INVESTIGATING THE EFFECT OF *ASPALATHUS LINEARIS* AS A CARDIO-PROTECTIVE THERAPY IN ISOLATED RAT CARDIOMYOCYTES

Phiwayinkosi Vusi Dludla

(200707612)

Supervisors: Dr. R. Johnson

Prof. A.R. Opoku

Co-Supervisor: Dr. R. Salie

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Declaration

I declare that the entirety of the work contained herein is my own and that it has not been submitted for any degree or examination at any other University and that all the sources I have used have been acknowledged by complete references.

Full Name: Phiwayinkosi V. Dludla

Signature:

Date:

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Abstract

Diabetic cardiomyopathy (DCM) is a common disorder of the heart muscle and it contributes significantly to cardiovascular related deaths in the diabetic population. Excess generation of free radical species due hyperglycaemia has been directly implicated in the pathogenesis of DCM. Current studies focus on therapies to protect a diabetic heart at risk of cardiovascular disease, and also on prolonging lives of diabetic patients. In recent years, the use of *Aspalathus linearis* (commonly known as rooibos tea), as a therapeutic, has gained popularity worldwide. *Aspalathus linearis* (rooibos) is rich in antioxidants and may have a cardio-protective effect in combating oxidative stress induced by a diabetic state. However, its role in protection against diabetic-induced cardiomyopathy has not been fully elucidated.

Six month old Wistar rats weighing in the range 350-450 g were used for this study. Rats were divided into a non-diabetic and diabetic group. Diabetes was chemically induced by STZ injection, while rats in the non-diabetic group were treated with citrate buffer. Non-diabetic and diabetic rats were terminated and hearts removed 2 weeks after STZ injection. Cardiomyocytes were isolated and sequentially digested with collagenase before prepared for long term culture. Cultured cardiomyocytes were treated with ARC 61 (1 and 10 µg/ml), vitamin E (50 µg/ml), and n-acetyl cysteine (NAC) (1 mM) for 6 hours, before exposed to either 24 hours of oxidative stress (H_2O_2 solution) or 2 hours of ischaemia (ischaemic solution). After the pre-determined exposure time, metabolic activity (ATP assay) and relevant bio-assays (apoptosis, H-FABP membrane leakage, intracellular ROS determination and total GSH content) were done to assess the protective effect of rooibos against diabetic-induced cardiomyocytes injury.

Results obtained showed that cardiomyocytes from diabetic hearts were far more susceptible to oxidative damage compared to myocytes obtained from healthy rats. Cardiomyocytes exposed to either H_2O_2 or an ischaemic solution showed a decrease in metabolic activity and GSH content with a concomitant increase in apoptosis, intracellular ROS and membrane leakage. Pre-treatment with 1 µg/ml of ARC 61 was able to ameliorate this effect.

In conclusion, this study provides evidence that an aqueous extract of ARC61 has a potential cardio-protective effect against diabetic induced-cardiomyopathy in an *in vivo* cardiomyocyte model.

List of Abbreviations

NCD	Noncommunicable disease
CVD	Cardiovascular disease
DM	Diabetes mellitus
MeS	Metabolic syndrome
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
H-FABP	Heart-fatty acid binding protein
LDL	Low-density lipoprotein
IR	Insulin resistance
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus
ROS	Reactive oxygen species
IHD	Ischaemic heart disease
AMPK	5’ adenosine monophosphate-activated protein kinase
DCM	Diabetic cardiomyopathy
PFK2	Phosphosfructokinase 2
PKC	Protein kinase C
PKB	Protein kinase B
Ca	Calcium
AGEs	Advanced glycation end-products
HDL	High density lipoprotein
NAD	Nicotinamide adenine dinucleotide
SERCA2a	Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
GTFAT	Glutamine fructose-6-phosphate amidotransferase
ATP	Adenosine triphosphate
PUFAs	Polyunsaturated fatty acids
DAG	Diacylglycerol
GSH	Glutathione
CD-3	Cluster of differentiation 3
RO_2-	Hydroperoxyl radical
NAC	N-acetyl cysteine
HO^-	Hydroxyl radical

NF-kB	Nuclear factor kappa-B
P38-MAPK	P38 mitogen-activated protein kinases
JAK-STAT	Janus Kinase-Signal transducer and activator of transcription
SOD	Superoxide dismutase
H_2O_2	Hydrogen peroxide
O_2-	Superoxide radical
GSHPx	Glutathione peroxidase
CAT	Catalase
TGFβ	Transforming growth factors β
PI-3 kinase	Phosphoinositide 3-kinase
VEGF	Vascular endothelial growth factor
eNOS	Endothelial nitric oxide synthase
H9c2	Rat hear myogenic cell line
Pen/Strep	Penicillin-streptomycin amphoterin
FBS	Fetal bovine serum
BW	Body weight
STZ	Streptozotocin
PBS	Phosphate buffer saline
BrDu	5-bromo-2-deoxyuridine
DMSO	Dimethyl sulfoxide
HBSS	Hanks balanced saline solution
BSA	Albumin from bovine serum
BDM	2, 3 Butanedione monoxime
NSB	Non specific binding tubes
DCFH-DA	Dichlorodihydrofluorescin diacetate
H&E	Haematoxylin and eosin
PI	Propidium iodide
RIA	Radioimmunoassay
H-FABP	Heart-fatty acid binding protein
DMEM	Dulbecco’s modified Eagle’s medium
Vit E	Vitamin E
BDM	2, 3 butanedione monoxime
ddH_2O	Double distilled water

BSA	Bovine serum albumin
NaOH	Sodium hydroxide
Pen-Strep	Penicillin-streptomycin amphoterin B
HPLC	High-performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography with diode- array detection
MRC	South African medical research council
WHO	World health organisation
IDF	International diabetes federation
DSA	Diabetes South Africa
DDP	Diabetes discovery platform
ECRA	Ethics committee for research on animals
ARC	Agricultural research council

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Chapter 1

Literature Review

1.1. Global burden of disease

There is a continued shift in the burden of disease away from communicable to noncommunicable diseases (NCDs) (World Health Organisation, 2012b). Noncommunicable diseases account for 43% of the disease burden and 63% of deaths worldwide (World Health Organisation, 2012a). According to the World Health Organisation (WHO, 2012), death rates for NCDs will increase by 15% in the next decade except for Africa, South-East Asia and Eastern-Mediterranean where the increase is estimated to be around 20%. Currently, the countries with the highest mortality rates due to NCDs include Bosnia Herzogovinia (95%), Serbia (95%), Montenegro (95%), San Marino (95%), Bulgaria (94%), Croatia (95%) and Germany (92%). The 2012 WHO health statistics report revealed that the NCD mortality rate in South Africa has increased by 28% and that this increase encompasses an increase in the four major leading NCDs; i.e. cardiovascular disease (CVD) (48%), cancer (21%), chronic respiratory diseases (8.4%) and diabetes mellitus (DM) (2.6%) (World Health Organisation, 2010).

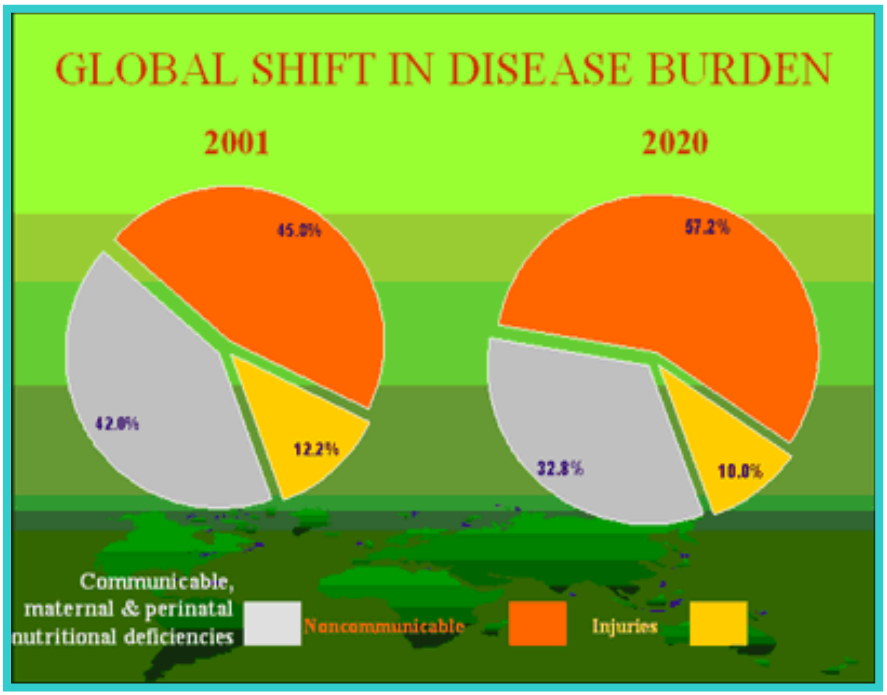


Figure 1.1. Representative graphs of the projected shift in the burden of disease from 2001 to 2020 (http://www.ceche.org/programs/globalhealth/globalhealth.html).

1.2. Risk factors associated with NCD

Urbanisation, per se, is often a positive development due to prospective economic opportunities luring more people from rural areas to big cities (Oucho, 1995). Though the prevalence of metabolic risk factors is evident in rural areas, it is strongly associated with an urbanised population (Al-Moosa et al, 2006). Rapid urbanisation can result in a swift transformation in life style. This transformation is favoured by a Western diet and this diet is accompanied by a high calorie intake and physical inactivity (Fig. 1.2) (Brons et al, 2009). Thus, making urbanisation and a change in lifestyle two of the major risk factors associated with the aforementioned shift in the global burden of disease (Allender et al, 2011;Mirmiran et al, 2009). A change in lifestyle results in, or is associated with various behavioral risk factors that include tobacco and alcohol use, physical inactivity and an unhealthy diet (Hu, 2011). These behavioral risk factors are often associated with an increased development of metabolic risk factors that include hypertension, increased blood triglycerides, abdominal obesity and high blood cholesterol (Park et al, 2003).

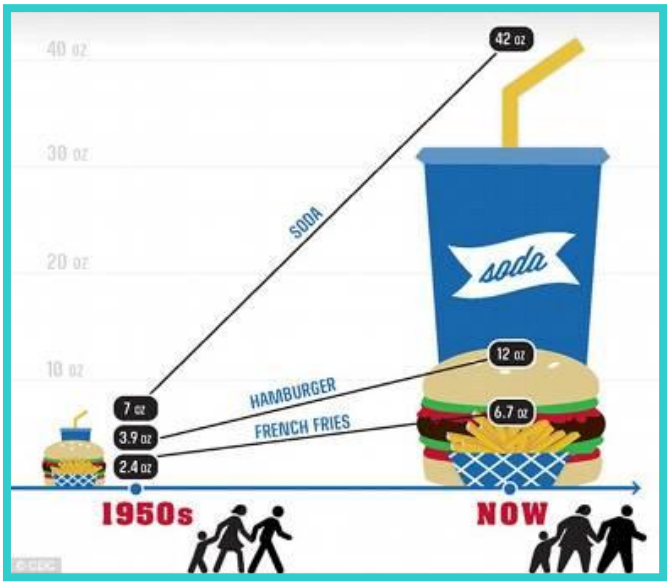


Figure 1.2. Representative image of an increased calorie size in fast foods over the past few decades (http://www.washingtonpost.com/blogs/wonkblog/post/a-fast-food-soda-is-six-times-bigger-than-it-was-60-years-ago/2012/05/24/gJQA23JxmU_blog.html).

1.3. Metabolic syndrome

Metabolic syndrome (MeS) encompass a group of risks factors that when occurring together increases the risk of developing CVD and diabetes (Grundy et al, 2004). Insulin resistance (IR) is one of the cornerstones of MeS (Pansuria et al, 2012).

1.3.1. Diabetes mellitus

Diabetes mellitus is a NCD that occurs either when the pancreas does not produce sufficient insulin or when the body cannot adequately use the insulin it secretes (WHO, 2012). Diabetes is associated with an impaired carbohydrate, fat and protein metabolism (American Heart Association, 2013). According to the latest International Diabetes Federation (IDF) report, 366 million people are affected by DM worldwide, and it has been estimated that the total number of individuals diagnosed with DM will increase to 552 million by the year 2030 (Garg et al, 2012). A recent report by WHO states that China has surpassed India to become the country with the highest incidence of DM with a reported figure of 92.4 million affected individuals (World Health Organisation, 2012b). In developing countries, the prevalence of DM has increased with South Africa ranking 18th in the world (World life expectancy, 2012). Furthermore, in South Africa, one in five individuals over the age of 35 years has been diagnosed with DM, and Diabetes South Africa (DSA) reported that over 50% of the people with DM remained undiagnosed in the country (Diabetes South Africa, 2010).

Diabetes mellitus can be classified as type 1 (T1D), type 2 (T2D) or gestational DM (Buchanan et al, 2012;Rosenbloom, 2007). Type 2 is the most common form of DM and is a result of interplay between environmental factors, lifestyle and genetics (Langenberg et al, 2011). Type 2 DM comprises of approximately 90% of DM incidents globally and is characterised by an attenuated metabolic response to insulin referred to as IR (Morrison et al, 2012) (World Health Organisation, 2012b). Insulin resistance results in insulin-stimulated decreased glucose uptake in the muscle and adipose tissue, and increased gluconeogenesis and glycogenolysis in the liver (Samuel and Shulman, 2012). Physical inactivity and increased intake of sugars and saturated fats has been shown to be the most notable risk factors for T2D (Petersen et al, 2012). Exercise is known to decrease insulin resistance by providing muscles with alternative pathways for glucose uptake (Kadowaki et al, 2003). Thus, healthy diet and exercise remain the best therapy for the management of T2D (Gregg et

al, 2012). Furthermore, T2D is characterised by elevated blood glucose, termed hyperglycaemia (WHO, 2012). Hyperglycaemia, in a diabetic state, is implicated in the observed increase in production of free radicals, especially reactive oxygen species (ROS) (Inoguchi et al, 2000). In a disease state, the production of free radicals may override the scavenging effect of the body's antioxidant system leading to oxidative stress (West, 2000). Oxidative stress is a major inducer of endothelial dysfunction (Pashkow, 2011). The endothelium plays an important role in the optimal functioning of the vascular system, and its dysfunction may lead to the development of CVD (Mattu and Randeva, 2013).

1.3.2. Cardiovascular diseases (CVDs)

Cardiovascular diseases accounts for 17 million deaths worldwide and an estimated 23.6 million people will die of cardiovascular complications by year 2030 (World Health Organisation, 2012b). The prevalence of CVDs increases with age and is higher in males compared to females (Lawlor et al, 2002;World Health Organisation, 2012b). In Sub-Saharan Africa, ischaemic heart disease and cardiomyopathies account for up to 75% of deaths related to cardiovascular complications (Amoah and Kallen, 2000). Furthermore, in the diabetic state, the risk of developing cardiovascular complications increases up to 80% in those with diabetes compared to non-diabetic individuals (Thandavarayan et al, 2011;Voulgari et al, 2010). In the 19th century, the first association between DM and CVD was made in the Framingham heart study (Rubler et al, 1972). In this study, diabetic individuals presented with consistent left ventricular hypertrophy without the existence of coronary artery disease or hypertension. This suggests that diabetic cardiomyopathy (DCM) exist independent of coronary heart disease. This observation has been supported by various other studies showing that hyperglycaemia can be an independent risk factor for the development of diastolic dysfunction (Jarnert et al, 2012;Mohty et al, 2012). According to Khavandi et al (2009), up to 37% of individuals with diastolic dysfunction are reported to be diabetic. Diabetic cardiomyopathy may be subclinical for a long period of time before the onset of clinical symptoms appears (Danielsen et al, 1987;Mohty et al, 2012).

1.4. Diabetic cardiomyopathy pathology

The precise mechanisms explaining altered cardiac structure and function in DCM still remains poorly understood (Battiprolu et al, 2010;Opie et al, 1979). However, current dogma suggest that hyperglycaemia exerts its damaging effect via various pathways, including (i) formation of advanced glycation end-products (AGEs), (ii) the polyol pathway, (iii) the hexosamine pathway and (iv) activation of protein kinase C (PKC) (Fig. 1.3) (Daroux et al, 2010;Jiang et al, 2006;Kaneto et al, 2001).

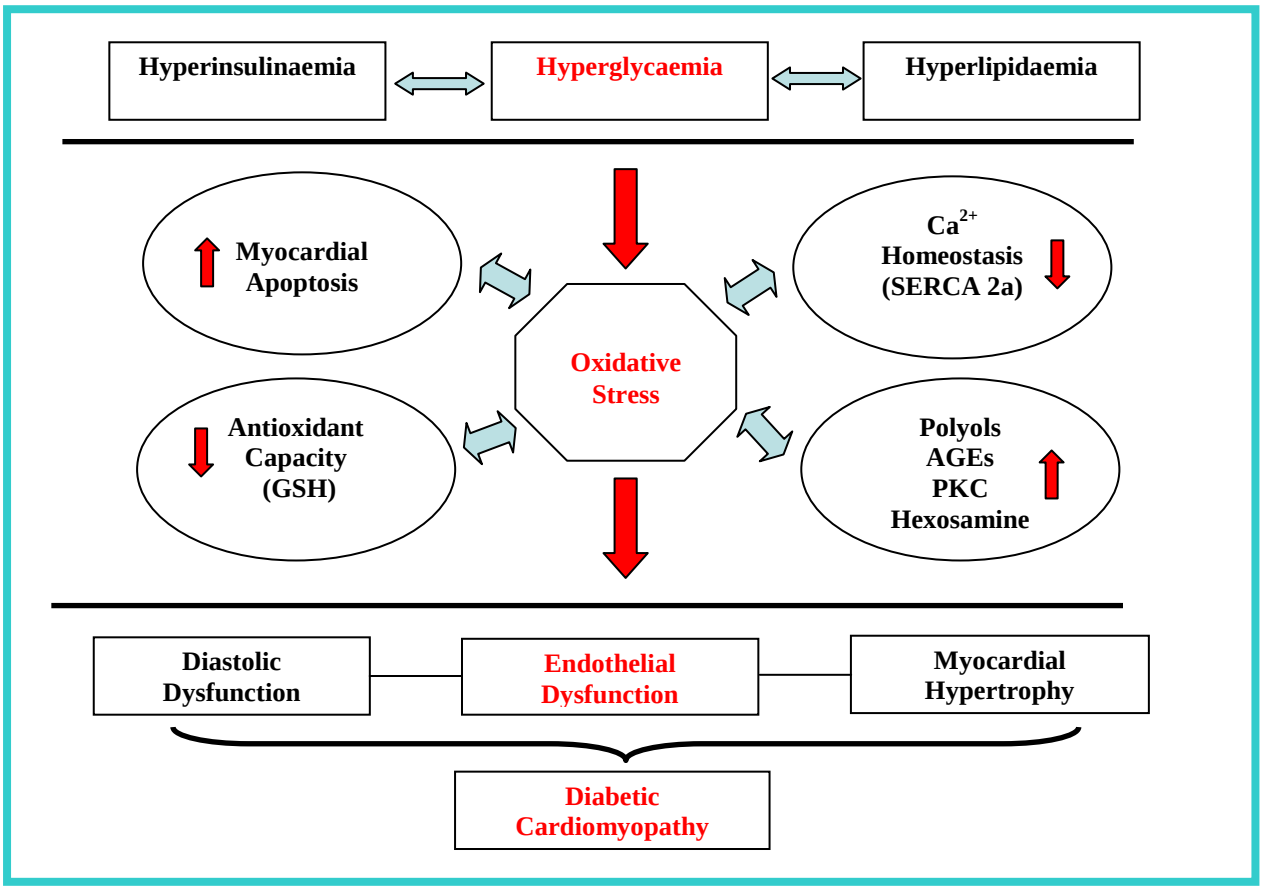


Figure 1.3. Schematic representation of myocardial damage as a result of hyperglycaemia. Modified from Battiprolu et al (2010).

1.4.1. Hyperglycaemia and the polyol pathway

The first mechanism associated with the effect of hyperglycaemia on the diabetic heart was described in 1966 (Gabbay et al, 1966). Today, it is known that the affinity of aldose reductase, a rate-limiting and an important enzyme in the polyol pathway, increases with an increase in glucose substrate (Tang et al, 2010). The overall reaction favors the production of sorbitol by reducing high intracellular glucose, and as a result aldose reductase consumes the cofactor nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) (Hinder et al, 2012;Lyons, 2002). Sorbitol, in excess, is known to be detrimental to the heart and is associated with endothelial dysfunction (Burg and Kador, 1988;Gaens et al, 2012). In the hyperglycaemic state, sorbitol activates nonenzymatic AGEs that blocks the activity of nitric oxide (NO) (Ansley and Wang, 2012). This activation of AGEs is reversible but is destructive in uncontrolled hyperglycaemia (Joshi et al, 2009).

1.4.2. Excess generation of advanced glycation end-products

Chronic hyperglycaemia can activate pathways that lead to the formation of AGEs (Rubin et al, 2012). It has been well documented that intracellular AGE precursors exert detrimental effects on cells via three mechanisms, i.e. modification of intracellular proteins involved in gene transcription, modification of intracellular matrix molecules involved in signaling and modification of circulating proteins in the blood (Cao et al, 2010;Kamioka et al, 2011;Sourris et al, 2010). AGEs are implicated in the deactivation of NO which plays a major role in endothelial tissue relaxation (Amezcua et al, 1989;Ghosh et al, 2012). Excess generation of AGEs may result in fibrosis and hypertrophy of cardiac cells (Grossin et al, 2008). As a result Ca^{2+} homeostasis is impaired, causing contractile dysfunction and ultimately myocardial apoptosis (LaRocca et al, 2012).

1.4.3. Hyperglycaemia and the hexosamine pathway

Under normal conditions, in the hexosamine pathway, fructose-6-phosphate is converted to N-acetylglucosamine-6-phosphate by the rate-limiting enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT) (Hsieh et al, 2012). The hexosamine biosynthetic pathway has been hypothesised to induce its detrimental effect via overexpression of GFAT (James et al, 2002). The overexpression of GFTAT has been found to promote obesity and

impair glucose tolerance in transgenic mice (Veerababu et al, 2000). Furthermore, excess formation of glucose through the hexosamine pathway induces increased transcription of genes for inflammation, oxidative stress and endothelial dysfunction (Back and Kaufman, 2012;Ceriello and Testa, 2009).

1.4.4. Hyperglycaemia and protein kinase C activation

The effects of the PKC are known to be hyperglycaemia-driven and are significant in vascular dysfunction (Koya and King, 1998). The actions of PKC are stimulated by the accumulation of metabolites in the glycolysis pathway that increase the synthesis of diacylglycerol (DAG) (Schmitz-Peiffer and Biden, 2008). Diacylglycerol, in turn activates PKC in the plasma membrane, which in turn affects gene expression of endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF β) and NADPH oxidase (Giacco and Brownlee, 2010;Jornayvaz and Shulman, 2012). Literature confirms the association between PKC activation and impairment in endothelial insulin signaling, vascular cell apoptosis and diabetic retinopathy in T2D patients (Geraldes et al, 2009;Tabit et al, 2013).

1.5. Effective treatment strategies to prevent diabetic complications

The quality of diabetes care remains suboptimal regardless of an array of effective interventions to prevent DM and its complications (Arbeeny, 2004;Polonsky, 2012). Long-term effective interventions that include changing diet and lifestyle are required to prevent DM and its complications (Carles and Raucoules-Aime, 2011). According to results obtained from the Diabetes Control and Complication Trial, tight control of blood glucose could reduce diabetic complications (National Diabetes Information Clearinghouse, 2012). In this clinical study, it was showed that keeping blood glucose levels as close to normal slowed the onset and progression of diabetic complications. However, optimal control of blood glucose levels still remains a big challenge (Sekhar et al, 2011). Thus, the use of antioxidant therapy to reduce oxidative stress might be an effective treatment strategy to reduce diabetic complications.

1.6. Oxidative stress

As defined earlier, oxidative stress is the imbalance between excess generation of free radicals and the inability of the body's antioxidant defense system to eliminate the radicals (Jones, 2006). Reactive oxygen species such as superoxide anion (O_2-), superoxide hydroxyl (OH^-) , peroxyl radical (RO_2-) and hydrogen peroxide (H_2O_2) are just some of the major free radical species that are implicated in the pathogenesis of various diseases, including DCM (Mackenzie et al, 2013;Rosca et al, 2012). Excessive generation of reactive oxygen species can have detrimental effects, including damage to lipids, DNA or proteins (Airaki et al, 2011;Stohs, 1995). In the diabetic state, generation of oxidative stress can result in the activation of several damaging pathways; i.e. the formation of AGEs, hexoamine pathway and activation of PKC, all of which are involved in micro- and macrovascular complications (Folli et al, 2011). Furthermore, superoxide and H_2O_2 generation can stimulate various signaling mechanisms. Some of which include, Janus Kinase signalling transducer and activator of transcription (JAK-STAT), p38 mitogen-activated protein kinase (p38-MAPK) and the nuclear factor kappa B (NF-kB) pathways. Activation of these destructive pathways can result in the structural and functional modification that contributes to vascular dysfunction (Pierce et al, 2009;Wu et al, 2012;Yamada et al, 2012).

1.7. The use of antioxidants as a protective therapy against oxidative stress

Hyperglycaemia, nitric oxide synthase (NOS) and the mitochondrial respiratory chain are just some of the major sources of oxidative stress generation (Giacco and Brownlee, 2010;Lobo et al, 2010). However, the human body is equipped with a defence system that controls free radical species and ameliorates oxidative damage via various enzymatic and nonenzymatic mechanisms (Bjelakovic et al, 2011). Oxidative stress can be ameliorated by a number of enzymatic antioxidant mechanisms (Kasperczyk et al, 2002). The most important intracellular antioxidant enzymes present in most cells, include superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT). Glutathione (GSH), vitamin C (vit C), vitamin E (vit E) and alpha-lipoic acid are just some of the nonenzymatic antioxidants that ameliorate oxidative stress (Aggarwal and Nesaretnam, 2012; Dakhale et al, 2011).

1.7.1. Enzymatic antioxidants

Superoxide dismutase rapidly converts O_2- to H_2O_2, which is then detoxified to water either by CAT or GSHPx in the lysosomes and mitochondria respectively (Marklund and Marklund, 1974). This enzyme is crucial to the body due to its strong antioxidant effect (Quiroz et al, 2009). Increased level of SOD in tissues is associated with longer life span (Van Raamsdonk and Hekimi, 2009). Superoxide dismutase can also be taken orally as an antioxidant supplement, but evidence supporting its absorption in the body is limited (Lamprecht and Williams, 2012). It can be used commercially as an injection to stop pain, inflammation and gout (Wang et al, 2004). Literature further reports on the augmented defects on the activity and protein expression of this enzyme in the diabetic state, thus causing a suppressed defense response (Bilginoglu et al, 2009;Sindhu et al, 2004).

Glutathione peroxidases are a group of enzymes that neutralises H_2O_2 and lipid hydroperoxides to their non-toxic forms (Bhabak and Mugesh, 2010). These enzymes can exist in different forms solely in the cytosol and mitochondria (Mari et al, 2012). The active site of GSHPx is cysteine mediated and this contributes to its strong antioxidative properties (Cho et al, 2010;Miller et al, 2012). It is reported that this enzymes protects red blood cells from H_2O_2-induced heme degradation (Nagababu et al, 2003). Furthermore, GSHPx is involved in the protection against H_2O_2-induced β-cell toxicity in diabetic mice, and its reduced levels are associated with the development of CVD (Buijsse et al, 2012;Harmon et al, 2009).

Catalase is one of the major antioxidant enzymes in the human body and it acts by converting H_2O_2 to water (Glorieux et al, 2011). The activity of catalase is associated with many diseases, including diabetes, and is also associated with aging (Goth, 2008). The target expression of catalase in mice reduces age associated mitochondrial dysfunction and insulin resistance (Lee et al, 2010). The reported down regulation of this enzyme is associated with an increased production of ROS which is implicated to be driving force in disease progression (Gomes et al, 2012;Min et al, 2010).

1.7.2. Nonenzymatic antioxidants

Glutathione is one of the major antioxidants in the body and is important for strong antioxidant status of the body (Jani et al, 2012). It can act as a direct scavenger or as a co-factor for GSHPx in neutralising free radical species (Liang et al, 2005). Glutathione in its reduced form plays an important role in the removal of H_2O_2 and peroxidation of membrane lipids (Dumaswala et al, 2000). A decrease in the reduced form of GSH is further associated with progression of diabetic complications (Chertow, 2004;Tachi et al, 2001). Furthermore, a study by Sekhar et al (2011) reports on the decrease in the reduced form of GSH in T2D patients with prolonged hyperglycaemia.

Vitamin E is a fat-soluble micronutrient that plays an important role in lipid peroxidation (Gupta et al, 2011b). According to literature, eight different forms of vit E exist, with alpha-tocopherol being the most active form in humans (Aggarwal and Nesaretnam, 2012). Vitamin E exerts its effect by directly reacting with free radical species (Flora, 2009). If supplemented with insulin, vit E protects against deterioration in renal function in an STZ-induced diabetes rat model (Haidara et al, 2009). In addition, it ameliorates oxidative stress and improves the antioxidant defense system in T1D patients (Gupta et al, 2011a). The beneficial effects of vit E may be related to the degree of oxidative stress and subsequently to the severity of hyperglycaemia (Giacco and Brownlee, 2010).

Vitamin C is a strong antioxidant and also acts as a co-factor in some enzyme reactions (Rahman, 2007). It has been reported that vit C attenuates the degree of atherosclerosis in an *in vivo* rat model by decreasing generation of oxidative stress (Ekuni et al, 2009). In a randomised trial, administration of metformin and vit C reduced hyperglycaemia and improved glycosylated hemoglobin in T2D patients (Dakhale et al, 2011).

Alpha-lipoic acid is a hydrophilic antioxidant and it plays an important role in mitochondrial dehydrogenase reactions (Johansen et al, 2005). The reduced form of alpha-lipoic acid, dihydrolipoate, directly reacts with ROS and is able to regenerate other antioxidants such as vit C and vit E (Packer et al, 1997). Through its strong antioxidant effect, it ameliorates hyperglycaemia and IR in T2D diabetic patients (Ansar et al, 2011). In addition, intravenous treatment with alpha-lipoic acid, reportedly improved endothelium-dependent vasodilatation in T2D patients (Heinisch et al, 2010). Furthermore, in an STZ-induced diabetes rat model, it prevents nerve dysfunction (Kanter et al, 2010).

1.8. The use of antioxidants as a cardio-protective therapy against diabetic-induced stress

1.8.1. Vitamin E as a cardio-protective therapy

To date, contradicting literature is available on the beneficial effect of vit E. In an infarcted rat heart model, vit E is reported to prevent elevation of malondialdehyde content, as well as the depression of left ventricular function (Dhalla et al, 2000). The beneficial effect of vit E is reported in reducing the risk of CVD in rats and also in diabetic individuals (Blum et al, 2010;Shirpoor et al, 2009;Ward et al, 2007). This highlights the potential use of vit E in ameliorating oxidative stress induced by diabetes. In contrast, in a study extending over ten years, involving 39 815 healthy women, vit E supplementation did not affect the overall risk of MI (Chae et al, 2012). Thus, the possible beneficial effect of vit E on cardio-protection requires further confirmation in large population studies.

1.8.2. Alpha-lipoic acid as a cardio-protective therapy

Literature reports on the strong cardio-protective effect of alpha-lipoic acid in both *in vitro* and *in vivo* models (Adeghate et al, 2010;Ghibu et al, 2012). In an *in vivo* rat model, an adequate dose of alpha-lipoic acid is reported to be effective in preventing myocardial reperfusion and this effect is strongly associated with its strong antioxidative effect (Oh et al, 2009). The synergic use of alpha-lipoic acid with melatonin is reported (Ghibu et al, 2012;Mukherjee et al, 2011). In this study, they reported that alpha-lipoic acid in collaboration with melatonin had a greater cardio-protective effect against oxidative injury than either of them alone.

1.8.3. Resveratrol as a cardio-protective therapy

Resveratrol is a natural phenolic compound found in red wine, grape skins and several other plants (Fremont, 2000). It has been found to possess several health properties, including cardio-protection (Netticadan, 2012;Smoliga et al, 2011). In an *in vivo* rat model, resveratrol is reported to improve lipopolysaccharide-induced cardio-toxicity (Sebai et al, 2011). In addition, resveratrol improves cardiac function in DCM by upregulating SERCA2a (Sulaiman et al, 2010;Zhang et al, 2010). The mechanism by which resveratrol exerts its

strong cardio-protective activity has been proposed to be via activation of p38-MAPK and phosphoinositide-3 kinase (PI-3-kinase) (Davies and Roulleau, 2012).

1.8.4. Herbal teas as an adjunct to pharmaceuticals

Herbal tea due to its health promoting properties is one of the most consumed beverage in the world (Liu, 2011). Herbal teas consist of various phenolic compounds, mostly flavonoids with strong antioxidant activity, and some of these phenolic compounds have been studied extensively in animal models (Erejuwa et al, 2011;Stephens et al, 2011). To date, various literature has reported on the antioxidant properties found in herbal teas and their efficacy in ameliorating diet-induced DM complications (Soory, 2012). Some of the herbal teas that are currently found on the market with known antioxidant properties include green tea, black tea, oolong tea, honeybush, and rooibos (McKay and Blumberg, 2007;van der Merwe et al, 2006).

1.8.4.1. *Camellia sinensis* (Green, Oolong and Black teas)

Camellia sinensis is a green shrub that is native to Southeast Asia (Fig. 1.4). Green, oolong and black teas are three of the major types of teas processed from *Camellia sinensis* plant (Ng et al, 2008). The leaves and buds of this plant are used to produce tea (Cabrera et al, 2003). Oolong tea is produced by partial oxidation of plant leaves, while black tea is produced from complete fermentation of the plant leaves, and green tea is unfermented tea (Balentine, 1992). The efficacy of green, oolong and black tea has been extensively studied and it shows strong pharmacological potential (Grove and Lambert, 2010). High-performance liquid chromatography analysis reveal the presence of catechins, theaflavins and thearubigins as some of the major phenolic compounds in these teas (Del et al, 2004). A study, investigating the efficacy of green tea, reported on its stronger antibacterial activity against *Salmonella*, a well-documented food borne pathogen (Ciraj et al, 2001). Other researchers have reported on the anticarcinogenic, anti-adipogenic, anti-hypercholesterolaemic as well as the cardio-protective of the plant (Maron et al, 2003;Pekal et al, 2012;Pekal et al, 2011).

Black tea, which is fermented tea, has been shown to possess the same antioxidant capacity as green tea (Leung et al, 2001). Black tea shows strong ameliorative effect on endothelial dysfunction through its activation of endothelial nitric NO synthase (eNOS) (Jochmann et al, 2008;Lorenz et al, 2009). In contrast to most findings, the lack of a feasible mechanism that accounts for improved endothelial function or reduced cardiovascular risk in patients after consumption of black tea has been reported (Widlansky et al, 2005). In addition, oolong tea has been shown to have anti-obesity properties in diet-induced overweight subjects (Grove et al, 2012;Grove and Lambert, 2010). It also possesses antimutagenic, antioxidant, antidiabetic, hyperlipidaemic and cardio-protective properties (Ankolekar et al, 2011;Chan et al, 2011).

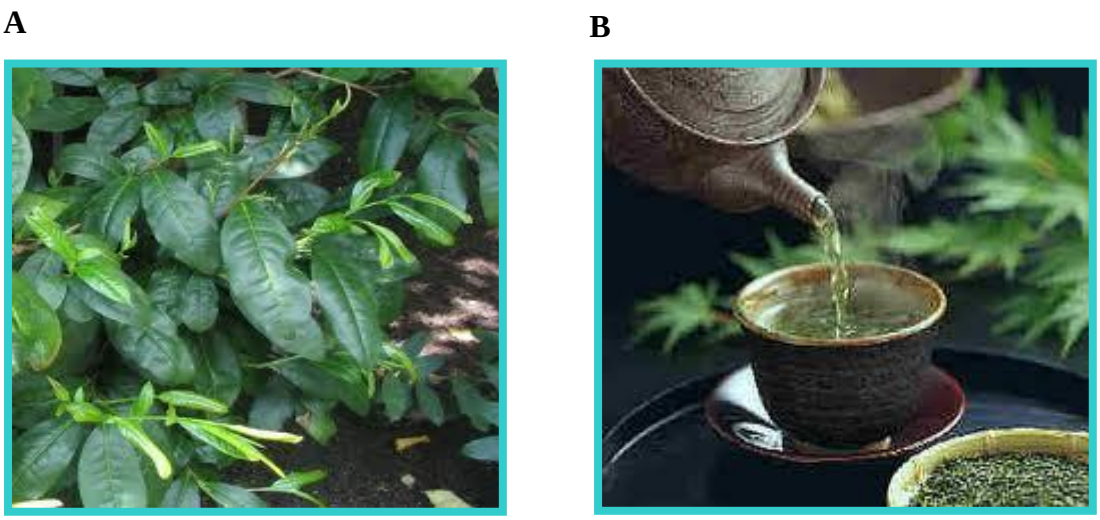


Figure 1.4. Representative images of *Camellia sinensis*. (A) A representative image showing green leaves of *Camellia sinensis* plant (http://www.growyourowntea.com/camellia-sinensis-1-quart-pots/). (B) A representative image of unfermented *camellia sinensis* (green tea) (http://everythingfibromyalgia.com/will-green-tea-keep-fibro-symptoms-at-bay/).

1.8.4.2. *Cyclopia* (Honeybush)

Cyclopia (honeybush) is a genus compromising of flowering plants and is indigenous to South Africa (Fig. 1.5) (McKay and Blumberg, 2007). Twenty three species of *Cyclopia* have been discovered thus far, and research has shown that the major bioactive compound in honeybush is mangiferin followed by hesperidin (Joubert et al, 2006).

Figure 1.5. A representative image of *Cyclopia* (honeybush) plant (http://www.capehoneybushtea.co.za/about.htm).

The fermented leaves and stems of the plant are used to produce tea (Kokotkiewicz and Luczkiewicz, 2009). An aqueous extract of honeybush has been shown to be effective in reducing hyperglycaemia in a diabetic rodent model (Muller et al, 2011). It ameliorated hyperglycaemia, hyperinsulinaemia and IR in the *in vivo* studies (Ariffin et al, 2011;van der Merwe et al, 2006). Other studies also confirm the anti-diabetic and chemo-protective properties of honeybush (Larsen et al, 2012;Marnewick et al, 2009).

1.8.4.3. *Aspalathus linearis* (rooibos)

Aspalathus linearis (commonly known as rooibos) is a plant that grows in the Cederberg mountain area of the Western Cape Province, South Africa (Fig. 1.6 A) (Morton, 1983). Rooibos tea is indigenous to South Africa and was discovered about 300 years ago (Fig 1.6 B). Rooibos tea is becoming popular in Western countries due to its growing health benefits (Joubert et al, 2008). As a result, the past few decades has seen an increased demand in the export of rooibos tea to European countries (Raynolds and Ngcwangu, 2010). This is a key socioeconomic factor to a developing country like South Africa.

A) B)

Figure 1.6. Representative images of *Aspalathus linearis* (rooibos tea). (A) An image of a plantation of rooibos tea that grows in the Cederberg Mountain of the Western Cape (http://www.foodwithastory.co.za/View-Business/299/Carmin-Organic-Rooibos-Tea). (B) An image of an aqueous extract of fermented *Aspalathus linearis* (Brum.f) Dahlg. (fabaceae) (Joubert et al, 2008).

The tea exists in two forms, a fermented and unfermented tea. In the fermented rooibos tea, which is produced by a process of oxidising the plant leaves, the leaves are picked and left to ferment (Joubert and Schultz, 2006). This fermentation process gives rooibos its distinctive reddish-brown colour (Gadow et al, 1997). Green rooibos (unfermented rooibos tea) is produced in the same way but is not fermented. Green rooibos tea is reported to be higher in antioxidant when compared to the traditional fermented red rooibos extract (van der Merwe et al, 2006). Rooibos tea contains abundant levels of phenolic compounds and antioxidants, including aspalathin, orientin, iso-orientin and rutin (Fig. 1.7).

The effectiveness of polyphenols in disease prevention has been questioned due to their limited bioavailability (D'Archivio et al, 2010). However, research continues to provide evidence on the availability of polyphenols in rooibos following human consumption (Courts and Williamson, 2009;Joubert et al, 2009a;Villaño et al, 2010). Literature has reported on an observed increase in the measurement of antioxidant in healthy human subjects following rooibos consumption (Breiter et al, 2011;Villaño et al, 2010). Aspalathin has been found to be unique to rooibos (Jaganyi and Wheeler, 2003). Research reports on the ameliorative effect of aspalathin on oxidative stress and in *Caenorhabditis elegans*, and hypoglycaemic effect in db/db mice (Chen et al, 2012;Kawano et al, 2009). Furthermore, rooibos ameliorates

metabolic disturbances in mice through its strong phenolic content (Beltran-Debon et al, 2011;Hesseling et al, 1979;Joubert et al, 2009b;Nakano et al, 1997). Its anti-inflammatory and anti-diabetic effect has also been reported in Wistar rats (Awoniyi et al, 2012;Muller et al, 2012). Literature shows the efficacy of rooibos is centralised to its strong antioxidative potential when tested in both *in vitro* and *in vivo* studies (Joubert and de Beer, 2011).

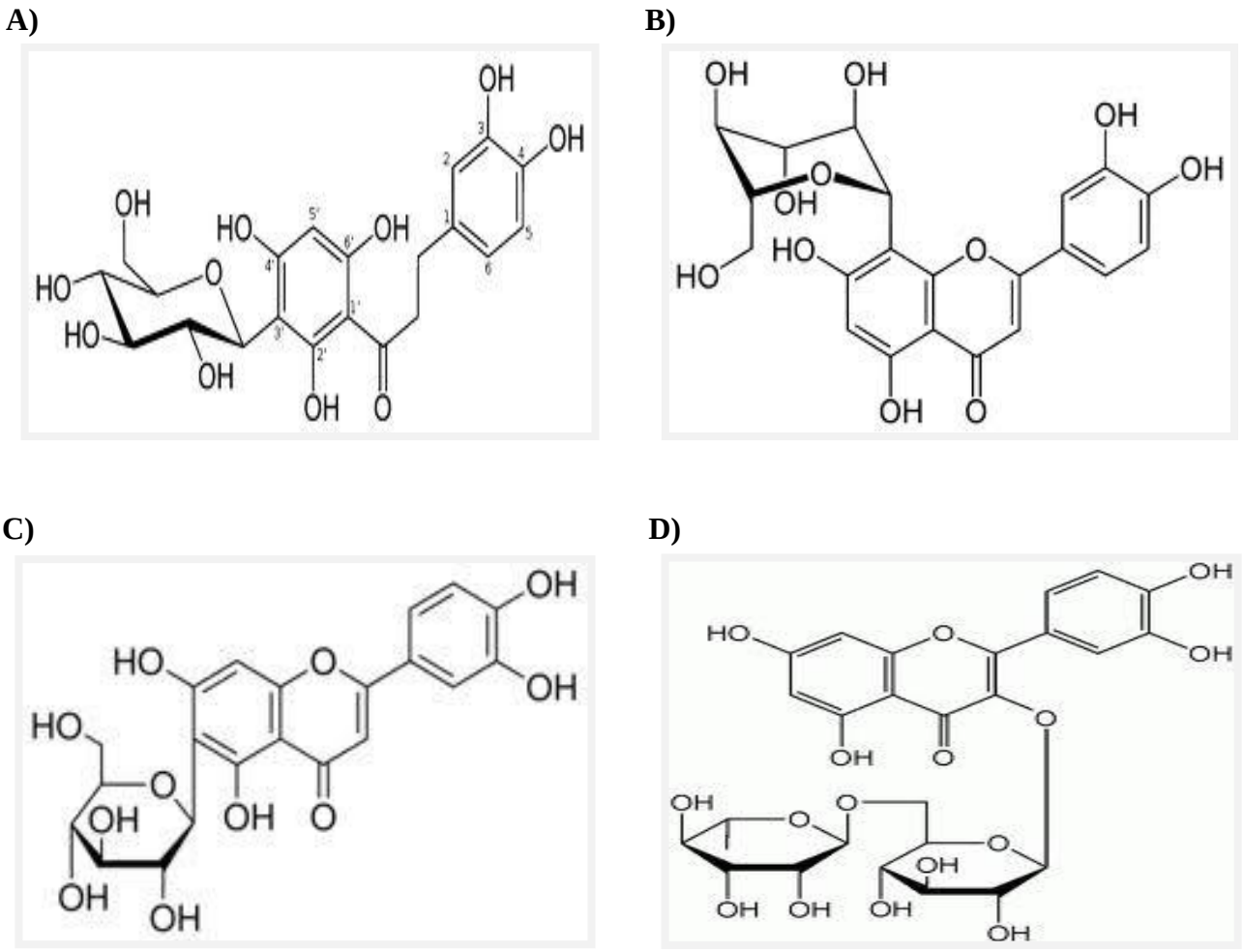


Figure 1.7. The major phenolic compounds present in rooibos. (A) The chemical structure of aspalathin (http://en.wikipedia.org/wiki/File:Aspalathin.svg), (B) orientin (Zhang et al, 2008), (C) iso-orientin (Yuan et al, 2012)and (D) rutin (dos Santos et al, 2008).

1.9. Experimental models to study the effect of natural compounds on diabetic induced cardiomyopathy

A number of *in vitro* and *in vivo* models have been used to study the effect of diabetic induced cardiomyopathy on cardiac cells (El-Helou et al, 2009;Wold and Ren, 2004). Through these models, a better understanding of the pathophysiology that occurs in the myocardial cells and molecular mechanism related to the disease state can be identified. Animal models, immortal and primary cell models are some of the integral models researchers use in the study of CVDs and their complications (Alpers and Hudkins, 2011;Colligan et al, 2002).

1.9.1. Primary cells

Acutely isolated primary cells are structural and functionally more relevant to the disease state and like immortal cells, can provide researchers with a tool to adequately study morphological changes that occur in the middle of a heart muscle (Greulich et al, 2012). Over the years models have been successfully established for the isolation of primary rat cardiomyocytes (Fischer et al, 1991). In 1977, adult cardiomyocytes were cultured for the first time (Wada et al, 1975). However, 33 years later it still remains a challenge due to alteration in cardiac phenotype with long term culture (Louch et al, 2011;Xu and Colecraft, 2009). Long-term cultured cardiomyocytes are known to change morphology in a process termed cardiomyocyte "redifferentiation" and this remain a obstacle to most pathophysiological studies (Allen et al, 2011).

Previous work on long term culture of cardiomyocytes reports on the importance of establishing a primary cardiomyocyte model showing a good transition from isolation to culture, with delayed morphological changes in cardiac cells (Gallina et al, 2012). Effective isolation of myocytes is the key to successful culture of rod shape cardiomyocytes (Banyasz et al, 2008). Although optimal preparation of primary cardiomyocytes may be achieved, successful culture of rod shaped cardiomyocytes with minimal alteration in phenotype still remains a big challenge (Tonne et al, 2011).

1.9.2. Immortal cell lines

H9c2 and HL-1 myoblasts cells are just some the immortalised cell lines that are commercially available, and they play an important role in the study of cardiac muscle research (Zordoky and El-Kadi, 2007). Furthermore, immortal cell lines provide researchers with a homogeneous population of cells that can be studied without the interference of nonmyocytic cells like fibroblast, endothelial cells and smooth cells (Fahey and Wallace, 2011). The study of immortal cell lines has provided and continues to provide value to the experimental studies on the heart muscle. However, immortal cell lines do not display characteristics of normal cardiac morphology and this, might limit their use in cardiovascular research (Allen et al, 2005;Ebert and Svendsen, 2010).

1.9.3. Animal models

Animal models (rodents and primates), due to their genotypic homology to human are extensively used as an alternative means to study the human pathology of various diseases, including diabetic cardiomyopathy (Cefalu, 2006). Animal models allow researchers to induce conditions, similar to humans, in order to study the pathology of the intact heart (Woodruff et al, 2011). However, the limitations of animal models lies within the inability to study morphological structure within an intact heart or to analyse the precise location related to an infarction (Zaragoza et al, 2011). This limits the use of animal models and highlights the importance of using isolated cardiomyocytes (Chlopcikova et al, 2001;Meng et al, 2012).

1.10. Study objectives

In this study we hypothesise that the antioxidants present in fermented rooibos extract can protect cardiomyocytes against oxidative injury associated with diabetic cardiomyopathy. Thus in this study, the aim was to establish an *in vitro* cardiomyocyte model in order to evaluate the cardio-protective effect of a fermented rooibos extract on diabetic induced cardiomyopathy.

The study aims were divided into three phases:

1.10.1. Phase 1

- To establish a rodent heart perfusion system.
- To isolate and establish a robust and viable e*x vivo* diabetic cardiomyocyte model.

1.10.2. Phase 2

- To expose cardiomyocytes for 3-24 hours to a dose equivalent of six cups of rooibos tea in order to elucidate the protective effect over time.

1.10.3. Phase 3

- To determine if hyperglycaemia and ischaemia results in an increased production of ROS, that leads to oxidative myocardial injury.
- To determine if a dose equivalent of six cups of rooibos has a protective effect and is able to ameliorate the modification of myocardial injury in diabetes.

Chapter 2

Experimental Design

2.1. Plant extract

2.1.1. Source and Rooibos extract preparation

A freeze dried aqueous extract of fermented rooibos (ARC 61) was supplied by Prof. Joubert of the Post-Harvest and Wine Technology Division, Agricultural Research Council (ARC). The extract was prepared as follows: Fermented rooibos (400 kg) was extracted on a large scale by percolating purified water (preheated to 93°C) and the resulting extract was circulated for 35 minutes with a ratio 1:10 of plant material to water. The final heated extract (~2.96% soluble matter) was centrifuged and concentrated with a plate evaporator under vacuum at less than 55°C. The extract was sterilised at 121-123°C with a contact time of approximately 68 seconds. The sterilised concentrate (~23% soluble matter) was cooled to less than 25°C and vacuum-dried for 24 hours. The final product was sealed in containers and stored in a decanter at room temperature until required.

2.1.2. High-performance liquid chromatography (HPLC) for ARC 61

High performance liquid chromatography with diode- array detection (HPLC-DAD) analysis was used to determine the chemical composition of phenolic compounds present in ARC 61 (Beelders et al, 2012). Briefly, analyses were performed on the Agilent 1200 system (Agilent Technologies, Waldbronn, Germany) at 37°C. Ultraviolet-visible spectra were recorded between 200-700 nm with selective wavelength monitoring at 288 and 350 nm.

2.2. Animals

Six month old adult male Wistar rats (350-450g) were used for the study. The animals were housed at the Medical Research Council (MRC) Primate Unit in an environmental controlled animal facility with a 12-hour light dark cycle in a temperature range of 23ºC-25ºC (relative humidity: ~50%). All animals had free access to standard rat chow and tap water. All procedures were performed in accordance with the MRC guidelines for care and use of laboratory animals (http://www.mrc.ac.za/ethics/ethicsbook3.pdf). Ethical approval for this study was granted by the MRC ethics committee for research on animals (Project ethical number: ECRA 11/03/G).

2.2.1. Non-diabetic control rats

Rats for the non-diabetic control group were injected with 0.1 M citrate buffer (588 mg of sodium citrate was dissolved into 15 ml ddH_2O to make a 0.1 M citrate buffer; pH 4.6) (without streptozotocin (STZ)) at an equivalent volume of 3ml/kg body weight (BW).

2.2.2. STZ diabetic rat model

Adult male Wistar rats (350-450g) were injected intraperitoneally with 40 mg/kg BW STZ in 0.1 M citrate buffer to induce hyperglycaemia (Fig. 2.1). Five days later (following a 4 hour fast) plasma glucose concentrations were determined. Rats with fasting plasma glucose concentrations higher than 14 mmol/l were considered as diabetic and included in the study.

Figure 2.1. Method of administering intraperitoneal STZ injection.
Source:http://www.procedureswithcare.org.uk/intraperitoneal-injection-in-the-mouse/.

2.3. Plasma glucose determination

After a 4 hour fast, tail pricks were performed on rats to measure plasma glucose levels using a glucometer (Accu-check®, Roche Diagnostics).

2.4. *In vitro* dose experiment

Based on a previous study done by Marnewick et al (2011), whereby they showed that 6 cups of rooibos tea had a cardio-protective effect, for this study cardiomyocytes were exposed to an equivalent dose of 6 cups of rooibos tea (ARC 61) *in vitro*.

2.4.1. Estimation of the human to rat equivalent dose translation of ARC 61

Human equivalent dose

Average human weight = 70 kg (Holmes et al, 2009)
1 cup of rooibos tea = 200 mg in 200 ml (Grassi et al, 2009)
i.e. Human equivalent dose = 2.9 mg/kg

Formula for dose translation from human to rat (Reagan-Shaw et al, 2008).

$$\text{Human equivalent dose (mg/kg)} = \text{Animal equivalent dose (mg/kg)} \times \frac{\text{Rat Km}}{\text{Human Km}}$$

$$2.9 \text{ mg/kg} = \text{Animal equivalent dose (mg/kg)} \times \frac{6}{37}$$

= 18.13 mg/kg (1 cup of tea)

Therefore, 6 cups of tea = 18.13 mg/kg x 6
= 108.78 mg/kg

Metric conversion (1 L H2O = 1 kg ARC 61)
109 mg/kg= 109 µg/ml (*in vitro* dose used in the study that is an equivalent to 6 cups of tea)

2.5. Experimental design

The present study will be divided into 3 subsections, namely phase 1, phase 2 and phase 3 (Fig. 2.2).

2.5.1. Phase 1

In this phase, the culture conditions were optimised to maximise long term culturability of rod shaped cardiomyocytes isolated from non-diabetic rats. Cell viability was assessed using trypan blue assay, metabolic activity was assessed using ATP assay and cell morphology was assessed using haematoxylin and eosin stain. This phase was needed for the successful completion of phase 2.

2.5.2. Phase 2

In this phase, the optimal time and dose effect of ARC 61 and vit E was determined using non-diabetic rats. The optimal time and dose of ARC 61 and vit E was selected based on the minimum dose at which cell death was induced after exposure to H_2O_2. In this phase, an ATP assay was used as an endpoint measurement of metabolic activity from which the optimal time and dose was selected. Successful completion of this phase was needed to complete phase 3.

2.5.3. Phase 3

In this phase, non-diabetic and diabetic rats were used to investigate the cardio-protective effect of ARC 61 against diabetic-induced cardiomyopathy. In this phase, various biochemical measures were measured (Fig. 2.2).

Study design

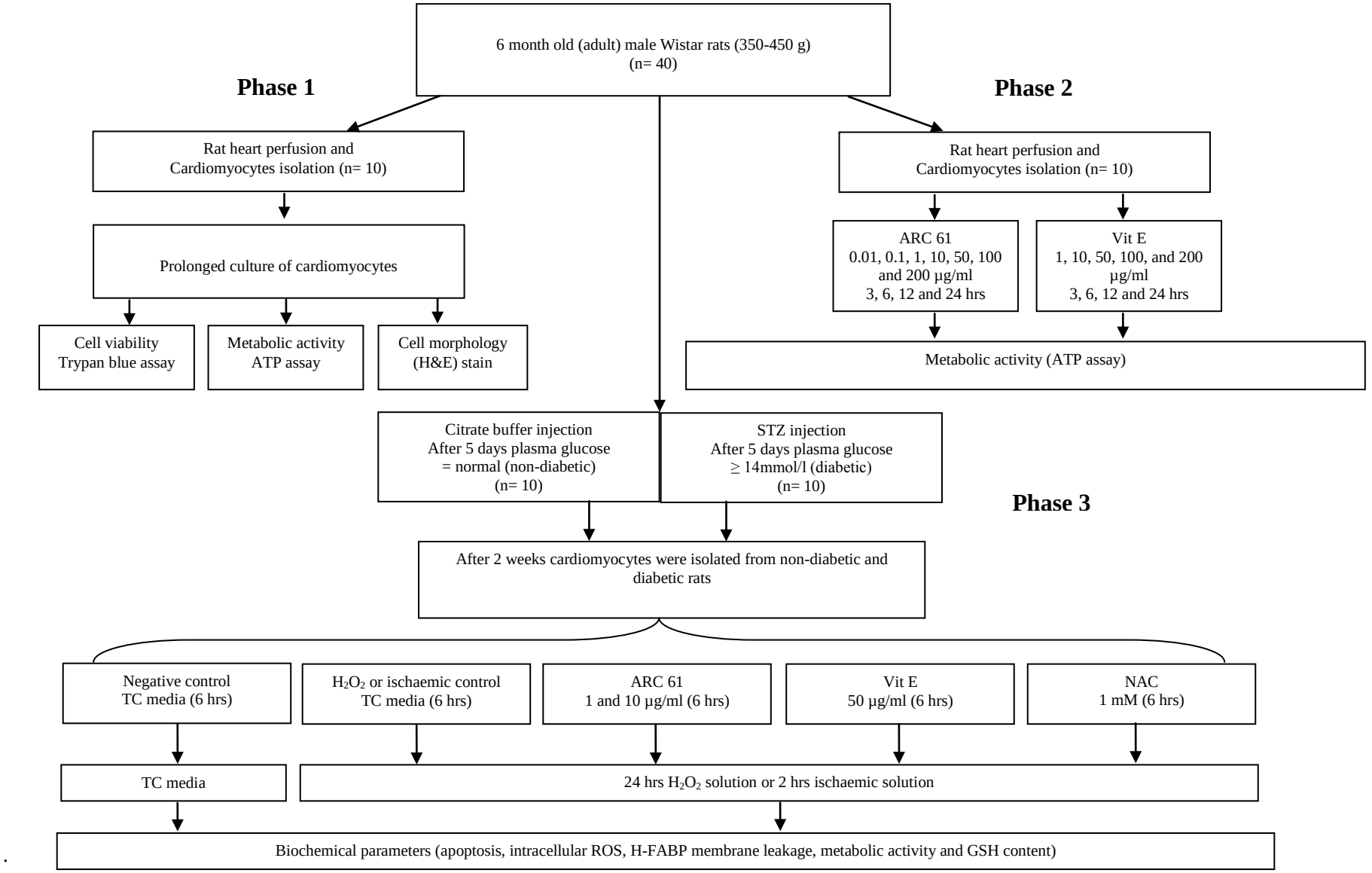


Figure 2.2. Representation of phase 1, phase 2 and phase 3 of the study. Abbreviations: STZ-streptozotocin, hrs- hours, H&E-haematoxylin and eosin, ATP-adenosine tri-phosphate, vit E- vitamin E, NAC-n-acetyl cysteine, TC-tissue culture, H_2O_2- hydrogen peroxide and ROS- reactive oxygen species.

Chapter 3

Material and Methods

3.1. PHASE 1

3.1.1. Cardiomyocytes isolation

Rat cardiomyocytes were isolated by modifying and merging two protocols (Fischer et al, 1991; Xu and Colecraft, 2009). Rats were anaesthetised with sodium pentobarbital (40 mg/kg) and hearts removed. The hearts were rapidly rinsed in calcium free ice-cold Kreb's-Henseleit buffer (prepared in H_2O containing: 119 mM NaCl, 24.9 mM $NaHCO_3$, 4.74 mM KCl, 1.19 mM KH_2PO_4, 0.6 mM $MgSO_4$; 0.59 mM $NaSO_4$; 1.25 mM $CaCl_2$ and 11 mM glucose, pH 7.4) and transferred to a Langendorff perfusion apparatus, where the aorta was fixed to a cannula and perfusion was initiated (Fig. 3.1). Pressure was kept above 60 kpa whilst perfusion solutions were continuously gassed with medical oxygen and the temperature was kept constant at 37ºC using a circulating water bath. The hearts were perfused with solution A (prepared in H_2O containing: 6 mM KCl, 1 mM NA_2HPO_4, 0.2 mM NaH_2PO_4, 1.4 $MgSO_4$, 128 mM NaCl, 5.5 mM glucose, 10 mM HEPES, pH 7.4) for 5 minutes to wash out residual blood. After 5 minutes, hearts were digested with solution B (Dissolved in "Solution A" containing: 2 mM glutamic acid, 0.1% collagenase, 5 mM BDM, 2 mM carnitine, 5 mM taurine and 100 mM $CaCl_2$) for a total of 35 minutes. During that period the calcium concentration of solution B was increased as follows; after 10 minutes 37 µl of 100 mM $CaCl_2$ was added followed by 50 µl of 100 mM $CaCl_2$ after 15 minutes. The digestion was continued for an additional 10-15 minutes. The digestion was complete when the solution flowed freely from the heart.

The heart was removed from the Langendorff apparatus, the atrium cut-off and the heart ventricles were minced into small pieces using scissors. The minced heart tissue was then submersed into an oxygenated solution C (Dissolved in 50 ml of Solution A + 50 ml of Solution B" containing: 1% BSA, 1% BSA (fatty acid free) and 100 mM $CaCl_2$) and then placed in a shaking water bath at 37ºC for 15 minutes. The Ca^{2+} concentration during incubation was raised by adding 100 µl of 100 mM $CaCl_2$ after 16, 17, 18, 19 and 20 minutes respectively, to obtain a final concentration of 1.25 mM $CaCl_2$. The myocytes were filtered through a 200 µm pore size nylon mesh and gently spun down at 30 x g for 3 minutes. The supernatant was aspirated and the pellet was resuspended by overlaying in 25 ml of solution D (Dissolved in "Solution A" containing: 0.1% BSA (fatty acid free) and 100 mM in $CaCl_2$) at room temperature. After 5 minutes, solution D was aspirated and the pellet was resuspended in 10 ml of supplemented medium 199 (5 mM carnitine, 5 mM taurine, 0,1 mM

BrdU, 5 mM creatine monohydrate, 5% FBS and 0.5% penicillin-streptomycin amphoterin B). Before continuation of the experiment, cell viability count was determined by trypan blue assay and a cell viability ≥ 70% was required for subsequent experiments.

Cells were pre-plated in a 60 mm Petri dish for 1 hour under standard tissue culture conditions (CO_2 incubator with 5% CO_2 at 37ºC with relative humidity ± 80%) to remove debris and nonmyocytic cells. After 1 hour of incubation, the non-adherent cells (cardiomyocytes) were removed and transferred to a sterile 50 ml tube. Cell viability was done and a cell viability of ≥ 70% was used for subsequent culturing.

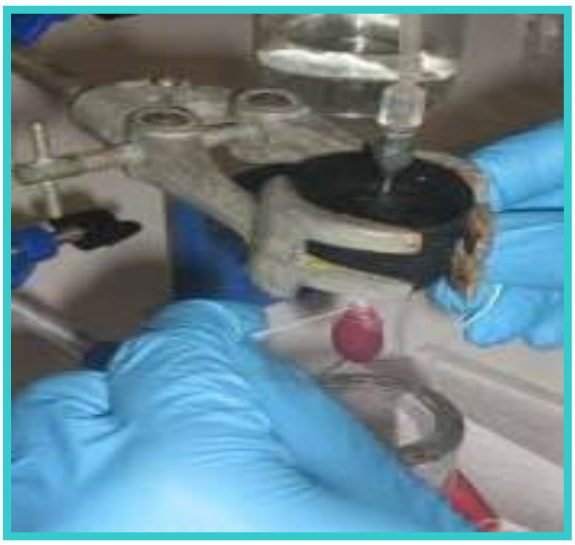


Figure 3.1. A representative image demonstrating cannulation of a rat heart onto a Langendorff perfusion system.

3.1.2. Optimisation and long term culturing conditions

Laminin coated plates were prepared a day prior to culture. Laminin was prepared according to the manufacturer's instructions. Tissue culture plates were coated with laminin at a final of concentration of 2 µg/cm^2.

Cells were plated at a required density (Table 3.1) on laminin coated plates and incubated under standard tissue culture conditions for 4 hours in supplemented media 199 (5 mM carnitine, 5 mM taurine, 0,1 mM BrdU, 5 mM creatine monohydrate, 5% FBS and 0.5% penicillin-streptomycin amphoterin B (pen/strep)). After 4 hours, the media was removed and the cardiomyocytes cultured overnight in supplemented media 199 with no FBS (5 mM carnitine, 5 mM taurine, 0,1 mM BrdU, 5 mM creatine monohydrate and 0.5% pen/strep).

Table 3.1. Seeding density

Plate	Growth area	Seeding density
6 well	**9.5 cm^2**	**5.94 x 10^5**
24 well	**3.8 cm^2**	**2 x 10^5**
96 well	**0.32 cm^2**	**1.9 x 10^5**

The following day (24 hours after isolation), a long term culture was setup by incubating cardiomyocytes under standard tissue culture conditions in media 199 with or without 5% FBS (5 mM carnitine, 5 mM taurine and 5 mM creatine monohydrate).

For each experiment, cells were divided into 2 experimental groups: group one, cells cultured with FBS and group two, cells cultured without FBS. In both group one and group two, cells were cultured in 24 and 96 well TC plates to assess cell morphology and cell viability respectively. Media was refreshed every other day. Metabolic activity (ATP assay), cell viability (trypan blue assay), and morphology (haematoxylin and eosin (H&E) stain) were assessed daily until cardiomyocyte redifferentiation occurred. Plates were discarded after each of the aforementioned measurements.

3.2. Cardiomyocyte contraction measurements

The contractions were measured by randomly focusing on one cardiomyocyte and contractility was recorded for 1 minute using Nikon eclipse Ti Inverted microscope (Nikon, Japan). The cardiomyocyte contractions were recorded for each plate before an end point measurement of each experiment was done.

3.3. Cell viability assessment (trypan blue dye)

The trypan blue dye was used to determine cell viability as an end point measurement of membrane integrity (due to exclusion of trypan blue dye). After aspiration of the media, cells were stained with 0.4% (v/v) trypan blue in PBS. Viable (unstained) and nonviable (stained) cells were counted using Nikon eclipse Ti Inverted microscope (Nikon, Japan). For statistical analysis, 5 fields were taken for each individual well.

3.4. Metabolic activity (ATP assay) of cardiomyocytes

The ATP assay was done using ViaLight™ plus kit (Lonza, Basel, Switzerland), following manufacture's instructions. Luminescence was measured using an ELISA plate reader BioTek FLx800 (Bio-Tek Instruments, Inc., Winooski, VT, USA).

3.5. Morphometric determination (H & E stain) of cardiomyocytes

Haematoxylin and eosin staining was done in clear 24 well tissue culture plates. After the predetermined experimental procedure, the culture media was aspirated and cells were fixed in 200 µl of a 4% (v/v) formaldehyde solution in PBS, pH 7.4 for 20 minutes. After fixation the formaldehyde solution was removed and replaced with 200 µl of 1% (v/v) Triton X-100 in PBS for 5 minutes. The Triton X-100 solution was aspirated and the cells were washed 5 times with PBS before staining with 200 µl Mayer's haematoxylin solution at room temperature. After 12 minutes the haematoxylin solution was aspirated and the cells were washed with ddH_2O before the eosin solution was added for 2 minutes. The cells were once again washed in ddH_2O followed by de-staining in 95% (v/v) ethanol before microscopic analysis.

3.6. PHASE 2

3.6.1. ARC 61 extract preparation for cardiomyocytes treatment

A stock solution of 0.1 mg/µl ARC 61 was prepared in ddH_2O and filter sterilised using a 0.22 µm filter. Serial dilutions of the stock solution (200 µg/ml, 100 µg/ml, 50 µg/ml, 10 µg/ml, 1 µg/ml, 0.1 µg/ml and 0.01 µg/ml) were prepared in supplemented media 199 (5 mM carnitine, 5 mM taurine and 5 mM creatine monohydrate).

3.6.2. Vitamin E solution preparation for cardiomyocytes treatment

Tissue culture grade vit E solution was prepared by dissolving 1 mg/ml of vit E in 100% (v/v) ethanol. A stock solution of 0.1 mg/µl vit E was prepared in media 199 before filter sterilisation using 0.22 µM syringe filter. Serial dilutions were prepared (200 µg/ml, 100 µg/ml, 50 µg/ml, 10 µg/ml, and 1 µg/ml) using supplemented media 199 (5 mM carnitine, 5 mM taurine and 5 mM creatine monohydrate).

3.6.3. Preparation of H_2O_2 for exogenous oxidative stress induction

H_2O_2 solution was prepared in supplemented media 199 (5 mM carnitine, 5 mM taurine and 5 mM creatine monohydrate) to make a 1000 µM stock solution. The stock solution was filter sterilised using 0.22 µM syringe filter before serial dilutions (512 µM, 256 µM, 128 µM, 64 µM and 32 µM) in media 199 were prepared.

3.6.4. Treatment of cardiomyocytes

Cardiomyocytes were cultured in 96 well tissue culture plates under standard tissue culture conditions in supplemented media 199 (5 mM carnitine, 5 mM taurine, 5 mM creatine monohydrate and 5% FBS). On the day of the experiment cultured cells were exposed to various concentrations of ARC 61 and vit E (Table 3.2). The negative controls were exposed to the vehicle only. After the time intervals as per table 3.2 the metabolic activity was assessed with the ATP assay (see section 3.4). Three independent biological experiments were done and each experiment had 3 technical replicates per time point.

Table 3.2. The optimal time and dose tested for the treatments

Treatment	Dose (µg/ml)	Time (hours)
ARC 61	0.01, 0.1, 1, 10, 50, 100 and 200	3, 6, 12 and 24
Vitamin E	1, 10, 50, 100 and 200	3, 6, 12 and 24

3.7. PHASE 3

3.7.1. *In vitro* experimental procedure for cardiomyocyte treatment

3.7.1.1. Diabetic rat model

A total number of 20 Wistar rat hearts were used for this phase of the study. Diabetes was induced in 6 month old adult male Wistar rats (n=10) by single intraperitoneal injection at a dose of 40 mg/kg BW STZ (see section 2.2.2). Non-diabetic control rats (n=10) received citrate buffer without STZ (see section 2.2.1). Diabetes was confirmed 5 days after STZ administration by measuring blood glucose as per section 2.3. Rats with blood glucose ≥ 14 mmol/l were considered diabetic and were included in the study. Diabetic rats were left for 2 weeks for the diabetic changes to develop in the heart. After 2 weeks diabetic and non-diabetic rats were euthanised using sodium pentobarbital at 35 mg/kg BW before hearts were removed.

3.7.1.2. Exposure and treatment of cardiomyocytes

After removal and perfusion of the hearts, cardiomyocytes were isolated and cultured overnight in 6 well tissue culture plates as per plate layout Fig. 3.2. The following day cardiomyocytes were exposed to different experimental groups and treated with ARC 61, vit E, NAC prepared in Dulbecco's modified eagle medium (DMEM) (4.5 g/L glucose, 3.7 g/L $NaHCO_3$, 1% (w/v) BSA (fatty acid free and no phenol red) respectively (as per plate layout Fig.3.2).

Cardiomyocytes were preconditioned by exposure to ARC 61, vit E and NAC for 6 hours. The negative and untreated controls (H_2O_2 and ischaemic controls) were exposed to DMEM only. After 6 hours, media was removed and replaced with either H_2O_2 or an ischaemic solution (prepared in ddH_2O containing: 116 mM NaCl; 50 mM KCl; 1.8 mM $CaCl_2$; 2 mM $MgCl_2 \cdot 6H_2O$; 26 mM $NaHCO_3$; 1 mM $NaH_2PO_4 \cdot 2H_2O$) for 24 hours and 2 hours respectively (see experimental plate layout Fig.3.2). The negative controls were not exposed to H_2O_2 or an ischaemic solution but received fresh DMEM.

3.7.2. NAC (n-acetyl cysteine) preparation for cardiomyocytes treatment

A 613 mM NAC stock solution was prepared by dissolving 100 mg of NAC into 1 ml ddH_2O. A working stock solution was prepared by dissolving the 613 mM stock solution into DMEM to a make a 1 mM NAC working stock solution. The working stock solution was filter sterilised using 0.22 µM syringe before use.

3.7.3. Radioimmunoassay (RIA) for rat insulin

3.7.3.1. Blood collection to determine rat insulin

Before removal of the heart, 1 ml blood was collected from the inferior vena cava into chilled heparinised tubes. Plasma was separated by centrifugation at 1400 x g (RCF) for 15 minutes at 4ºC and blood plasma was stored at -20ºC until RIA analysis.

3.7.3.2. RIA insulin determination

Rat insulin was determined using a radioimmunoassay kit (RI-13K) as per manufacturer's instruction (Linco, USA). The insulin concentration was determined using Perkin Elmer automatic gamma counter (Wellesley, MA, USA), and was expressed as ng/ml.

3.7.4. Assessment of cell viability, metabolic activity, oxidative stress and apoptosis in treated cardiomyocytes

3.7.4.1. Protein concentration determination for normalising cell counts

Protein concentrations were determined using the RCDC assay as per manufacturer's instructions Biorad (Hercules, CA, USA). Protein assay was done to normalise cell numbers per well to relevant assays.

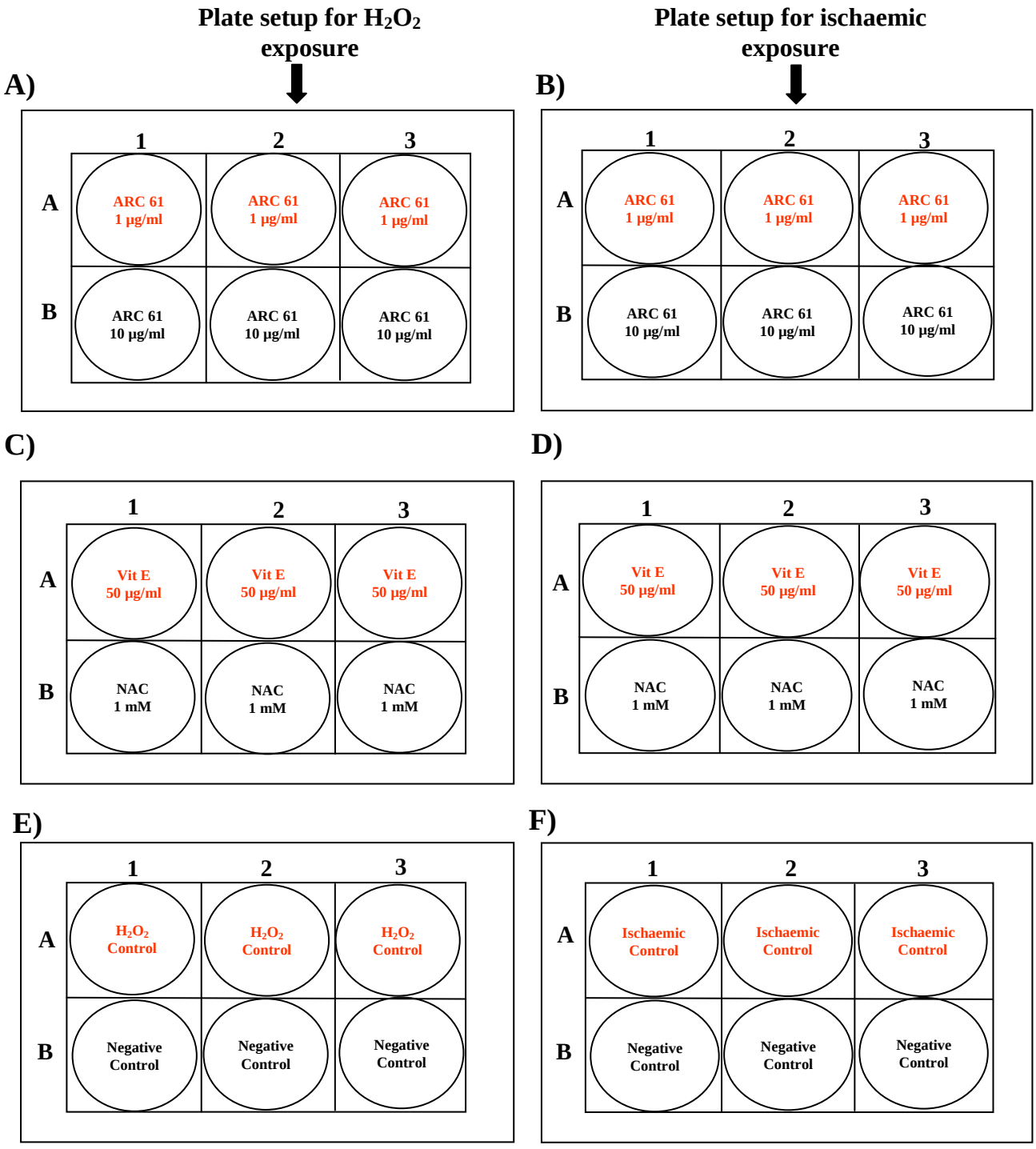


Figure 3.2. Experimental plate layout for cardiomyocytes exposed to H_2O_2 conditions. (A) Treated for 6 hours with 1 and 10 µg/ml ARC 61 respectively, (C) 6 hours with 50 µg/ml vit E and 1 mM NAC and (E) H_2O_2 control (only exposed to DMEM for 6 hours). After pre-treatments for 6 hours media was removed and cells were exposed for 24 hours to 32 µM H_2O_2.

Experimental plate layout for cardiomyocytes exposed to ischaemic conditions. (B) Treated for 6 hours with 1 and 10 µg/ml ARC 61 respectively, (D) 6 hours with 50 µg/ml vit E and 1 mM NAC and (F) Ischaemic control (only exposed to DMEM for 6 hours). After pre-treatments for 6 hours media was removed and cells were exposed for 2 hours to an ischaemic solution. For both H_2O_2 and ischaemic experiments, 3 technical replicates and 3 independent biological experiments were performed.

3.7.4.2. Annexin V and propidium iodide

Cardiomyocytes were washed once with PBS and apoptosis was evaluated by labelling cells with 10% annexin V (Invitrogen, Carlsbad CA) and 20 µg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO) dissolved in PBS. The cells were incubated at room temperature for 15 minutes with an annexin V/PI solution before it was removed and cells were resuspended in PBS. Fluorescence intensity of each cardiomyocyte was determined using NIS elements software with a Nikon Eclipse Ti Inverted microscope (Nikon, Japan). For statistical analysis, 5 fields were taken per individual well.

3.7.4.3. Determination of membrane leakage of cardiomyocytes

The myocardial membrane leakage was detected using a rat H-FABP ELISA kit according to manufacture's instructions (Hycult biotech, Uden, The Netherlands). Absorbance was measured using ELx800™ Absorbance microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Concentrations were determined using the Gen5 software. The readings were normalised to the protein concentration and expressed as mg/ml.

3.7.4.4. Determination of intracellular ROS

The ROS concentration of cardiomyocytes was estimated using 2', 7'-dichlorfluorescein-diacetate (DCFH-DA) dye. For the determination of ROS media was aspirated and replaced with 1 µM DCFH-DA dissolved in Hank's buffered salt solution (HBSS). After 30 minutes of incubation in a CO_2 incubator at 37ºC, the dye was aspirated and the cells were resuspended in HBSS. The fluorescence intensity of each cardiomyocyte was analysed using NIS elements software with a Nikon eclipse Ti Inverted microscope (Nikon, Japan). For statistical analysis, 5 fields were taken per individual well.

In order to determine the protective effect against oxidative stress, the cell numbers (y axis) were plotted against DCFH-DA fluorescence intensity (x axis). Three different scoring categories were used: low intensity (10000-29000), medium intensity (30000-39000) and high intensity (40000-59000). An increase in the amount of DCFH-DA fluorescence intensity was an indication of oxidative stress.

3.7.4.5. Total glutathione content

Total Glutathione content of cardiomyocytes was detected using CellTracker^{TM} Blue CMAC dye, according to manufacture's instructions with minor changes. Briefly, media was aspirated and replaced with 2.5 µM CellTracker dye dissolved in DMEM without phenol red. After 30 minutes of incubation in a CO_2 incubator at 37 ºC, the dye was aspirated and the cells were resuspended in PBS. The intracellular fluorescence intensity of each cardiomyocyte was determined using NIS elements software with a Nikon Eclipse Ti Inverted microscope (Nikon, Japan). For statistical analysis, 5 fields were taken per individual well.

Glutathione content was detected by measuring the fluorescent content of a CellTracker blue dye. Increase in GSH content was measured by plotting the cell number (y axis) against the GSH fluorescent intensity on the (x axis). Three different scoring categories were used low intensity (10000-29000), medium intensity (30000-39000) and high intensity (40000-59000).

3.7.5. Statistical analysis

Results were expressed as the mean of 3 independent biological experiments and each biological experiment was done in triplicate. For microscopic analysis, 5 random fields at x10 magnification were analysed per well. Statistical analysis between groups was performed using one way ANOVA analysis followed by a Tukey post hoc test or an unpaired student t-test where appropriate. A p-value of ≤ 0.05 was deemed as statistically significant.

Chapter 4

Results

4. Results

4.1. PHASE 1: Model Establishment

The results in this phase establish the optimised culture conditions for long term culture of rod shaped cardiomyocytes.

4.1.1. Characterisation of isolated cardiomyocytes: morphological analysis and cell viability assessment

Initial microscopic examination of freshly isolated cardiomyocytes cell suspension revealed two types of cell morphologies: rod shape cells and rounded-up cells (Fig. 4.1 A, B). These morphologies were also observed after pre-plating of isolated cells. Within 4 hours of initial plating, cardiomyocytes started to adhere to the laminin coated plates and exhibited spontaneous rhythmic contractions, as visualised using light microscopy (data not shown). Fetal bovine serum had no effect on the contractile ability of cardiomyocytes after 24 hours in culture as no difference was observed for cells grown in the presence and absence of FBS. Similar results were observed for cells cultured for 2 and 3 days. However, cells cultured in media containing FBS had slower beating activity (3 beats per minute) compared to cells cultured with no FBS (5 beats per minute) after 3 days. Furthermore, distinct morphological changes were observed after 3 days in culture. Cells cultured without FBS maintained their rod-shaped morphology up to 5 days, whereas cells cultured in media containing FBS lost their rod-shape phenotype and flattened into fibroblastic-like cells as ascertained using H&E staining (Fig. 4.2). In addition, after 4 days in culture, cells grown in the presence of FBS became elongated with prominent nuclei (Fig. 4.2).

A)

B)

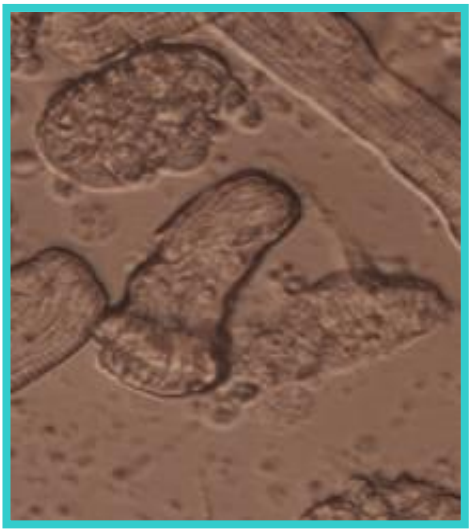


Figure 4.1. Initial microscopic examination of freshly isolated cardiomyocytes before culture. (A) Rod shape structured cardiomyocytes and (B) cardiomyocytes displaying a contracted structure.

Results from here onwards will be expressed as the mean (%) ± SEM (%). Cell viability of 70% ± 12% variation was obtained after 24 hours of culture; with 80% ± 4% of cells being rod shaped irrespective of culture medium (Fig. 4.3 and Fig. 4.4). On day 3, a decline in cell viability was observed, with a 59% ± 6% and 53% ± 7% cardiomyocytes cell viability, in cells cultured with FBS compared to without FBS respectively (Fig. 4.3 and Fig. 4.4). This result correlated with results obtained for ATP metabolic activity with cardiomyocytes in culture having a metabolic activity above 65% ± 8% irrespective of culture media. After 3 days, a diminished effect in cardiomyocyte viability was observed (Fig. 4.4). A significant decrease in metabolic activity between day 1 and day 5 of cells cultured in media with FBS ($p<0.04$) was observed. A similar effect was observed in cells cultured in media without FBS ($p<0.01$). However, the effect on cell viability was not as distinct in cells cultured with FBS whose viability remained above 55% compared to 37% ± 5% in cells cultured without FBS for 5 days (Fig. 4.3).

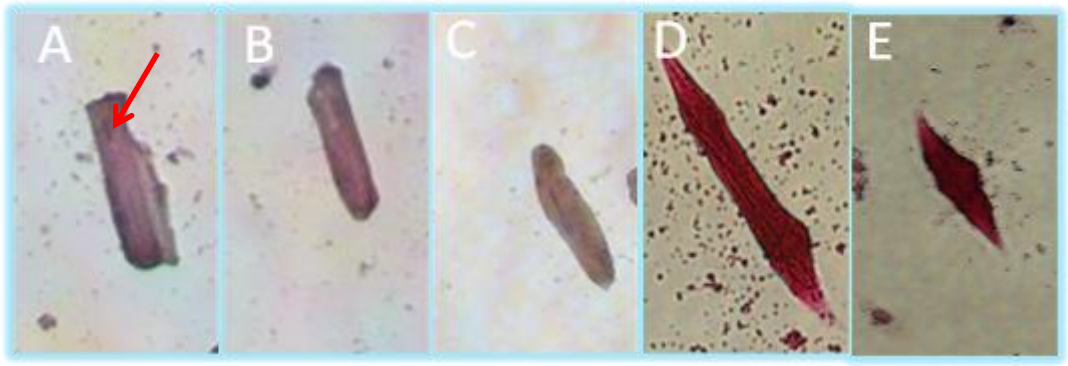
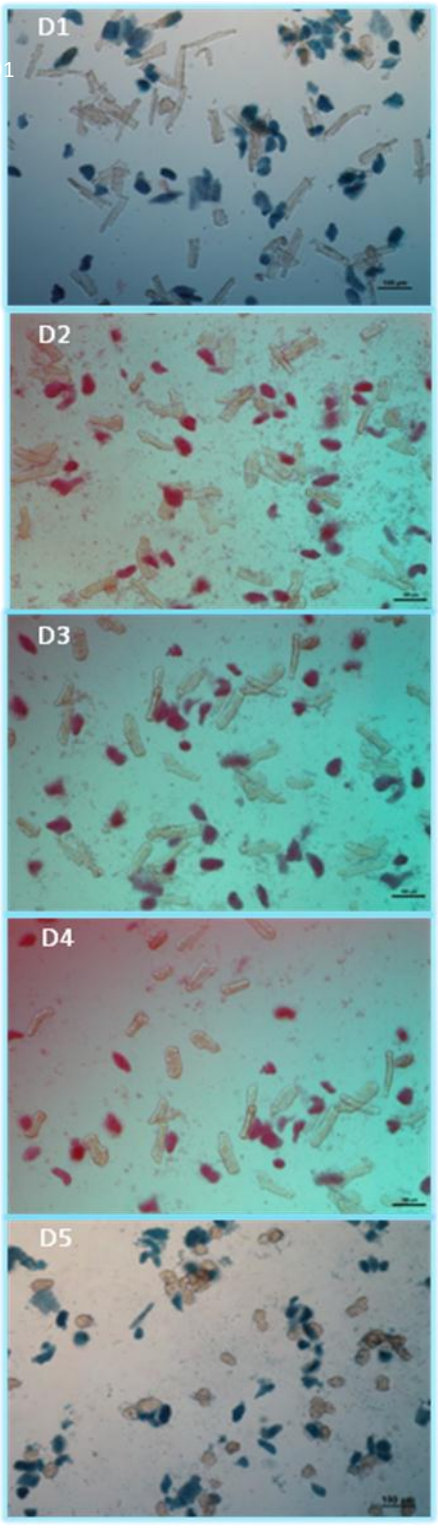
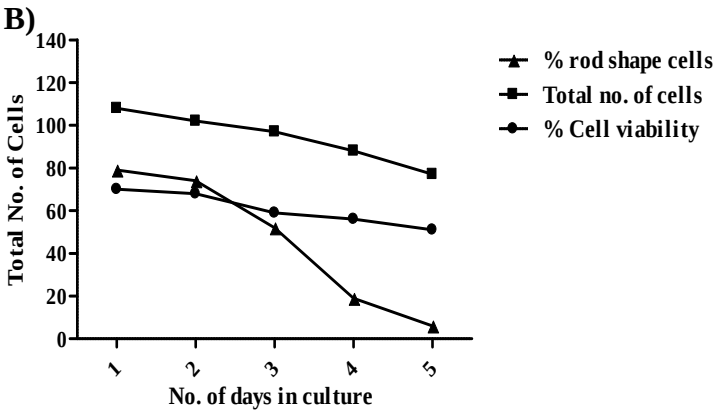


Figure 4.2. Morphological analysis of cardiomyocytes over a 5 day period using H&E stain. Representative images of morphological transformation of cardiomyocytes through a 5 day culture period. (A) Denotes 24 hours in culture, (B) 48 hours in culture, (C) 72 hours in culture, (D) 96 hours in culture and (E) 120 hours in culture. Red arrow-nucleus.

A)



B)



C)

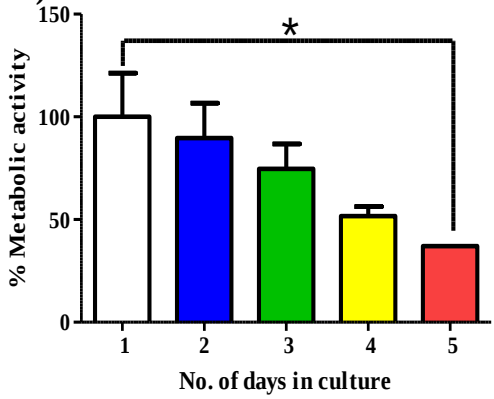


Figure 4.3. Cell viability assessment of cardiomyocytes cultured in media supplemented with 5% FBS for 5 days. (A) Representative images of cardiomyocytes cultured up to 5 days (D1-D5) and stained with trypan blue dye, (B) trypan blue cell viability count of cardiomyocytes grown in culture and (C) ATP metabolic activity of cardiomyocytes in culture. The results are the mean ± SEM n=3 independent experiments. A p value < 0.05 was deemed as significant. * Represents a statistical significance when compared to 24 hours of culture.

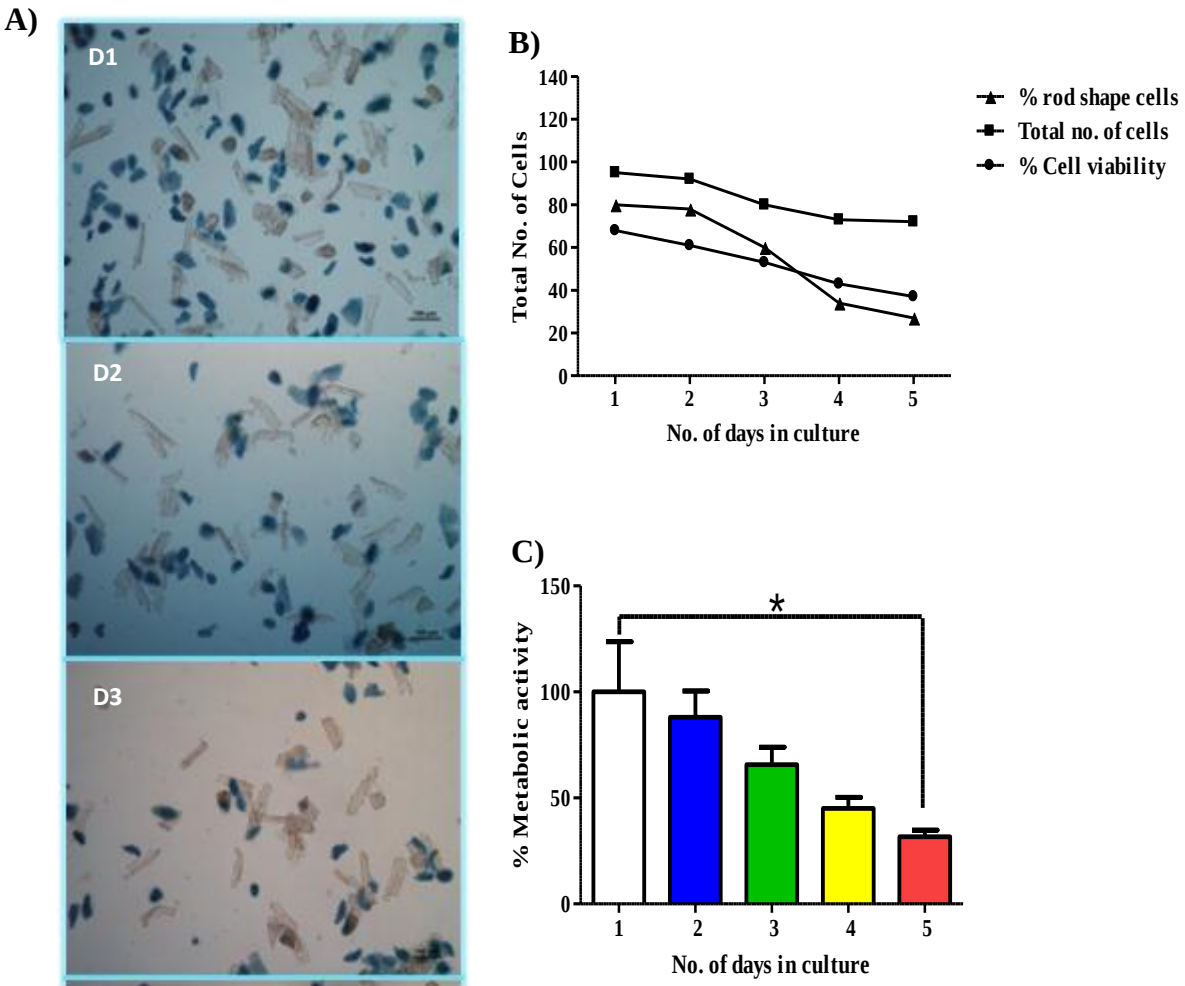


Figure 4.4. Cell viability assessment of cardiomyocytes cultured in media containing no FBS for 5 days. (A) Representative images of cardiomyocytes cultured for up to 5 days (D1-D5) and stained with trypan blue dye, (B) trypan blue cell viability count of cardiomyocytes grown in culture and (C) ATP metabolic activity of cardiomyocytes in culture. The results are the mean ± SEM n=3 independent experiments. A p value < 0.05 was deemed as significant. * Represents a statistical significance when compared to 24 hours of culture.

4.2. PHASE 2

Results for this phase of the study establish the optimal time and dose response of ARC 61 using the ATP assay. These results were used to determine the optimal time and doses for ARC 61 required in phase 3.

4.2.1. HPLC chromatogram analysis for phenolic acid content of ARC 61

The major phenolic content of the fermented rooibos extract, ARC 61, was determined using HPLD-DAD and is shown in Table 4.1 and Fig. 4.5. HPLC characterisation of ARC 61 showed that the fermented rooibos extract had high levels of iso-orientin (0.94 g/100 g), orientin (0.721 g/100 g), z-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG) (0.713 g/100 g) and quercetin (0.446 g/100 g) (Table 4.1). Aspalathin, a C-linked dihydrochalcone glucoside, is unique to rooibos and the HPLC analysis showed that the ARC 61 extract used in this study contained 0.364 g/100 g of this compound.

Table 4.1. The phenolic compounds and their composition in ARC 61

Compound	Concentration (g/100g) of ARC 61
z-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG)	0.713
Aspalathin	0.364
Nothofagin	0.070
Iso-orientin	0.924
Orientin	0.721
Quercetin-3-o-robinobioside	0.446
Vitexin	0.152
Hyperoside	0.087
Rutin	0.185
IsoVitexin	0.140
Isoquercitrin	0.063
Luteolin-7-glucoside	0.069

* The phenolic compounds of ARC 61with the highest concentration are highlighted.

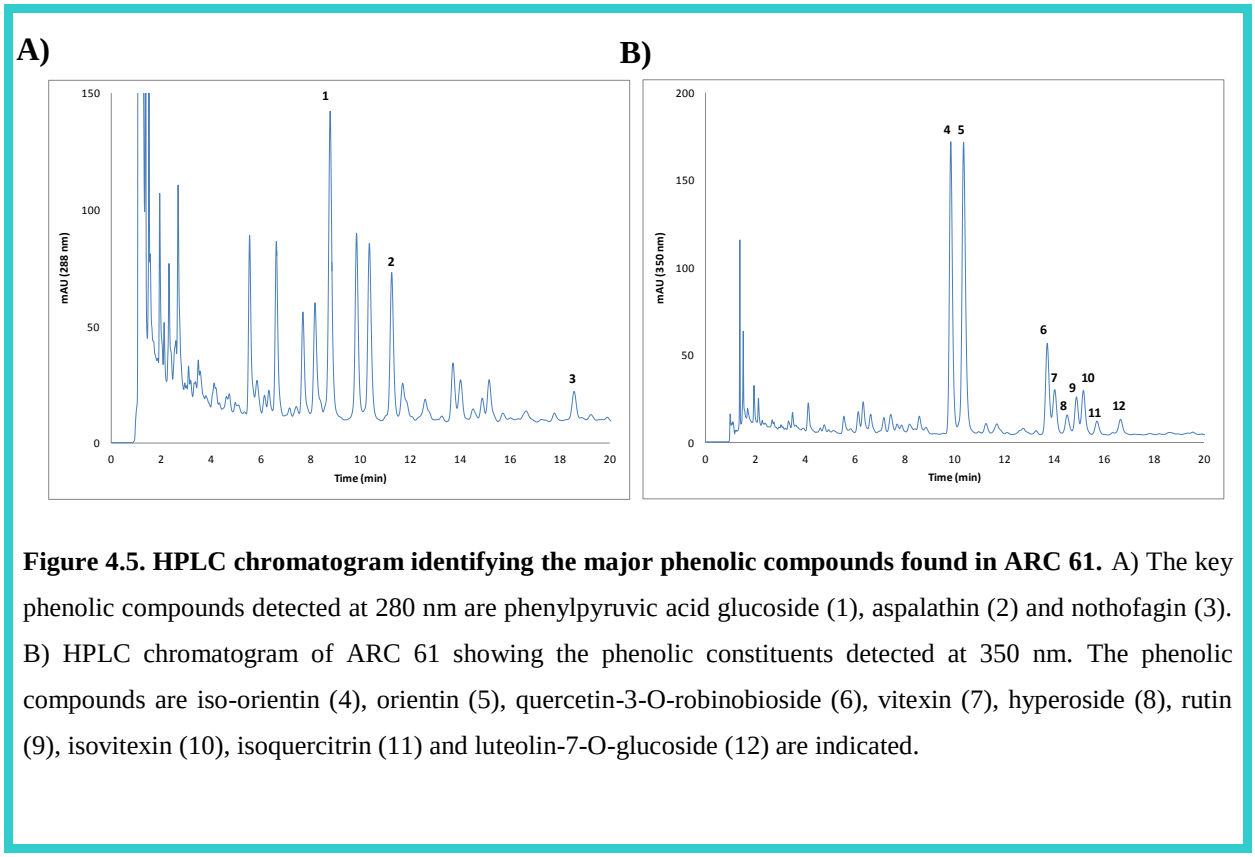


Figure 4.5. HPLC chromatogram identifying the major phenolic compounds found in ARC 61. A) The key phenolic compounds detected at 280 nm are phenylpyruvic acid glucoside (1), aspalathin (2) and nothofagin (3). B) HPLC chromatogram of ARC 61 showing the phenolic constituents detected at 350 nm. The phenolic compounds are iso-orientin (4), orientin (5), quercetin-3-O-robinobioside (6), vitexin (7), hyperoside (8), rutin (9), isovitexin (10), isoquercitrin (11) and luteolin-7-O-glucoside (12) are indicated.

4.2.2. The effect of varying exposure times on cardiomyocytes

4.2.2.1. ARC 61

The metabolic activity of cardiomyocytes showed the highest activity after 3 hours exposure to ARC 61 (166% ± 12%) (Fig. 4.8 A). After 6 hours and 12 hours respectively, the activity was still increased although the increase was not significant and was less than that of 3 hours (143% ± 11%) and 12 hours (131% ± 10%) respectively (Fig. 4.6 A, B, C and Fig.4.8 A). After 24 hours, ARC 61 lost its effect (89% ± 14%) (Fig. 4.6 D and Fig. 4.8 A).

4.2.2.2. Vit E

Results showed that compared to the negative control, vit E treatment increased ATP metabolic activity after 3 hours of exposure (100% ± 9% to 166% ± 9%, $p<0.012$) (Fig. 4.7 A

and Fig. 4.8 B). After 6 and 12 hours respectively, the increase in metabolic activity was lost (105% ± 3% and 100% ± 9% respectively) (Fig. 4.7 B, C and Fig. 4.8 B). Twenty four hours later, the metabolic activity was reduced compared to the negative control (100% ± 9% to 40% ± 21%) (Fig. 4.7 D and Fig. 4.8 B).

4.2.3. The effect of varying doses on metabolic activity in cardiomyocytes

4.2.3.1. ARC 61

Estimation of the efficacy of ARC 61 on ATP concentrations using log dilutions at 3 hours yielded an EC50 value of 0.002 µg/ml (see appendix II). The peak activity was shown to be 1 µg/ml (166% ± 12%) at this time (Fig. 4.6 A and 4.8 A). Upon increasing the dose of ARC 61 to 200 µg/ml, a decrease in metabolic activity was observed (89% ± 14%) (Fig. 4.6 A). At the highest dose (200 µg/ml) this decrease was shown to be time dependent (Fig. 4.6 A, B, C, D and Fig. 4.8 A). At 24 hours, ARC 61 had no effect on ATP concentrations at the lower doses <1 µg/ml. However, increased doses of ARC 61 decreased ATP concentrations in a dose dependant manner (from 89% ± 14% to 2% ± 0.1%) (Fig. 4.6 D and 4.8 A).

4.2.3.2. Vit E

The peak activity for vit E was achieved at a dose of 50 µg/ml after 3 hours of exposure (166% ± 9.1%, p>0005) (Fig. 4.7 A). No effect of vit E was observed at any of the doses tested at 6 and 12 hours (Fig. 4.8 B). However, at 24 hours there was a dose dependant decrease in ATP concentration (from 40% ± 4% to 17% ± 16%) (Fig. 4.7 D and Fig. 4.8 B).

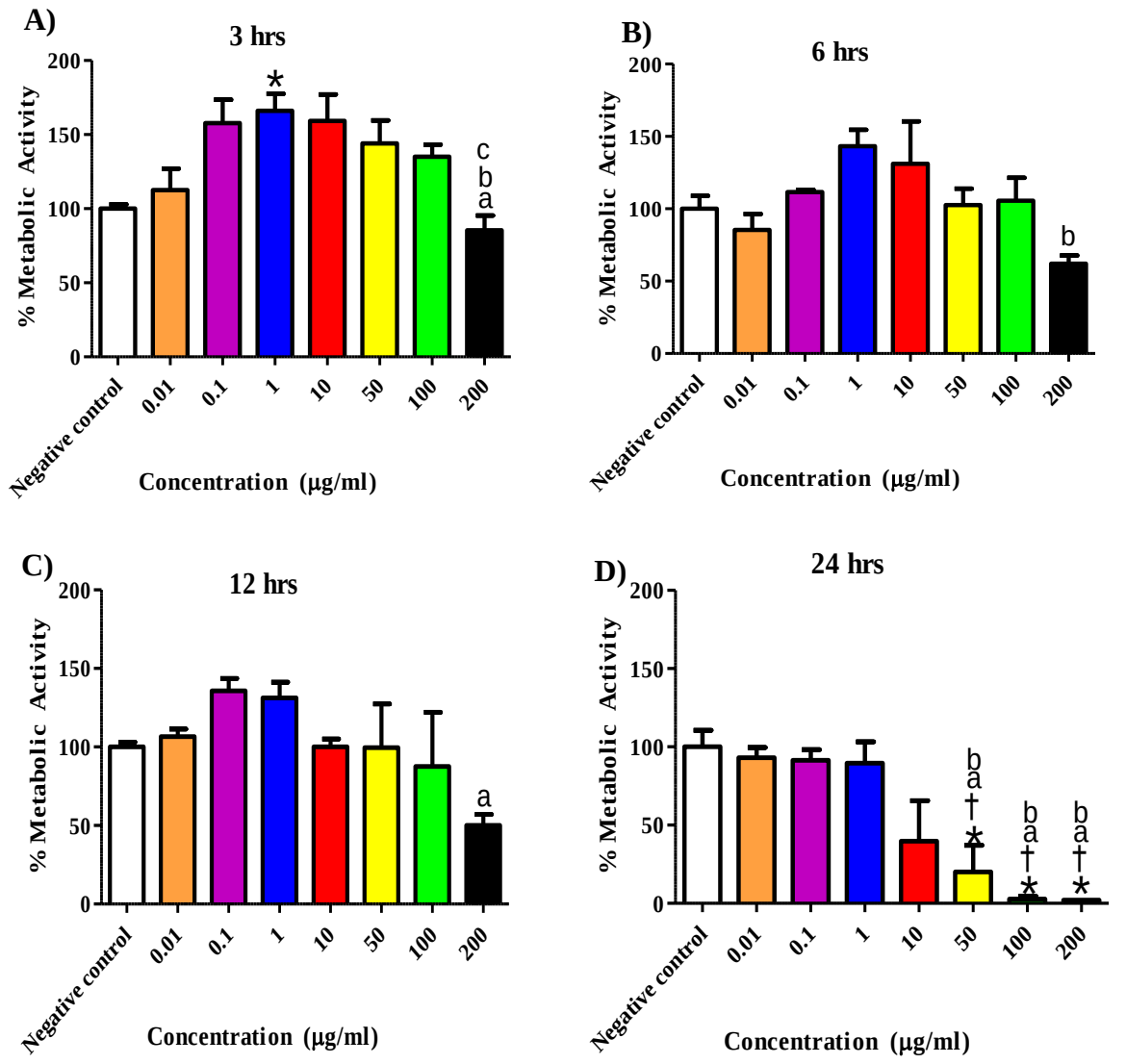


Figure 4.6. The metabolic activity of cardiomyocytes after exposure to varying time and concentrations of ARC 61. An ATP assay was utilised to determine metabolic activity. (A) Represents cardiomyocytes exposed to ARC 61 for 3 hours, (B) 6 hours, (C) 12 hours and (D) 24 hours, respectively. Results are expressed as the mean ± SEM of 3 independent biological experiments with each experiment having 3 technical replicates. * indicates significance difference when compared to a negative control, † compared to 0.01 µg/ml ARC 61, [a] compared to 0.1 µg/ml ARC 61, [b] compared to 1 µg/ml ARC 61 and [c] compared to 10 µg/ml ARC 61.

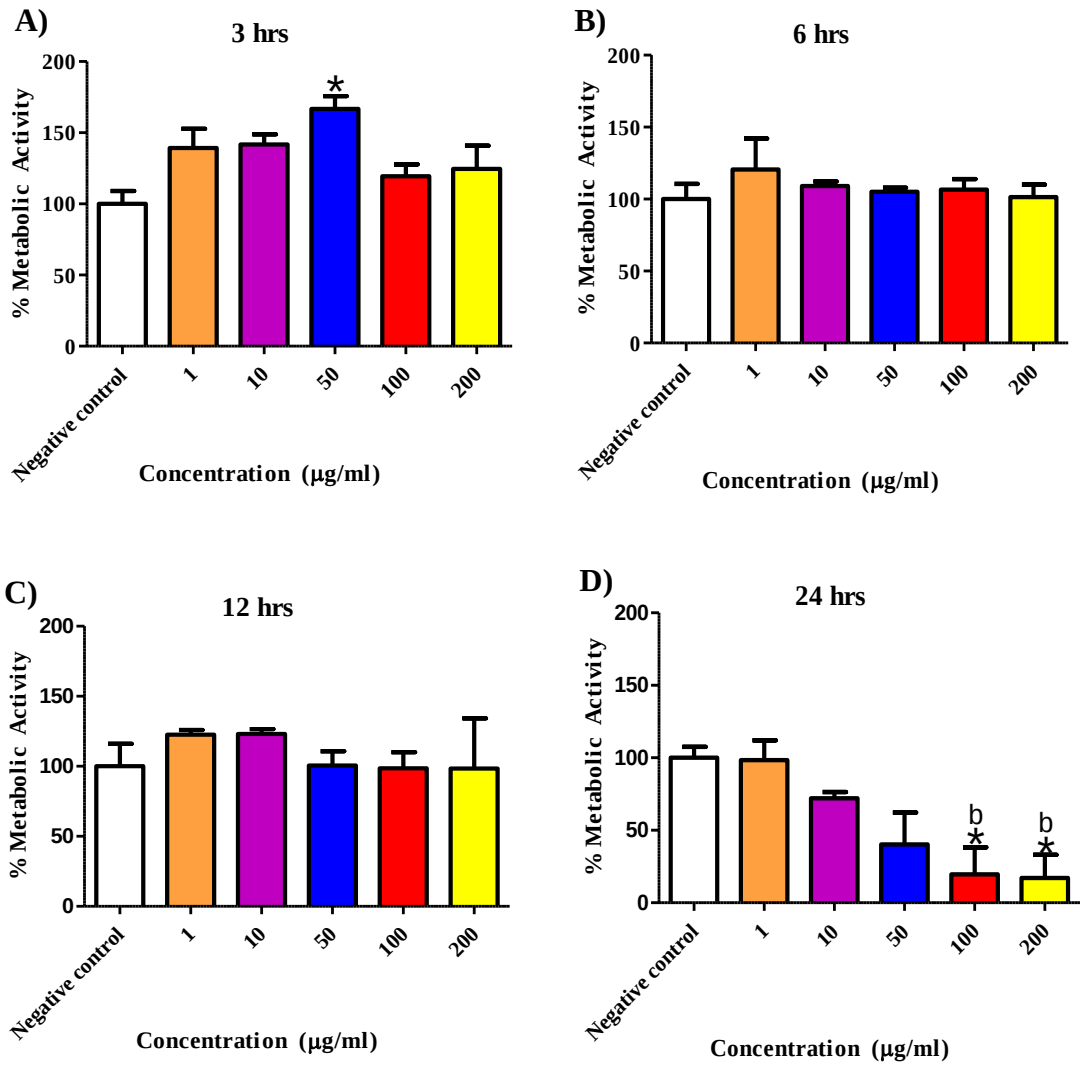


Figure 4.7. The effect of varying time and concentrations of vit E on the metabolic activity of cardiomyocytes. Metabolic activity was determined by using an ATP assay. (A) Represents cardiomyocytes exposed to vit E for 3 hours, (B) 6 hours, (C) 12 hours and (D) 24 hours. Results are expressed as the mean ± SEM of 3 independent biological experiments with 3 technical replicates. * indicates significance difference when compared to a negative control and [b] compared to 1 µg/ml.

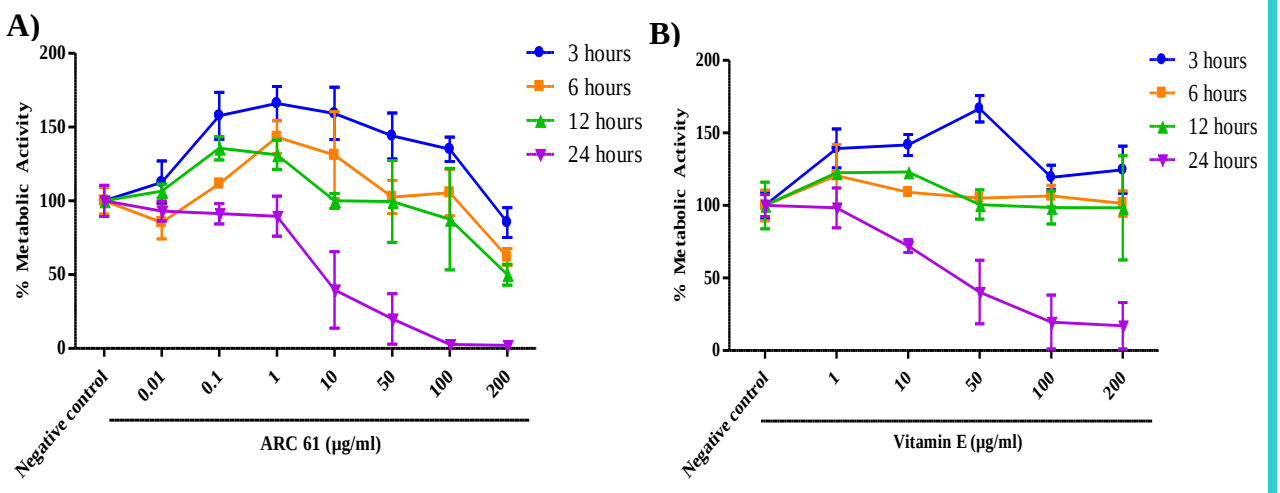


Figure 4.8. Time and dose effects of ARC 61, vit E and H_2O_2 on metabolic activity of cardiomyocytes. Metabolic activity was determined by using the ATP assay. (A) Represents the effect of varying concentrations of ARC 61 and (B) vit E on cardiomyocytes at different time points. Results were expressed as the mean ± SEM of 3 independent biological experiments and 3 technical replicates.

4.2.4. Time and dose confirmation of cardiomyocytes pre-treated with ARC 61 and vit E

4.2.4.1. Pre-treatment of cardiomyocytes isolated from non-diabetic rats with 1 µg/ml of ARC 61 for 3 and 6 hours

Based on dose and time experiments, doses of 1 µg/ml of ARC 61 and 50 µg/ml of vit E at 3 and 6 hours respectively were selected for further assessment (Fig. 4.6, Fig. 4.7 and Fig. 4.8 A, B).

Results in Fig. 4.9 A, B shows that pre-treatment with ARC 61 increased metabolic activity when compared to the negative control and the H_2O_2-induced controls at 3 and 6 hours respectively (274% ± 38%, $p<0.0001$ and 183% ± 20%, $p>0.05$). At 3 hours, H_2O_2 had no measurable effect on the ATP concentration; however, at 6 hours a significant decrease in ATP concentrations in comparison to the negative control was apparent (Fig. 4.9 A, B). These results confirm that H_2O_2 induced oxidative stress results in the depletion of ATP. Pre-

treatment of cardiomyocytes with ARC 61 and vit E for 6 hours was able to ameliorate this effect (Fig. 4.9 A, B). Thus, based on this result the pre-treatment period of 6 hours was selected for phase 3.

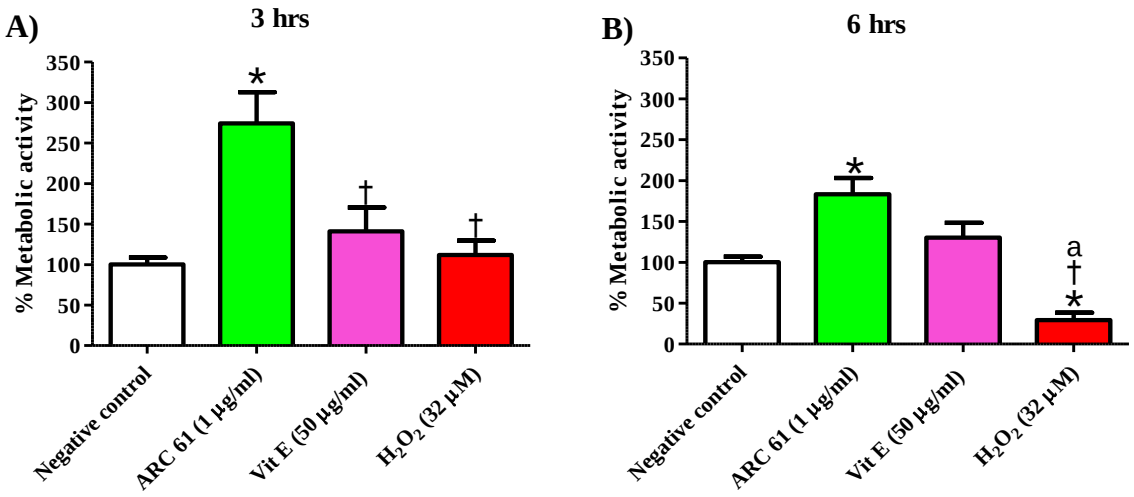


Figure 4.9. The metabolic activity of cardiomyocytes pre-treated with ARC 61 for 3 and 6 hours. Metabolic activity was measured by using the ATP assay. (A) Represents cardiomyocytes pretreated for 3 hours and (B) 6 hours, respectively. Results were expressed as the mean ± SEM of 3 independent biological experiments, each performed in triplicate. * indicates a significant difference when compared to a negative control. † compared to ARC 61 (1 µg/ml) and [a] compared to vit E (50 µg/ml).

4.3. PHASE 3

Results for this phase of the study demonstrate the putative antioxidant effect of 1 and 10 µg/ml of ARC 61 against H_2O_2 and ischaemic-induced stress.

4.3.1. The diabetic cardiomyopathy model

Hyperglycaemia was induced in 6 month old adult male Wistar rats by STZ injection. After 2 weeks blood was collected from the fasted diabetic rats (glucose >14 mmol/l). Fasting plasma glucose and random serum insulin concentrations were determined to confirm the diabetic status of the rats (Table 4.2 and 4.3). STZ administration induced hyperglycaemia and hypoinsulinaemia resulting in a reduction of the insulin/glucose ratio from 0.7 ± 0.1 to 0.02 ± 0.003 after 2 weeks of STZ injection.

Table 4.2. Plasma glucose- and insulin levels of non-diabetic rats*

Treatment group	Rat number	Glucose (mmol/L)	Insulin (ng/ml)	INS/GLU Ratio
Normal control rats	1	5.20	4.47	0.93
	2	6.10	3.02	0.51
	3	5.40	4.48	0.84
Average		**5.66**	**3.93**	**0.71**
SEM		**0.31**	**0.50**	**0.10**

* Plasma glucose was measured in all rats (n=10). However, 3 rats were randomly selected to determine insulin levels. INS/GLU- insulin/glucose ratio. SEM-Standard error of the mean.

Table 4.3. Plasma glucose- and insulin levels of STZ-treated rats*

Treatment group	Rat number	Glucose (mmol/L)	Insulin (ng/ml)	INS/GLU Ratio
STZ induced control Rats	1	16.00	0.54	0.03
	2	20.00	0.24	0.02
	3	18.00	0.59	0.03
	4	19.00	0.39	0.02
Average		**18.25**	**0.39**	**0.02**
SEM		**0.85**	**0.13**	**0.00**

* Plasma glucose was measured in all rats (n=10). However, 4 rats were randomly selected to determine insulin levels. INS/GLU- insulin/glucose ratio. SEM-Standard error of the mean.

4.3.2. ARC 61 protects isolated cardiomyocytes from apoptosis

The protective effect of ARC 61 against H_2O_2- and ischaemic-induced apoptosis was determined using annexin V and propidium iodide.

Contradictory to expectations, cardiomyocytes isolated from the STZ-induced diabetic rats only had slightly increased apoptosis of their isolated cardiomyocytes compared to the cardiomyocytes from non-diabetic rats (Fig 4.10 negative controls). However, these marginal increases were not statistical significant.

4.3.2.1. H_2O_2 exposure

A significant increase (from 42% ± 5% to 67% ± 5%, $p<0.007$ and from 46% ± 3% 74% ± 5%, $p< 0.0003$) in annexin V and propidium iodide staining was observed in isolated cardiomyocytes from non-diabetic and diabetic rats exposed to H_2O_2 when compared to a negative controls, respectively (Fig. 4.10 A, C). Pre-treatment with 1 µg/ml and 10 µg/ml of ARC 61 protected against H_2O_2-induced apoptosis in cardiomyocytes from non-diabetic rats (47% ± 3%, $p<0.02$ and 49% ± 4%, $p<0.02$) as well as diabetic (56% ± 5%, $p<0.03$ and 63% ± 4%) (Fig. 4.10 A, C and Fig. 4.11 C). In the cardiomyocytes from diabetic rats, treatment with ARC 61 at 10 µg/ml and vit E failed to significantly reduce the H_2O_2-induced apoptosis (Fig. 4.10 C and Fig. 11 D, F). However, pre-treatment with 1 µg/ml of ARC 61 was comparable to the known antioxidant NAC (positive control) in cardiomyocytes from both non-diabetic and diabetic rats (56% ± 5%, $p<0.007$ and 63% ± 2%, $p<0.005$ respectively) (Fig. 4.10 A, C and Fig. 4.11 E).

4.3.2.2. Ischaemic exposure

Exposing cardiomyocytes from non-diabetic and diabetic rats to 2 hours of ischaemic conditions resulted in a significant increase in apoptosis when compared to the negative control (84% ± 3%, $p<0.0001$ and 74% ± 2%, $p<0.0001$) (Fig. 4.10 B, D and Fig. 4.12 B). Pre-treatment of non-diabetic and diabetic derived cardiomyocytes with both concentrations of ARC 61, vit E and NAC ameliorated this increase (Fig. 4.10 B, D and Fig. 4.12 C, D, E, F). Vitamin E failed to significantly reduce ischaemic-induced apoptosis in cardiomyocytes from non-diabetic rats but was effective in cardiomyocytes from diabetic rats (Fig. 4.10 B, D and Fig. 4.12 F).

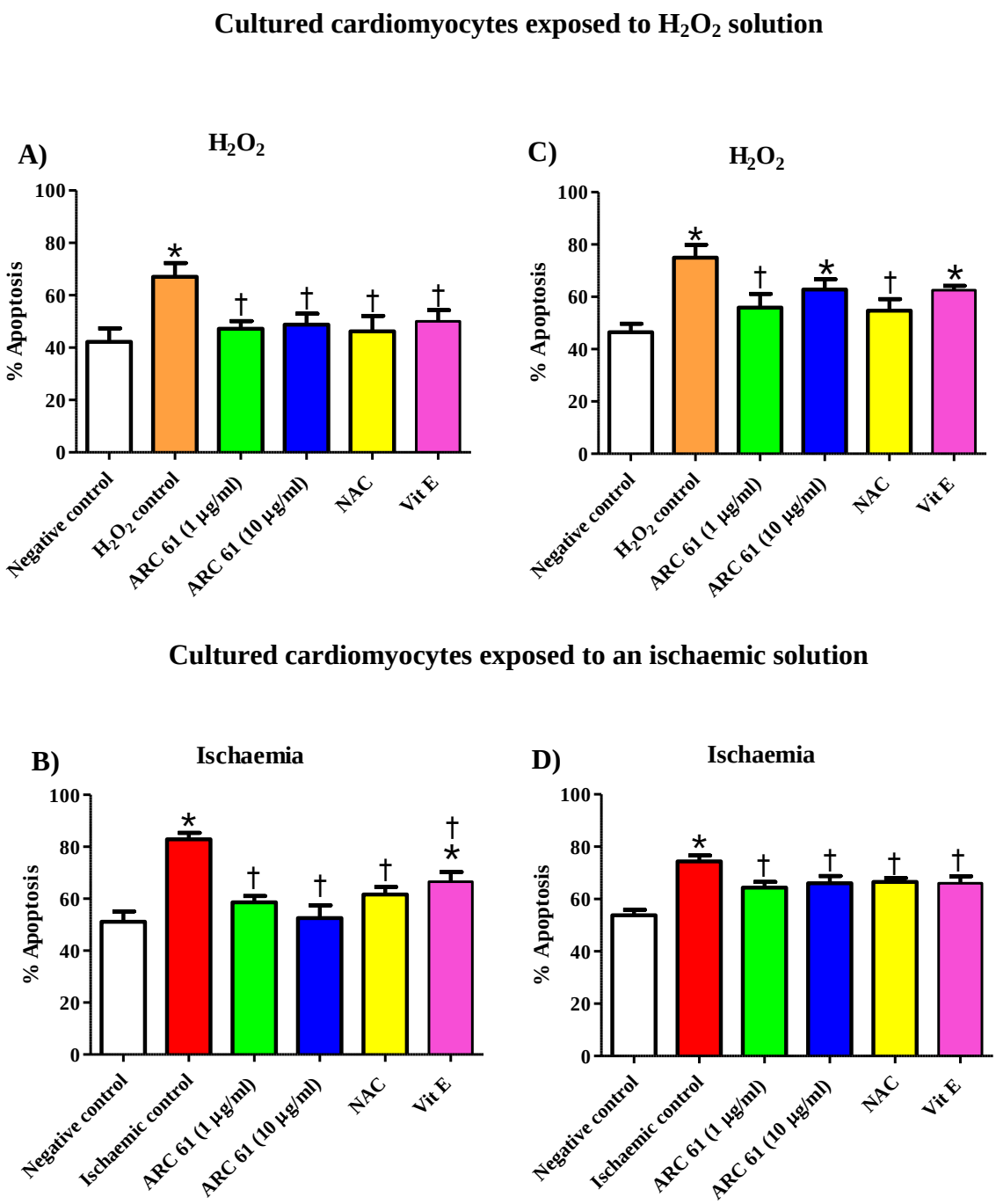


Figure 4.10. The effect of ARC 61 on apoptosis in cardiomyocytes isolated from non-diabetic (A and B) and diabetic (C and D) rats. Apoptosis was detected by staining cardiomyocytes with annexin V and propidium iodide. Panels represent cardiomyocytes from non-diabetic and diabetic rats exposed to: (A and C) H_2O_2 and (B and D) an ischaemic solution. Results are given as the mean ± SEM of 3 independent biological experiments. * Indicates a significant difference when compared to the negative control and † compared either H_2O_2/ischaemic control.

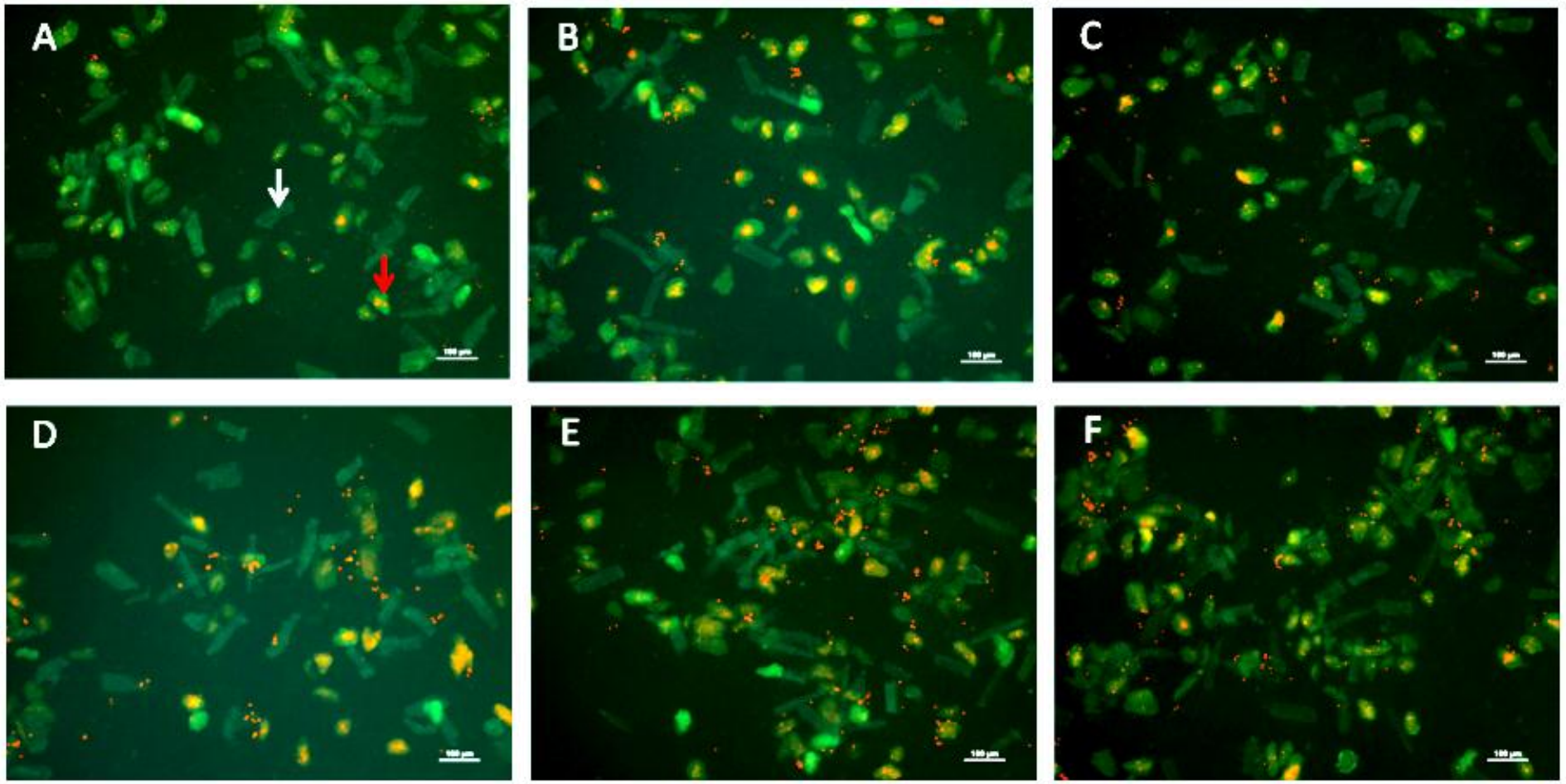


Figure 4.11. Cardiomyocytes isolated from diabetic rats stained with annexin V and propidium iodide after 24 hours exposure to H_2O_2. Panels (A) represent cardiomyocytes exposed to a vehicle used as the negative control, (B) cardiomyocytes exposed to H_2O_2 only, (C) pre-treated with 1 µg/ml of ARC 61, (D) pre-treated with 10 µg/ml of ARC 61, (E) pre-treated with vit E used as positive control and (F) pre-treated with vit E used as a positive control. A red arrow indicates non-viable cells that were stained with both annexin V and propidium iodide while a white arrow indicates a viable rod shape cardiomyocytes that was not stained with either stain.

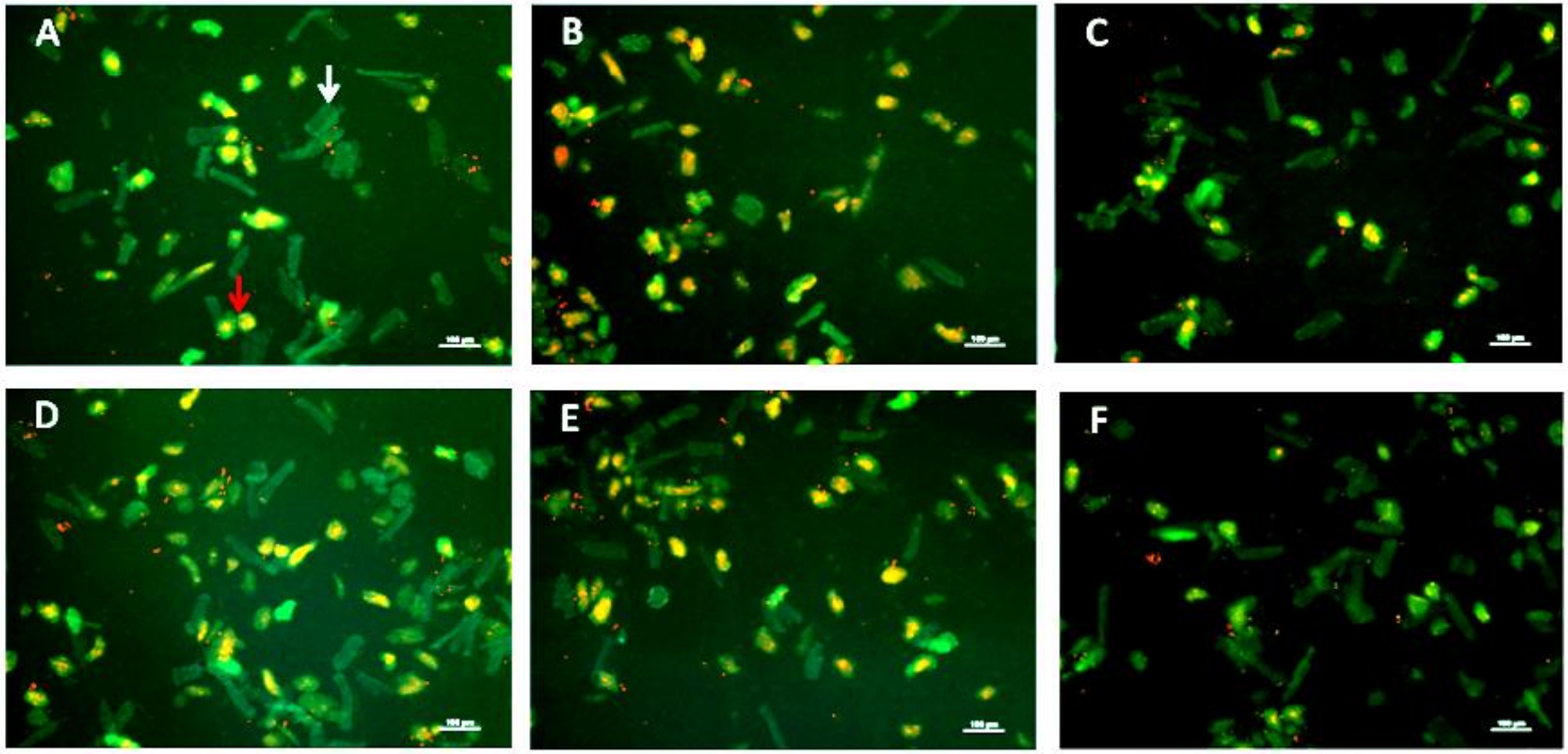


Figure 4.12. Cardiomyocytes isolated from diabetic rats stained with annexin V and propidium iodide after 2 hours exposure to an ischaemic solution. Panels represent: (A) primary cardiomyocytes exposed to a vehicle used as a negative control, (B) exposed to an ischaemic solution only, (C) pre-treated with 1 µg/ml of ARC 61, (D) pre-treated with 10 µg/ml of ARC 61, (E) pre-treated with NAC utilised as the positive control and (F) pre-treated with vit E and utilised as the positive control. A white arrow indicates a cell that was stained with both annexin V and propidium iodide while a white arrow indicates a viable rod shape cell.

4.3.3. Myocardial membrane leakage (H-FABP)

Heart-fatty acid binding protein (H-FABP), a cardiac specific cytosolic protein was used as a marker for membrane leakage incurred due to cellular stress.

4.3.3.1. H_2O_2 exposure

Heart-fatty acid binding protein concentrations in the media showed a greater than 2-fold increase in the media of cardiomyocytes from diabetic rats compared to non-diabetic rats (18670 ± 69 and 9425 ± 372) (Fig. 4.13 A, C). Exposure to H_2O_2 induced an increase in H-FABP leakage in cardiomyocytes from non-diabetic rats which was prevented by both concentrations of ARC 61, NAC and vit E pre-treatment for 6 hours (Fig. 4.13 A). In cardiomyocytes from diabetic rats H_2O_2 administration did not have a marked effect on an already increased H-FABP leakage levels in the negative control (Fig 4.13 C). However, pre-treatment of cardiomyocytes appeared to slightly lower the H_2O_2-induced effect (Fig 4.13 C).

4.3.3.2. Ischaemic exposure

Under ischaemic conditions, H-FABP leakage of cardiomyocytes from non-diabetic rats was increased ~10 fold (18600 ± 69) (Fig 4.13 B). Pre-treatment with ARC 61, NAC and vit E did not appear to have any effect on H-FABP leakage (Fig 4.13 B). In cardiomyocytes derived from diabetic rats ischaemia induced a slight increase in already elevated H-FABP levels (Fig. 4.13 D). Pre-treatment with ARC 61 appeared to have a slight ameliorative effect on H-FABP leakage (Fig 4.13 D).

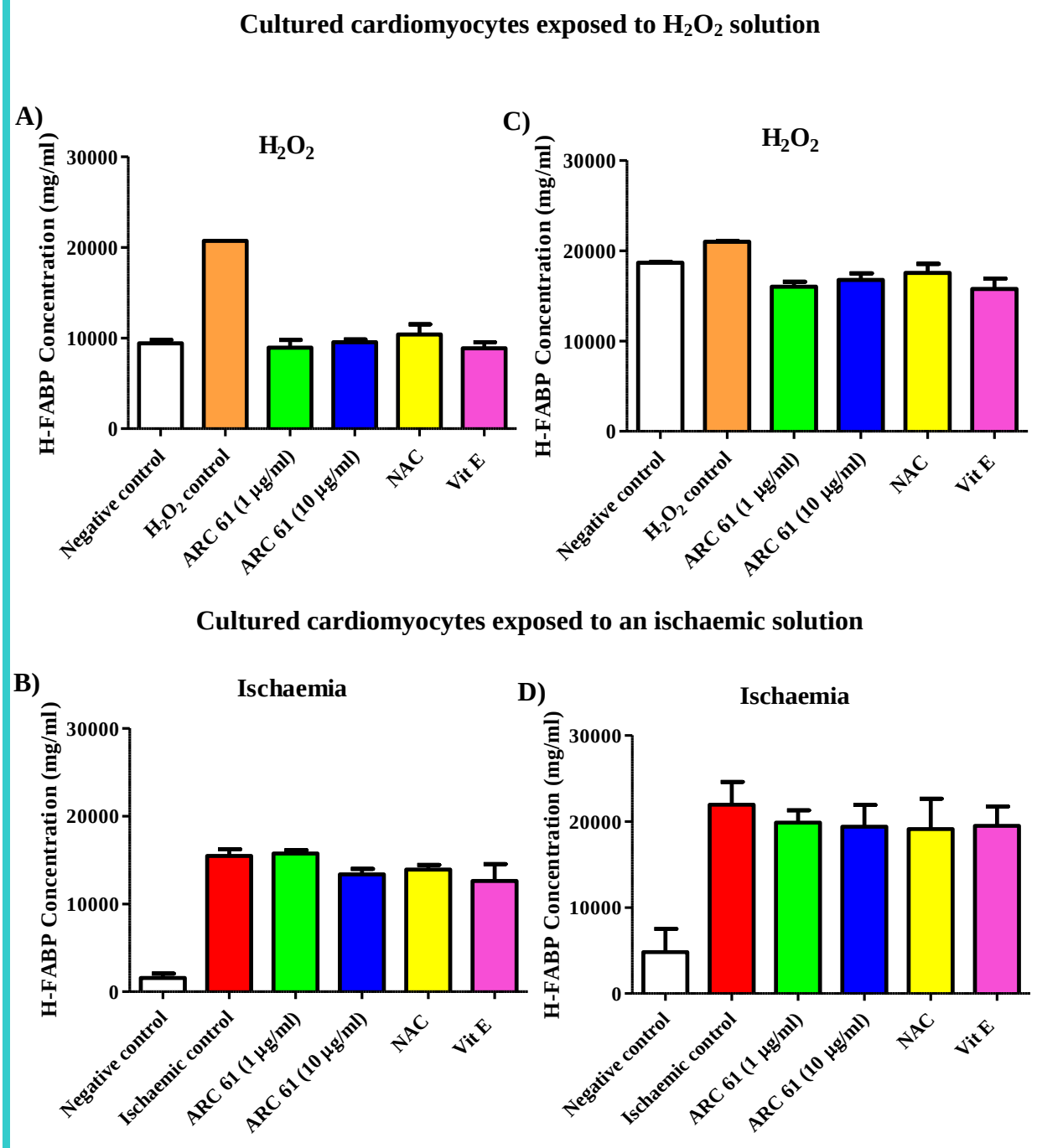


Figure 4.13. The H-FABP concentration in the medium of cardiomyocytes derived from non-diabetic (A and B) and diabetic rats (C and D) after exposure to H_2O_2 and ischaemia for 24 hours or for 2 hours respectively. Panels represent cardiomyocytes from non-diabetic and diabetic rats exposed to: (A and C) H_2O_2 and (B and D) an ischaemic solution. Results are given as the mean ± SEM of 2 independent biological experiments of which each biological experiment had 3 technical replicates. Statistical analysis was not done due to the analysis only having 2 independent biological experiments.

4.3.4. Detection of Oxidative Stress

Generation of intracellular ROS in cardiomyocytes was measured using a cell-permeable fluorescent probe: DCFH-DA.

4.3.4.1. The amount of DCFH-DA produced by cardiomyocytes during H_2O_2 exposure

Exposing cardiomyocytes from diabetic rats to H_2O_2 resulted in an increased production of intracellular ROS in comparison to cardiomyocytes from non-diabetic rats (149% ± 4%, $p<0.05$ and 123% ± 8% respectively) (Fig 4.14 A, C). In cardiomyocytes from non-diabetic rats, H_2O_2 failed to significantly increase intracellular ROS (123% ± 8%) (Fig. 4.14 A). In cardiomyocytes from diabetic rats there was an increase in intracellular ROS production in the H_2O_2 control (149% ± 4%, $p<0.05$) (Fig. 4.14 C and Fig. 4.15 B). Pre-treatment with 1 μg/ml ARC 61, NAC and vit E was able to reduce this effect (108% ± 2%, $p<0.02$; 111% ± 1%, $p<0.02$ and 113% ± 2%, $p<0.05$ respectively) (Fig. 14 C and Fig 4.15 B, C, E, F). However, ARC 61 at a higher dose of 10 μg/ml was ineffective (Fig. 4.15 D).

4.3.4.2. The amount of DCFH-DA produced by cardiomyocytes during ischaemia

Elevated levels of intracellular ROS were detected after exposing cardiomyocytes from non-diabetic and diabetic rats to ischaemic conditions (142% ± 3%, $p<0.039$ and 166% ± 7%, $p<0.032$ respectively). Pre-treatment with ARC 61, NAC and vit E reduced this effect (Fig. 4.14 B, D and Fig. 4.16 B). In the cardiomyocytes from non-diabetic rats, intracellular ROS levels were reduced to near normal values (142% ± 3%) (Fig. 4.14 B).

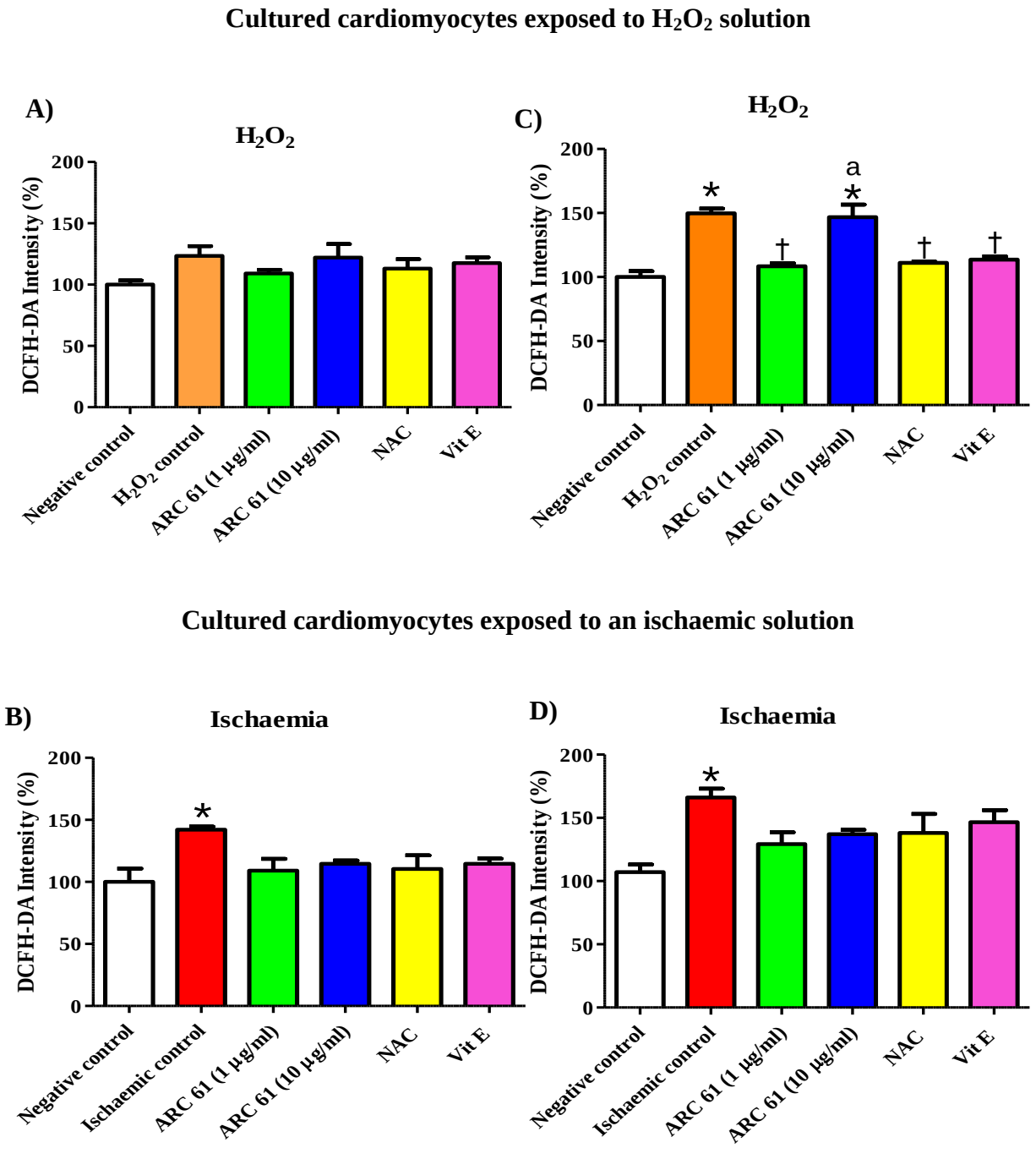


Figure 4.14. ARC 61 protects cardiomyocytes from non-diabetic (A and B) and diabetic rats (C and D) against intracellular ROS. Intracellular ROS was detected by staining cardiomyocytes with DCFH-DA. Panels represent cardiomyocytes from non-diabetic and diabetic rats exposed to: (A and C) H_2O_2 and (B and D) an ischaemic solution. Results are given as the mean ± SEM of 3 independent biological experiments of which each biological experiment had 3 technical replicates. * Indicates significant difference when compared to a negative control, † compared to either H_2O_2 control and ^{a} compared to 1 µg/ml of ARC 61.

4.3.4.3. The effect of ARC 61 on DCFH-DA intensity of cardiomyocytes from diabetic rats

To assess the effectiveness of ARC 61 on ameliorating intracellular ROS, DCFH-DA intensity was measured and categorised into low, medium and high intensity. The addition of H_2O_2 shifted the number of cells from low intensity (Fig. 4.17 A) to high DCFH-DA intensity (Fig 4.17 B). Pre-treatment with ARC 61 prevented this shift in DCFH-DA intensity (Fig 4.17 C). This decrease can be equated to an improved reactive oxygen scavenging effect of the extract. At a lower dose, ARC 61 was more effective than either NAC or vit E in reducing intensity levels of DCFH-DA (Fig. 4.17 C, E, F).

Under ischaemic conditions, DCFH-DA fluorescence intensity shifted towards the high intensity (Fig 4.18 B). After pre-treatment this effect was ameliorated by both concentrations of ARC 61, NAC and vit E (Fig. 4.18 C, D, E, F). However, ARC 61 at both concentrations elicit a similar protective effect compared to that of the known antioxidant, NAC and vit E.

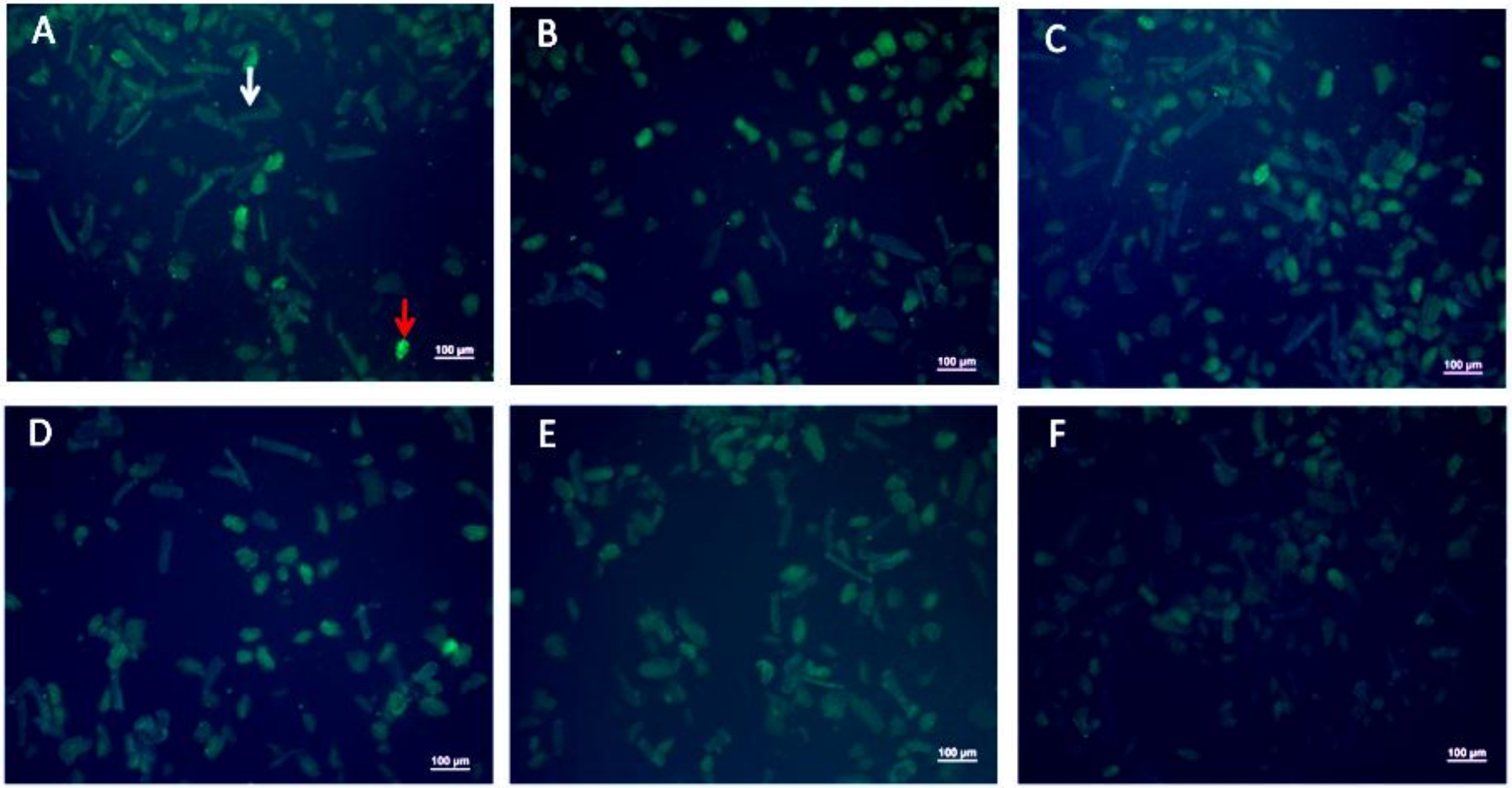


Figure 4.15. Cardiomyocytes isolated from diabetic rats stained with DCFH-DA to detect the production of ROS after 24 hours exposure to H_2O_2. Panels represent: (A) primary cardiomyocytes exposed to a vehicle used as a negative control, (B) exposed to the H_2O_2 solution only, (C) pre-treated with 1 µg/ml of ARC 61, (D) pre-treated with 10 µg/ml of ARC 61, (E) pre-treated with NAC utilised as the positive control and (F) pre-treated with vit E and utilised as the positive control. A red arrow indicates a contracted cell with high fluorescence intensity while a white arrow indicates a viable rod shape cell with low fluorescence intensity.

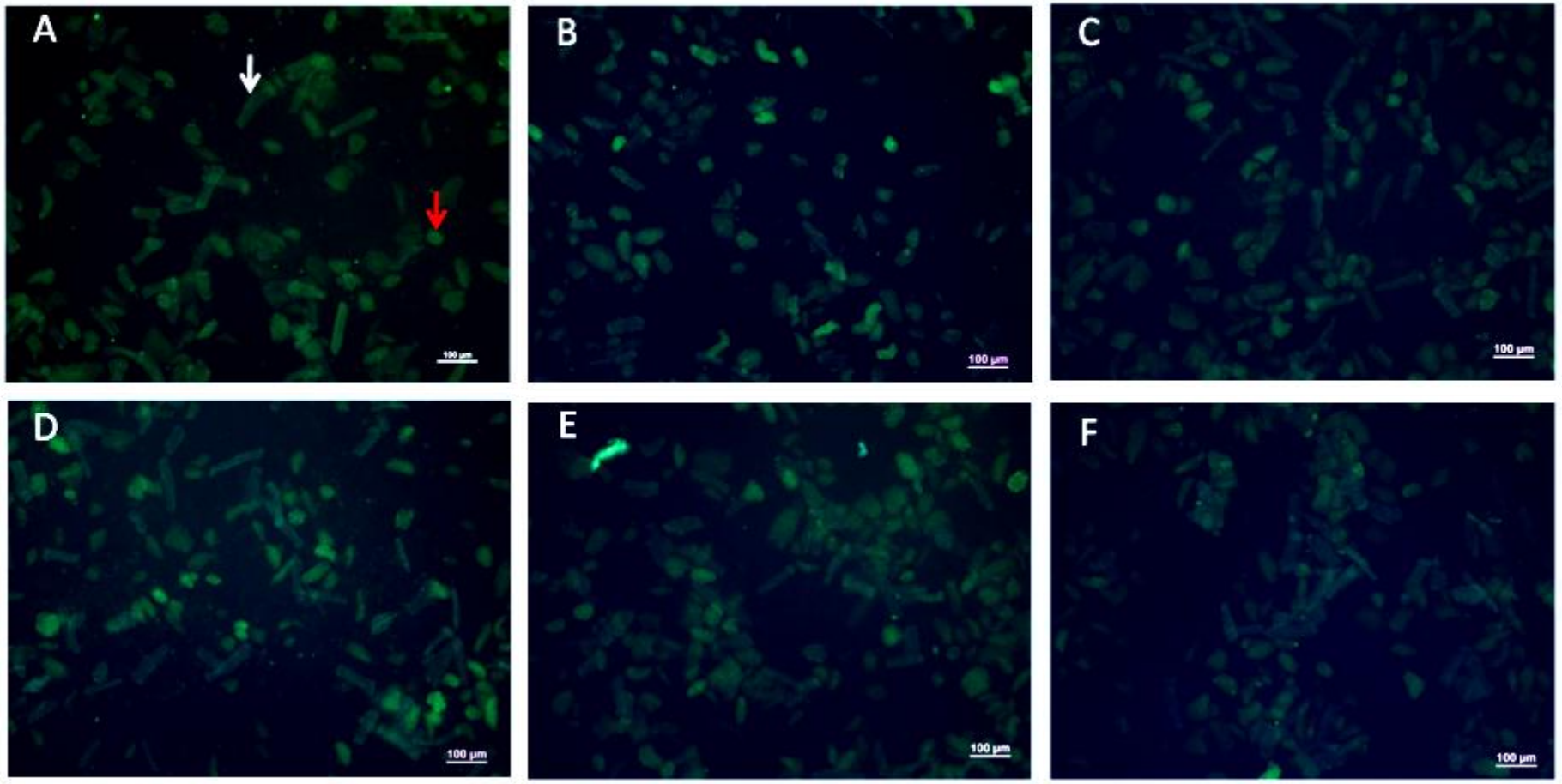


Figure 4.16. Cardiomyocytes isolated from diabetic rats stained with DCFH-DA to detect the production of ROS after 2 hours exposure to ischaemia. Panels represent: (A) primary cardiomyocytes exposed to a vehicle used as a negative control, (B) exposed to an ischaemic solution only, (C) pre-treated with 1 µg/ml of ARC 61, D) pre-treated with 10 µg/ml of ARC 61, (E) pre-treated with NAC utilised as the positive control and (F) pre-treated with vit E and utilised as the positive control. A red arrow indicates a contracted cell with high fluorescence intensity while a white arrow indicates a viable rod shape cell with low fluorescence intensity.

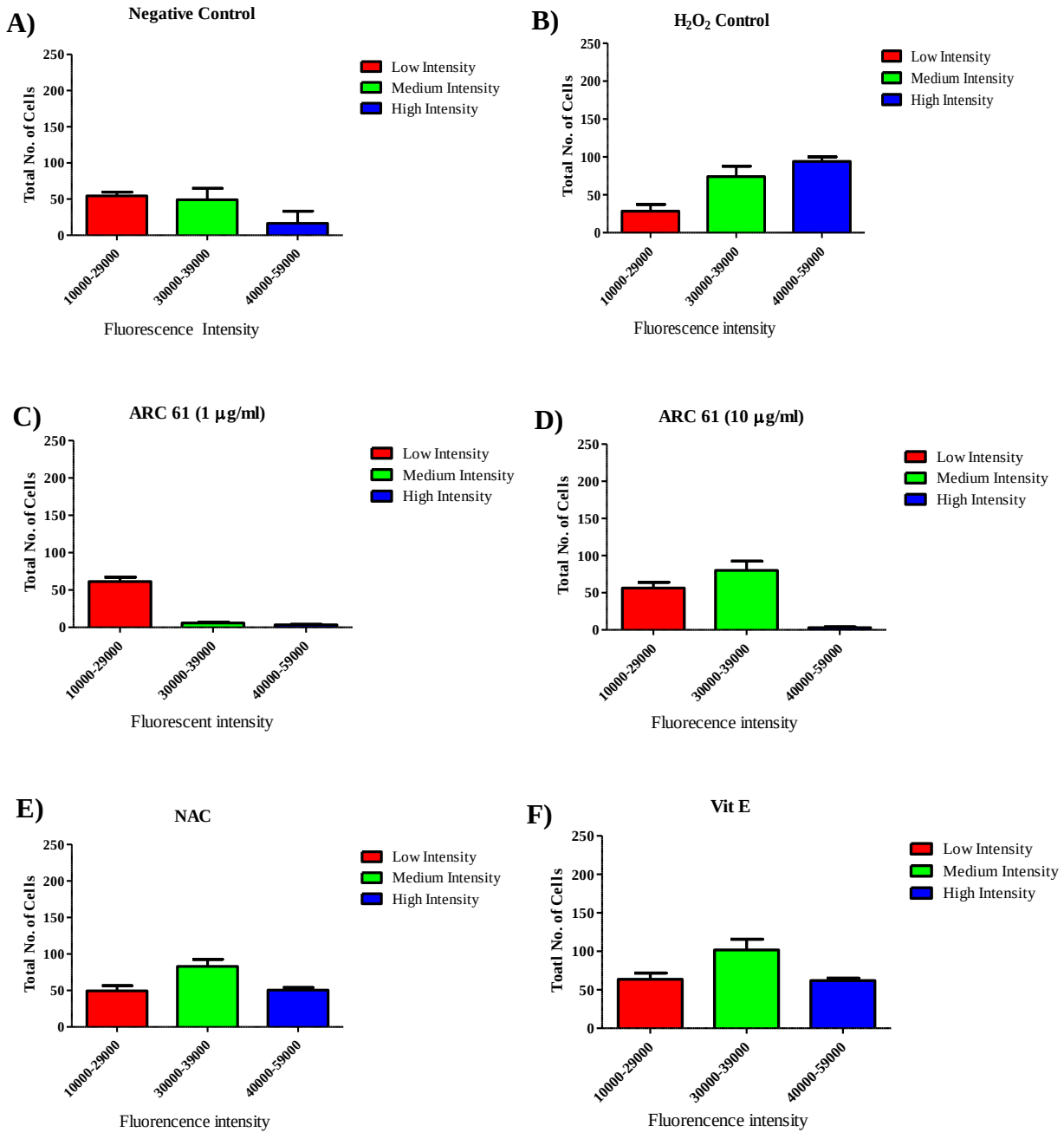


Figure 4.17. DCFH-DA fluorescence intensity of cardiomyocytes derived from diabetic rats after 24 hours exposure to H_2O_2. Low, medium and high fluorescence intensity of (A) negative control, (B) H_2O_2 control, (C) 1 µg/ml ARC 61, (D) 10 µg/ml ARC 61, (E) 1 mM NAC and (F) 50 µg/ml vit E. The result are a mean of n=3 independent experiments. Fluorescence intensity is arbitrary fluorescence units.

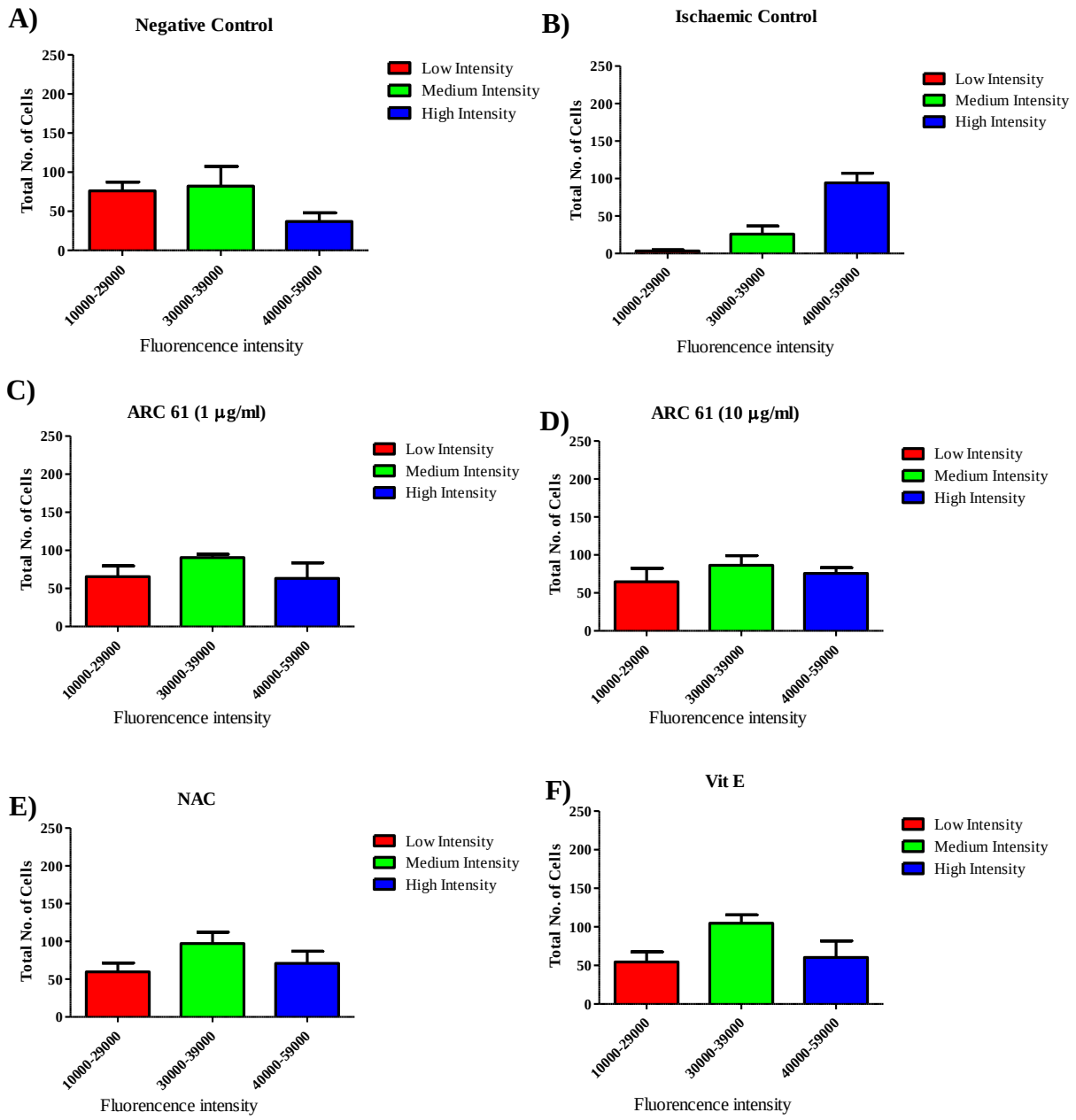


Figure 4.18. DCFH-DA fluorescence intensity of cardiomyocytes derived from diabetic rats after 2 hours exposure to ischaemia. Low, medium and high fluorescence intensity of (A) negative control, (B) ischaemic control, (C) 1 µg/ml ARC 61, (D) 10 µg/ml ARC 61, (E) 1 mM NAC and (F) 50 µg/ml vit E. The result are a mean of n=3 independent experiments. Fluorescence intensity is arbitrary fluorescence units.

4.3.5. Metabolic activity

Metabolic alteration in cardiomyocytes subjected to oxidative stress or ischemic insult was assessed by measuring metabolic activity (ATP production).

4.3.5.1. H_2O_2 exposure

Exposure of cardiomyocytes derived from non-diabetic and diabetic rats to H_2O_2-induced stress resulted in a significant decrease in intracellular ATP concentration (12% ± 0.1%, $p<0.001$ and 1% ± 0.3%, $p<0.0001$) (Fig. 4.19 A, C). Pre-treatment with 1 and 10 µg/ml of ARC 61 as well as NAC and vit E prevented the H_2O_2-induced intracellular ATP depletion (increased from 12% ± 0.3% to 114% ± 11%, $p<0.0001$ and (109% ± 5%, $p<0.0001$ as well as 106% ± 11%, $p<0.0001$ and 120% ± 23%, $p<0.0001$ respectively) (Fig. 4.19 A).

4.3.5.2. Ischaemic exposure

Under ischaemic condition, a decrease in ATP concentration was observed after cardiomyocytes from non-diabetic and diabetic rats were exposed to an ischaemic solution (12% ± 2%, $p<0.0002$ and 10% ± 2%, $p<0.0002$) (Fig. 4.19 B, D). This effect was completely abolished after pre-treatment with ARC 61, NAC and vit E for cardiomyocytes derived from diabetic rats (Fig. 4.19 D). A lower dose, ARC 61 (92% ± 2%, $p<0.0001$) was more effective than a higher dose of ARC 61, NAC and vit E (64% ± 5%, $p<0.0002$; 77% ± 5%, $p<0.0002$ and 69% ± 7%, $p<0.0002$) (Fig 4.19 D).

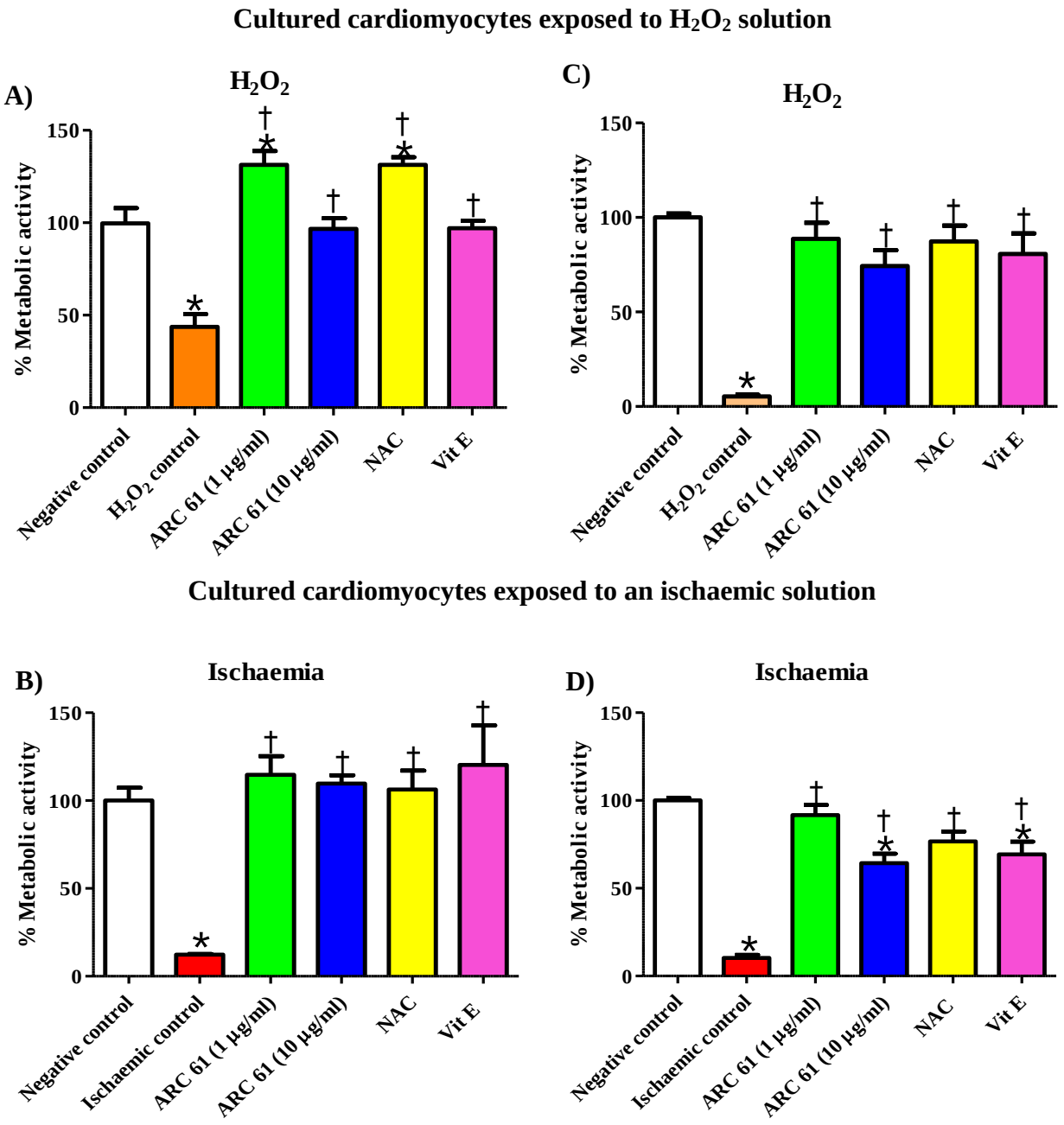


Figure 4.19. The metabolic activity (ATP production) of cardiomyocytes derived from non-diabetic (A and B) and diabetic rats (C and D) exposed to H_2O_2 and ischaemia. Panels represent cardiomyocytes from non-diabetic and diabetic rats exposed to: (A and C) H_2O_2 and (B and D) an ischaemic solution. Results are the mean ± SEM of 3 independent biological experiments. * Indicates significant difference when compared to a negative control and † compared either H_2O_2/ischaemic control.

4.3.6. Total glutathione content

Assessment of GSH content is an important indicator of cellular antioxidant capacity. GSH content of cardiomyocytes of non-diabetic and diabetic rats was measured using a CellTracker dye.

Pre-treatment with ARC 61 preserved GSH content

4.3.6.1. H_2O_2 exposure

In non-diabetic rats H_2O_2 did not significantly affect GSH content when compared to cardiomyocytes derived from diabetic rats (Fig. 4.20 A, C). In cardiomyocytes from diabetic rats, H_2O_2 induced a significant decrease in GSH content (37% ± 17%, $p<0.02$) (Fig. 4.20 C and Fig. 4.21 B). Pre-treatment with ARC 61 for 6 hours at a concentration of 1 μg/ml was able to preserve the GSH content (99% ± 7%, $p<0.02$) (Fig. 4.20 C and Fig. 4.21 C). One microgram per milliliter of ARC 61 was more effective than ARC 61 at a higher dose, NAC or vit E (Fig. 4.20 C and Fig. 21 B, C, D, E, F).

4.3.6.2. Ischaemic exposure

A decrease in GSH content was observed after cardiomyocytes from both non-diabetic and diabetic rats were exposed to an ischaemic solution (25% ± 11%, $p<0.05$ and 18% ± 3%, $p<0.05$) (Fig. 4.20 B, D). However, this effect was ameliorated by pre-treatment with both concentrations of 1 and 10 μg/ml of ARC 61 in non-diabetic (70% ± 31% and 59% ± 16% respectively) and diabetic rats (72% ± 13% and 71% ± 3%) respectively (Fig. 4.20 B, D and Fig. 4.22 C, D). Similar effect was observed after pre-treatment with NAC and vit E (88% ± 9% and 70% ± 11%) (Fig. 4.20 B, D and Fig. 4.22 E, F).

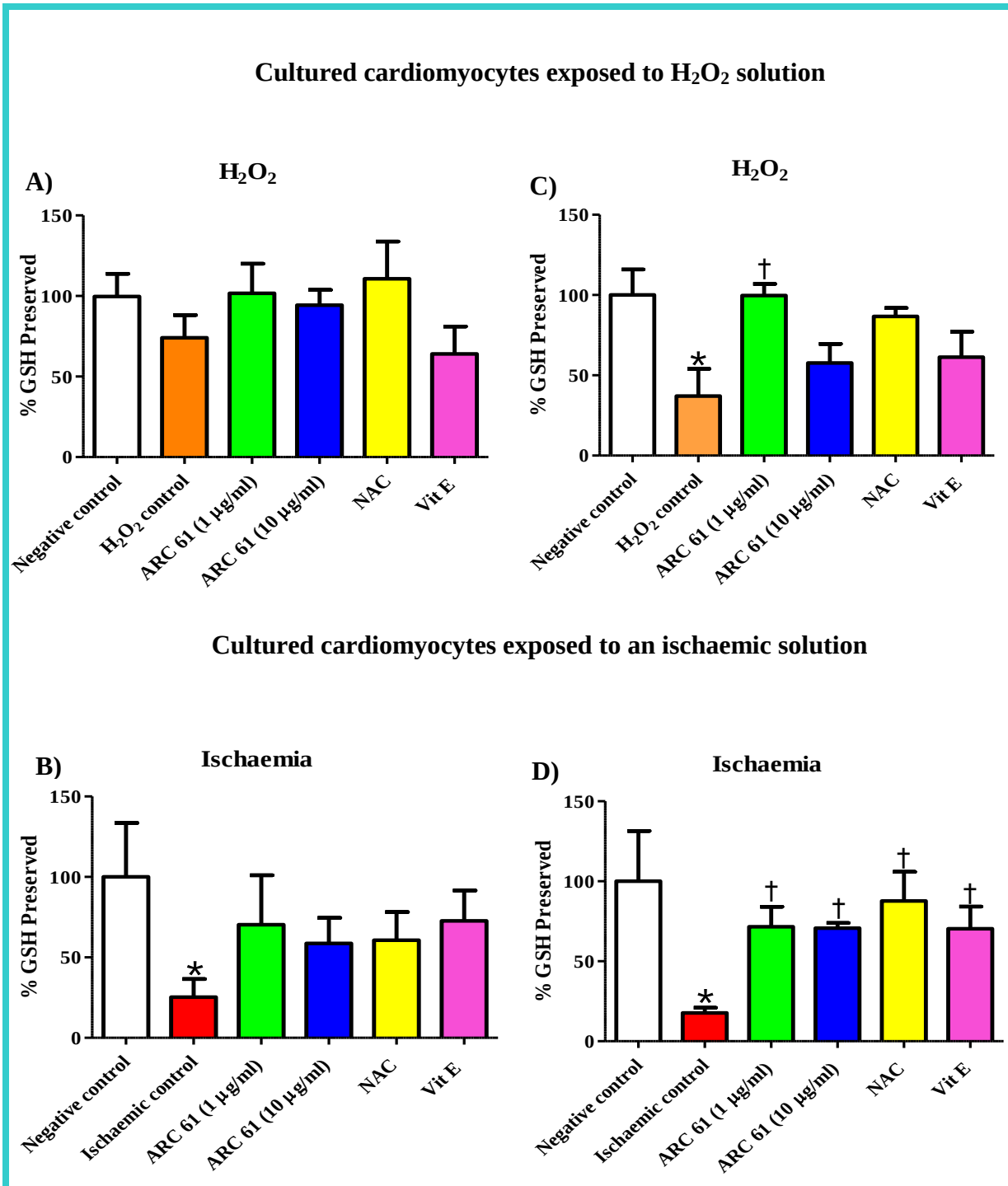


Figure 4.20. ARC 61 pre-treatment preserve GSH content in cardiomyocytes derived from non-diabetic (A and B) and diabetic rats (C and D) exposure to H_2O_2 and ischaemia. Panels represent cardiomyocytes from non-diabetic and diabetic rats exposed to: (A and C) H_2O_2 and (B and D) an ischaemic solution. Results are the mean ± SEM of 3 independent biological experiments. * Indicates significant difference when compared to a negative control and † compared to H_2O_2/ischaemic control.

4.3.6.3. ARC 61 protects cardiomyocytes against oxidative stress

As previously described, the fluorescent intensity of GSH was measured and categorised into low, medium and high intensity. The addition of H_2O_2 to cardiomyocytes abolished high intensity staining for GSH and induced concomitant increase in cells with low intensity fluorescent staining (Fig. 4.23 B). Pre-treatment of ARC 61 at a dose of 1 μg/ml prevented this shift from high to low intensity in GSH staining (Fig. 4.23 C). The distribution of GSH fluorescence was similar to that of negative control (Fig. 4.23 A). At a higher dose of 10 μg/ml, ARC 61 treatment preserved some high intensity staining but could not prevent staining of low intensity in cardiomyocytes (Fig. 4.23 D). The positive controls (NAC and vit E) protected GSH intensity but mostly in the medium intensity range (Fig. 4.23 E, F).

Two hours of ischaemia completely abolished the high intensity and severely diminished medium intensity staining for GSH (Fig. 4.24 B). A similar protective effect of ARC 61, NAC and vit E of medium to high fluorescence intensity for GSH was observed (Fig. 4.24 C, E, F). However, at a higher concentration, ARC 61 was less effective at protecting the high and medium intensity (Fig. 4.24 D).

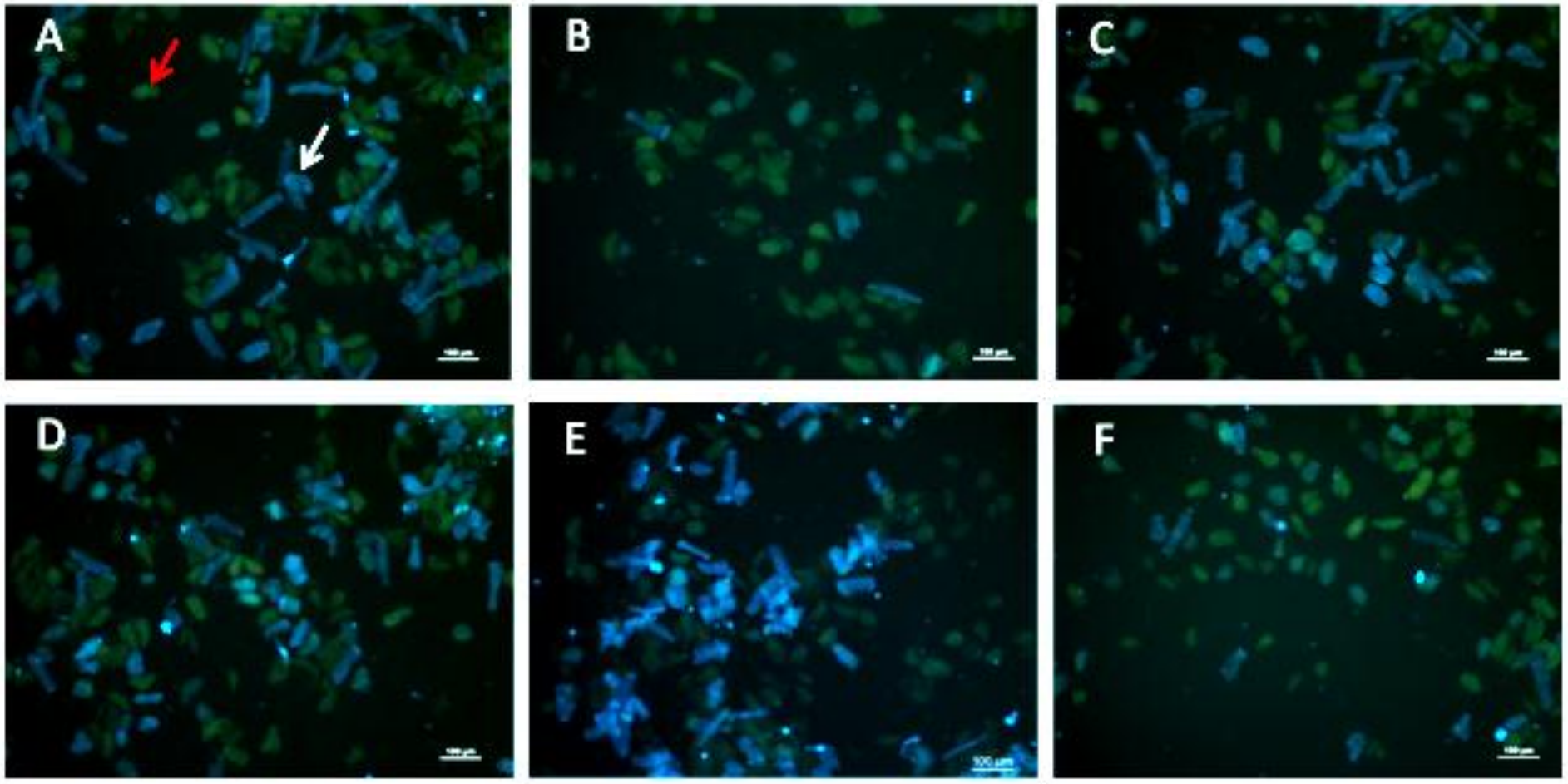


Figure 4.21. Cardiomyocytes from diabetic rats stained with CellTracker to detect GSH content after 24 hours exposure to H_2O_2. Panels represent: (A) primary cardiomyocytes exposed to a vehicle used as a negative control, (B) exposed to the H_2O_2 solution only, (C) pre-treated with 1 µg/ml of ARC 61, (D) pre-treated with 10 µg/ml of ARC 61, (E) pre-treated with NAC utilised as the positive control and (F) pre-treated with vit E and utilised as the positive control. A red arrow indicates a contracted cell with high fluorescence intensity while a white arrow indicates a viable rod shape cell with low fluorescence intensity.

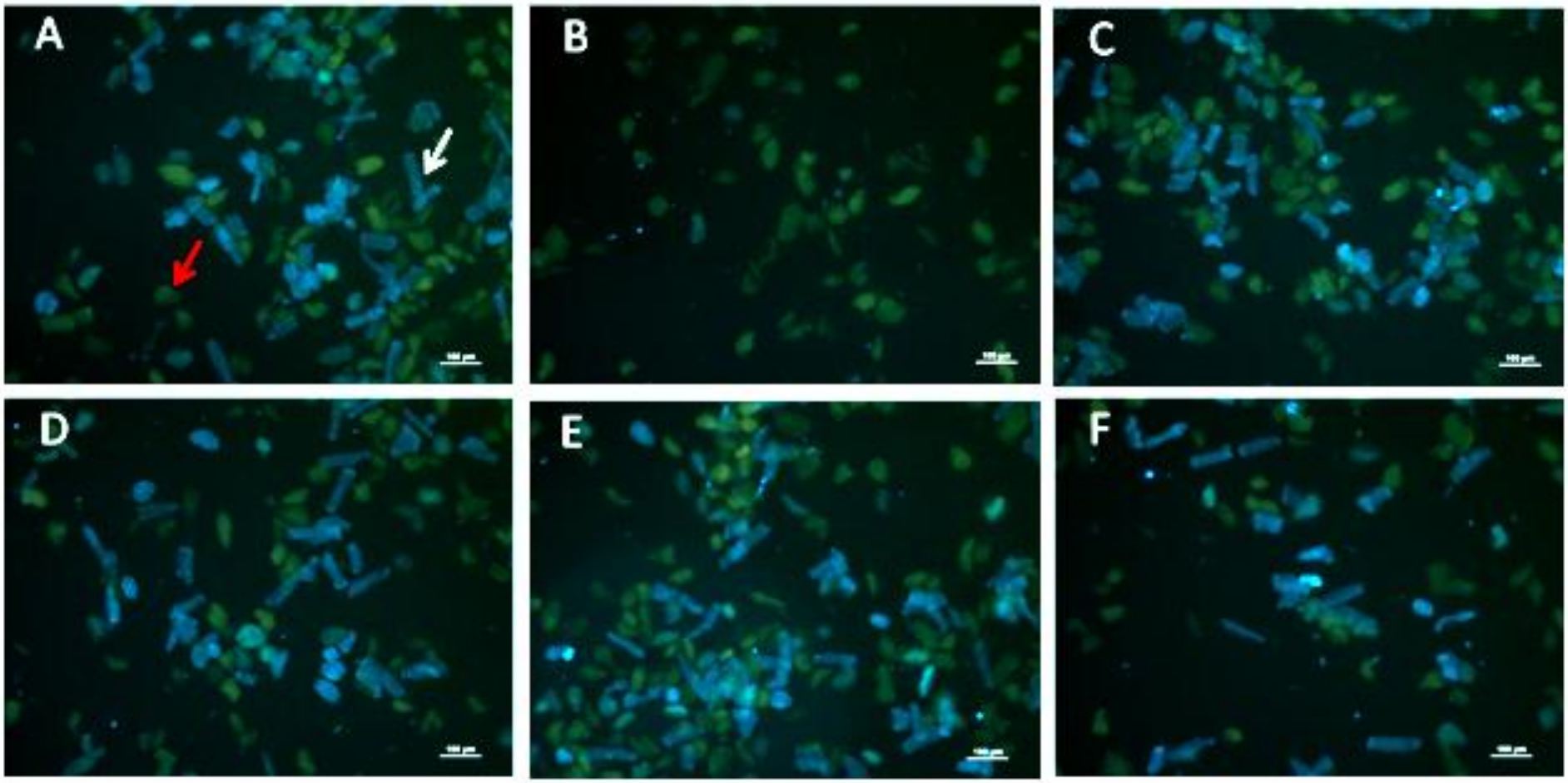


Figure 4.22. Cardiomyocytes from diabetic rats stained with CellTracker to detect total GSH content after 2 hours exposure to ischaemia. Panels represent: (A) primary cardiomyocytes exposed to a vehicle used as a negative control, (B) exposed to an ischaemic solution only, (C) pre-treated with 1 µg/ml of ARC 61, (D) pre-treated with 10 µg/ml of ARC 61, (E) pre-treated with NAC utilised as the positive control and (F) pre-treated with vit E and utilised as the positive control. A red arrow indicates a contracted cell with high fluorescence intensity while a white arrow indicates a viable rod shape cell with low fluorescence intensity.

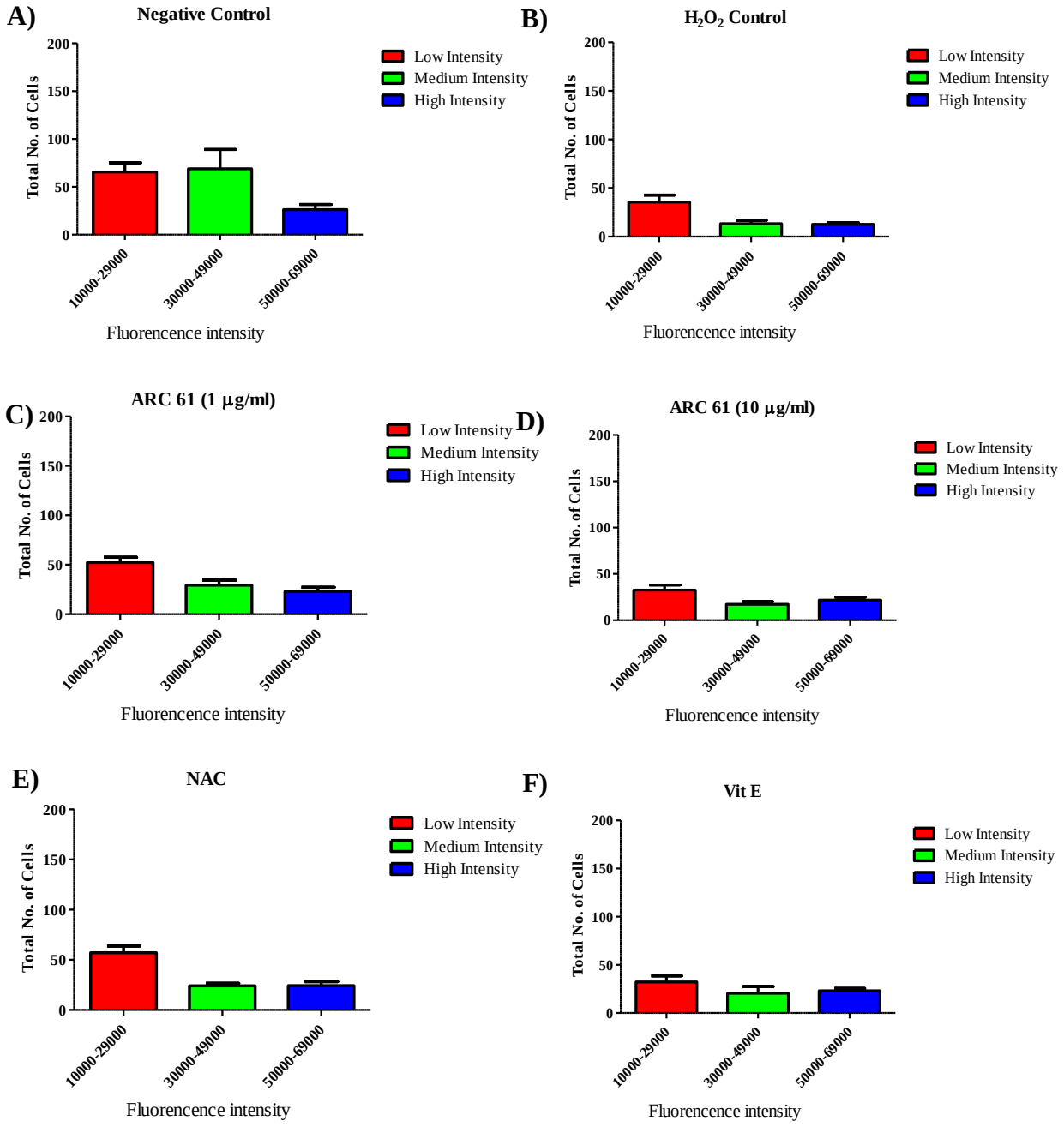


Figure 4.23. The GSH fluorescence intensity of cardiomyocytes to assess the protective effects of ARC 61 after 24 hours exposure to H_2O_2. Low, medium and high fluorescence intensity of (A) negative control, (B) H_2O_2 control, (C) 1 µg/ml ARC 61, (D) 10 µg/ml ARC 61, (E) 1 mM NAC and (F) 50 µg/ml vit E. The result are a mean of n=3 independent experiments. Fluorescence intensity is arbitrary fluorescence units.

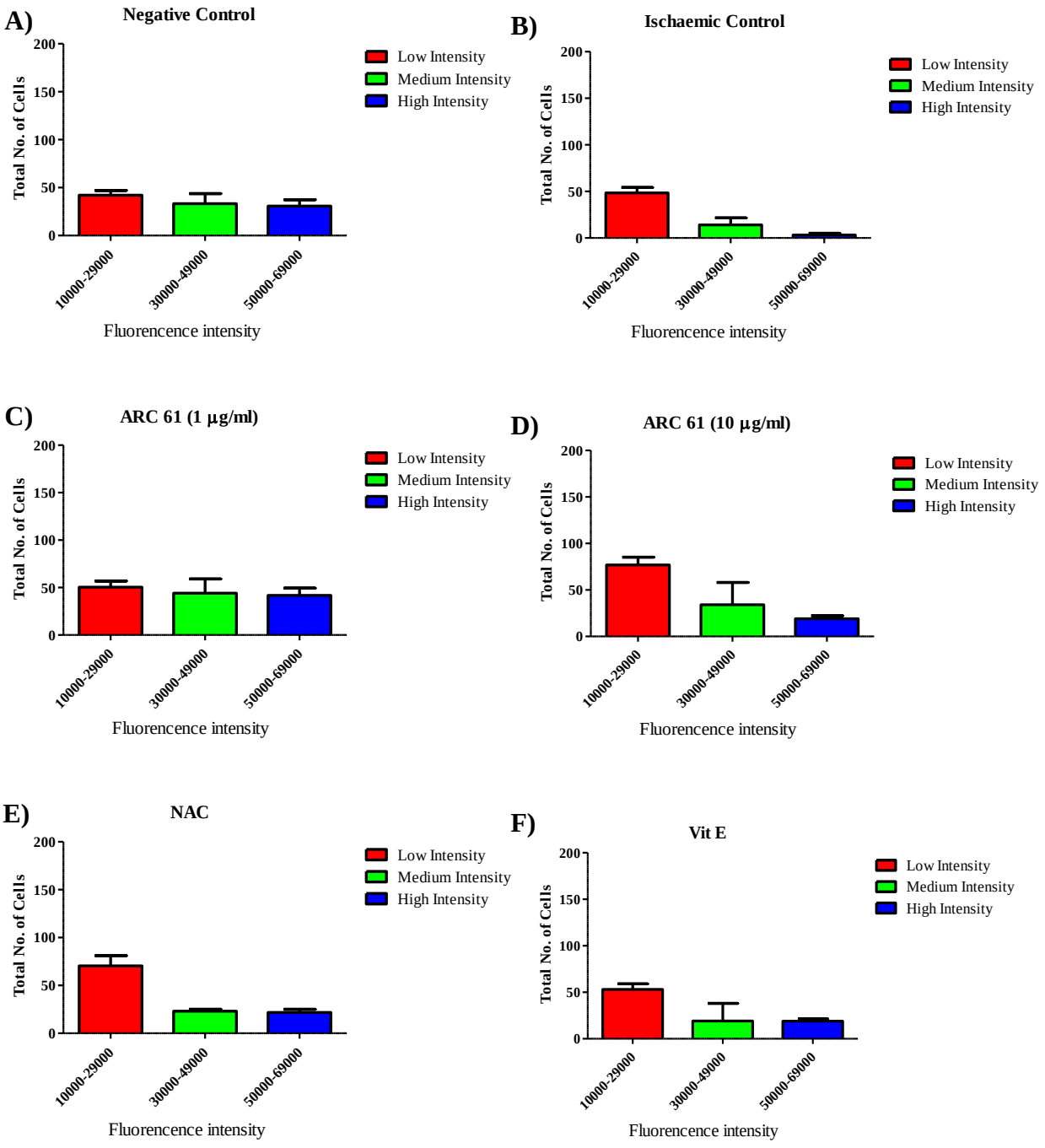


Figure 4.24. The GSH fluorescence intensity of cardiomyocytes to assess the protective effects of ARC 61 after 2 hours exposure to ischaemia. Low, medium and high fluorescence intensity of (A) negative control, (B) ischaemic control, (C) 1 µg/ml ARC 61, (D) 10 µg/ml ARC 61, (E) 1 mM NAC and (F) 50 µg/ml vit E. The result are a mean of n=3 independent experiments. Fluorescence intensity is arbitrary fluorescence units.

Chapter 5

Discussion

5. Discussion

5.1. Rat cardiomyocyte model establishment

The prevalence of T2D continues to increase at an alarming rate throughout the world (Best et al, 2012;Fonseca et al, 2012). Hyperglycaemia, characteristic of a diabetic state, exacerbates the development and progression of microvascular and macrovascular complications in patients with DCM. Cardiomyopathy is induced through an adaptive response of the heart to a mechanical stress or tissue injury. This stress or injury in DCM is due to the myocardial exposure to chronic hyperglycaemia, dyslipidaemia aberrant insulin concentrations and other factors (Karnik et al, 2007; van de Weijer et al, 2011). Myocardial remodeling, accompanied by contractile dysfunction, are the end results of these diabetic complications.

Although research continues to report on diseases that induce heart muscle deterioration, it is clear that an adequate model to study morphology and biochemical changes of cultured cardiac cells is important. In this part of the study, long term culturability of cardiomyocytes was investigated and several critical factors to improve cardiomyocytes yield were identified. A protocol used for the isolation of adult rat cardiomyocytes yielded a heterogeneous population of cells with rod shape cardiomyocyte morphology of approximately 70%. Other cells in the isolate included fibroblasts, endothelial cells and smooth muscle cells. These are cells that make up 60-70% of the heart cells (Banerjee et al, 2007). Measures employed to remove non-myocytic cells included pre-plating of isolated cells for a short period to separate the heavier, intact cardiomyocytes from non-myocytic cells and cell debris. After pre-plating, the harvested cells were co-cultured with BrdU, a synthetic thymidine analog which inhibits actively proliferating interstitial cells (Ambrosone et al, 2012). Laminin, fibronectin, collagen and gelatin are some of the known cell attachment methods (Ramos et al, 1990). According to various literatures, laminin is the preferred cell attachment matrix for culturing of adult rat cardiomyocytes (Damodaran et al, 2009; Koch-Schneidemann et al, 1994). In this study, we made use of laminin coated plates as they provided enhanced cell attachment. After overnight culture, cells were refreshed with supplemented media 199 with and without the addition of FBS. Addition of FBS initially had no effect on the morphology of the cardiomyocytes. However, after 3 days the presence of FBS promoted cell redifferentiation and improved viability of cardiomyocytes for up to 5 days in culture. Similar results were reported by other studies (Chang et al, 2011; Mitcheson

et al, 1998). In this study, cardiomyocyte nuclei became more prominent after 4 days in culture. Chang et al (2011) reported that the distance between nuclei in single cardiomyocytes decreased if cultured with 5% FBS whereas it was unchanged in cardiomyocytes cultured without FBS for 6 days. Although we did not measure the distance between nuclei in this study, the effect of FBS on structural rearrangement of the cardiomyocytes was similar. In terms of contractility and morphology, we observed a modulating activity in the presence of FBS. Fetal bovine serum, important for providing minerals and growth factors, has been shown to promote cell attachment and redifferentiation properties in cultured cardiomyocytes (Rauch et al, 2011). The effect of increased FBS supplementation on cardiomyocyte redifferentiation is also shown on human adult cardiomyocytes (Bird et al, 2003). In addition, FBS increases cell survival of cultured cells (Jochems et al, 2002). Our study showed that the addition of FBS improved cell viability of cardiomyocytes in terms of their membrane integrity i.e. ability to exclude trypan blue dye, induced cell redifferentiation and enhanced ATP content. Furthermore, the addition of FBS to long-term cultures may contribute to the preservation of intracellular energy levels (Qian et al, 2004). The increased levels of ATP support this hypothesis (Figure 4.2 C). Decreased ATP production in cultured cardiomyocytes is associated with impaired mitochondrial bioenergetic capacity and contractility due to the ruptured sarcoplasmic reticulum (Hammer et al, 2010; Korte et al, 2011). Although not investigated in this study, it is known that the sarcoplasmic reticulum rupture results in the disruption of Ca^{2+} homeostasis of cardiomyocytes (Montaigne et al, 2012).

5.2. The phenolic acid content of ARC 61

Initial screening of ARC 61, an aqueous extract of fermented rooibos, showed high polyphenolic compound content. The extract contained high amounts of iso-orientin, PPAG, orientin, quercetin and aspalathin (Table 4.1). The extract contains a combination of phenolic compounds which have been reported to contribute to its strong pharmacological effects (McKay and Blumberg, 2007). Due to the proven antioxidant properties of most of these compounds, ARC 61 can be considered a useful natural product for human health. Iso-orientin and orientin are classified as C-glycosylated flavonoids and they have been reported to show strong antioxidant properties and a potential to suppress postprandial hyperglycaemia in T2D through the inhibition

of alpha-glucosidase (Li et al, 2009; Snijman et al, 2009). The phenolic content of PPAG was also very high, not much has been reported on this compound, but a recent study reports on its association with the taste of rooibos tea (Joubert et al, 2013). Furthermore, Muller et al (2012) demonstrated that an aspalathin enriched green rooibos extract exhibited antidiabetic activity in STZ-induced diabetic Wistar rats. Kawano et al (2009) confirmed the antidiabetic effect of aspalathin in db/db mice. Rutin, also shown to have antidiabetic effects, protects neonatal rat cardiomyocytes and H9c2 cells against H_2O_2 induced apoptosis (Nassiri-Asl and Hosseinzadeh, 2009). Other flavonoids present in ARC 61 have also demonstrated cardio-protective effects (Liebgott et al, 2000; Zern and Fernandez, 2005; Zern et al, 2003). Quercetin has been shown to protect cardiomyocytes against lipid peroxidation during reperfusion by lowering malondialdehyde concentration (Ikizler et al, 2007). Orientin protects H9c2 cardiomyocytes against ischemia/reperfusion injury via inhibiting mitochondrial calcium uniporter opening, and PI3K/Akt signaling pathway may be involved in these effects of orientin (Lu et al, 2011). The aforementioned literature provides evidence on the potential protective effect of polyphenols in ARC 61 against DM and its complications. However, in this study, individual compounds were not tested. Thus, the observed effect must have been synergistic.

5.3. The effective dose of ARC 61

This study was based on a study by Marnewick et al (2011) which, showed that a daily intake equivalent to six cups of rooibos tea has cardio-protective effects. Based on our calculations from human to rat conversion this dose is equivalent to ~100 µg/ml. Results from this study, after testing a range of doses (from 0.01 to 200 µg/ml), demonstrated that an *in vitro* dose of 1 µg/ml of ARC 61 had the greatest beneficial effect on the isolated cardiomyocytes after 3 hours (Fig. 4.6 A). Increases in ATP concentrations, albeit not significant, were still observed at 6 and 12 hours (Fig. 4.6 B, C). The stability of the active flavonoids, particularly aspalathin, in aqueous solutions at a pH of 7 could have contributed to the decrease in ATP relative to the negative controls. At the highest dose, ARC 61 decreased the ATP concentrations at 3, 6 and 12 hours respectively. At 24 hours, a dose related decrease in ATP concentration was observed from 10 to 200 µg/ml of extract. This suggests that higher doses do not offer protection to cardiomyocytes during extended periods of culture. Toxicity of some polyphenols at higher doses is well

documented (Mennen et al, 2005; Pandey and Rizvi, 2009). Furthermore, an *in vitro* dose cannot be equated to an *in vivo* dose, as bioavailability, absorption and metabolism are not taken into account.

In this study, the efficacy of a known antioxidant, vit E, was also confirmed. Vitamin E, as a positive control, showed an increase in ATP production of cardiomyocytes after 3 hours relative to the negative control (Fig. 4.7 A). However, this effect was lost at 6 and 12 hours. After 24 hours, in a similar manner to ARC 61, ATP decreased with the increase in vit E concentrations. This study corroborate the findings of various other studies that shows the pro-oxidant effect of vit E if used at too high a concentration for an extended period of time (Benedetti et al, 2008; Bouayed and Bohn, 2010). This data provides evidence for the critical importance in determining the optimal time and dose for such experiments. In this study, time and dose determination was critical in the detection of optimal activity and also projecting possible toxic levels of both ARC 61 and vit E. Based on this and a subsequent pilot study, ARC 61 at a dose of 1 μg/ml and vit E at a dose of 50 μg/ml was deemed to offer protection against H_2O_2-induced oxidative stress and was used in the 3rd phase of the study. In addition a dose of 10 μg/ml was included for comparative purposes.

5.4. Diabetic cardiomyopathy model and the cardio-protective effects of ARC 61

Streptozotocin is a well-established method to chemically induce diabetes in a dose dependent manner (Nacci et al, 2009). STZ enters the vulnerable pancreatic β-cells via glucose transporter 2 (GLUT2) and induce apoptosis in these cells (Lenzen, 2008). Alkylation of DNA has been shown to induce apoptosis of pancreatic β-cells to a state of insulin-dependent DM (Elsner et al, 2006). At a dose of 40 mg/kg the adult Wistar rats developed stable non-ketoacidotic diabetes. Due to low levels of residual insulin, these rats present with persistent hyperglycaemia without a need for exogenous insulin therapy. Literature has shown that an STZ rat model can effectively be used to mimic and study a human DCM condition since it mimics cardiac fibrosis, time-dependent systolic and diastolic performance deficits observed in DCM (Factor et al, 1981;Mihm et al, 2001). In this study, elevated levels of plasma glucose concentrations in STZ-induced rats were able to confirm DM model in the rats (Table 4.3).

Based on current literature, early myocardial changes related to chronic hyperglycaemia occurs around 2 weeks after STZ induced diabetes (Bidasee et al, 2003). In mice ventricular dysfunction, including increased heart beat (tachycardia), are established 2 weeks after STZ induction with no visible change in myocardial morphology (Cai et al, 2005). However, in a study done by Yu et al (2007), significant changes to myocardial morphology and function were established after 4 weeks of STZ treatment. These changes include systolic and diastolic dysfunction and decreased left ventricular wall thickness and a reduced ventricular ejection fraction (Khavandi et al, 2009;Yu et al, 2007). For this study the emphasis was on protecting the diabetic heart at risk, not treatment of an already existing DCM condition. Therefore, cardiomyocytes were isolated from diabetic rats 2 weeks after STZ induction.

Exposure of cardiomyocytes derived from diabetic rats showed more vulnerability to H_2O_2-induced apoptosis, had higher levels of intracellular ROS and reduced levels of ATP and GSH compared to the cardiomyocytes derived from non-diabetic rats (Fig. 4.14 A, C; Fig. 4.19 A, C and Fig. 4.20 A, C). Furthermore, ATP levels were decreased to almost zero in the H_2O_2 control group in cardiomyocytes obtained from diabetic rats (Fig. 4.19 C). Coinciding and explaining this decrease is an increase in apoptosis, H-FABP leakage, with a concomitant decrease in GSH was observed in the H_2O_2 control of cardiomyocytes derived from diabetic rats. This confirms that the STZ diabetic model yielded cardiomyocytes that were more susceptible to exogenous H_2O_2-induced oxidative stress. This result was confirmed by various literature that reports on how increased ROS production impose cardiomyocytes injury resulting in an increase in free fatty acids (FFAs), apoptosis, and depleted ATP concentrations in the hearts of diabetic patients (Tarquini et al, 2011;Wang et al, 2006). The diminished antioxidant capacity, due to lower GSH content, further demonstrated the vulnerability of these cardiomyocytes. The intracellular GSH content is important for the intracellular antioxidant defense system (Townsend et al, 2003). Thus, its depletion is strongly associated with generation of oxidative stress (Amado and Monserrat, 2010). Cardiomyocytes derived from diabetic rats exposed to ischaemic conditions demonstrated a similar increase in apoptosis, but with an increased H-FABP leakage and intracellular ROS (Fig. 4.13 B, D and Fig. 4.14 B, D). In contrast to what was expected, there was a slight increase in H-FABP leakage of untreated cells in cardiomyocytes derived from diabetic rats (Fig. 4.13 C). Given the sensitivity of the H-FABP kit, any technical error or artifact resulting from cell culture could have been responsible for the abnormal increased levels of H-

FABP leakage in these untreated cells. Free fatty acids are responsible for up to 70% of energy metabolism in a cardiac cell and their intracellular coupling carried-out by H-FABPs is crucial for survival of the cell. The membrane leakage of these proteins is associated with myocardial damage and more specifically myocardial ischaemia (Azzazy et al, 2006;Tanaka et al, 2006). ATP and GSH content was also reduced to a similar extent by ischaemia (Fig. 4.19 B, D and Fig. 4.20 B, D). Pre-treatment of cardiomyocytes derived from non-diabetic rats with ARC 61 for 6 hours at concentrations of 1 and 10 μg/ml prevented the H_2O_2-induced increase in apoptosis and these results were similar to both NAC and vit E (Fig. 4.10 A). Thus, this study suggests that pre-treatment of these cardiomyocytes with ARC 61 has protective effects against ischaemia. This protective effect of ARC 61 was further confirmed by the reduction in H-FABP leakage and preserved the ATP content as well as the GSH content of these cardiomyocytes (Fig. 4.13 A; Fig. 4.19 A and Fig. 4.20 A). In comparison, in cardiomyocytes derived from the diabetic rats, only pre-treatment with ARC 61 at the lower dose and NAC were able to reduce apoptosis and intracellular ROS, as well as preserve the GSH content (Fig. 4.13 C; Fig. 4.19 C and Fig. 4.20 C). Pre-treatment with ARC 61 at a higher dose failed to prevent the increased apoptosis and ROS but was able to preserve ATP and GSH, albeit less effective at the lower concentration (Fig. 4.10 C; Fig. 4.14 C; Fig. 4.19 C and Fig. 4.20 C). This result may explain the significance difference that is caused by different doses of ARC 61. Furthermore, in cardiomyocytes derived from diabetic rats NAC was shown to be more effective in protecting against H_2O_2-induced apoptosis compared to vit E. It therefore appears that in the vulnerable cardiomyocytes derived from diabetic rats a clear dose effect could be demonstrated whereby a dose of 1 μg/ml of ARC 61 yielded better protection. This confirms the earlier dose response findings in phase 2. Therefore, the present study reveals that as little as 1 μg/ml is required to prevent myocytic apoptosis triggered by oxidative stress.

Under normal conditions, a balance exists in cells between the production of ROS and the cell's natural ability to scavenge these free radicals through cellular antioxidants including GSH (Paradis et al, 1996). In disease conditions, including DM, myocardial infarction and ischaemia reperfusion injury, cardiomyocytes become more vulnerable to cell damage and apoptosis due to an imbalance in the antioxidant-oxidative stress status (Liang et al, 2005). The addition of exogenous H_2O_2 induces excess levels of ROS resulting in cellular damage mainly by oxidation redox sensitive proteins (Son et al, 2011). Using this model we were able to establish a

difference in the ability of cardiomyocytes derived from diabetic rats and non-diabetic rats in terms of their ability to protect themselves against H_2O_2 and apoptosis. This study shows that the diabetic effect on cardiomyocytes is not transient but is of more permanent nature. The depletion of GSH suggests that these cells have lower levels of intracellular antioxidant capacity and/or less effective in decreasing levels of ROS by enzymatic processes (Movahed et al, 2012). It therefore makes sense that addition of antioxidants such as NAC, vit E and plant products rich in polyphenols could be of benefit to protect against increased levels of oxidative stress. Although in this study vit E was less effective at preventing cell damage and apoptosis than NAC in cardiomyocytes derived from diabetic rats. This can be explained by contradicting reports on the cardio-protective effects of vit E (Cockey, 2006;Shirpoor et al, 2009). The cardio-protective effects of rooibos and its polyphenolic constituents have been described both *in vivo* and *ex vivo* (Marnewick et al, 2011;Pantsi et al, 2011). Marnewick et al (2011) showed that rooibos tea decreased lipid peroxidation, increased GSH and improved the lipid profile of human subjects at risk of CVD. Pantsi et al (2011) showed in the reperfusion heart model on normal rats pre-treated with rooibos GSH levels were increased and pro-apoptotic proteins were decreased by rooibos treatment. However, very little data is available on the potency of rooibos against DCM. In this study we demonstrate the protective effect of rooibos against H_2O_2-induced oxidative stress in cardiomyocytes derived from diabetic rats and non-diabetic rats. Rooibos decreased intracellular ROS, preserved ATP and GSH content and suppressed apoptosis (Fig. 4.14 A, C; Fig. 4.19 A, C and Fig. 4.20 A, C). An interesting finding in cardiomyocytes from diabetic rats was the relationship between dose and efficacy. Increasing the dose from 1 to 10 μg/ml nullified the decrease in ROS and the increase in GSH. This was not seen in cardiomyocytes derived from non-diabetic rats. Although this finding is unexpected it is known that polyphenols can become pro-oxidants and induce toxicity at higher concentrations which could be the case in these vulnerable cardiomyocytes (Lee et al, 2003).

Exposing these cardiomyocytes to ischaemic conditions induced comparable results to exogenous H_2O_2 in terms of the induction of apoptosis, increased H-FABP leakage, increased intracellular ROS and ATP depletion (Fig. 4.13 B, D; Fig. 4.14 B, D and Fig. 4.19 B, D). GSH levels appear to be severely reduced by ischaemia (Fig. 4.20 B, D). Rooibos treatment, apart from slightly lower ATP levels at the higher dose, was as effective at ameliorating these adverse effects induced by ischaemia, as was seen with exogenous H_2O_2-induced oxidative stress.

Deprivation of cardiomyocytes from oxygen results in hypoxia-acidosis-associated cell death related to increased anaerobic glycolysis (Burton and Gibson, 2009). Contrary to reperfusion experiments whereby cardiomyocytes are reperfused after a period of ischaemia, these cardiomyocytes were kept under ischaemic conditions. Various studies have reported that the polyphenols present in rooibos can activate the master regulator of cellular energy, AMPK (Beltran-Debon et al, 2011; Zang et al, 2006). In the ischaemic heart activation of AMPK plays an essential role in cell survival (Li et al, 2006). During periods of ischaemia, cellular energy levels become severely depleted (Amr Moussa and Ji Li, 2012). As ATP levels are depleted, AMP levels increase which triggers the activation of AMPK. Once activated, AMPK increases glucose uptake, glycolysis, fatty acid oxidation, and inhibits energy consuming pathways such as protein synthesis thereby preserving essential cellular energy levels (Paiva et al, 2011). There is also evidence that AMPK has anti-apoptotic activity via p38 MAPK pathway (Andersson et al, 2006; Hsu et al, 2010). Cardio-protective effects of metformin in T2D individuals is said to be mediated via activation of AMPK (Boyle et al, 2011). Our results would support such a mechanism for rooibos.

Our findings provide the first evidence that rooibos tea protects cardiomyocytes obtained from diabetic rats against oxidative injury. This was evident by the observed increase in the preservation of GSH content and decrease in apoptosis in cardiomyocytes pre-treated with the extract. Furthermore, the observed effect was synergistic and was not triggered by a single compound.

Chapter 6

Conclusion

6. Conclusion

Compelling evidence exists that exposure to prolonged hyperglycaemia is reponsible for cardiac muscle damage through the generation of oxidative stress. This increased generation of oxidative stress plays an important role in the development of DCM. Current insulin therapies that are used to control insulin resistance do not offer much protection against comorbidities, such as DCM. The use of plant-derived antioxidants as an adjunct to current therapies continue to show promise and various epidemiological and clinical studies are starting to support such therapies.

The current study confirms that ARC 61 might be used as a potential therapeutic to protect cardiac cells obtained from diabetic hearts against H_2O_2-induced stress. The cardiomyocytes from the diabetic heart were far more susceptible to oxidative damage compared to myocytes obtained from non-diabetic rats, suggesting that healthy individuals can tolerate and hadle oxidative stress better compared to the diabetic counterpart.

This study showed that, according to the dose equivilent extrapolation, an equivelent of less than a quarter cup of rooibos tea is required to protect cardiac cells against diabetic-induced stress. Furthermore, we proposed that the protective effect might be due to an interplay between genes and/or proteins involved in mitochondrial depolirisation and endoplasmic riticulum stress. However, this still need to be investigated and can be elucidated on in future studies.

6.1. Future prospects

In this study, the osmolarity of isolated cardiomyocytes was not tested, which could have contributed to the low viability seen during initial isolation and preparation of rod shape cardiomyocytes. Furthermore, it could be of interest to test the efficacy of ARC 61 after 3 hours of pre-treatment as it showed increased metabolic activity in phase 2 of the study.

In conclusion, the effect of polyphenols on mitochondrial dysfunction as a possible mechanism of inhibiting oxidative stress has been copiously documented. It would be of interest to test the effects of ARC 61 on mitochondrial dysfunction in order to unravel the precise mechanism by which rooibos exerts its cardio-protective effects.

7. Reference list

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8. Appendix

8.1. Appendix I-

Unless otherwise stated, all reagents were of a molecular grade.

Reagents supplied by Sigma-Aldrich Corporation:

- Magnesium sulfate heptahydrate
- L-Glutamic acid
- Potassium monobasic anhydrous
- Sodium bicarbonate
- Sodium phosphate monobasic dehydrate
- L-Carnitine hydrochloride
- HEPES free acid
- Potassium chloride
- Sodium phosphate mono anhydrous
- L-Glycine
- Magnesium chloride
- D-Glucose
- Sodium pyruvate
- Albumin from bovine serum (BSA)
- 2,3 Butanedione monoxime (BDM)
- Calcium chloride dehydrate
- Potassium phosphate dibasic: trihydrate
- Vitamin (α-tocopherol acetate)
- Laminin from Engelbreth-Holm-Swarm murine sarcoma basement
- Creatine hydrate crystalline
- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide) MTT
- 5-bromo-2-deoxyuridine (BrdU)
- Dimethyl sulfoxide (DMSO)
- Trypsin-EDTA solution

- N-acetyl cystein
- Propidium Iodide
- Sodium hydroxide
- Triton X-100
- Ethanol absolute
- Nylon mesh (200 µM pore size)

Other sources:

- Hydrogen peroxide (Merck)
- Taurine (Acros organics)
- Collagenase type II (Worthington Biochemical Corporation)
- Albumin from bovine serum (BSA) (free fatty acid) (Roche Diagnostics)
- Penicillin-streptomycin amphoterin B (Lonza)
- Medium 199 (Lonza)
- DMEM (Lonza)
- Hanks buffered saline solution (HBSS) (Lonza)
- Fetal bovine serum (GIBCO)
- Phosphate-Buffered Saline (GIBCO)
- Sodium Pentobarbitone (Kyron laboratories (Pty)Ltd)
- Annexin V (Invitrogen)
- Trypan blue dye (Invitrogen)
- Percoll (GE Health care Bio-sciences Corp)
- Sodium citrate (Merck)
- Hydrochloric acid (Merck)
- Eosin (Merck)
- Thymol crystals (Merck)
- Haematoxylin (Merck)
- Disposable sterile filter system unit (Merck-Millipore)
- Tissue culture plates (Greiner and SPL life sciences)

- Filter tips (Whitehead Scientific and Lasec SA)
- Sterile tubes (Greiner and Merck)
- Formalin buffered neutral (Merck)
- Potassium (Merck)
- Sodium iodate (Merck)

8.2. Appendix II-

Independent analysis: EC50 determination for ARC 61 extract.

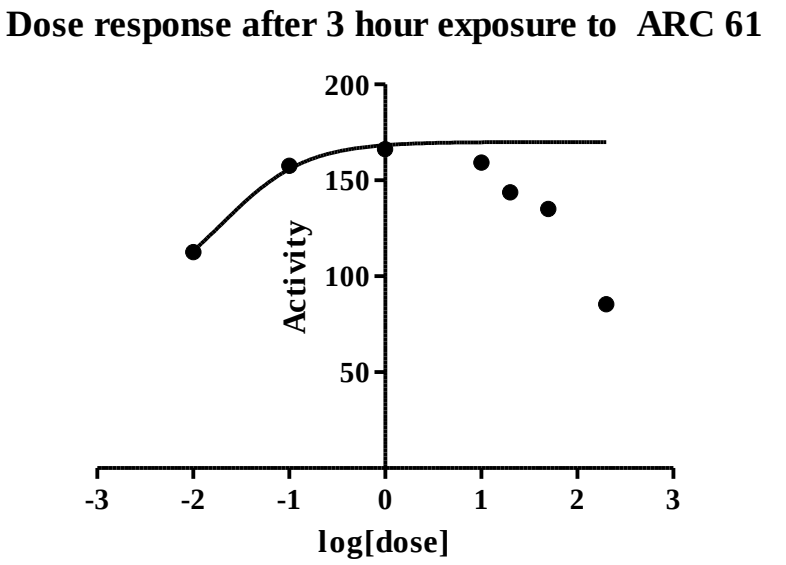

Figure 8.1. The half maximal effective concentration (EC50) of ARC 61 after 3 hours exposure.