cell does not increase the protein expression as a means of counteracting the malonyl-CoA inhibition, but rather as an attempt to deal with the high levels of fatty acids. The increase in protein expression to counteract the inhibition of an inhibitor might only be of relevance in other forms of inhibition, with the absence of a sensor like AMPK.

However the situation may change slightly when pharmacological inhibition (oxfenicine) of CPT-1 is induced. Now both oxfenicine and malonyl-CoA is present. These two agents will most probably compete for binding sites on CPT-1. Despite this, oxfenicine will inhibit CPT-1. When oxfenicine inhibits CPT-1, both the sensor (AMPK) malonyl-CoA is still active.

It should be remembered that the DIO rats were already obese at 8 weeks while the controls were not, due to diet differences. Concerning the diets, the level of obesity in the

DIO rats causes elevation of FFA levels, which have been shown to increase uncoupling protein (UCP) expression (Bugger & Abel, 2008). At 8 weeks, control rats were not obese and thus the cell/heart had no stimulus to increase UCP expression. Concerning oxfenicine; the drug has been shown to, under some circumstances, cause cardiac tissue lipid accumulation. During the last 8 weeks, the DIO rat hearts were already exposed to elevated fatty acids from adipose tissue as well as elevated fatty acids caused by oxfenicine treatment. As elevated levels of fatty acids are known to increase UCP expression, this would further increase UCP expression. This creates the condition where both the diet and the drug ultimately lead to increased UCP expression. In the control animals, this was not the case, since their UCP expression levels would be normal, after 8 weeks of their control diet.

It is only after another 8 weeks of oxfenicine treatment, that lipid accumulation, caused by oxfenicine would increase UCP expression in the control animals. However, this is not as severe as that of the DIO rats, but rather intermediate in severity. Thus the DIO rat hearts would have dramatically higher UCP levels than the intermediately increased UCP levels in the controls. UCP's are known to be able to export fatty acids out of the mitochondria and decrease lipotoxicity (Opie & Knuuti, 2009), in fact, UCP's are believed to exist as an inherent cardiac adaptive or protective mechanism (Bugger & Abel, 2008). Therefore in the DIO rats the severely increased UCP's could possibly decrease the fatty acid levels. This would be sensed by the sensor (AMPK) and the cell would be signalled to decrease fatty acid breakdown and lead to decreased CPT-1 expression.

This decrease is even further exacerbated by the already present inhibition of CPT-1 and signals to the cell to further decrease CPT-1 expression. This could explain the decrease in CPT-1 protein expression in our DIO rats treated with oxfenicine. This decrease in CPT-1 expression would not occur in the control animals, since the intermediate level of UCP expression, caused by the drug, might not be enough to cause a decrease in CPT-1 expression. Therefore no decrease in CPT-1 expression is detected.

Total MCAD expression

There were no differences in the MCAD levels between any of the groups (Fig. 3.3.2). Neither the diet nor the drug had an effect on MCAD expression. This corroborates data from a study done by Okere *et al.* (2007) who also found that oxfenicine had no effect on MCAD expression. It is known that the metabolic changes which occur during the state of ischemia and insulin resistance are similar to those that occur during hypertrophy/heart failure (Opie & Knuuti, 2009). In another study in a rodent model of progressive hypertrophy, in vivo expression of MCAD was maintained at control levels (Lionetti *et al.* 2005). The reason for this unchanged MCAD expression after oxfenicine treatment remains unclear. If it is agreed that oxfenicine does inhibit CPT-1 activity, then MCAD activity should also be inhibited. MCAD protein expression levels should thus remain unchanged, but MCAD activity should be decreased. Therefore MCAD activity should be assessed in future studies rather than mere total expression. Okere *et al.* (2007) found that oxfenicine increased MCAD activity in control animals fed a diet of high saturated fatty acids.

Phosphorylated and total IRS-1 expression

The phosphorylation of IRS-1 (Ser 641) was significantly increased in the DIO group when compared to the control group (Fig. 3.3.3). There were no differences in IRS-1 phosphorylation between the other groups. Total IRS-1 expression was significantly increased in the controls + oxfenicine when compared to the control without oxfenicine and decreased in the DIO + oxfenicine group compared to the DIO group (Fig. 3.3.4). There were no differences between the control group and DIO group. Guo and Tabrizchi (2006) proposed that a high circulation/uptake of free fatty acids (as during insulin resistance/pre-diabetes) is associated with incomplete beta oxidation and accumulation of fatty acid oxidation intermediates such as ceramide, acyl-CoA, and diacyl-glycerol. As a result, the expression and activity of protein kinase C- θ (PKC- θ) increase which changes the phosphorylation state of the insulin receptor substrate (IRS). Serine (641) binding site

phosphorylation is increased in insulin resistance/pre-diabetes. This reduces the ability of the IRS to stimulate PI3-kinase (Phosphatidylinositol-3-phosphate-kinase). The reduced PI3-kinase activity, ultimately suppresses the glucose uptake by preventing Glucose Transporter-4 (GLUT-4) translocation to the cell membrane (Guo & Tabrizchi, 2006; McCarthy *et al.* 2005).

The fact that we saw differences in the IRS-1 phosphorylation is in line with the concept proposed by Guo and Tabrizchi (2006). The drug increased the total IRS-1 expression in the control + oxfenicine group compared to the controls without oxfenicine. This could explain the negative effects of the drug in the control animals. Oxfenicine possibly induces insulin resistance in the control animal. Thus a situation is created that is similar to that present in the insulin resistant/pre-diabetic state (as described by Guo & Tabrizchi, 2006). Another possible explanation for the increase in total expression in the control + oxfenicine group could be that oxfenicine inhibits beta oxidation and thus increases the accumulation of toxic beta oxidation metabolites. Thus the drug may further increase elevated fatty acid circulation/uptake which causes the serine (641) phosphorylation of IRS-1. The drug decreased the total expression of IRS-1 in the DIO group + oxfenicine. When observed individually, it might appear that there was phosphorylation of IRS-1 (Ser 641) in the DIO groups. However, when the phosphorylated/total IRS-1 ratio was determined we could see no differences. Therefore oxfenicine did not influence the activation of IRS-1 even though it influenced the total expression.

Total Glut 4 expression

Our data showed no differences in the expression of total GLUT- 4 in any of the groups (Fig. 3.3.6). This could be due to the fact that a more direct measure of glucose uptake and activation of GLUT- 4 would be the assessment of GLUT- 4 translocation. Thus if oxfenicine did increase glucose uptake, mere GLUT- 4 protein expression would not reflect this change or the possible effect of the drug. This is in line with our current understanding of GLUT- 4 and glucose uptake.

Total and phosphorylated ERK (42/44) and PKB/Akt expression

These kinases form part of the RISK pathway, a prosurvival signaling pathway which protects the myocardium against reperfusion-induced injury (Hausenloy & Yellon, 2004). We could not find any significant differences in the phosphorylated nor total ERK-44 and ERK 42 levels (Fig. 3.3.7 and Fig. 3.3.8) or the phosphorylated and total PKB/Akt. The ratio for phosphorylated/total ERK-44 and ERK-42 did not differ between groups, neither did the ratio for phosphorylated/total PKB/Akt. This ratio gives an indication of how much of the expressed protein is in actual fact phosphorylated. The fact that no differences were observed at 30 minutes does not necessarily mean there were no differences early in reperfusion. These kinases usually peak at about 15 minutes of reperfusion and then return towards basal levels (Hausenloy & Yellon, 2004, Hausenloy & Yellon, 2007). The conclusion is that the RISK pathway was not activated in any of the groups at the time point we investigated (i.e. 30 minutes reperfusion).

CHAPTER 5

CONCLUSION

From this study we can conclude that:

1. Chronic oxfenicine treatment protects the obese heart during ischemia/reperfusion.
2. Oxfenicine induced cardioprotection in obese animals appear to be due to the improvement of mitochondrial respiratory parameters (QO_2 , ADP: O ratios and ADP phosphorylation rates) without activation of the RISK pathway.
3. Chronic oxfenicine treatment has adverse effects on the hearts of control lean animals.
4. Oxfenicine induced adverse effects in control animals may be due to worsening of mitochondrial respiratory parameters (QO_2 , ADP: O ratios and ADP phosphorylation rates) but does not involve the RISK pathway.
5. There were no differences in the expression/activation of ERK, IRS-1 and PKB/Akt. MCAD and GLUT-4 expression remained unchanged whilst CPT-1 expression was decreased in the DIO + oxfenicine group compared to the DIO group.

Oxfenicine causes an increase in mitochondrial oxygen consumption in the control rats when compared to the normal controls. This indicates oxygen wastage and thus a possible mechanism by which oxfenicine causes its negative effects. In addition to this it might also be that the negative effects seen in the control rats + oxfenicine and the positive effects seen in the DIO rats + oxfenicine is not merely related to only CPT-1 inhibition but also an additional influence such as oxfenicine causing organ (liver, kidney) hypertrophy. The pronounced negative effects of oxfenicine or its pronounced positive effects might possibly be due to a systemic influence, additional to its local effects on the heart (Bachmann & Weber, 1988).

Based on the results of this study, it appears that chronic oxfenicine treatment may be an effective intervention if administered to metabolic syndrome animals. The treatment appears to protect the obese heart against ischemic injury and could therefore be used as a possible prophylactic treatment. At this stage, the toxicity of this drug remains unclear as many studies have reported a wide variety of consequences in various animal models post oxfenicine treatment, whether short term or long term. Extensive research is however still required before oxfenicine could be applied in clinical studies.

CHAPTER 6

LIMITATIONS OF THE STUDY

During this study we did not measure the direct effects of oxfenicine on myocardial glucose and beta oxidation. This could have been done by perfusions with radio-labelled glucose and palmitate. As this drug has never been tested (according to our knowledge) in a model such as ours, it may very well be that oxfenicine had an uncharacterised effect on myocardial fuel metabolism. This was however beyond the scope of this study.

We could have assessed mitochondrial respiration with palmitate, but with a bigger sample size. This would have given us a better indication of respiration, as fatty acids are the major fuel of the adult mammalian heart. Again limited availability of animals dictated that group numbers were sometimes sub optimal.

We could have done experiments on the working heart perfusion system, which may have given us a better indication of the effects of oxfenicine on myocardial mechanical function. This is a more complex model which requires more training and practise.

Because this was a chronic treatment study (8 weeks) it is difficult to know whether these drug effects observed are due to CPT-1 inhibition in the heart or due to indirect (systemic) effects on other organs such as the liver, kidney or skeletal muscle.

It would have been interesting to see what the effects of the drug would be on parameters such as fasting plasma glucose, plasma lipids, and plasma insulin as well as GLUT-4 translocation. These are all parameters that would have to be documented in a subsequent study but was beyond the scope of the present project.

We could not see activation of the RISK pathway because we had to record cardiac function at 30 minutes. At this time activity of ERK and PKB are in our experience already decreased. Activation could be seen when hearts were freeze clamped at 5 or 10

minutes reperfusion in previous studies in our laboratory. We believe documenting RISK pathway protein changes earlier in reperfusion may have provided different data.

CHAPTER 7

FUTURE ENDEVORS

Oxfenicine appears to achieve its positive effects, in the DIO rats, by improving mitochondrial respiration and activation of the RISK pathway. Furthermore, the ADP: O ratios were lower in the DIO rats when compared to the control rats. This is in line with the rest of our data which showed a decrease in cardiac function after ischemia/reperfusion, an increase in infarct size and an increase in oxygen consumption (QO_2) but no change in ADP phosphorylation rates. This suggests that the level of obesity or the high caloric diet might be sufficient to cause oxygen wastage but not sufficient to influence ADP phosphorylation rates (and thus not sufficient to diminish ATP production). However this is in accordance with literature which indicates that the negative effects of diet induced obesity are not related to diminished ATP production, but more likely a mechanism related to tissue damage or cell death/necrosis. The positive effects of oxfenicine observed in the DIO rats were decreased infarct size, improved cardiac mechanical function, decreased oxygen consumption (QO_2), increased ADP phosphorylation rates and increased ADP: O ratios these findings suggest that the underlying mechanism is related to ATP levels/production and possible reversal of tissue damage/cell death. Future studies could aim to investigate how oxfenicine (i) affects pathways involved in necrosis/apoptosis, (ii) whether it affects mitochondrial permeability transition pore (mPTP), Bad, Bax, Bcl-2, cytochrome-C, mitochondrial genes/DNA and (iii) whether oxfenicine affects Na^+/Ca^{2+} exchange and SERCA-2a.

A different mechanism appears to underlie the negative effects of oxfenicine observed in the control rats. Oxfenicine inhibits beta oxidation and would most probably cause accumulation of fatty acids, triglycerides and fatty acid oxidation intermediates which may lead to lipotoxicity. This has been shown in the literature (Jodalen *et al.* 1988; Greaves *et al.* 1984; Higgins, Faccini & Greaves, 1985; Okere *et al.* 2007, Finck *et al.* 2005). This could then serve as a possible explanation for the negative effects of oxfenicine in control hearts, particularly with regards to the increase in oxygen

consumption (oxygen wastage). The question might arise as to why the control hearts are negatively affected by oxfenicine induced accumulation of fatty acids/metabolites and/or lipotoxicity, while DIO rats fare better. This could be explained by the fact that DIO rats have adapted to cope with elevated supply of fatty acids to the myocardium, -lipotoxicity and -accumulation of intermediates, while this may not be the case for the control hearts (Morse, Gluati & Reisin, 2010; Steifelhagen, 2010). Uncoupling proteins have been shown to have paradoxical functions and could export fatty acids out of the mitochondria and lead to attenuation of lipotoxicity in the DIO rats and not in the control rats (Opie & Knuuti, 2009). The DIO rats might have severely increased UCP levels and control rats not or if it is, UCP levels are only increased intermediately. While the DIO rats has adapted to the lipotoxicity, the control heart is not. Future studies could investigate how oxfenicine influences UCP expression in the metabolic syndrome, during and post ischemia/reperfusion. These studies could also investigate how oxfenicine affects tissue lipid content in the DIO rats and controls.

CHAPTER 8

ADDENDUM TABLES

Addendum 1: Effects of oxfenicine up to date (Based on literature research):

Effect	Animal Model	Author (s)	Date (year)
Effective treatment for patients with obstructive coronary artery disease & angina	Human	Bergman <i>et al.</i>	1980
Has a potent effect on lactate metabolism, without any hemodynamic effects	Human	Naqvi <i>et al.</i>	1980
Protect hearts from necrotic tissue damage during ischemia	Rat	Higgins <i>et al.</i>	1980
Effectively increases pyruvate oxidation	Rat	Higgins <i>et al.</i>	1981
Can switch the heart from the oxidation of fat to glucose or lactate as fuel	Dog	Drake-Holland & Passingham	1983
Protects ischemic stressed myocardium	Canine	Korb <i>et al.</i>	1984
Induce cardiac hypertrophy in rats and dogs, after 1-2 years of treatment at various dosages	Rat & Dog	Greaves <i>et al.</i>	1984
Protects against ischemic damage	Rat	Higgins <i>et al.</i>	1985

Effect	Animal Model	Author (s)	Date (year)
Reduce the accumulation of long-chain acyl-carnitine in the ischemic myocardium after coronary artery occlusion and reduce infarct size	Dog	Vik-Mo <i>et al.</i>	1986
Has beneficial effects on cardiac contractility and enzyme release in the ischemic heart	Dog	Burges <i>et al.</i> ; Molaparast-Sales <i>et al.</i>	1981-1987
Oxfenicine induced cardiotoxicity	Rat	Bachmann & Weber	1988
Induces the accumulation of lipid in the rat myocardium	Rat	Jodalen <i>et al.</i>	1988
Have beneficial effects on hypoxic rat atria	Rat	Carregal <i>et al.</i>	1995
Reduced concentrations of the endogenous malonyl-CoA	Rat	Kennedy <i>et al.</i>	2000
Significantly blocked cell death induced by the combination of palmitate and carnitine	Cardiomyocyte	Kong <i>et al.</i>	2002
Dramatically decrease palmitate oxidation, increase glucose oxidation and improve cardiac efficiency	Swine	Chavez <i>et al.</i>	2003
Improves regional contractile power and efficiency during demand induced ischemia	Swine	Chandler <i>et al.</i>	2003
Increase recovery of systolic pressure, cardiac output and ventricular contraction	Rats	Broderick & Glick	2004

Effect	Animal Model	Author (s)	Date (year)
Might be effective in slowing the progression of clinical heart failure	Dog	Lionetti <i>et al.</i>	2005
Increase mRNA levels of genes regulated by PPAR α	Rat & Human	Rupp <i>et al.</i>	2005
Reduced recovery in fasted control animals, increased their lactate production and attenuated contracture in fed rats	Rat	Prendes <i>et al.</i>	2006
Not associated with cardiac hypertrophy in rats fed a high fat diet	Rat	Okere <i>et al.</i>	2007
Induce a switch in cardiac energy metabolism and thereby increasing left ventricular mechanical efficiency, without affecting cardiac energy expenditure	Swine	Zhou <i>et al.</i>	2008
Increased peripheral aorta resistance	Rat	Chang <i>et al.</i>	2010

Addendum 2: Results of clinical trials and animal studies on inhibition of fatty acid oxidation, with inhibitors other than oxfenicine:

Effects/Results	Animal Model	Author (s)	Date (year)
Reduces gluconeogenesis and improves economy of cardiac work	Rat	Rupp <i>et al.</i>	2002
Has a potent action on myosin heavy chain expression in the heart	Rat	Rupp <i>et al.</i> ; Stanley <i>et al.</i>	2002
Improves left ventricular function in dogs with chronic heart failure	Dog	Sabbah <i>et al.</i>	2002
Increases theoretical ATP:O ₂ -ratio and lowers myocardial oxygen consumption (MVO ₂) for glucose and lactate in comparison to long chain fatty acids	Swine	Chavez <i>et al.</i> ; Chandler <i>et al.</i>	2003
Increases cardiac mechanical efficiency under aerobic conditions	Swine	Chavez <i>et al.</i>	2003
Reduces the symptoms of demand induced ischemia and results in improved regional systolic function	Swine	Chandler <i>et al.</i>	2003
Prevents left ventricular remodelling and delays decompensation in pacing-induced heart failure	Dog	Lioveti <i>et al.</i>	2005

Effect	Animal Model	Author (s)	Date (year)
Long-term treatment improves functional class and left ventricular function in patients with HF	Rats, human, dog & swine	Fragasso <i>et al.</i>	2006
Reduces myocardial infarct size without affecting hemodynamic	Rat	Sesti <i>et al.</i>	2006
Improves cardiac pump function and slows the progression of heart failure	Rat	Lopaschuk	2006
Reducing the formation of reactive oxygen species (ROS) and improves reperfusion mechanical function	Human	Bhandari <i>et al.</i>	2007
Is <i>not</i> associated with cardiac hypertrophy in rats fed a high fat diet	Rat	Okere <i>et al.</i>	2007
Decreases myocardial oxygen consumption and improves cardiac efficiency in demand-induced ischemic heart	Rat	Wu <i>et al.</i>	2008
Improves left ventricular mechanical efficiency by increasing left ventricular power for a given rate of myocardial energy expenditure	Dog	Zhou <i>et al.</i>	2008

Effect	Animal Model	Author (s)	Date (year)
Increases efficiency of ATP production, decreases the production of lactate and protons and decreases the accumulation of harmful fatty acid metabolites	Rat & human	Lopaschuk; Dyck & Lopaschuk	2008
Had no effects on the post-MI occurrence of LV dysfunction or remodelling	Rat	Mouquet <i>et al.</i>	2009

CHAPTER 9

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