

RESVERATROL MEDIATED CARDIOPROTECTION IN OBESE RATS

by

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ABSTRACT

Obesity is an independent risk factor for the development of heart disease. Food derived compounds such as resveratrol have been reported to have strong medicinal properties and have shown potential in both preventing and reversing heart diseases. This study investigated the cardioprotective properties of resveratrol in an animal model of diet-induced obesity and the possible cellular mechanisms responsible for these effects.

In the first study, obese prone (OP) and obese resistant (OR) rats were fed with high fat (HF) diet (55% of energy from fat) while, sprague dawley (SD) rats that served as control were fed with standard chow (SC) diet (13% energy from fat) for a total of 17 weeks. During the last 5 weeks of the study, the treatment group received resveratrol daily by oral gavage at a dosage of 2.5 mg/kg body weight and the vehicle group received 1mL of 50% ethanol (vehicle for resveratrol). Body weight, cardiac structure and function and blood pressure, total visceral fats, glucose, insulin, lipids, inflammatory and oxidative stress markers were measured. Body weight, blood pressure, total visceral fat, glucose, insulin, thiobarbituric acid reactive substance (TBARS), tumor necrosis factor α (TNF- α), interleukin 6 (IL-6) triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), adiponectin and cardiac isovolumic relaxation time (IVRT) were significantly higher in OP HF control rats when compared to SD SC control rats. Treatment with resveratrol significantly improved IVRT, TBARS, TNF- α , IL-6, triglycerides, glucose, LDL and increased insulin in OP HF rats. OR HF rats had significantly higher blood pressure, IVRT, TNF- α , IL-6, glucose and leptin. Resveratrol treatment significantly decreased TNF- α , glucose and leptin levels in OR HF rats. Expression levels of cardiac calcium handling proteins such as sarcoplasmic reticulum Ca^{2+} -ATPase 2a (SERCA2a), phospholamban (PLB) and phosphorylated PLBthr17 and PLBser16 were measured. There was no change in the expression levels of these proteins between experimental groups.

In the second part of the thesis, the potential for resveratrol to prevent cardiac dysfunction by improving calcium handling (responsible for cardiomyocyte contractile function) was investigated. Cardiomyocytes were incubated for 24 hours with different doses (10, 100 and 200 μ M) of palmitic acid (PA). One group of cardiomyocytes was pretreated with resveratrol for 45 minutes prior to addition of PA. After a 24 hour incubation, cell morphology, apoptosis, expression of calcium handling proteins SERCA2a, PLB, phosphorylated PLB^{thr17}, PLB^{ser16} and cardiac troponin were measured. Cardiomyocyte contractility and TBARS were assessed after 4 hours of incubation with PA. Incubation with 200 μ M PA significantly increased the number of rounded cardiomyocytes and apoptosis. Resveratrol treatment prevented these changes. Incubation with 200 μ M PA resulted in a reduced rate of relaxation in cardiomyocytes and resveratrol prevented this reduction. Western blot analysis showed that the PA induced decrease in SERCA2a expression and the SERCA2a:PLB ratio was prevented with resveratrol treatment.

In conclusion, high fat diet induced cardiac dysfunction in both OP HF and OR HF rats, while resveratrol treatment reversed these abnormalities in OP HF rats, but not in OR HF rats. Oxidative stress, inflammation, hyperglycemia and dyslipidemia were improved in OP HF rats. *In vitro* experimentation showed that lipid loading disrupts cardiomyocytes contractility and can also result in increased cell death. The change in the rate of relaxation in cardiomyocyte contractility might be due to changes in the SERCA2a expression levels and the SERCA2a:PLB ratio. Importantly, this study showed that resveratrol can act directly on cardiomyocytes and thereby, has the potential to be a cardioprotective agent in obesity and similar pathological conditions.

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DEDICATION

To my loving parents who gave me unconditional support

in all fronts of my life

&

My dearest wife and son

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LIST OF ABBREVIATIONS

AMPK - 5' Adenosine monophosphate-activated protein kinase

ATP – adenosine triphosphate

BAT – brown adipose tissue

BMI – body mass index

BP – blood pressure

cGMP - cyclic guanosine monophosphate

CO - cardiac output

CQS – calsequestrin

CVD – cardiovascular disease

EF - ejection fraction

ELISA - enzyme-linked immunosorbent assay

FA – fatty acids

FKBP-12.6 - FK-506 binding protein–12.6

HDL – high density lipoprotein

HF – high fat

IL-6 – interleukin-6

IVRt - isovolumetric relaxation time

IVSd - interventricular septal wall thickness at diastole

LDL - low density lipoprotein

LTCC - L-type calcium channel

LV – left ventricle

LVIDd - left ventricular internal dimensions at diastole

LVPWd - left ventricular posterior wall thickness at diastole

mPTP - mitochondrial permeability transition pore

NCX - Na^+ - Ca^{2+} exchanger

NO - nitric oxide

Nrf2 - nuclear factor erythroid 2-related factor 2

OP – obese prone

OR – obese resistant

PA – palmitic acid

PKG - protein kinase G

PLB – phospholamban

PP – phosphatases

RYR2 - ryanodine receptor

SD – sprague dawley

SERCA – sarcoendoplasmic reticulum Ca^{2+} -ATPase

SIRT1 - sirtuin 1

SR - sarcoplasmic reticulum

TBARS – thiobarbituric acid reactive substance

TNF- α – tumor necrosis factor alpha

TNF α - tumor necrosis factor- α

TnI – troponin I

WAT – white adipose tissue

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Cardiovascular disease (CVD) and subsequent heart failure claims the most number of lives every year around the globe. According to estimates available through the World Health Organization (WHO), around 17.3 million died of CVD in 2008 and this number is expected to exceed 23 million per year by 2030 (WHO, 2013a). There are a number of risk factors for the development of CVD such as hypertension, diabetes, hyperlipidemia, coronary disease, valvular disease and certain gene mutations (Berry et al., 2012; Lloyd-Jones et al., 2004; Murabito et al., 2004). In combination with neurohormonal and cellular changes, the heart is capable of compensating for the stress arising from these pathophysiological conditions through structural and functional remodeling (Cohn, Ferrari, & Sharpe, 2000). However, prolonged stress leads to the permanent loss of structure and function of the heart and culminates in heart failure (Grossman & Paulus, 2013). There are a number of therapeutic strategies being used to prevent, abate or reverse development of heart failure (S. S. Liu, Monti, Kargbo, Athar, & Parakh, 2013). For mild cases, lifestyle modifications are the first step of therapy and pharmaceutical agents are prescribed for use if either changes in lifestyle do not have any effect or the disease has already progressed beyond being manageable with lifestyle changes (Guyatt & Devereaux, 2004; Wexler, Pleister, Raman, & Borchers, 2012). In certain cases such as valvular disease, electrical abnormalities and structural disabilities of the heart by birth, surgeries are used as the first step in treatment (S. S. Liu et al., 2013). However, despite all of the modern biomedical inventions and the development of modern therapeutic methodologies, heart failure still claims millions of lives every year. This scenario leads us to think of alternative strategies to either prevent the development or arrest the progression of CVD into overt heart failure and mortality.

The obesity epidemic is directly and indirectly associated with millions of deaths every year (Bastien, Poirier, Lemieux, & Despres, 2014). Obesity is a condition wherein excess fat gets deposited in the body, under the skin (subcutaneous fat) and in other major organs (visceral fat) of the body. This further increases the risk of development of diabetes, hypertension, vascular diseases and other independent risk factors of cardiovascular disease (Guh et al., 2009). Heart failure is a major endpoint in obese patients and the millions becoming obese every year are in the risk of developing some form of heart disease (Artham, Lavie, Patel, & Ventura, 2008).

Family genetics, spontaneous gene abnormalities, diet and other environmental factors are considered to be the major risk factors of obesity (Dasouki, Youngs, & Hovanes, 2011; Hebebrand & Hinney, 2009; Walley, Blakemore, & Froguel, 2006; Youngson & Whitelaw, 2011). A genetic predisposition together with the personal choice of diet play significant roles in the development of obesity (Drewnowski, 2004). The increased availability of energy dense foods has certainly boosted the incidences of obesity around the globe (Drewnowski & Darmon, 2005). High caloric intake accompanied by low physical activity results in increasing lipids stored as fat. After a certain point, cells that store fat (adipocytes) cannot keep up with the demand, which results in their dysfunction (Bluher, 2013). Adipocyte dysfunction leads to the release of adipokines into the circulation which is accompanied by increasing circulating lipids. High levels of circulating lipids results in the increased intake of lipids by other organs. However, an increased deposition of lipids contributes to cellular stresses resulting in organ dysfunction (Drosatos & Schulze, 2013). The heart is one of these target organs and the resulting lipotoxic stress causes cardiac dysfunction that can culminate in heart failure (Ussher, 2014).

Earlier forms of therapies for human ailments were all naturally derived. Different types of plants were used to treat all human ailments (Fabricant & Farnsworth, 2001; Mosihuzzaman,

2012). Many of these medications were either served as food itself or mixed with other foods (Bhamarapravati, Pendland, & Mahady, 2003; Santos et al., 2010). Nutraceutical is the modern term for compounds that are naturally derived and together with nutrition deliver medicinal effects (Brower, 2005). Resveratrol can be called a nutraceutical as it is a polyphenol that is mainly found in grapes and other berries and can be consumed in the form of pills. Over the years, research has shown that resveratrol has strong medicinal properties against a variety of human ailments including cancer, cardiovascular diseases, diabetes and some type of infections (Raederstorff, Kunz, & Schwager, 2013). The cardioprotective properties of resveratrol have been well documented in pre-clinical models and recently it has also been shown to be beneficial in humans (Raj et al., 2014; Tome-Carneiro, Larrosa, et al., 2013).

CHAPTER 2

REVIEW OF LITERATURE

2.1 OBESITY

Obesity is a condition wherein excess fat accumulates in the body. Individuals can be classified using body mass index (BMI) which is calculated by dividing body weight (in kg) by the square of that individual's height (meters) (Romero-Corral et al., 2008). For adults the classification is as follows,

BMI	Classification
$\leq 24.9\text{kg/m}^2$	Normal
25 – 29.9 kg/m^2	Overweight
$\geq 30\text{kg/m}^2$	Obese
$\geq 39\text{ kg/m}^2$	Severely obese

Table 2.1: BMI classification. This table shows how the individuals are classified based on their BMI (body weight divided by square of height).

However, BMI does not directly measure fat stores or adiposity and hence additional measurements are required to determine body fat (Rothman, 2008). Waist circumference, the skin fold test, waist to hip ratio calculation and modern techniques such as whole-body air displacement plethysmography, underwater weighing, dual energy X-ray absorptiometry and even ultrasound techniques are used to determine body fat composition (Carmienke et al., 2013; S. K. Das, 2005; de Koning, Merchant, Pogue, & Anand, 2007; Deurenberg & Deurenberg-Yap, 2002; Schneider et al., 2010). A small percentage of the obese population is apparently completely healthy despite being classified as obese (Hamer & Stamatakis, 2012). However, in most cases obesity is accompanied by several comorbidities and obese individuals experience a

poor quality of life and suffer psychologically from social stigma (Gouveia, Frontini, Canavarro, & Moreira, 2014; J. Wang, Sereika, Styn, & Burke, 2013). Obesity is a manageable condition and in some cases completely reversible with strong changes in lifestyle including physical activity and diet, medications and in some cases with surgery (Dalle Grave, Calugi, & El Ghoch, 2013; Lagerros & Rossner, 2013; Picot et al., 2009). Irrespective of the status of economic development (underdeveloped, developing or developed), the growing incidence of obesity and increased dependence on the health care systems has become a major concern for most countries (Pawloski, Ruchiwit, & Markham, 2011; Ramachandran & Snehalatha, 2010; A. G. Tsai, Abbo, & Ogden, 2011; Y. C. Wang, McPherson, Marsh, Gortmaker, & Brown, 2011; Withrow & Alter, 2011).

2.1.1 World statistics

At least 2.8 million deaths every year around the world that is associated with obesity or being overweight. During the period between 1980 and 2008, the worldwide prevalence of obesity nearly doubled. A total of more than 500 million people were considered to be obese or overweight. This number composed of 205 million men and 297 million women over the age of 20 were obese or overweight (WHO, 2014). The prevalence was highest in the Americas (approximately 62% overweight and 26% obese) and lowest in the South East Asia (approximately 14% overweight and 3% obese). Women are more prone to obesity when compared to men around the world (Borders, Rohrer, & Cardarelli, 2006). In some African and Mediterranean countries, the prevalence of obesity in women is almost double that of men. Income also seems to affect the prevalence of obesity (Drewnowski & Darmon, 2005). The prevalence of obesity in upper middle income countries is almost triple (24%) when compared to lower middle income countries (WHO, 2013b). According to 2008 estimates, almost half of the

world's overweight people reside in China and the United States. These two countries also had the highest increase in the prevalence of obesity in the last two decades. Brazil, Mexico, Indonesia, India, Turkey, Germany and Russia are other countries with a very high prevalence of overweight adults (Stevens et al., 2012; WHO, 2013b).

2.1.2 Canadian statistics

According to the latest estimates published by the Public Health Agency of Canada and the Canadian Institute for Health Information, 1 in 4 Canadian adults (24.3%-25.4%) are obese. Statistic Canada data in 2012 shows that 67% of men and 54% of women aged 18-79 are overweight or obese (Canada, 2013). These estimates are higher than the self-reported data in 2009 which showed 59.2% of Canadian men and 43.9% of women are in the overweight category (Canada, 2009). The number of obese children and youth is also increasing with 11.7% obese between 6 and 17 years of age (Karen C. Roberts, 2012). The geographic distribution of obesity varies within Canada. The lowest reported prevalence was 3.4% of the population in certain geographic areas while the highest is as much as 34.3%. Obesity is also more prevalent in some ethnic populations when compared to others (Bruce, Riediger, Zacharias, & Young, 2011; Ng, Corey, & Young, 2011). On-reserve Aboriginal adult populations have an obesity prevalence of 36% while off-reserve Aboriginal adult population is reported to be around 25.7%. This is still considerably higher than among non-Aboriginal adults in Canada (17.4%). However, these estimates are based on self-reported data and actual height and weight measurements will usually result in higher numbers than that shown by self-reporting data (Connor Gorber & Tremblay, 2010). These numbers also confirm that the prevalence of obesity has doubled between 1981 and 2009 (PHAC, 2013).

2.1.3 Obesity in children

Childhood obesity is another major area of concern as it possesses several health risks including the identification of cardiovascular disease and mortality in early adulthood (Franks et al., 2010; Must, 1996; K. C. Roberts, Shields, de Groh, Aziz, & Gilbert, 2012). Based on data from Statistics Canada in 2012, 32% of children and youth between the ages 5-17 years are overweight or obese (Canada, 2012). Obesity in children and youth is measured using a different set of BMI cut-offs. According to the International Obesity Task Force (IOTF), a BMI greater than 21.22 kg/m² and 26.02 kg/m² are the cut-off for 12 year old boys and girls, respectively, to be considered obese. There are other systems of BMI cut-offs and the estimates may vary accordingly. For example, in the Canadian Community Health Survey in 2004, based on the IOTF system, the obesity rate of 8.2% was reported among children and youth (2-17 years). However, based on the Centers for Disease Control cut-offs, the estimate increased to 12.7% whereas it was estimated to be 12.5% based on WHO system (PHAC, 2013).

2.1.4 Health costs

Obesity is associated with a number of co-morbidities. The risk of type 2 diabetes, hypertension, CVD and cancers increases significantly with being obese. Due to the social stigma attached to obese individuals, a number of psychological conditions are also prevalent among the obese (Dalrymple et al., 2011; Zimmerman et al., 2011). Premature mortality rates increase with the severity of obesity (Bastien et al., 2014). All of these factors contribute to an increased life time dependence on the health care system by these individuals, when compared to the non-obese. This directly results in higher than normal health care expenses per obese individual and puts a huge burden in the health care system. A conservative estimate made in 2008, on health care

costs related to obesity is approximately \$4.6 billion per year, which is up almost 19% from the estimates made in in year 2000 (PHAC, 2013). Some other more liberal estimates attribute to \$7.1 billion per year healthcare costs to obesity (Anis et al., 2010). These data show how important it is to increase the awareness and counter obesity among populations, especially among those who are in the higher risk categories (Finkelstein et al., 2012).

Overall, the increasing prevalence of obesity is a major concern and needs immediate attention in order to protect future generations. Race, sex, income and socioeconomic factors have been associated with an increased risk for obesity. Childhood obesity is also increasing which is alarming because usually the risk of obesity increases with age. Childhood obesity also results in an early onset of comorbidities such as diabetes, hypertension and reduced life expectancy which would also dramatically increase healthcare expenses.

2.2 PATHOLOGY OF OBESITY

2.2.1 Adiposity/obesity and adipose dysfunction

Obesity is a condition that develops due to excess fat deposition in the body over time. Generally, excess energy in the body is stored as fat. When the energy intake is chronically higher than the energy expenditure of the body, it results in obesity (**Figure: 2.1**). The human body stores excess circulating lipids in adipocytes (Fischer-Posovszky, Wabitsch, & Hochberg, 2007). These stored fats are kept as a reserve energy bank that is used in a state of fasting (Viscarra & Ortiz, 2013). Accordingly, adipocytes are normally an integral part of the human body and harmless. However, when circulating lipids are chronically higher than normal, the amount of fat deposits also increases. Over time, this increased adiposity results in pathological changes of obesity (Trayhurn, 2014). There are many factors such as an energy dense diet, physical inactivity, gene mutations and/or other pathophysiological conditions that contribute to an increase in circulating lipids (Lin et al., 2013; Nestruck & Davignon, 1986). Adiposity can be increased in two different ways, either by increasing the number of adipocytes (hyperplasia) or by increasing their size (hypertrophy) (Jo et al., 2009). The first is considered to be physiological while the second, wherein the adipocyte enlargement is considered pathological (Skurk, Alberti-Huber, Herder, & Hauner, 2007). Earlier, adipose tissues were considered as just a store of fat. However, later it was discovered that adipose tissues are also an endocrine organ and adipocytes secrete hormones (adiponectin, leptin and resistin), cytokines (tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6)) and proteins (cholesterol ester transfer protein, angiotensin II, plasminogen activator inhibitor 1) involved in the metabolism and function of liver, muscle, vasculature, the brain and other organs of the body (Hajer, van Haften, & Visseren, 2008). Cytokines secreted from adipocytes are generally known as adipokines (Coelho, Oliveira, & Fernandes, 2013).

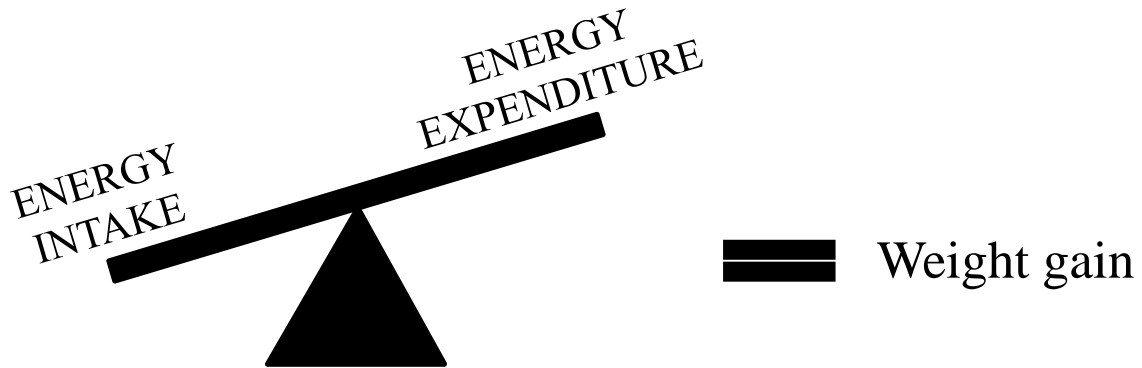


Figure 2.1: Graphical representation for energy balance equation. The equation shows that weight gain is the result of increased energy intake and lower energy expenditure.

Some adipokines are beneficial while others cause unhealthy effects on biological functions. In normal or healthy conditions, adipocytes secrete more adipokines which are beneficial, while in pathophysiological conditions where adipocyte dysfunction occurs, the balance will be shifted to increased release of detrimental adipokines (Coelho et al., 2013; Skurk et al., 2007). The origin of adipocyte dysfunction is mainly associated with the physiological demands of storing very high levels of fat. Macrophages are also present among the adipocytes and are involved in fat storage and secretion of cytokines. The adipocytes and macrophages are highly involved in the genesis of chronic inflammation in the adipose tissue and release of pro-inflammatory factors into the blood (Suganami, Nishida, & Ogawa, 2005). Consequently, there is also an increased lipolysis and a release of free fatty acids (FA) into the circulation. Accordingly, adipose tissue is considered to be the major source of pathophysiological effects in obesity (Bluher, 2009, 2013). This also makes adipocytes a potential drug target to ameliorate the metabolic disarray in obese conditions. To some extent targeting adipocyte dysfunction has shown promise in preventing or improving metabolic imbalances in obesity (G. X. Wang et al., 2014).

2.2.2 Obesity and cardiovascular disease

According to the Framingham Heart Study, the risk of developing heart failure in obese patients was 2 times higher than that of normal weight subjects (Hubert, Feinleib, McNamara, & Castelli, 1983). Cardiovascular complications are one of the major contributors to poor health and a lower life expectancy among obese individuals (Flegal, Graubard, Williamson, & Gail, 2007; Jiang, Ahn, Huang, & Hayes, 2013). Obesity, especially abdominal obesity, is an independent risk factor for CVD (Shields, Tremblay, Connor Gorber, & Janssen, 2012). Higher BMI is directly

associated with adipocyte dysfunction, increased release of certain adipokines, insulin resistance, hypertension, increased inflammation and oxidative stress that promotes the development of cardiovascular disease (Taube, Schlich, Sell, Eckardt, & Eckel, 2012). Although not unanimously accepted, the 'obese paradox' theory claims that obese individuals have a better prognosis with respect to CVD when compared to normal weight individuals (Lavie, Milani, & Ventura, 2009). A possible explanation for this paradoxical theory is that the BMI measurements are insufficient to assess the state of obesity in an individual (Chrysant & Chrysant, 2013). Additional measurements such as waist circumference and waist to hip ratio would better classify the subjects based on the levels of fat deposition. A study on the Monza population has shown that with every 1 kg/m² increase in BMI, the risk of developing left ventricle (LV) hypertrophy increases by 5.1%, and for every 1 cm increase in waist circumference the risk increases by 2.5% (Bombelli et al., 2011). Visceral adiposity and subcutaneous fat deposits contribute to an increased waist circumference and have been found to be an independent risk factor for the development of heart disease (Britton & Fox, 2011). The WHO cut-off values for waist circumference in men is 94 cm and women is 80 cm for an increased CVD risk whereas 102 cm in men and 88 cm in women is associated with a substantial increase in CVD risk (Klein et al., 2007).

Obesity exerts stress on the heart by increasing blood volume and cardiac output which simultaneously thereby places a larger work load on the heart (Bastien et al., 2014). This results in adverse changes to hemodynamics, cardiovascular structure and function. Obesity increases total body area and volume by the addition of fat tissue and the changes in the cardiovascular system are aimed at maintaining sufficient blood supply to the whole body (Bastien et al., 2014). Adipose tissue contains a large volume of fluid which is present in the interstitial spaces of the

tissue. The interstitial space composes approximately 10% of the total adipose tissue weight. Obesity also creates an increase in lean body mass which independently elevates cardiac output (Poirier et al., 2006). The combination of an increased lean and fat mass could account for a large increase in stroke volume and cardiac output. An expansion in the volume of blood increases the preload on the heart and shifts the Frank-Starling curve to the left. A significant change in vascular structure and function is also observed in obesity. Obesity causes arterial stiffness (Zebekakis et al., 2005), an increase in intima-media thickness (Iannuzzi et al., 2004; Ozcetin et al., 2012) and increases in calcification (Lee, Jacobs, Schreiner, Iribarren, & Hankinson, 2007). All of these vascular changes are also independent predictors of CVD. Further, these vascular changes may also contribute to the development of hypertension in obese individuals (Kotsis, Stabouli, Papakatsika, Rizos, & Parati, 2010).

These changes in hemodynamics increase wall tension and induce LV dilation and hypertrophy. Prolonged exposure to these conditions reduces LV wall compliance and diastolic dysfunction ensues. Initial adaptations by the LV help preserve LV systolic function in the early stages of cardiac remodeling. Over time, an impairment in systolic function will appear and this will initiate heart failure (Aurigemma, de Simone, & Fitzgibbons, 2013). It was also found that the fatty heart, as a result of increased fat deposits is prone to cardiomyopathy (Goldberg, Trent, & Schulze, 2012). Damage to heart muscles by fat accumulation can occur in two ways, metaplasia and lipotoxicity (Carpenter, 1962). In metaplasia, some cells (epithelial or mesenchymal) are replaced by fat cells which disrupts conduction in cardiac cells. In lipotoxicity, free fatty acid accumulation in cardiomyocytes induces cell death in the myocardium. In either case, damage to the cells results in a weakening of the myocardium, resulting in the development of a cardiomyopathy (Bastien et al., 2014). The increase in blood volume also induces left atria

enlargement. This increases the risk of developing atrial fibrillation and its complications. Based on the findings from the Women Health Study, obesity was associated with increased an risk for atrial fibrillation (Karasoy et al., 2013). Other types of arrhythmias and sudden cardiac death are also higher in obese populations (Chrostowska, Szyndler, Hoffmann, & Narkiewicz, 2013). Obesity and metabolic dysfunction increases the risk of coronary artery disease. The incidence of coronary atherosclerosis is very high in adult obesity and a major risk factor for heart disease (Steyn et al., 2005). Obesity is also directly linked to an increased incidence of stroke. The INTERSTROKE study found that waist to hip ratio was strongly associated with an increased risk for stroke (O'Donnell et al., 2010). Obstructive sleep apnea is another risk factor for hypertension and CVD (Drager, Togeiro, Polotsky, & Lorenzi-Filho, 2013). Obesity is one of the major risk factors for obstructive sleep apnea (Drager et al., 2013). In many, it is undiagnosed which increases the risk for the development of heart disease.

Adipose tissue is also an endocrine organ releasing a number of molecules into the bloodstream. TNF- α , IL-6, leptin, angiotensinogen, resistin and plasminogen activator inhibitor-1 are released from adipose tissues and have direct or indirect effects on promoting the development or progression of heart disease (Van de Voorde, Pauwels, Boydens, & Decaluwe, 2013). A significant proportion of the circulating concentrations of these molecules originate from adipose tissue. Most of these are mediators of an inflammatory response and may be involved in the progression of coronary artery disease (Luc et al., 2010).

An indirect effect of obesity on cardiovascular pathology involves impairment in kidney structure and function. Glomerular hyperfiltration, increased albumin loss, glomerulosclerosis and progressive loss of kidney function are associated with obesity-induced kidney damage (Wickman & Kramer, 2013). Population studies, PREVEND (de Jong, Verhave, Pinto-Sietsma,

Hillege, & group, 2002) and the Framingham Heart Study (Foster et al., 2011) have found a direct correlation between kidney damage and obesity. The accumulation of fat in higher amounts is one of the mechanisms of kidney damage in obese individuals (Poirier et al., 2006).

2.2.3 Lipids and heart

FA are the primary energy source of the heart. In normal conditions, approximately 60-80% of the energy is being derived from FA (Neely & Morgan, 1974). The remaining energy is derived from metabolism of glucose, lactate and ketones. Generation of adenosine triphosphate (ATP) from FA oxidation requires more oxygen demanding than from glucose, and the heart has the ability to switch to glucose as its major energy source in oxygen deficient environments such as ischemia, hypertension and other pathological conditions (Taegtmeyer, Sen, & Vela, 2010). This ability helps the heart to adapt to difficult conditions, preserve available oxygen and minimize the damage to the tissue. Fetal hearts also depend more on glucose and lactate for energy, while adult hearts shift to FA for energy (Taegtmeyer et al., 2010). Diet, hepatic FA synthesis and lipolysis in adipose tissue are the major sources of lipid for the heart. Heart tissues can use both non-esterified (free fatty acids) and esterified (bound to lipoproteins) FA. Circulating triglycerides undergo lipolysis mediated by endothelium-bound lipoprotein lipase and are then internalized via membrane receptors, transporters or by diffusion (Trent et al., 2014). Internalized free FA are then converted to fatty acyl-CoAs and then either stored as acyl glycerides (mono, di or tri) or transported to mitochondria for ATP generation (van der Vusse, van Bilsen, & Glatz, 2000). Triglycerides stored in the cell are processed to free FA by hormone sensitive lipase and adipose triglyceride lipase during lipolysis (Lopaschuk, Ussher, Folmes, Jaswal, & Stanley, 2010).

2.2.4 Lipotoxicity

The balancing act of energy homeostasis is much more complex than the previous equation (Figure 1). For example there is a significant difference between the white adipose tissue (WAT) and brown adipose tissue (BAT); both are fat deposits but their physiological roles are different. Increased WAT is associated with the genesis of metabolic syndrome whereas BAT contributes to thermogenesis (Saely, Geiger, & Drexel, 2012). Diet is the major source of FA, however some FA are also synthesized from through *de novo* lipogenesis (Ameer, Scandiuzzi, Hasnain, Kalbacher, & Zaidi, 2014). Depending on physiologic demands FA are released into the circulation from adipose tissue by lipolysis and will be used by other organs. FA can also be transported into the cells by different protein transport mechanisms (Schwenk, Holloway, Luiken, Bonen, & Glatz, 2010; van der Vusse et al., 2000). These FA are then used for a variety of cellular mechanisms involving the synthesis of membrane, signaling molecules, post-translational protein modification, transcriptional regulation and, more importantly, for energy production through beta oxidation (U. N. Das, 2006). Normally, a balance is maintained between lipid uptake and oxidation thereby preventing lipid accumulation. Metabolic disturbances often result in increased lipid levels in the circulation. Higher circulating FA in obesity and type 2 diabetes cause excess deposition of FA in non-adipose tissues such as kidney, liver, skeletal muscles and heart. The excess lipid accumulating inside the cell may cause cellular dysfunction through ER stress, mitochondrial dysfunction, oxidative stress and ultimately resulting in cell death (Turkieh et al., 2014). This is known as lipotoxicity (Eguchi & Manabe, 2014; Lake et al., 2014; Nolan, 2014).

2.2.5 Lipotoxicity in the heart

The heart is one of the major organ affected by lipid accumulation and cell dysfunction (Drosatos & Schulze, 2013). Lipid accumulation is also observed in cardiac pathologies wherein the myocardium shifts back to glucose as the source of energy (Taegtmeyer et al., 2010). Lipotoxic effects lead to cardiomyocyte dysfunction, contractile abnormalities, cell death and pathogenesis of heart failure (Schulze, 2009). Cardiomyopathies in type 2 diabetes and obesity are often a result of lipotoxic damage to the myocardium (Lopaschuk et al., 2010). Long chain FA like palmitate have been found to induce lipotoxicity in heart muscle cells when compared to short chain FA (de Vries et al., 1997; Hickson-Bick, Buja, & McMillin, 2000). The mechanism of lipotoxic effects in the heart has been unclear and research is ongoing. Some pathological cellular changes associated with lipid accumulation are endoplasmic reticulum stress, mitochondrial dysfunction and oxidative stress. Increased ceramide accumulation has been observed as a contributor towards cell death in the heart (Park et al., 2008). Insulin is involved in the regulation of glucose metabolism, activation of survival pathways in ischemia and also in intracellular Ca^{2+} handling. Lipotoxicity induces insulin resistance and thereby causing cardiomyocyte dysfunction. Activation of protein kinase C, mitogen activated protein kinases and reduced expression of peroxisome proliferator-activated receptors are also considered to be involved in the process of lipid accumulation and cellular responses in the cell (Drosatos & Schulze, 2013). Lipid accumulation also induces contractile abnormalities through the degeneration of myofibrils (Dyntar et al., 2001).

2.3 CONTRACTILE FUNCTION OF THE HEART

The primary function of the heart is to pump blood throughout the body. The heart receives deoxygenated blood from the circulation, oxygenates the blood via the lungs, and then pumps the oxygen rich blood back into circulation. All these happen in a matter of milliseconds and the heart has to keep working continuously throughout life. This demand by itself can induce wear and tear, weakening the heart muscle over time (Cheitlin, 2003). Molecular changes in cardiomyocytes are another contributor to reduced function of the heart (North & Sinclair, 2012).

Normal contractility of cardiomyocytes is essential to maintain proper cardiac function. The contractile mechanism of the heart involves calcium (Ca^{2+}) ions and proteins that regulate the inward and outward flow of Ca^{2+} across cell and organelle membranes (Bers, 2008). The process starts with the depolarization of the plasma membrane which activates the L-type Ca^{2+} channels allowing a small amount of calcium into the cytoplasm. This Ca^{2+} then binds and activates the ryanodine receptor (RYR2) channels which release stored Ca^{2+} from the sarcoplasmic reticulum (SR). The Ca^{2+} released from the SR then binds to troponin C which initiates actin-myosin cross-bridges resulting in cardiac contraction as a result of the sliding action of thin and thick cardiac filaments. Cardiac relaxation results from Ca^{2+} uptake by the sarcoplasmic reticulum Ca^{2+} -ATPase 2a (SERCA2a), and the remaining Ca^{2+} is removed by the action of the sarcolemmal Na^{+} - Ca^{2+} exchanger (NCX) (Bers, 2008; Marks, 2013a). Starting with depolarization of the membrane, the whole cycle is repeated again. This contraction and relaxation constitutes the systolic and diastolic function of the heart.

By controlling the intracellular Ca^{2+} levels, the SR plays a central role in regulating cardiac contractility. SERCA2a (110KDa) is the cardiac SR isoform and accounts for 75-90% of Ca^{2+}

uptake, while the remaining 10-25% is removed by the sarcolemmal NCX. The function of SERCA2a is regulated by phospholamban (PLB, 27 KDa, polymeric form). Unphosphorylated PLB remains bound to SERCA2a inhibiting Ca^{2+} uptake (Periasamy, Bhupathy, & Babu, 2008). RYR2 (565 KDa) mediates the release of Ca^{2+} from the SR and FK-506 binding protein-12.6 (FKBP-12.6) regulates the function of RyR2 by keeping the channel in its closed conformation (Marks, 2013a). Calsequestrin (CQS, 45 KDa) is a Ca^{2+} binding protein in the lumen of SR. It can store Ca^{2+} in high capacity and with less affinity enabling a rapid release during the contraction cycle. CQS also regulates RYR2 function through junction and triadin proteins (Beard, Laver, & Dulhunty, 2004). SERCA2a and RYR2 gene knockouts are embryonically lethal showing their importance in the normal function of the heart (Periasamy et al., 1999; Yang et al., 2002).

The functions of calcium proteins are regulated by phosphorylation and dephosphorylation. There are several protein kinases or phosphatases (PP) involved in this process. PLB phosphorylation is mediated by two endogenous kinases in the SR, cAMP-dependent protein kinase (CaMK) and Ca^{2+} -calmodulin-dependent protein kinase (PKA) (Bers, 2008). Phosphorylation of RYR2 by CaMK and PKA increases Ca^{2+} sensitivity and promotes the release of Ca^{2+} (Marks, 2013a). There are two classes of PP in the heart, PP1 and PP2. Endogenous PP1 and PP2 in the SR mediates dephosphorylation of PLB, thereby promoting SERCA inhibition by PLB (Carr et al., 2002).

2.3.1 Heart failure and SR

Defective SR Ca^{2+} handling plays an important role in the pathophysiology of heart failure. Decreased SERCA expression and function has been found to be significantly changed in a

diseased heart. Decreased SERCA activity limits the amount of calcium uptake after each systolic cycle, thereby reducing the Ca^{2+} stored in the SR. This process will result in lower Ca^{2+} stored and simultaneously reduce the force of contraction (Periasamy et al., 2008). Reduced Ca^{2+} influx through L-type Ca^{2+} channels and increased extrusion of intracellular Ca^{2+} through NCX are other factors affecting Ca^{2+} regulation (Goonasekera et al., 2012; Pott, Eckardt, & Goldhaber, 2011). Increased NCX expression has been detected in heart failure (L. Xu et al., 2012). Changes in PLB expression levels or phosphorylation will also have a direct effect on the function of SERCA. An increase in dephosphorylated PLB decreases Ca^{2+} uptake by SERCA2a. Both an increase and decrease in phosphorylated PLB has been reported in heart failure. Decreased phosphorylation was correlated with decreased SERCA Ca^{2+} sensitivity in failing hearts. The ratio of PLB to SERCA is also an important factor in regulating SERCA function. Increased PLB to SERCA ratio means higher SERCA inhibition and results in depressed cardiac contractility (Periasamy et al., 2008). A decrease in PLB expression is also detrimental to cardiac contractility and results in dilated cardiomyopathy. More recently, a leaky Ca^{2+} channel RYR2 was also correlated to heart failure. A leaky RYR2 is a result of a hyperphosphorylated state of RYR2 preventing the binding of FKBP12.6 and closure of the channel. This increases the diastolic SR Ca^{2+} leak and reduces Ca^{2+} content (Marks, 2013a). However, this hypothesis is yet to be universally accepted and more research is currently underway in this area. The importance of SR calcium regulation in heart failure is more evident from the success of experimental therapeutic approaches targeting SR proteins. Several studies have been successful in treating contractile dysfunctions by gene transfer of contractile proteins (Zouein & Booz, 2013). Restoring SERCA2a expression by gene transfer has been able to recover failing heart in animal models (Byrne et al., 2008; Kawase et al., 2008).

2.4 FUNCTIONAL FOODS AND NUTRACEUTICALS

“Let food be thy medicine and medicine be thy food”

Hippocrates

While food has always been used to satisfy hunger, it has also been used as a medicine from ancient times (Hamdan & Afifi, 2004; Pieroni, 2000; Serafini, Stanzione, Foddai, Anton, & Delmulle, 2012). Those days food was naturally produced and consumed without significant processing. With the arrival of modern times, the increase in population and the shortage of natural foods, our food consumption habits have considerably changed (Brubacher, Hornig, & Ritzel, 1981; Chaput, Klingenberg, Astrup, & Sjodin, 2011). Artificial foods have dominated our diet for a long time. However, during the past few decades there is an increasing trend in recognizing the medicinal value of food and modern scientific testing has supported this long forgotten truth (Alissa & Ferns, 2012; Hasler, 2000; Magrone et al., 2013). Apart from whole food, food derived compounds such as polyphenols that have medicinal properties have been isolated, concentrated and tested for a more potent therapeutic approach (Habauzit & Morand, 2012; Quinones, Miguel, & Aleixandre, 2013). Polyphenols serve a wide variety of functions in plants ranging from growth to pollination and defense. Depending on the number of phenol rings, and the structural properties of their binding to each other, polyphenols are divided into subclasses including phenolic acids, flavonoids, stilbenes and lignans. There are hundreds of polyphenolic compounds in foods, and many of them have potential medicinal properties (Landete, 2012). However, few have yet reached the clinical trial stage, and only a handful have shown beneficial effects in humans (Cardona, Andres-Lacueva, Tulipani, Tinahones, & Queipo-

Ortuno, 2013; Ding, Yao, Yao, Fai, & Zhang, 2013; Khurana, Venkataraman, Hollingsworth, Piche, & Tai, 2013; Maeda-Yamamoto, 2013; Magrone et al., 2013).

Nutraceutical is the term used to define nutrients, dietary supplements and herbal products in the form of pills that can provide health benefits (Kalra, 2003) whereas functional food represents the whole food. Many types of nutraceutical products are already available on the market and this field has grown into a multibillion dollar industry (Brower, 2005). Increased acceptance among individuals together with minimal regulation from government drug departments has accelerated the growth. The physiological benefits associated with nutraceuticals ranges from increased immunity, antioxidant activity, decreased inflammatory levels, improved stamina to protection against cancer and CVD (Del Rio et al., 2013; Dong et al., 2012; R. H. Liu, 2013). There is evidences from epidemiological, *in vivo*, *in vitro*, and clinical studies which indicating that nutraceuticals can reduce the risk of cardiovascular diseases (Alissa & Ferns, 2012). Dietary fibers, polyunsaturated fatty acids, phytosterols, vitamins and polyphenols are some of the groups of compounds that have been reported to have cardiovascular benefits. Among these, one of the most studied has been resveratrol which is structurally a stilbenoid (Raj et al., 2014).

2.5 RESVERATROL

Resveratrol is a phytoalexin compound produced by plants mainly in response to fungal infections, UV radiations and other environmental stresses such as cold temperatures (Juhasz et al., 2010). Resveratrol is present in significant amounts in grapes, peanuts, soybeans, pomegranates, mulberry and bilberry (Giovinazzo, Ingrosso, Paradiso, De Gara, & Santino, 2012) and to a lesser extent in pine, eucalyptus and spruce trees, and in a few flowering plants,

such as *Veratrum grandiflorum* and *Veratrum formosanum* (de Lorgeril, Salen, Guiraud, Boucher, & de Leiris, 2003; Pervaiz, 2003).

Resveratrol was discovered in the roots of white hellebore plants (Takaoka, 1940). Later it was also found in roots of *Polygonum cuspidatum*, a Japanese knotweed which was also called Ko-jo-kon and is the richest source of resveratrol. It was used in the preparation of Japanese and Chinese herbal medicines against skin infections like warts, dermatitis and athletes foot diseases (W. H. Tsai, Yang, Li, Chen, & Chien, 2013). This was followed by reports of the presence of resveratrol in eucalyptus and pine (Hillis & Isoi, 1965; Tyukavkina, Gromova, Lutskii, & Voronov, 1974). In 1976, Langcake and Price reported the presence of resveratrol in grape vines for the first time (Langcake & Pryce, 1976). During this period, resveratrol was mainly investigated for its anti-fungal properties and used to screen for disease resistant grape cultivars (M. Kubo, 1981; R.M. Pool, 1981). The first report linking resveratrol to potential cardiovascular benefits was from a Japanese group which showed that resveratrol administration reduced triglyceride synthesis in mice (Arichi et al., 1982). Later in 1992 moderate red wine consumption was linked to the reduced incidence of cardiovascular disease among the French population and this theory is now known as the ‘French Paradox’ (Renaud & de Lorgeril, 1992). At the same time Siemann and Creasy reported that resveratrol might be one of the bioactive ingredients in wine (Creasy, 1992). Further, Frankel *et. al.* showed that the phenolic component of red wine inhibited LDL oxidation which is a risk factor for atherogenesis and thereby heart disease (Frankel, Kanner, German, Parks, & Kinsella, 1993). The association of resveratrol to the French paradox generated a great interest in resveratrol research wherein either purified resveratrol or a food containing significant amounts of resveratrol were tested on a wide range of preclinical research models for human disease (Juhász et al., 2010). The highlights of resveratrol research

outcomes are its beneficial effects against different types of cancers, cardiovascular diseases and also against metabolic diseases such as diabetes and obesity (Raj et al., 2014; Tome-Carneiro, Gonzalvez, et al., 2013b; Q. Xu & Si, 2012).

2.5.1 Resveratrol chemistry

Resveratrol is a stilbene derivative, produced in plants by stilbene synthase. Stilbene synthase catalyzes the synthesis of resveratrol from one molecule of p-coumaroyl CoA and three molecules of malonyl CoA. Resveratrol exists in two structurally isomeric forms, *cis*- and *trans*-resveratrol (**Figure: 2.2**) (molecular weight: 228.24); both isomers are lipophilic in nature (Wallerath et al., 2002). Resveratrol has a melting point around 260°C. It is insoluble in water but soluble in ethanol and dimethyl sulfoxide. The *trans*-resveratrol is relatively more stable compared to *cis*-resveratrol, however, the *trans* form can be converted to the *cis* when exposed to heat or ultraviolet radiation (Opie & Lecour, 2007). *Trans*-resveratrol in the powder form is stable in normal atmosphere at room temperature and undergoes negligible oxidation in these conditions. Resveratrol is susceptible to photolysis if exposed to direct sunlight. Due to its structural similarity to the synthetic estrogen diethylstilbestrol, resveratrol, is also considered to be a phytoestrogen (Pervaiz, 2003).

2.5.2 Cardioprotection with resveratrol

Resveratrol is found in grape skins in high amounts and subsequently it is present in significant amounts in red wines. This led to the ‘French Paradox’ theory in which a lower incidence of cardiovascular disease in the French population was associated with the consumption of red wine (Opie & Lecour, 2007). Research confirmed the cardioprotective properties of resveratrol (Li & Forstermann, 2012). Resveratrol showed cardioprotective properties by reducing cardiac

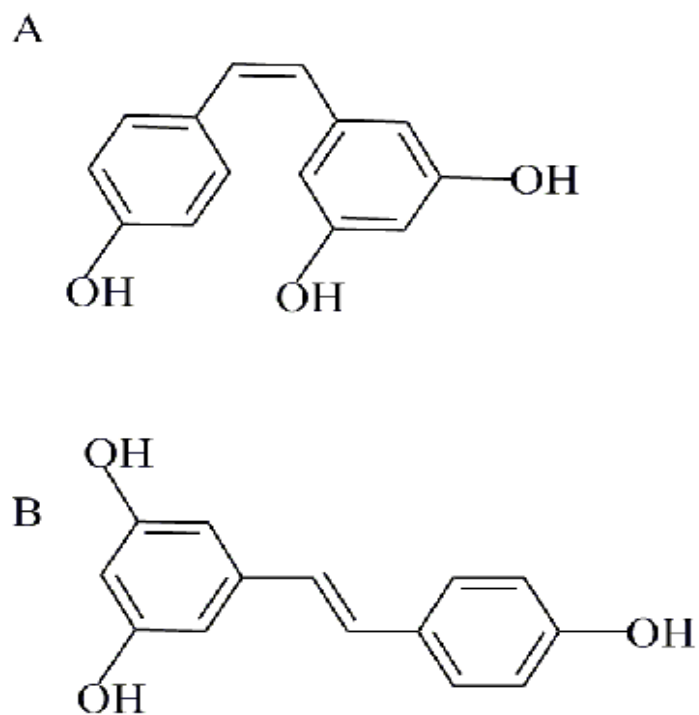


Figure 2.2: Structure of resveratrol isomers. Naturally found isomers of resveratrol, A. cis-resveratrol; B. trans-resveratrol.

abnormalities in hypertension, ischemic heart disease, obesity, diabetes and cardiomyopathies. Resveratrol has been shown to improve cardiac structure and function in animal models of heart disease (Raj et al., 2014). It also improves vascular structure and function improving hypertension and coronary diseases (Carrizzo et al., 2013; Zheng et al., 2013). Major cellular mechanisms that mediate the effects of resveratrol range from improving cardiovascular risk factors such as hyperlipidemia and insulin resistance to reducing oxidative stress, inflammation and regulating cellular protein activities (**Figure: 2.3**). Among the many molecules identified as resveratrol targets in the heart, 5' Adenosine monophosphate-activated protein kinase (AMPK), sirtuin 1 (SIRT1) and nitric oxide (NO) are the ones most frequently reported (Yu, Fu, & Wang, 2012). Resveratrol is found to enhance AMPK activity and thereby its downstream signaling which indirectly results in increased NO production (Chan et al., 2008; Thandapilly et al., 2011). AMPK activation could also be involved in a resveratrol mediated decrease in fibrosis (Beauloye, Bertrand, Horman, & Hue, 2011). An increase in SIRT1 expression is associated with resveratrol administration. SIRT1 could improve cardiac function by increasing SERCA2A expression and thereby improving Ca^{2+} handling. SIRT1 could also induce AMPK activation and thereby improve mitochondrial function (Price et al., 2012). Anti-inflammatory and antioxidant activities were the first to be identified and associated with the health benefits of resveratrol. Resveratrol prevent nuclear factor kappa B (NFkB) activation and translocation into nucleus leading to the transcription of a variety of genes detrimental to the cell (Penumathsa & Maulik, 2009). Resveratrol also helps preserve major antioxidant enzyme activities while reducing nicotinamide adenine dinucleotide phosphate oxidase (NOX) activity. Nuclear factor erythroid 2-related factor 2 (Nrf2) is involved in maintaining an antioxidant environment inside the cells and resveratrol is found to promote Nrf2 activation (Li, Xia, & Forstermann, 2012). Resveratrol

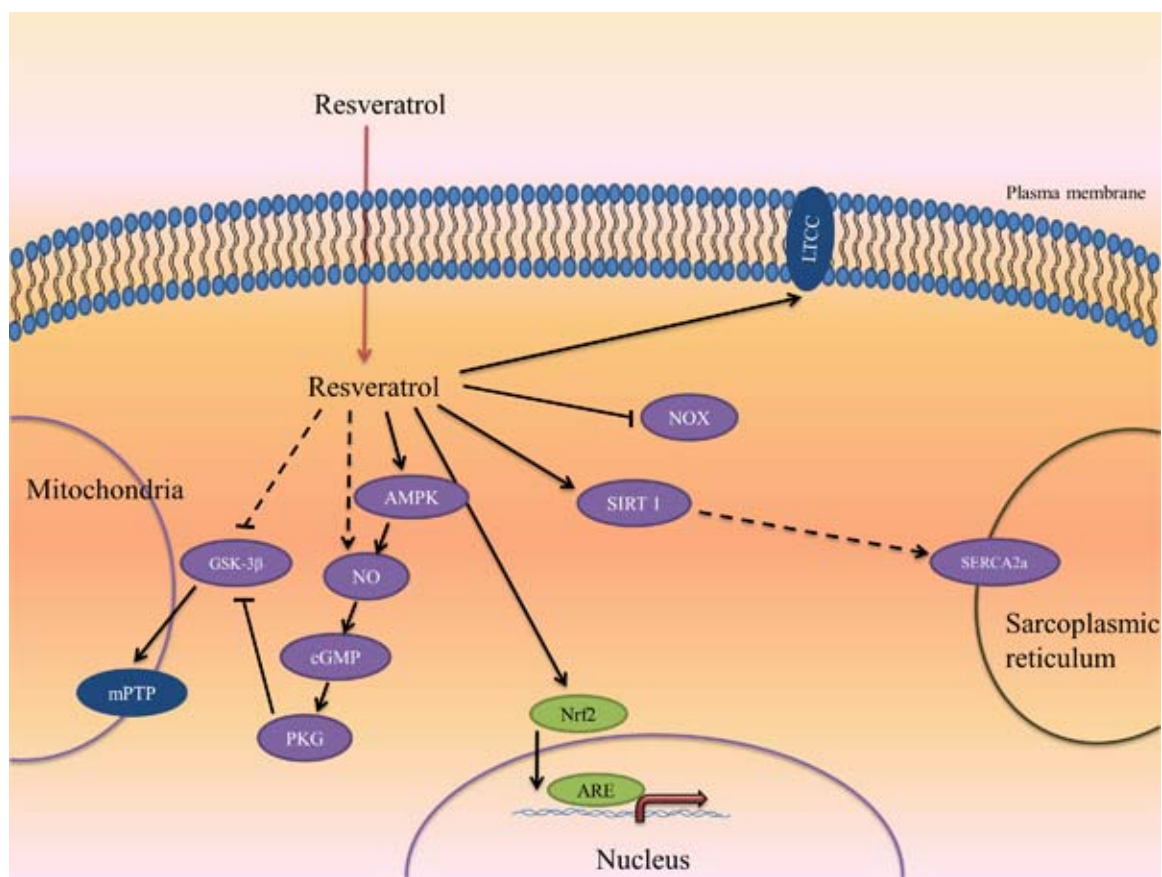


Figure 2.3: Potential mechanisms of resveratrol mediated cardioprotection. AMPK, 5' Adenosine monophosphate-activated protein kinase; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G; GSK3 β , glycogen synthase kinase 3 β ; mPTP, mitochondrial permeability transition pore; NOX, nicotinamide adenine dinucleotide phosphate oxidase; SIRT1, sirtuin 1; SERCA2a, sarco endoplasmic reticulum ATPase 2a; LTCC, L-type calcium channel; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element.

also affects mitochondrial function by preventing mitochondrial permeability transition pore (mPTP) activation and the subsequent increase in reactive oxygen species release and ATP depletion. Glycogen synthase kinase 3 β is an activator of mPTP. Inactivation of glycogen synthase kinase 3 β through NO/ cyclic guanosine monophosphate (cGMP) / protein kinase G (PKG) is the proposed mechanism of resveratrol action (Xi, Wang, Mueller, Norfleet, & Xu, 2009). Modulation of the L-type calcium channel (LTCC) is also a potential mechanism by which resveratrol could improve Ca²⁺ irregularities in cardiac cells (L. P. Zhang et al., 2006).

Resveratrol has also been tested in humans for potential cardioprotective activities. In a major studies using resveratrol, diastolic heart function was improved in post-infarcted patients (Magyar et al., 2012). Another study in a larger cohort showed a reduction in cardiovascular risk factors in patients at high risk for heart disease (Zamora-Ros et al., 2012). Yet another study showed an improvement in inflammatory and fibrinolytic markers with resveratrol administration in patients (Tome-Carneiro et al., 2012). Resveratrol was also tested in obese individuals and showed positive effects on metabolic parameters (Timmers et al., 2011). More testing on humans is currently underway and a better picture of the potential for resveratrol as a cardioprotective agent will be available in the near future (<https://clinicaltrials.gov/ct2/results?term=resveratrol&Search=Search>).

2.5.3 Toxicity of resveratrol

Evidence to date show that resveratrol has potential for use in humans against various ailments. Few reports have also shown that resveratrol have some unpleasant effects. Mild to moderate renal toxicity is the major side effect associated with resveratrol administration. However, nephrotoxicity was mostly associated with very high doses of resveratrol (>1g/kg/day) in animals (Crowell, Korytko, Morrissey, Booth, & Levine, 2004). Minor side effects associated with

resveratrol consumption in humans were headache, diarrhea, joint pain, small increase in blood bilirubin, skin rash and myalgia (Cottart, Nivet-Antoine, Laguillier-Morizot, & Beaudeux, 2010). A study using different doses of resveratrol in humans showed that upto 5g/day was generally safe, except for mild gastrointestinal problems in 2.5g/day and 5g/day doses (Brown et al., 2010). Recently, a study on pregnant primates reported that resveratrol administration to mothers induced pancreatic mass increase and exocrine proliferation in fetus pancreas (V. H. Roberts et al., 2014). Nevertheless, more studies need to be conducted to confirm these observations.

2.5.4 Resveratrol in obesity-induced heart disease

There is sufficient evidence showing that resveratrol improves metabolic abnormalities in animals (Poulsen, Jorgensen, Jessen, Richelsen, & Pedersen, 2013). However, there are only a few studies that explored the cardioprotective property of resveratrol in the setting of obesity. An early study reported that 5 mg/kg/day of resveratrol administration for 2 weeks reduced infarct size and cardiac apoptosis in ischemic-reperfused hearts (Lekli et al., 2008). Resveratrol (20 mg/kg/day for 8 weeks) also prevented an increase in blood pressure and preserved vascular function in an animal model of diet-induced obesity (Aubin et al., 2008a). Recently, Qin *et al.* showed a significant decrease in cardiac hypertrophy and an improvement in diastolic heart function in obese mice exhibiting characteristics of early stage type II diabetes, when they were treated for 4 months with 130 mg/kg/day resveratrol (Qin et al., 2012). Diabetes is an independent risk factor for cardiovascular disease and also a common comorbidity in obesity. There are only a few studies that reported cardioprotective effects of resveratrol in animal models of type 2 diabetes (Raj et al., 2014).

Accordingly, there is limited evidence on resveratrol mediated protection against obesity induced cardiovascular abnormalities. Given that diet and genetic predisposition are major contributors

towards an increased prevalence of obesity, more research is necessary to determine if resveratrol can protect the heart in the setting of diet-induced obesity. It is also important to know if resveratrol can improve diastolic dysfunction in obese animals and thereby prevent progression into heart failure. Finally, more evidence on the cellular mechanisms involved in the cardioprotective action of resveratrol will certainly be useful when resveratrol is administered in combination with other drugs or as a supplement.

CHAPTER 3

RESVERATROL MEDIATED REVERSAL OF DIASTOLIC DYSFUNCTION IN OBESE RATS

3.0 RATIONALE AND HYPOTHESIS

The prevalence of obesity has reached epidemic proportions (Bastien et al., 2014) and seriously impacts an individual's social and private life. There are several comorbidities such as hypertension, diabetes, cardiovascular disease and even some cancers that are associated with obesity (Guh et al., 2009). These conditions lead to a poor quality of life for the individual. Heart failure is a major end point in the obese population (Lavie et al., 2013; Pi-Sunyer, 2009). In obesity, many cellular and molecular changes are accompanied by diastolic dysfunction of the heart that trigger the onset of heart failure (Mittendorfer & Peterson, 2008). Adipose tissue dysfunction leads to the release of several detrimental adipokines such as leptin, IL-6, TNF- α and increased circulating lipids resulting in lipotoxic conditions in the muscle tissues, these are factors that trigger the development of cardiac dysfunction (Taube et al., 2012). The presence of hypertension and diabetes accelerates the progression into heart failure (Artham, Lavie, Milani, & Ventura, 2009; Paneni, Costantino, & Cosentino, 2014).

Conventional treatments to prevent or manage cardiovascular disease in obesity include lifestyle changes for weight loss, pharmacotherapy and in some cases surgery may also be used (Dodson et al., 2013). However, many of these drugs have non-specific effects and may worsen the overall quality of life (Dodson et al., 2013). Accordingly, newer classes of drugs or therapeutic approaches need to be developed that are much safer with fewer undesirable side effects. Recently, food derived molecules have been gaining momentum as alternative therapies to human ailments (Power & Pratley, 2011). There are many compounds that have proven cardioprotective properties (Andriantsitohaina et al., 2012). Resveratrol is a prominent food based molecule that has been extensively studied in the last couple of decades and shown promise as a cardioprotective agent (Raj et al., 2014). Resveratrol has been reported to be

beneficial in preventing or reversing cardiac dysfunction in models of hypertension in rats (Juric, Wojciechowski, Das, & Netticadan, 2007b; Thandapilly et al., 2013; Thandapilly et al., 2010a; Wojciechowski et al., 2010b). There is limited knowledge about the potential for resveratrol to reverse cardiac complications associated with obesity. A few earlier studies have reported a reduction in hypertension associated with obesity in animal models of obesity (Aubin et al., 2008b; Rivera, Moron, Zarzuelo, & Galisteo, 2009). Many more studies have reported beneficial effects of resveratrol in reducing insulin resistance (Bruckbauer et al., 2012; Jimenez-Gomez et al., 2013), inflammatory signaling (Bruckbauer et al., 2012; Jimenez-Gomez et al., 2013), oxidative stress (Franco et al., 2013; Gomez-Zorita et al., 2012), mitochondrial dysfunction (Beaudoin et al., 2013), adipose dysfunction (Beaudoin et al., 2013), body fat (Gulvady, Ciolino, Cabrera, & Jolly, 2013), lipotoxicity (Alberdi et al., 2013), and improving liver function (Alberdi et al., 2013; Franco et al., 2013; Gomez-Zorita et al., 2012) which are associated with obesity and are potential risk factors for the development of cardiovascular abnormalities. However, there is lack of studies examining the effectiveness of resveratrol on obese animal models with established cardiovascular dysfunction.

Diet induced obese models are more appropriate than genetic models of obesity because in humans diet is considered to be a major cause of obesity. Genetic models have a single gene defect while the diet induced obese model factors changes in multiple genes, which is similar to human conditions. Obese prone (OP) and obese resistant (OR) are selectively bred from Sprague Dawley (SD) rats wherein, due to genetic variability when fed high energy diet, some rats are prone to be obese while some are resistant to obesity (Madsen et al., 2010). OP and OR model have been extensively used in obesity studies. OP rats are presented with insulin resistance, hyperlipidemia, mild hypertension, increased resistin and decreased adiponectin which are also

seen in obese humans (Buettner, Scholmerich, & Bollheimer, 2007; J. Wang et al., 1998). The diet used in this study contains lard as the source of fat. This diet is rich in saturated fatty acids (43% of total FA), monounsaturated fatty acids (47% of total FA) and n-6 polyunsaturated fatty acids (PUFAs) while contains very less n-3 PUFAs with a n-6 to n-3 ratio of 9:1. Accordingly, this diet is obesogenic induce obesity symptoms similar to those in humans. High fat feeding induces similar characteristics in animals as seen in obese humans. Increased glucose uptake, adipocyte morphology changes, dysfunction of pancreatic cells, mild hypertension, cardiac complications, oxidative stress, inflammation are all observed in high fat fed animal models (Buettner et al., 2007). Most of these changes will be measured in this study. In this study rats will be fed with high fat diet for 17 weeks and starting at week 13, some animals will receive resveratrol for 5 weeks. The dietary and treatment regimen matches with earlier reported studies. Earlier studies have reported that high fat fed animals develop cardiac abnormalities within 12 weeks on diet (Schwartz, Young, & Landsberg, 1983; Zbinden & Rageth, 1978) and from our earlier studies 5 weeks of resveratrol administration was sufficient to prevent and reverse cardiac abnormalities in rats (Juric et al., 2007b; Wojciechowski et al., 2010b). Accordingly, this study will explore the effects of resveratrol in reversing established cardiac abnormalities associated with diet induced obesity in OP and OR rats.

We hypothesize that 12 weeks of high fat (HF) (55% of energy from fat) feeding will induce cardiovascular dysfunction in OP rats and 5 weeks of resveratrol treatment will reverse these abnormalities. Resveratrol will also have a positive effect on the metabolic abnormalities associated with obesity in this animal model.

3.1 MATERIALS AND METHODS

The experimental protocols used in this project were approved by the University of Manitoba Animal Care Committee and are in agreement with the Canadian Council on Animal Care and Use of Experimental Animals (Olfert ED, 1993).

3.1.1 Model of high fat feeding - Four week old, male OP and obese resistant (OR) rats purchased from Charles River, St Constant, Quebec, Canada, were used in this study; a set of normal Sprague Dawley (SD) rats obtained from Central Animal Care Services at the University of Manitoba, Manitoba, Canada, served as controls. For acclimatization, all animals were kept in a temperature and humidity-controlled room with a 12-h dark and 12-h light period cycle for 1 week prior to the start of the HF diet. OP and OR rats were fed a HF diet (energy from fat 55%, carbohydrate 30% and protein 15%) and control SD rats were fed a standard chow (SC) diet (Prolab® RMH 3000; 14% energy from fat) for a period of 17 weeks. All rats received tap water *ad libitum*. Body weight was determined weekly by weighing.

3.1.2 Resveratrol treatment – OP, OR and SD rats were randomly assigned to either resveratrol treated or control groups. There were 6 groups in total (SD SC control; SD SC resveratrol; OR HF control; OR HF resveratrol; OP HF control and OP HF resveratrol) and 10 rats per group in OP and OR groups whereas there were 8 rats per group in the SD group. Trans-resveratrol (>99% pure; Sigma-Aldrich Ltd, Ontario, Canada) was dissolved in 100% ethanol and then further diluted to a final concentration of 50% ethanol with double distilled water. Resveratrol and vehicle (50% ethanol) was administered daily by oral gavage (1 mL/rat) at a dosage of 2.5 mg/kg body weight, at the same time of the day starting at week 13 on diet and

continued until the end of 17 weeks (end of the study). The study was terminated at the end of 17 weeks on HF diet (**Figure : 3.1**).

3.1.3 Blood pressure measurements - A CODA multi-channel, computerized non-invasive blood pressure system (Kent Scientific, Torrington, CT) with a tail-cuff sphygmomanometer was used to measure systolic and diastolic blood pressure of conscious rats. Blood pressure measurement was carried out on all groups of animals at 12 and 17 weeks, as described previously (Cipolla, Smith, Bishop, Bullinger, & Godfrey, 2008).

3.1.4 Echocardiography – Echocardiographic measurements were conducted in all animal groups at 6, 12 and 17 week time points in the study. A Sonos 5500 ultrasound system (Agilent Technologies, Andover, MA, USA) with a 12 MHz (s12) transducer was used for transthoracic two-dimensionally (2D) guided M-mode and Pulse-Wave Doppler measurements (Cantor, Babick, Vasanji, Dhalla, & Netticadan, 2005). 2D M-mode measurements included percentage of left ventricular fractional shortening, left ventricular ejection fraction (EF), cardiac output (CO), left ventricular mass, heart rate, interventricular septal wall thickness at diastole (IVSd) and systole, left ventricular posterior wall thickness at diastole (LVPWd) and systole and left ventricular internal dimensions at diastole (LVIDd) and systole. Doppler measurements included isovolumetric relaxation time (IVRt), aortic ejection time and early diastolic filling velocity.

3.1.5 Tissue collection – At the end of 17 weeks on diet, overnight fasted rats were euthanized by CO₂ asphyxiation. Heart was collected, separated into LV, right ventricle and septal tissues, weighed and flash frozen in liquid nitrogen. All samples were stored at -80°C until further analyses. Plasma and serum samples were separated from blood collected at sacrifice by

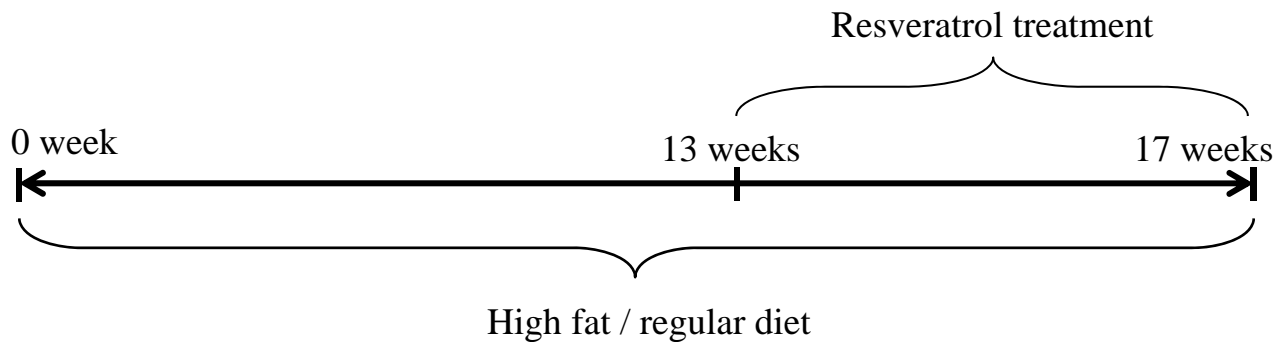


Figure 3.1: *In vivo* study design and timeline based on weeks in high fat diet. OP and OR rats received high fat diet while SD rats received regular lab chow diet for 17 weeks. Starting week 13, treatment groups of OP, OR and SD were administered with resveratrol until end of week 17.

centrifuging at 2500 rpm for 20 min and stored at -80°C for further analyses. Body weight and fat pad mass (epididymal, perirenal and mesenteric) were recorded for assessing adiposity.

3.1.6 Measurement of TBARS

Lipid peroxidation levels in blood plasma were estimated as the amount of malondialdehyde using the Oxiselect TBARS Assay Kit (Cell Biolabs, San Diego, CA) as per manufacture's protocol. Briefly, to prevent any further oxidation, 1X 5% butylated hydroxytoluene (BHT) was added to a fraction of plasma samples collected after centrifugation during sacrifice before storing in -80°C freezer. All samples were assayed in duplicate. 100µL plasma samples were mixed with 100µL sodium dodecyl sulfate (SDS) lysis solution (supplied with the kit), 250µL thiobarbituric acid reagent (5.2g/mL, pH 3.5) and incubated for 60 minutes at 95°C. After incubation, samples were cooled to room temperature in ice for 5 minutes. Samples were then centrifuged at 3000 rpm for 15 minutes. The supernatant was transferred to a fresh tube. 200µL was transferred into a 96 well plate and absorbance was read at 532nm.

3.1.7 Measurement of TNF- α and IL-6

TNF- α and IL-6 levels were estimated using corresponding enzyme-linked immunosorbent assay (ELISA) kits (Thermo Scientific, ON, Canada). Plasma samples were diluted 1:1 and assayed in duplicate. 50µL diluted sample was added to each well in a pretreated strips. The whole plate was covered and incubated at room temperature for an hour. After incubation, wells were vigorously washed 3 times. After wash, 50µL biotinylated antibody was added and incubated at room temperature for an hour. Wells were again washed 3 times. 100µL streptavidin-HRP reagent was added to each well and incubated for 30 minutes at room temperature. Wells were washed 3 times and 100µL TMB substrate solution was added to each well. It was then

incubated at room temperature in the dark for 10 minutes. Finally, the reaction was stopped by adding 100 μ L of stop solution to each well. Absorbance at 450 and 550nm was recorded using a plate reader (FLUOstar Omega, BMG LABTECH Inc., NC, USA).

3.1.8 Measurement of triglycerides

Triglyceride (TG) levels in serum samples were measured using a colorimetric method. A triglyceride assay kit (Genzyme Diagnostics P.E.I. Inc., Charlottetown, PE Canada) was used for the measurement. All solutions were supplied with the kit. Sample and TG assay reagents were mixed in the ratio 1:75 and absorbance was measured at 505nm. A positive control supplied with the kit was used to confirm the accuracy of the assay.

3.1.9 Measurement of glucose

Glucose levels in serum samples were measured using a colorimetric method. A glucose assay kit (Genzyme Diagnostics P.E.I. Inc., Charlottetown, PE Canada) was used for the measurement. All solutions were supplied with the kit. Sample and glucose assay reagents were mixed in the ratio 1:100 and absorbance was measured at 505nm.

3.1.10 Measurement of insulin

Insulin levels in serum samples were estimated using an ELISA kit (Alpco Diagnostics, Salem, NH, USA). All solutions used in this assay were provided with the kit. Test strips were equilibrated to room temperature before assay. 25 μ L serum samples, control and standards were pipetted into respective wells. All samples were measured in duplicate. 100 μ L of detection antibody was then added to each well and incubated at room temperature with shaking (700-900rpm). After incubation, each well was washed 6 times with 350 μ L wash solution. After

adding 100 μ L TMB substrate solution microplate was incubated for 15 minutes at room temperature as described above. After incubation, 100 μ L stop solution was added to each well and the absorbance was read at 450nm using a plate reader.

3.1.11 Measurement of leptin

Leptin levels in serum samples were estimated using an ELISA kit (Alpco Diagnostics, Salem, NH, USA). Samples were diluted 1:5 and assayed in duplicate. 100 μ L sample, control and standards were pipetted into respective wells. The plate was sealed and incubated at room temperature for 1hour with shaking (350rpm). After incubation, contents in the well were aspirated and the wells were washed 3 times with 250 μ L wash buffer. 100 μ L antibody conjugate was added to each well and incubated for 1 hour as described above. After incubation, wells were washed, 100 μ L of enzyme conjugate was added to each well and incubated for 30 minutes as described above. Wells were washed, 100 μ L of TMB substrate solution was added and incubated for 30 minutes in the dark. Reaction was stopped by adding 100 μ L stop solution and absorbance was measured at 450nm using a plate reader.

3.1.12 Measurement of adiponectin

Adiponectin levels in serum samples were estimated using an ELISA kit (Alpco Diagnostics, Salem, NH, USA). Samples were diluted 1:1000 and assayed in duplicate. 100 μ L sample, control and standards were pipetted into respective wells. The plate was sealed and incubated at room temperature for 1hour with shaking (350rpm). After incubation, contents in the well were aspirated and the wells were washed 3 times with 300 μ L wash buffer. 100 μ L antibody-POD-conjugate was added to each well and incubated for 1 hour as described above. After incubation, wells were washed, 100 μ L of TMB substrate solution was added and incubated for 30 minutes in

the dark. The reaction was stopped by adding 100 μ L stop solution and absorbance was measured at 450nm using a plate reader.

3.1.13 Measurement of HDL

HDL was measured using a colorimetric assay kit (Sekisui Diagnostics, MA, USA). Serum samples and reagent 1 from the assay kit were mixed in the ratio 5:210 and absorbance was read at 660nm after 7 min. 70 μ L reagent 2 was then added and absorbance was read at 600nm after 5 min. The concentration was calculated in mg/dL.

3.1.14 Measurement of LDL

LDL was measured using a colorimetric assay kit (Sekisui Diagnostics, MA, USA). Serum samples and reagent 1 from the appropriate kit were mixed in the ratio 5:210 and absorbance was read at 660nm after 5 min. 70 μ L reagent 2 was then added and absorbance was read at 660nm after 5 min. The concentration was calculated in mg/dL.

3.1.15 Preparation of SR vesicles

Sarcoplasmic reticulum vesicles were isolated from SD and OP rat heart tissues as described earlier (Babick et al., 2004; Chohan, Singh, Dhalla, & Netticadan, 2006; Singh, Chohan, Dhalla, & Netticadan, 2004; Vasanji, Cantor, Juric, Moyen, & Netticadan, 2006). Briefly, 100mg of left ventricular tissue was chopped into small pieces using scissors after adding 1mL of buffer containing (mM): 10 NaHCO₃, 5 NaN₃ and 15 Tris-HCl at pH 6.8. It was then homogenized with a polytron homogenizer (Brinkmann, Westbury, NY, USA) and centrifuged at 10,000g for 20 min. The supernatant was again centrifuged at 43,000g for 45 min (JA 20, Beckman, ON, Canada). The pellet was resuspended in buffer containing 0.6 M KCl and 20 mM Tris-HCl (pH

6.8) using a pestle and glass homogenizer. It was then centrifuged at 43,000g for 45 min. The resulting pellet is the SR fraction and was suspended in a buffer containing 250 mM sucrose and 10 mM histidine, pH 7.0. The SR fractions were aliquoted, flash frozen in liquid nitrogen and stored at -85°C . A protease inhibitor cocktail containing (μM): 1 leupeptin, 1 pepstatin and 100 phenylmethylsulfonyl fluoride was added to all buffers to prevent protein degradation during the isolation procedure. Protein concentrations were estimated using Lowry's method (DC protein assay kit, Bio-Rad laboratories, ON, Canada).

3.1.16 Measurement of SERCA2A, PLB, pPLBser16 and pPLBthr17 expression

Protein expression levels of SERCA2A, PLB, pPLBser16 and pPLBthr17 were estimated by Western blotting as previously described (Babick et al., 2004; Chohan et al., 2006; Singh et al., 2004; Vasanji et al., 2006). Protein samples were prepared at a concentration of 1mg/mL and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It was then transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were then probed with a monoclonal anti-SERCA2a antibody obtained from Affinity Bioreagents Inc., monoclonal anti-PLB antibody obtained from Upstate Biotechnology (Lake Placid, NY, USA), a polyclonal anti-phospho-PLB (Ser16) and a polyclonal anti-phospho-PLB (Thr17) antibody (Santa Cruz Biotechnology, Inc., Texas, USA). After incubating with appropriate secondary antibodies, proteins were detected using chemiluminescence kit (ECL prime western blotting detection reagent, GE Life Sciences, Quebec, Canada). Protein bands were scanned using a densitometer (GS-800, Bio-Rad Laboratories, Hercules, CA, USA). Band densities were quantified using the Quantity One 4.5.0 software (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as the loading control to normalize protein levels.

3.1.17 Statistical analysis – All statistical analyses were performed using a SAS statistical package (version 9.1; SAS Institute Inc., Cary, NC, USA). One-way repeated measures analysis of variance (ANOVA) was used to analyze weekly body weight. For all other parameters, two-way ANOVA was used to assess the significant main effects of model (genotype + diet; SD, OR, OP), treatment (\pm resveratrol) and their interactions. Significance was defined as $P < 0.05$ for the main effects and $P < 0.1$ for interactions. Data were assessed for normality using the Shapiro-Wilk's test and homogeneity of variance by Levene's test. Log transformation was used when necessary to normalize data sets. For post-hoc testing, significance ($P < 0.05$) among means was determined by Duncan's multiple range test. One way ANOVA was used to assess significance in leptin and adiponectin and Western blot data. Significance was defined as $P < 0.05$. All values are expressed as mean \pm s.e.

3.2 RESULTS

3.2.1 General observations

An overall mortality of 14% was observed in OR rats whereas there was no mortality in any other groups in the study. There was no difference between resveratrol treated and vehicle treated group in mortality. Vehicle (50% ethanol) treatment had no effect in any of the parameters measured in this study.

3.2.2 Effect of resveratrol treatment on body weight

OP rats had significantly higher body weight at the end of the study when compared to the OR rats. Standard chow fed SD rats had body weights similar to that of OP rats. The body weight increase was 3.7 fold in SD and OP rats, while OR rats had a 2.8 fold increase. Body weights were unchanged with resveratrol treatment (**Figure: 3.2**).

3.2.3 Effect of resveratrol treatment on total visceral fat mass

Total visceral fat to body weight ratio was significantly higher in OP HF rats. However, total visceral fat to body weight ratio of SD SC and OR HF rats were similar. Visceral fat deposit weights were unchanged with resveratrol treatment (**Figure: 3.3**).

3.2.4 Effect of resveratrol treatment on heart to body weight ratio

OP HF rats had significantly lower heart to body weight ratio when compared to SD SC control rats. OR HF rats had heart to body weight ratio similar to that of SD SC rats. Heart to body weight ratios was unchanged with resveratrol treatment (**Figure: 3.4**).

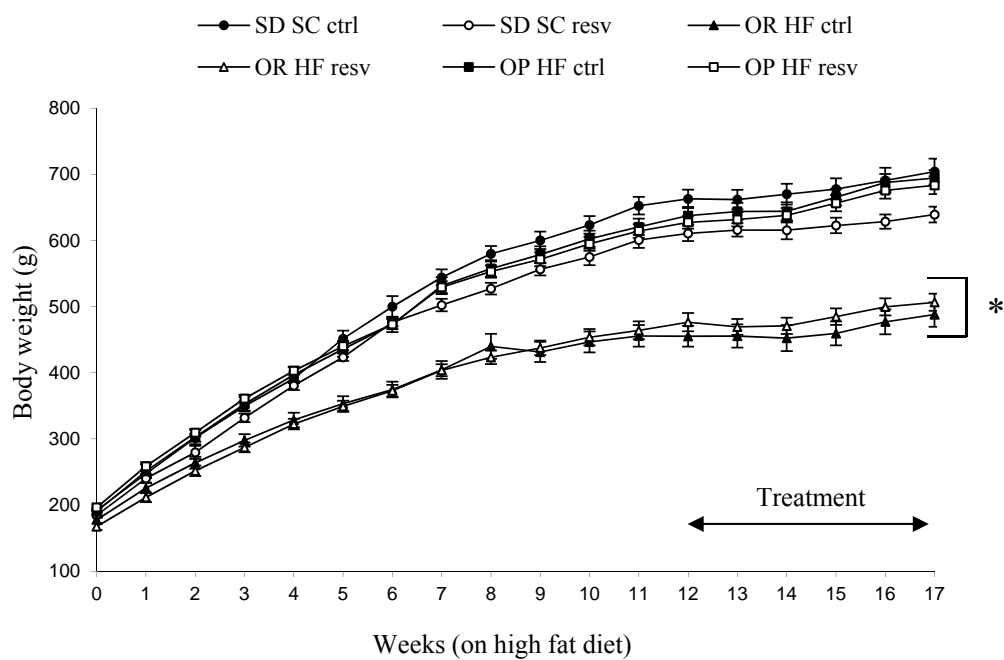


FIGURE 3.2: Weekly body weight of SD, OR and OP rats. Data are means \pm S.E., $n=6-10$. Significant main effect of model. * $P<0.05$ vs. other two models at 17 week time point. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

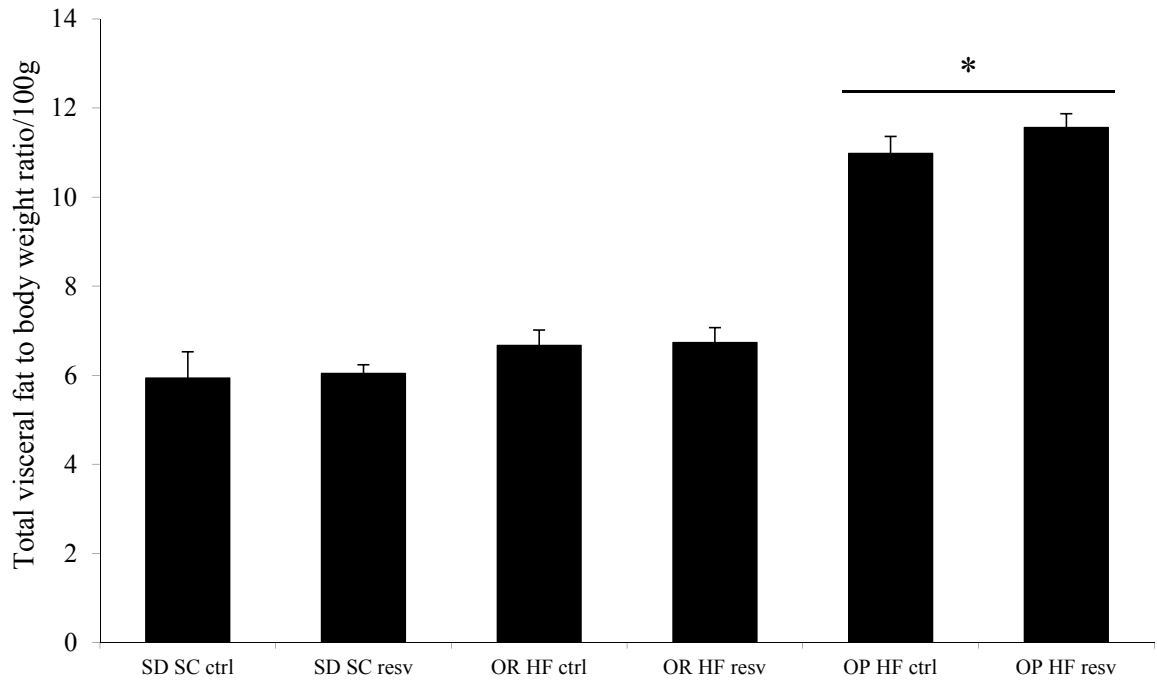


FIGURE 3.3: Total visceral fat weight (visceral fat equals sum of epididymal, perirenal and mesenteric fat pads) to body weight ratio of SD, OR and OP rats. Data are means \pm S.E., $n=6-10$. Significant main effect of model. * $P<0.05$ vs. other two models. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

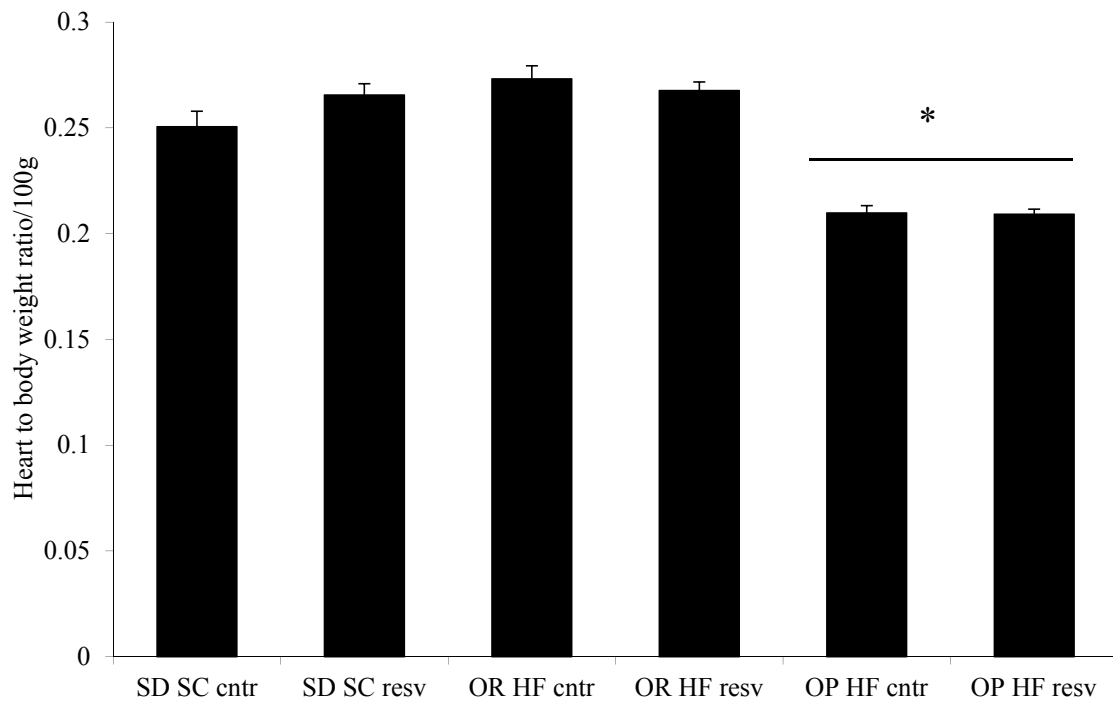


FIGURE 3.4: Heart weight to body weight ratio of SD, OR and OP rats. Data are means±S.E., $n=6-10$. Significant main effect of model. * $P<0.05$ vs. other two models. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

3.2.5 Effect of resveratrol treatment on systolic blood pressure

Systolic blood pressure was significantly higher in OP HF and OR HF rats after twelve weeks of HF feeding, when compared to the corresponding SD SC control rats. Blood pressure did not increase any further at the end of 17 weeks. Blood pressure was unchanged with resveratrol treatment (**Figure: 3.5**).

3.2.6 Effect of resveratrol treatment on cardiac function

At the 12 week time point, IVRt, a diastolic function parameter was significantly increased in OP and OR groups fed a HF diet when compared to standard chow fed SD rats. After 5 weeks of resveratrol treatment, IVRt was lowered back to normal in OP HF resveratrol treated rats. However, IVRt of OR HF rats treated with resveratrol remained significantly higher when compared to SD SC rats at the 17 week time point (**Figure: 3.6**). CO was not affected in HF fed OP rats at 6, 12 and 17 week time points when compared to corresponding SD SC rats. However, CO was significantly lower in OR HF rats at 6, 12 and 17 week time points when compared to corresponding SD SC rats. CO was not affected with resveratrol treatment in SD SC, OR HF and OP HF rats (**Figure: 3.7**). EF was significantly higher in OR HF and OP HF rats at 6 and 17 week time points. EF was unchanged with resveratrol treatment (**Figure: 3.8**).

3.2.7 Effect of resveratrol treatment on cardiac structure

IVSd was unchanged with HF feeding at 6, 12 and 17 week time points (**Figure: 3.9**). OR HF and OP HF rats had significantly lower LVIDd and LVPWd at 6, 12 and 17 weeks when compared to corresponding SD SC control rats (**Figure: 3.10 and 3.11**). IVSd, LVIDd and LVPWd were unchanged with resveratrol treatment in SD SC, OP HF and OR HF rats (**Figure:**

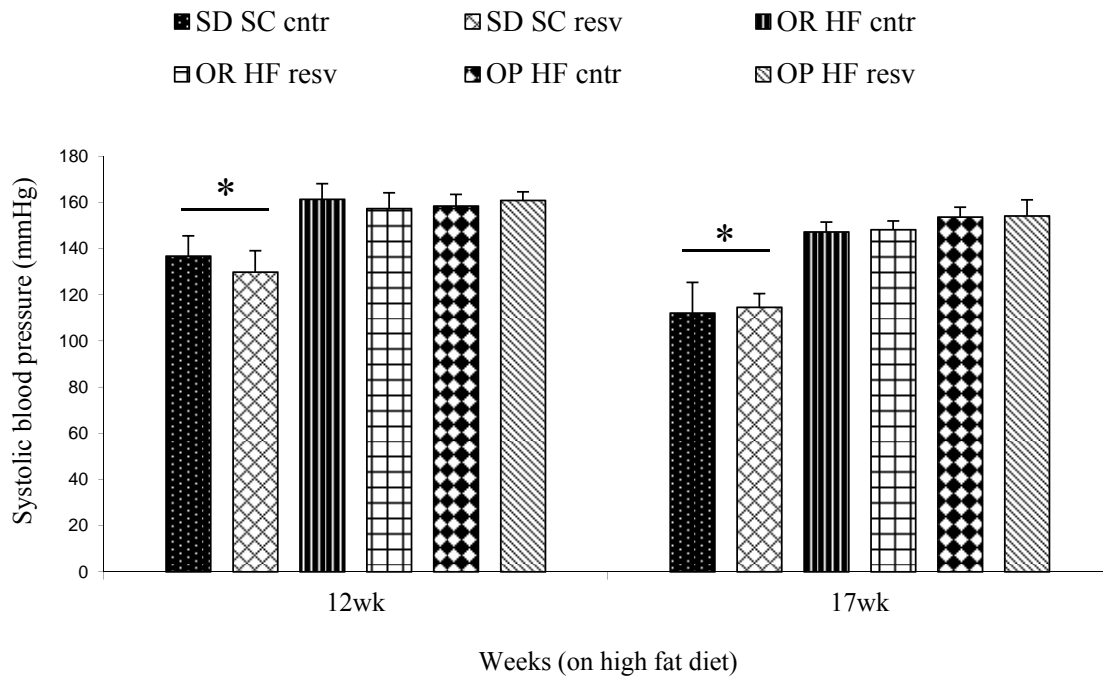


FIGURE 3.5: Systolic blood pressure of SD, OR and OP rats. Data are means \pm S.E., $n=6-9$. Significant main effect of model. * $P<0.05$ vs. other two models. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

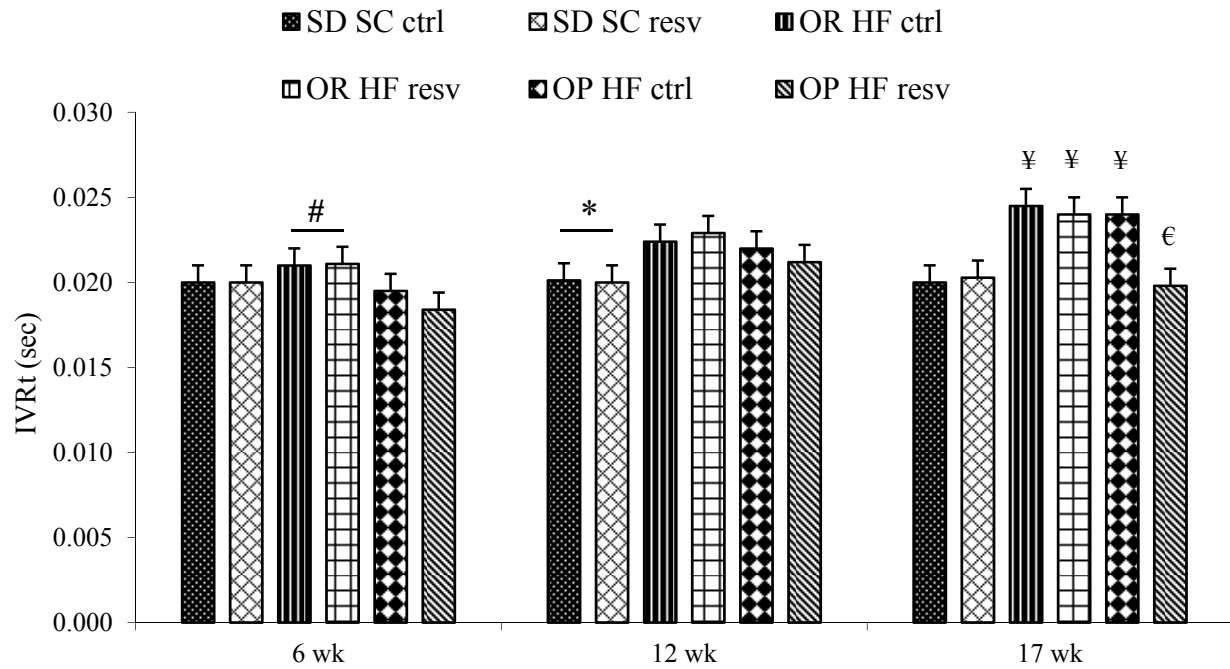


FIGURE 3.6: Echocardiographic data showing isovolumic relaxation time (IVRT) cardiac function measurements of SD, OR and OP rats. Data are means±S.E., $n=6-10$. Significant main effects of model for IVRt (6, 12 weeks) and significant model×treatment interaction for IVRt (17 weeks). * $P<0.05$ vs. other two models; # $P<0.05$ vs. OP; ¥ $P<0.05$ vs. SD SC ctrl, € $P<0.05$ vs. OP HF ctrl. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

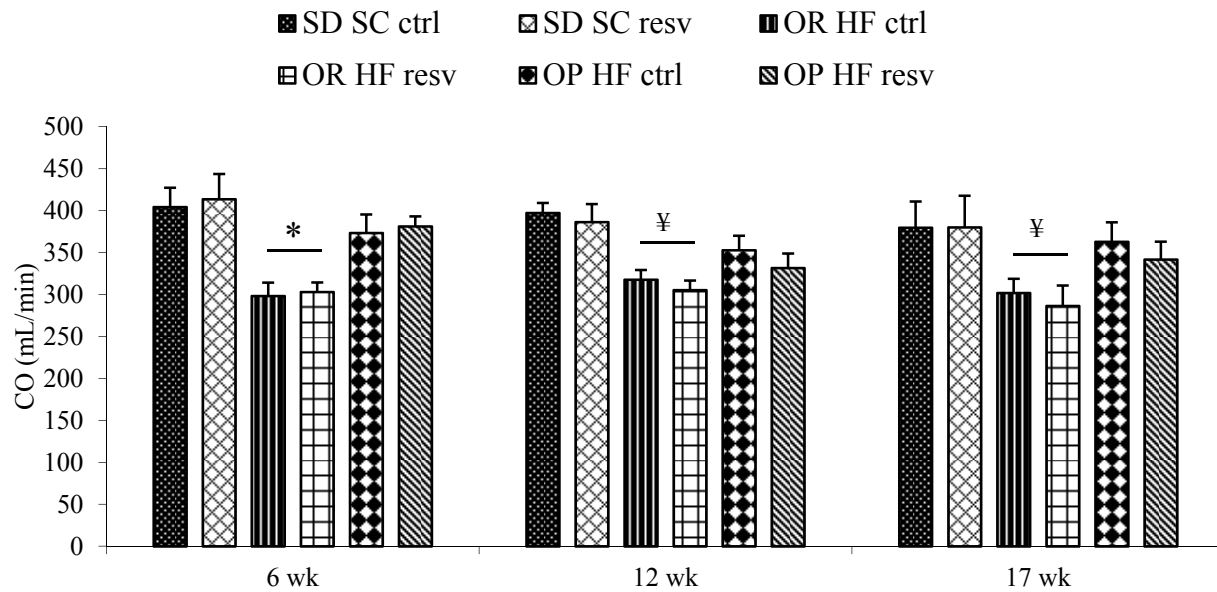


FIGURE 3.7: Echocardiographic data showing cardiac output (CO) measurements of SD, OR and OP rats. Data are means±S.E., $n=6-10$. Significant main effects of model for CO (6, 12, 17 weeks). * $P<0.05$ vs. other two models; ¥ $P<0.05$ vs. SD. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

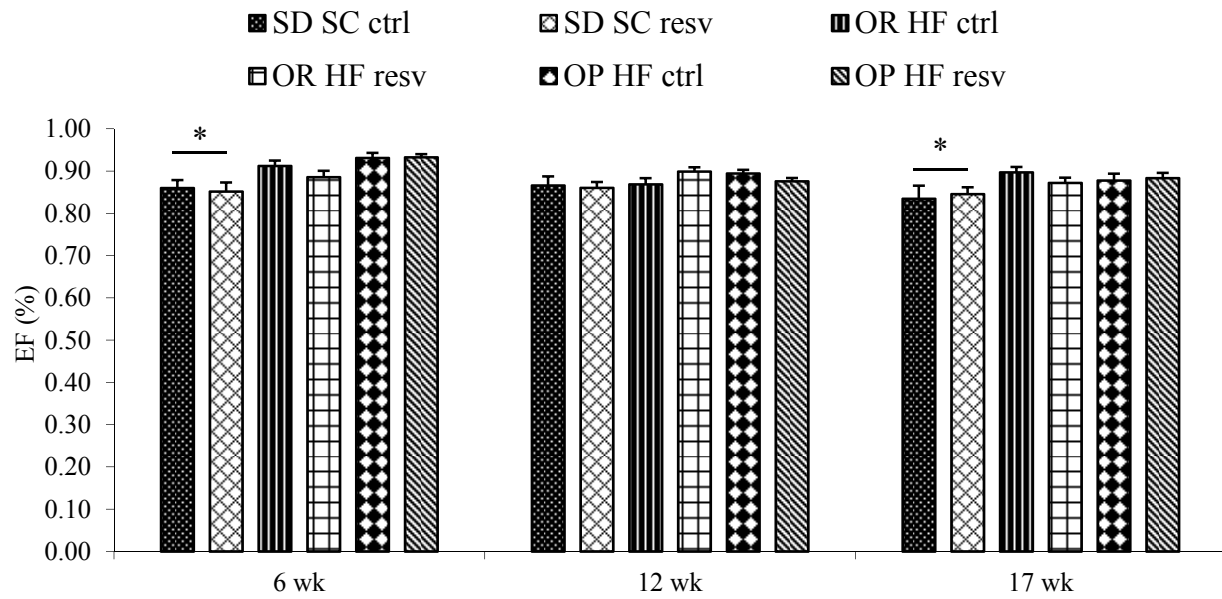


FIGURE 3.8: Echocardiographic data showing ejection fraction (EF) of SD, OR and OP rats. Data are means±S.E., $n=6-10$. Significant main effects of model for EF (6, 17 weeks). * $P<0.05$ vs. other two models. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

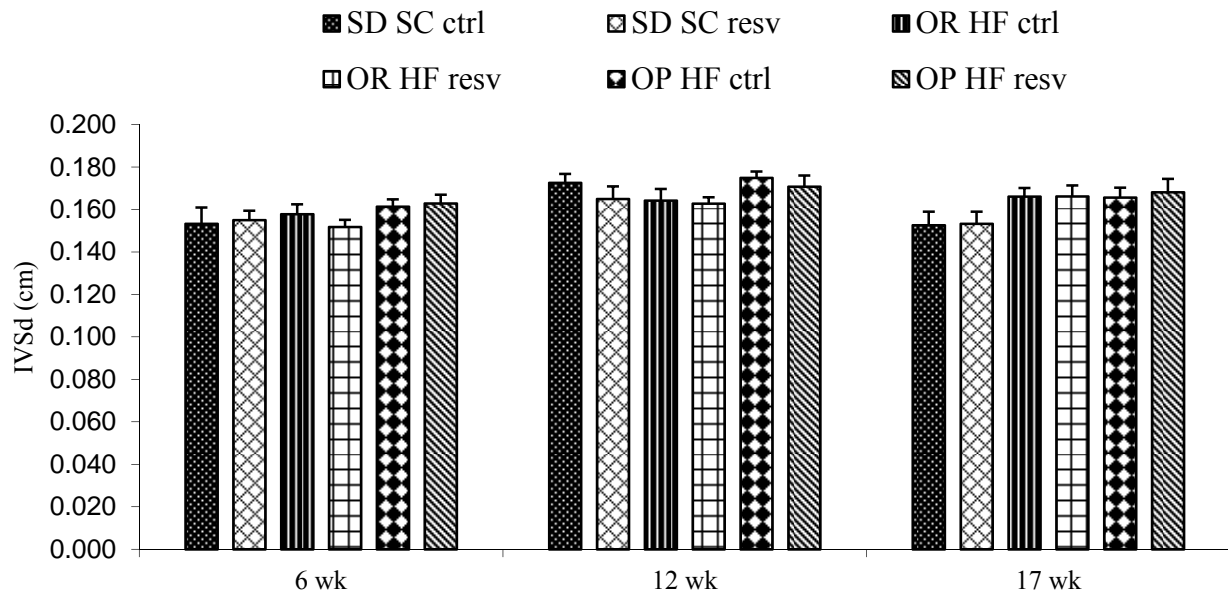


FIGURE 3.9: Echocardiographic data showing interventricular septal wall thickness at diastole (IVSd) measurements of SD, OR and OP rats. Data are means \pm S.E., $n=6-10$. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

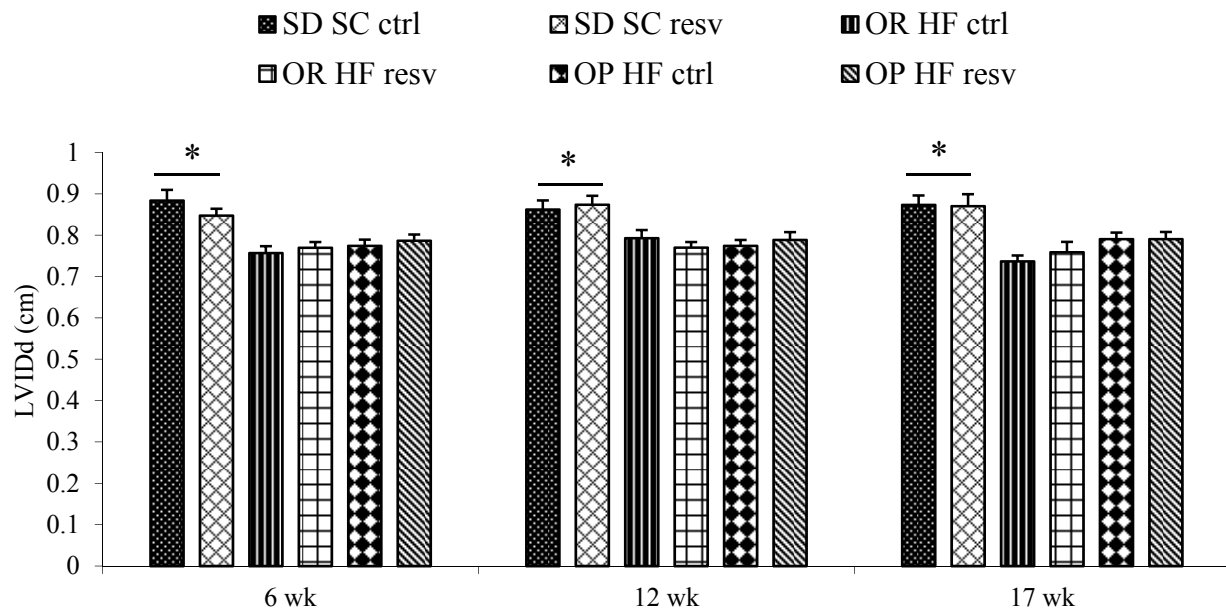


FIGURE 3.10: Echocardiographic data showing left ventricular internal dimension at diastole (LVIDd) measurements of SD, OR and OP rats. Data are means \pm S.E., $n=6-10$. Significant main effects of model for LVIDd (6, 12, 17 weeks). * $P<.05$ vs. other two models. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

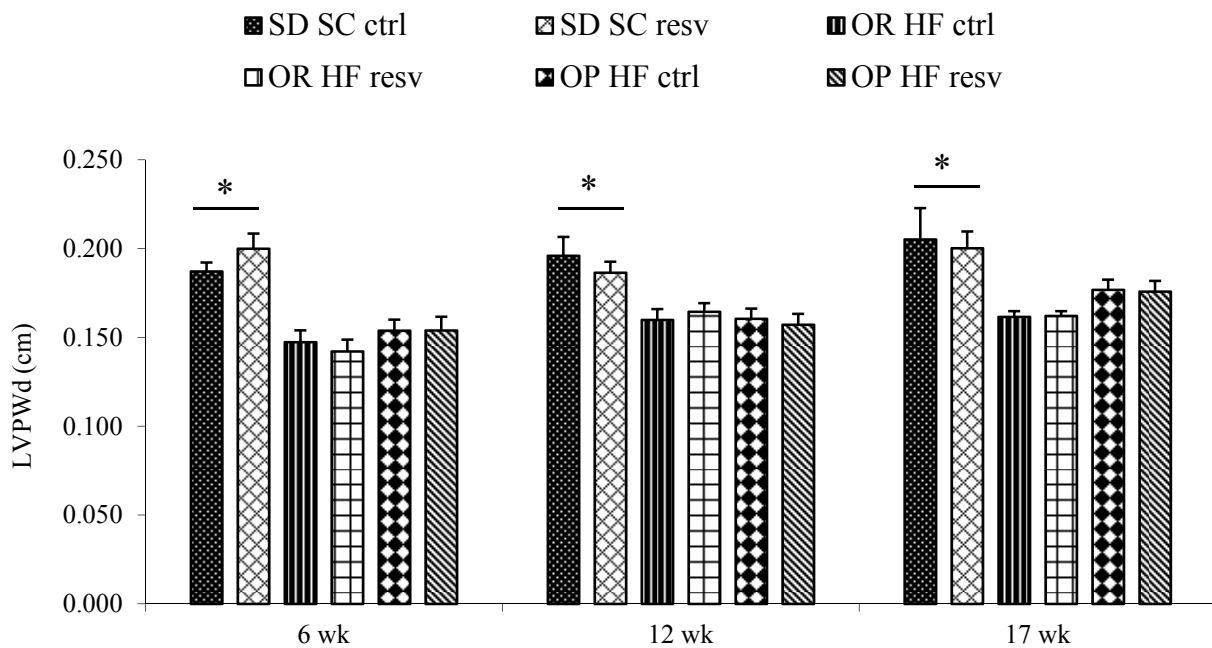


FIGURE 3.11: Echocardiographic data showing left ventricular posterior wall thickness at diastole (LVPWd) measurements of SD, OR and OP rats. Data are means \pm S.E., $n=6-10$. Significant main effects of model for LVPWd (6, 12, 17 weeks). * $P<.05$ vs. other two models. Data for LVPWd (6 weeks) were log transformed for statistical analysis, but untransformed data are presented. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

3.9, 3.10 and 3.11). representative 2D M-mode echocardiographic images are presented here (**Figure: 3.12**).

3.2.8 Effect of resveratrol treatment on serum TBARS levels

There was a significant increase in TBARS levels in OP rats fed HF diet for 17 weeks when compared to their age matched SD control rats. OR and SD rats had similar TBARS levels. Resveratrol treated OP rats had TBARS levels similar to that of SD rats. Resveratrol treatment had no effect on OR and SD rats (**Figure: 3.13**).

3.2.9 Effect of resveratrol treatment on serum TNF- α

TNF- α level was significantly higher in OP and OR rats fed a HF diet. TNF- α levels were increased by 6-fold and 3-fold in the serum of OR and OP rats respectively, when compared to SD control rats. Resveratrol treated OR and OP rats had serum TNF- α level similar to that of SD rats (**Figure: 3.14**).

3.2.10 Effect of resveratrol treatment on serum IL-6

Serum IL-6 level was increased 4-fold in OP rats while there was a 2-fold increase in OR rats when compared to SD control rats. Resveratrol treated OP rats had IL-6 levels similar to that of SD rats. IL-6 level in resveratrol treated OR rats were similar to that of untreated OR rats (**Figure: 3.15**).

3.2.11 Effect of resveratrol treatment on serum triglycerides

A 3-fold increase in fasting serum TG levels was observed in OP rats when compared to their corresponding SD controls. Resveratrol treated OP rats had significantly lower TG levels when

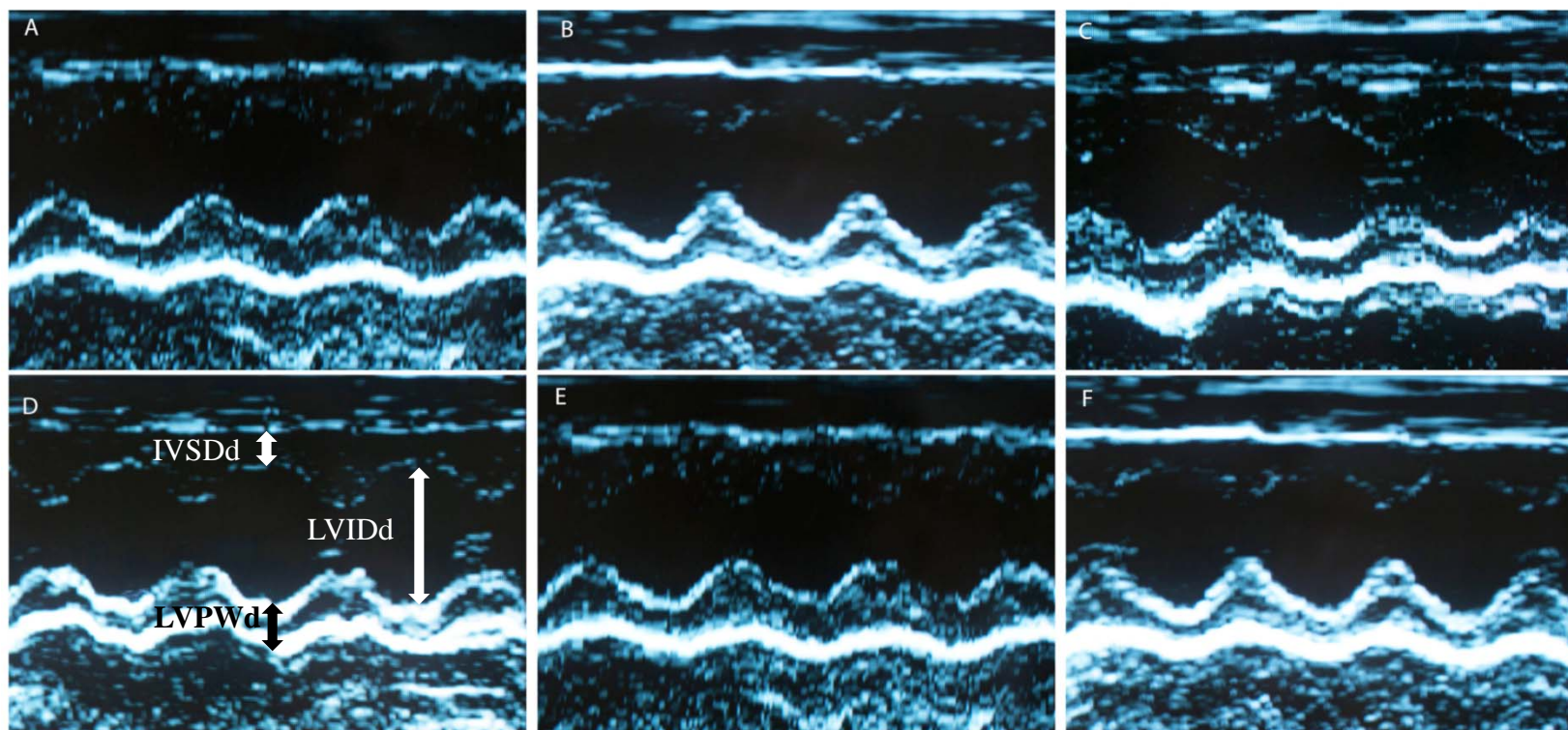


Figure 3.12: Representative echocardiograph images from 2D M-mode measurements for cardiac structure analysis at week 17. A. SD SC ctrl; B. SD SC resv; C. OR HF ctrl; D. OR HF resv; E. OP HF ctrl; F. OP HF resv. IVSDd, interventricular septal wall thickness at diastole; LVIDd, left ventricular internal dimension at diastole; LVPWd, left ventricular posterior wall thickness at diastole; SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

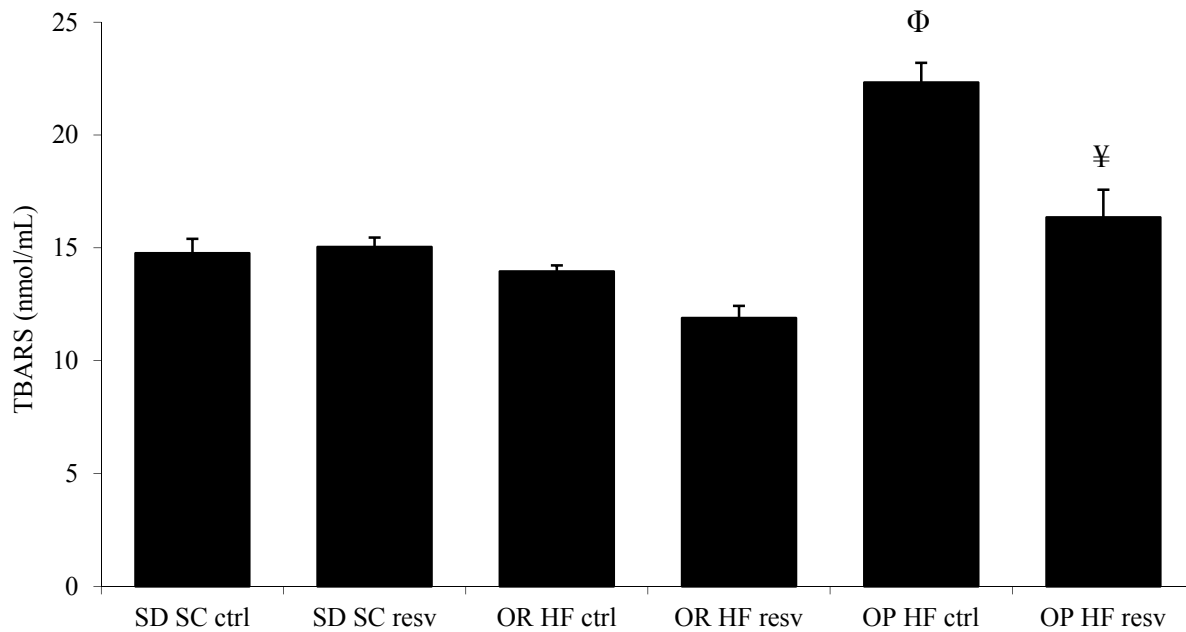


FIGURE 3.13: Serum thiobarbituric acid reactive substance (TBARS) levels in SD, OR and OP rats at week 17. Data are means \pm S.E., $n=5-10$. Significant model \times treatment interaction ($P<.1$) for TBARS. $\Phi P<0.05$ vs SD SC ctrl, $\text{¥}P<0.05$ vs OP HF ctrl. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

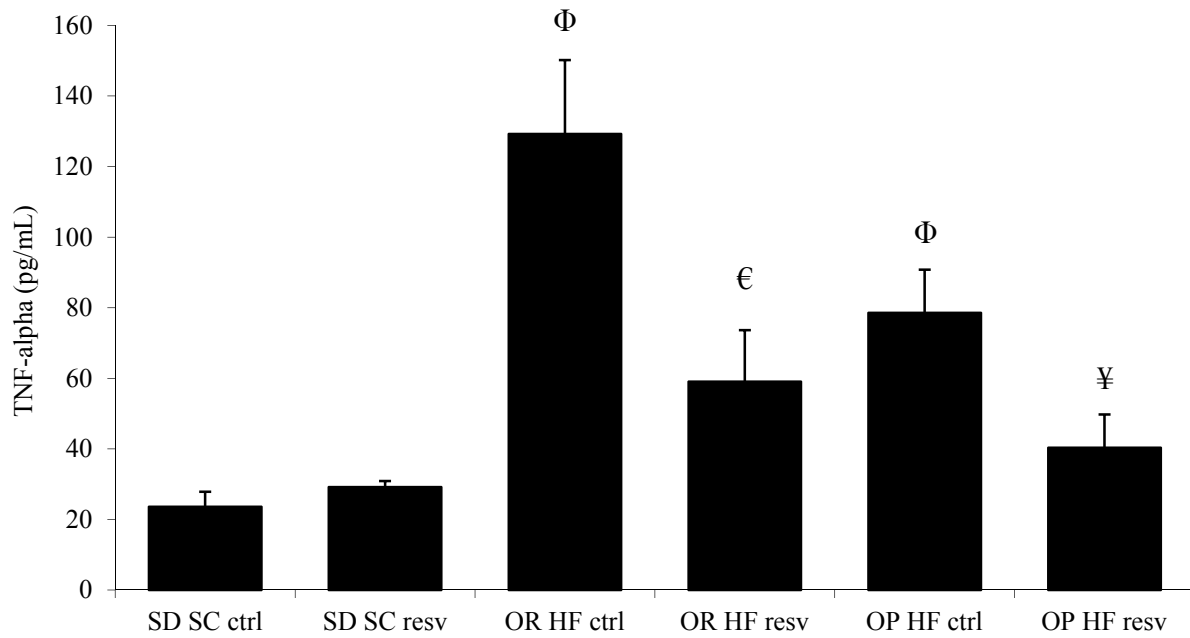


FIGURE 3.14: Serum tumor necrosis factor-alpha (TNF- α) levels in SD, OR and OP rats at week 17. Data are means \pm S.E., $n=5-10$. Significant model \times treatment interaction ($P<0.1$) for TNF- α . $\Phi P<0.05$ vs SD SC ctrl, $\epsilon P<0.05$ vs OR HF ctrl, $\text{¥} P<0.05$ vs OP HF ctrl. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

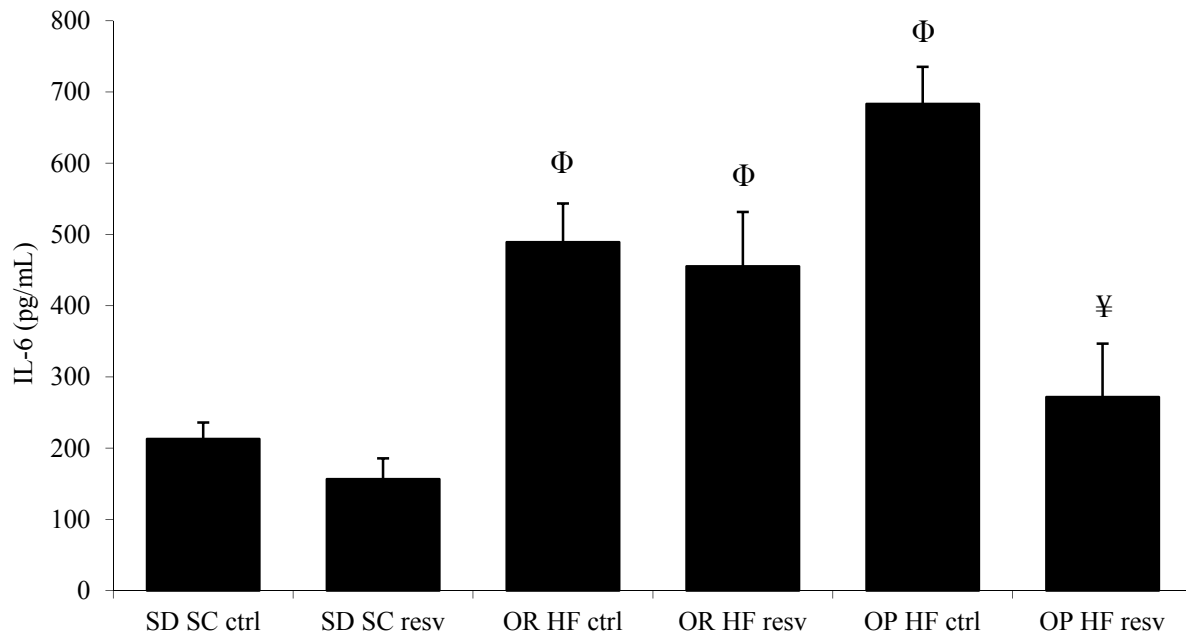


FIGURE 3.15: Serum interleukin-6 (IL-6) levels in SD, OR and OP rats at week 17. Data are means±S.E., $n=5-10$. Significant model×treatment interaction ($P<0.1$) for IL-6. $\Phi P<0.05$ vs SD SC ctrl, $\text{¥}P<0.05$ vs OP HF ctrl. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

compared to vehicle treated OP HF rats. TG levels in OR HF rats were similar to that in SD SC controls (**Figure: 3.16**).

3.2.12 Effect of resveratrol treatment on serum glucose

Glucose levels were increased 1.9 and 2.4 fold in OR HF and OP HF control groups, respectively, when compared to SD SC controls. Resveratrol-treated OP HF and OR HF rats had significantly lower glucose levels when compared to corresponding vehicle treated rats (**Figure: 3.17**).

3.2.13 Effect of resveratrol treatment on serum insulin

OP HF control rats had a 2.4 fold increase in insulin levels when compared to SD SC control rats. Insulin levels in OR HF control rats were similar to that in SD SC control rats. Resveratrol treated OP HF and OR HF rats had significantly higher insulin levels when compared to corresponding vehicle-treated OR HF and OP HF rats (**Figure: 3.18**).

3.2.14 Effects of resveratrol treatment on serum HDL

HDL levels were significantly higher in OP HF control rats when compared to SD SC control rats. OR HF and SD SC control rats had the same LDL levels. Resveratrol treatment had no effect on OP HF, OR HF and SD SC rats (**Figure: 3.19**).

3.2.15 Effect of resveratrol treatment on serum LDL

OP HF control rats had significantly higher LDL levels when compared to SD SC and OR HF control rats. Resveratrol treated OP HF rats had significantly lower LDL levels when compared to vehicle treated OP HF rats. Resveratrol treatment had no significant effect on LDL levels in SD SC and OR HF rats (**Figure: 3.20**).

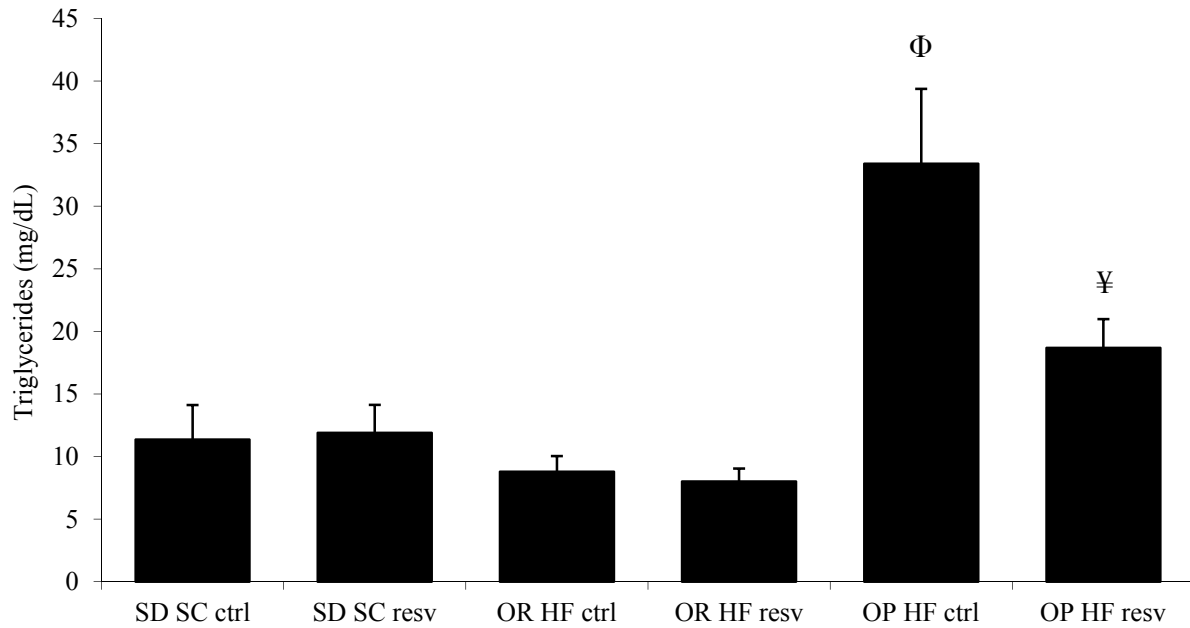


FIGURE 3.16: Serum triglyceride levels in SD, OR and OP rats at week 17. Data are means±S.E., $n=6-10$. Significant model×treatment interaction ($P<0.1$) for triglyceride. $\Phi P<0.05$ vs SD SC ctrl, $\text{¥}P<0.05$ vs OP HF ctrl. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

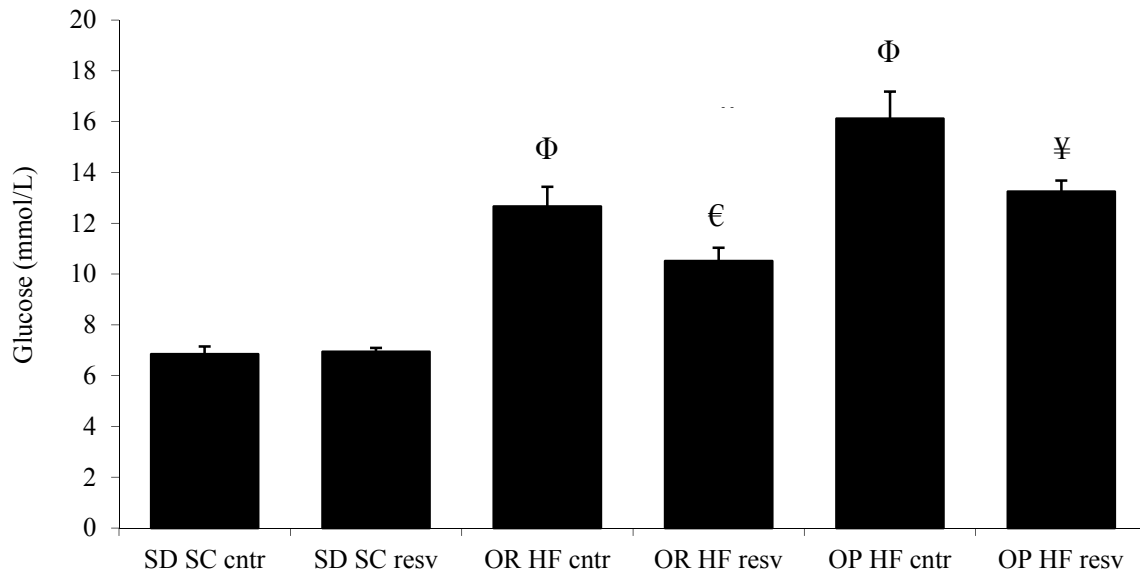


FIGURE 3.17: Serum fasting glucose levels in SD, OR and OP rats at week 17. Data are means±S.E., $n=6-10$. Significant model×treatment interaction ($P<0.1$) for glucose. $\Phi P<0.05$ vs SD SC ctrl, $\epsilon P<0.05$ vs OR HF ctrl, $\yen P<0.05$ vs OP HF ctrl. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

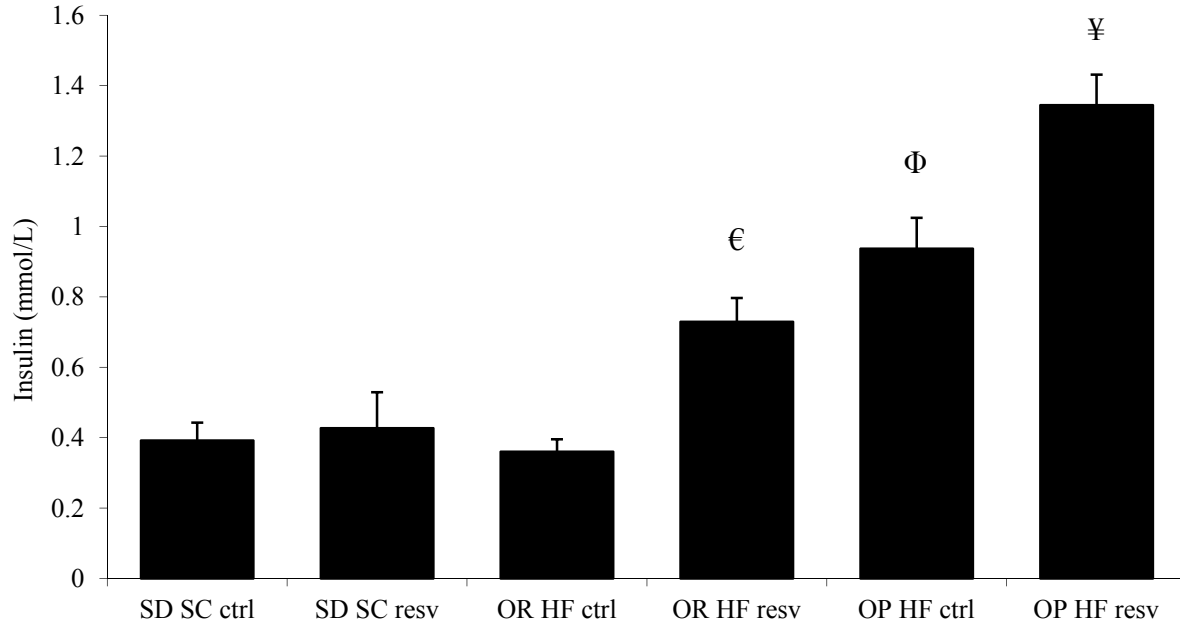


FIGURE 3.18: Serum fasting insulin levels in SD, OR and OP rats at week 17. Data are means \pm S.E., $n=6-10$. Significant model \times treatment interaction ($P<0.1$) for insulin. $\Phi P<0.05$ vs SD SC ctrl, $\epsilon P<0.05$ vs OR HF ctrl, $\yen P<0.05$ vs OP HF ctrl. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

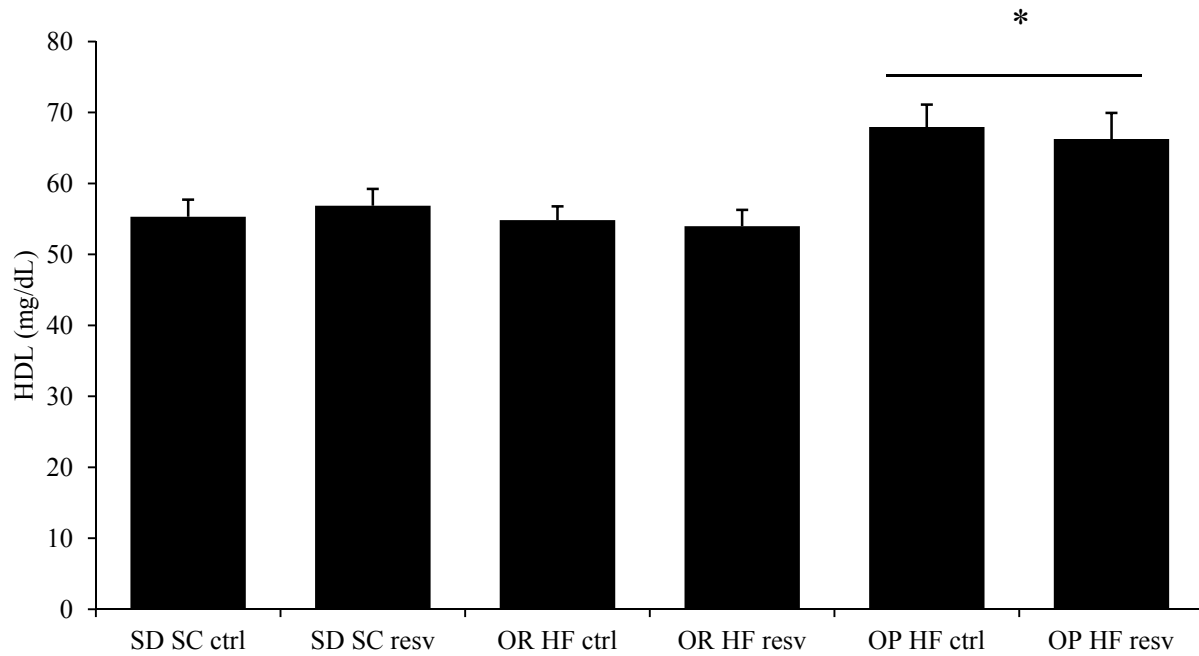


FIGURE 3.19: Serum high density lipoprotein (HDL) levels in SD, OR and OP rats at week 17. Data are means \pm S.E., $n=5-10$. Significant main effects of model for HDL.

* $P<0.05$ vs other two models. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

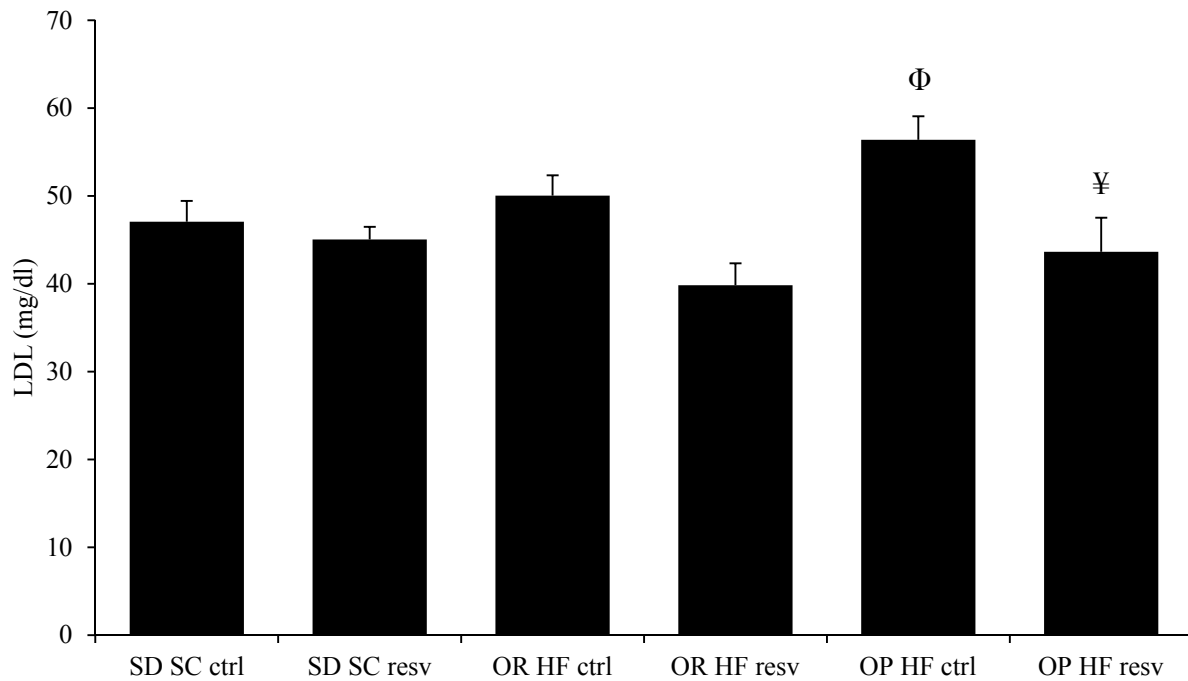


FIGURE 3.20: Serum low density lipoprotein (LDL) levels in SD, OR and OP rats at week 17. Data are means±S.E., $n=5-10$. Significant model×treatment interaction for LDL. $\Phi P<0.05$ vs SD ctrl, $\text{¥}P<0.05$ vs OP ctrl. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

3.2.16 Effect of resveratrol treatment on LDL to HDL ratio

LDL to HDL ratio was unchanged in resveratrol or vehicle treated OP HF, OR HF and SD SC rats and not significantly different between rat strains (**Figure: 3.21**).

3.2.17 Effect of resveratrol treatment on leptin

Leptin levels were significantly higher in OR HF control rats when compared to SD SC control rats. The changes in OP HF control rats were not statistically significant. Resveratrol treated OR HF rats had significantly lower leptin levels, while leptin levels in OP HF rats were not significantly lowered when compared to corresponding vehicle treated rats (**Figure: 3.22**).

3.2.18 Effect of resveratrol treatment on adiponectin

Adiponectin levels were significantly higher in OP HF control rats when compared to SD SC control rats. There was no significant difference between adiponectin levels in OR HF control and SD SC control rats. Resveratrol treatment had no significant effects adiponectin levels in OP HF, OR HF and SD SC rats (**Figure: 3.23**).

3.2.19 Expression levels of SERCA2A, PLB, pPLBser16 and pPLBthr17

Protein expression levels of SERCA2A, PLB, pPLBser16 and pPLBthr17 were not changed in HF fed animals. Expression levels of these proteins in resveratrol treated animal heart tissues were not significantly different from the vehicle treated animals. (**Figure: 3.24, 3.25, 3.26 and 3.27**).

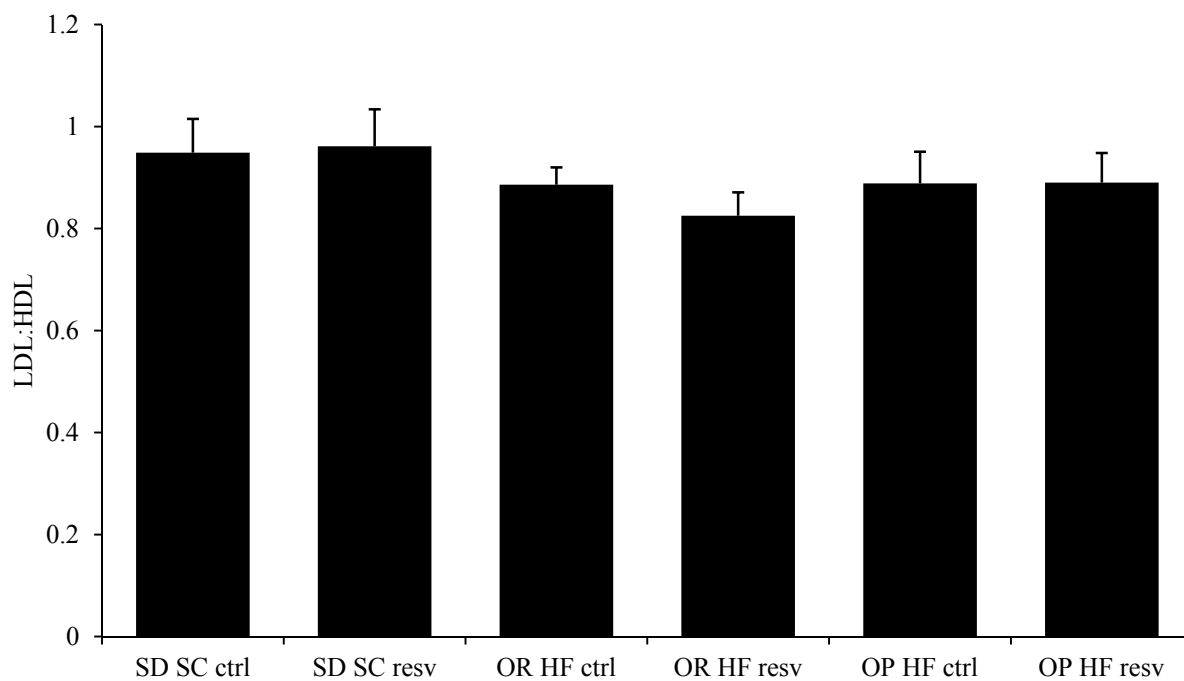


FIGURE 3.21: LDL to HDL ratio in SD, OR and OP rats at week 17. Data are means \pm S.E., $n=5-10$. LDL, low density lipoprotein; HDL, high density lipoprotein; SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

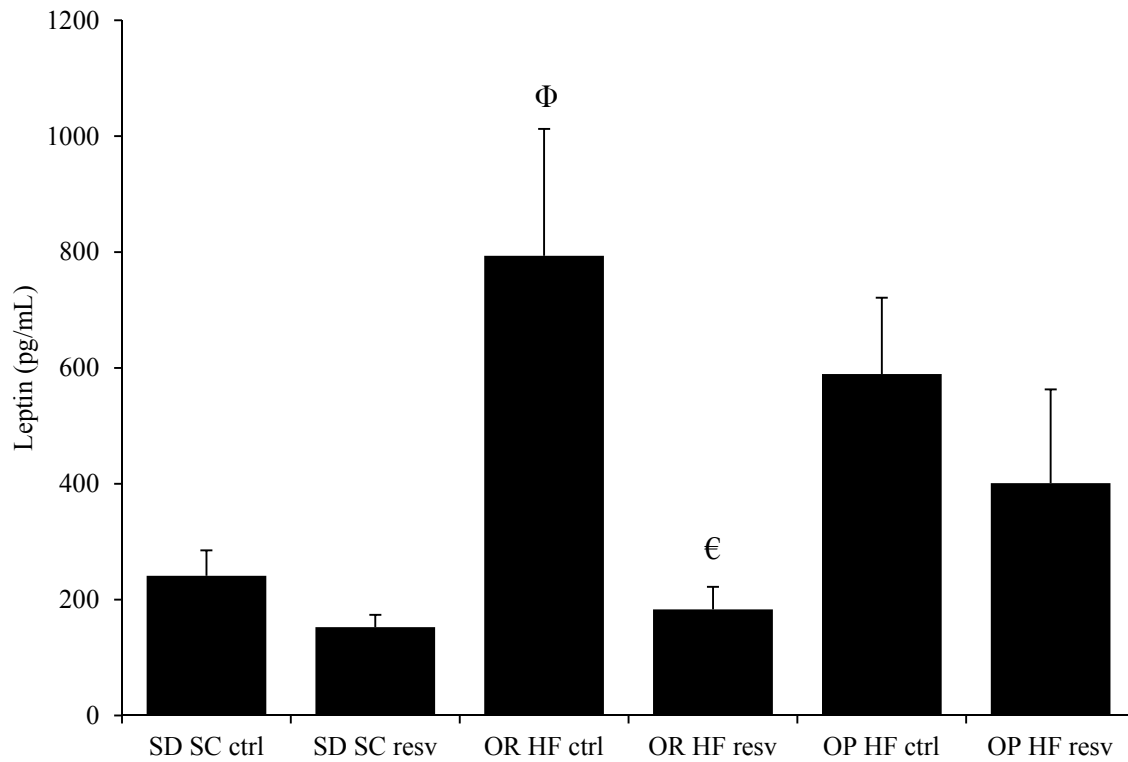


FIGURE 3.22: Serum leptin levels in SD, OR and OP rats at week 17. Data are means±S.E., $n=5-10$. Significant model×treatment interaction for leptin. $\Phi P<0.05$ vs SD SC ctrl, $\epsilon P<0.05$ vs OR HF ctrl. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

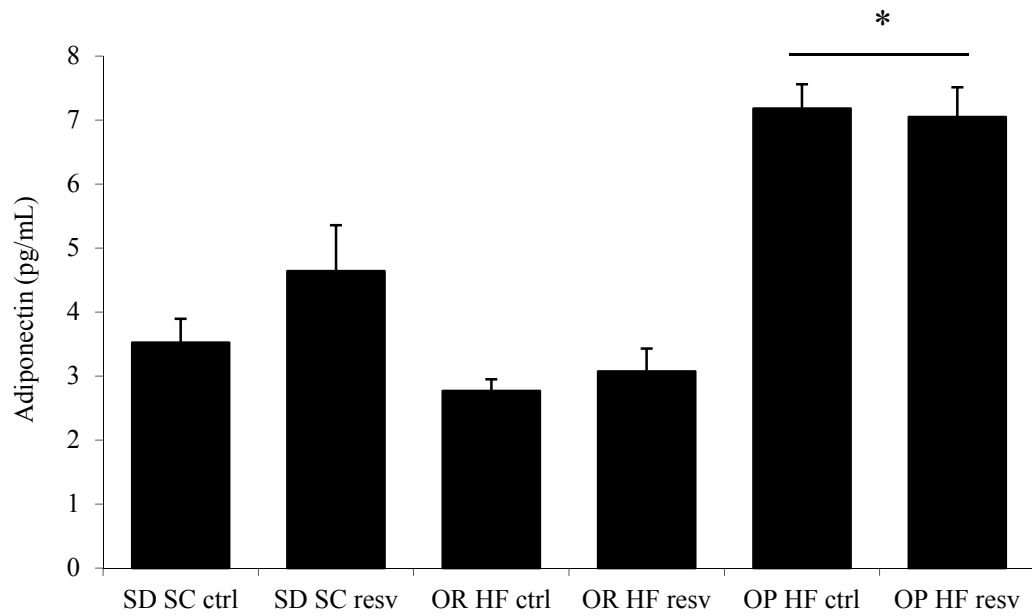


FIGURE 3.23: Serum adiponectin levels in SD, OR and OP rats at week 17. Data are means \pm S.E., $n=5-10$. Significant main effects of model for adiponectin. * $P<0.05$ vs other two models. SD, Sprague Dawley; OP, obese prone; OR, obese resistant; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

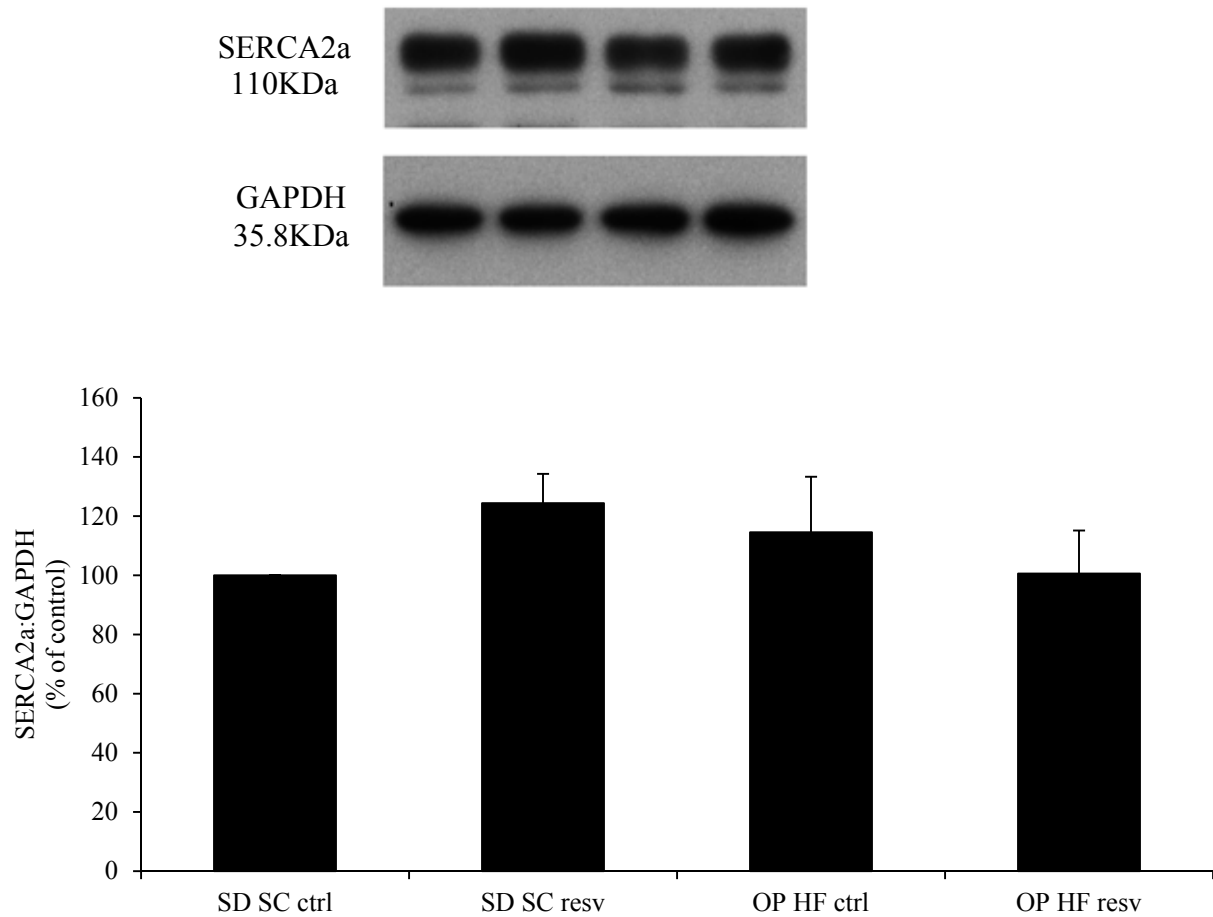


FIGURE 3.24: Sarcoendoplasmic reticulum calcium ATPase 2a (SERCA2a) expression levels heart tissue of SD and OP rats at week 17. Data are means \pm S.E., $n=5-6$. SD, Sprague Dawley; OP, obese prone; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

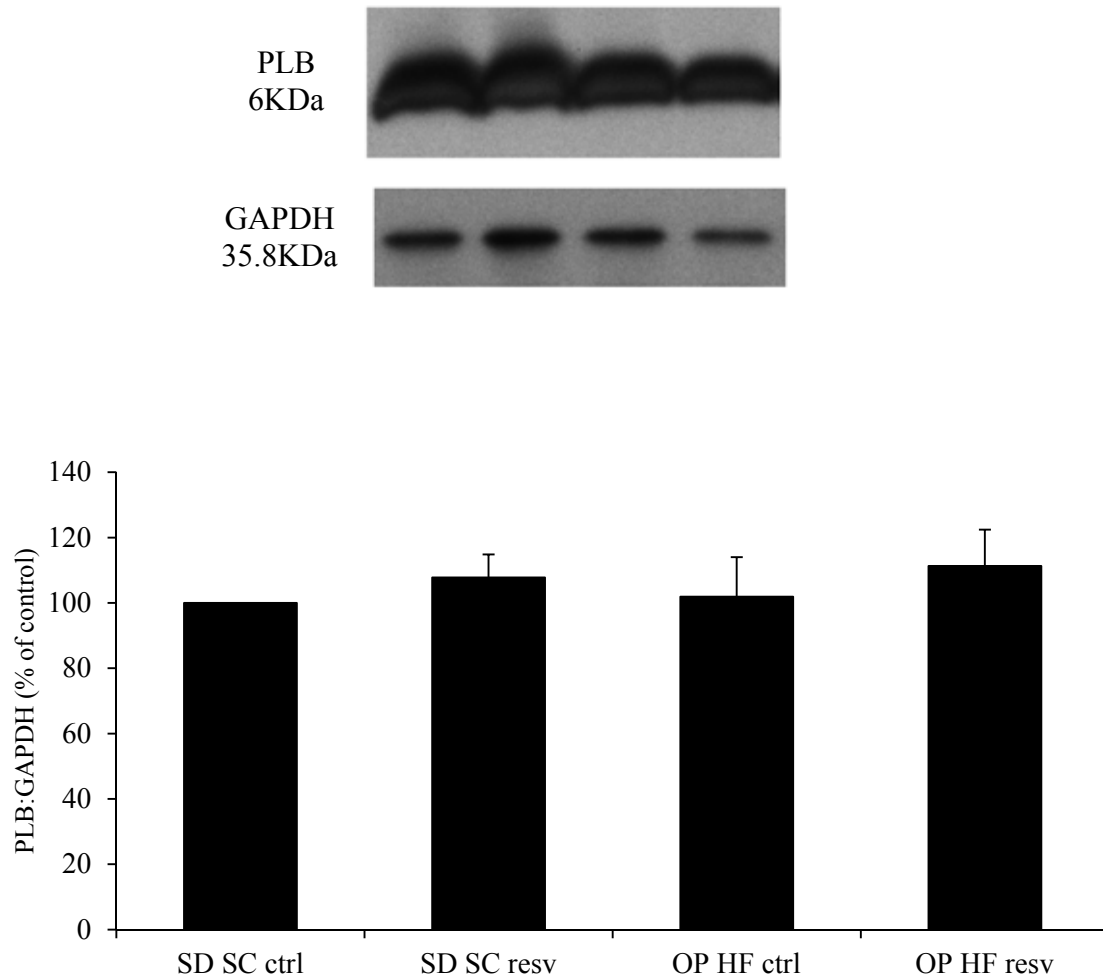


FIGURE 3.25: Phospholamban (PLB) expression levels heart tissue of SD and OP rats at week 17. Data are means±S.E., $n=5-6$. SD, Sprague Dawley; OP, obese prone; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

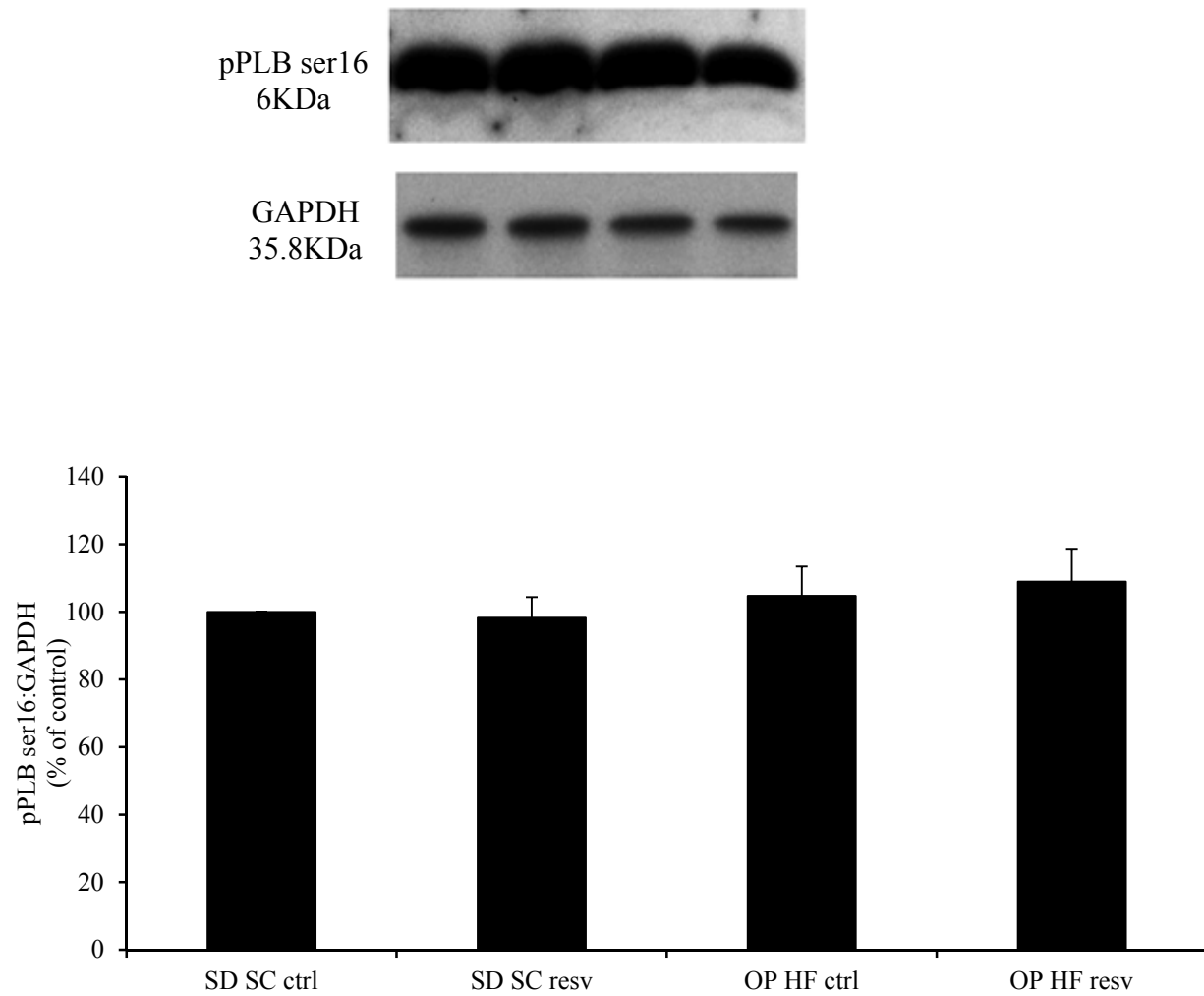


FIGURE 3.26: Phosphorylated phospholamban serine16 (pPLBser16) expression levels heart tissue of SD and OP rats at week 17. Data are means \pm S.E., $n=5-6$. SD, Sprague Dawley; OP, obese prone; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

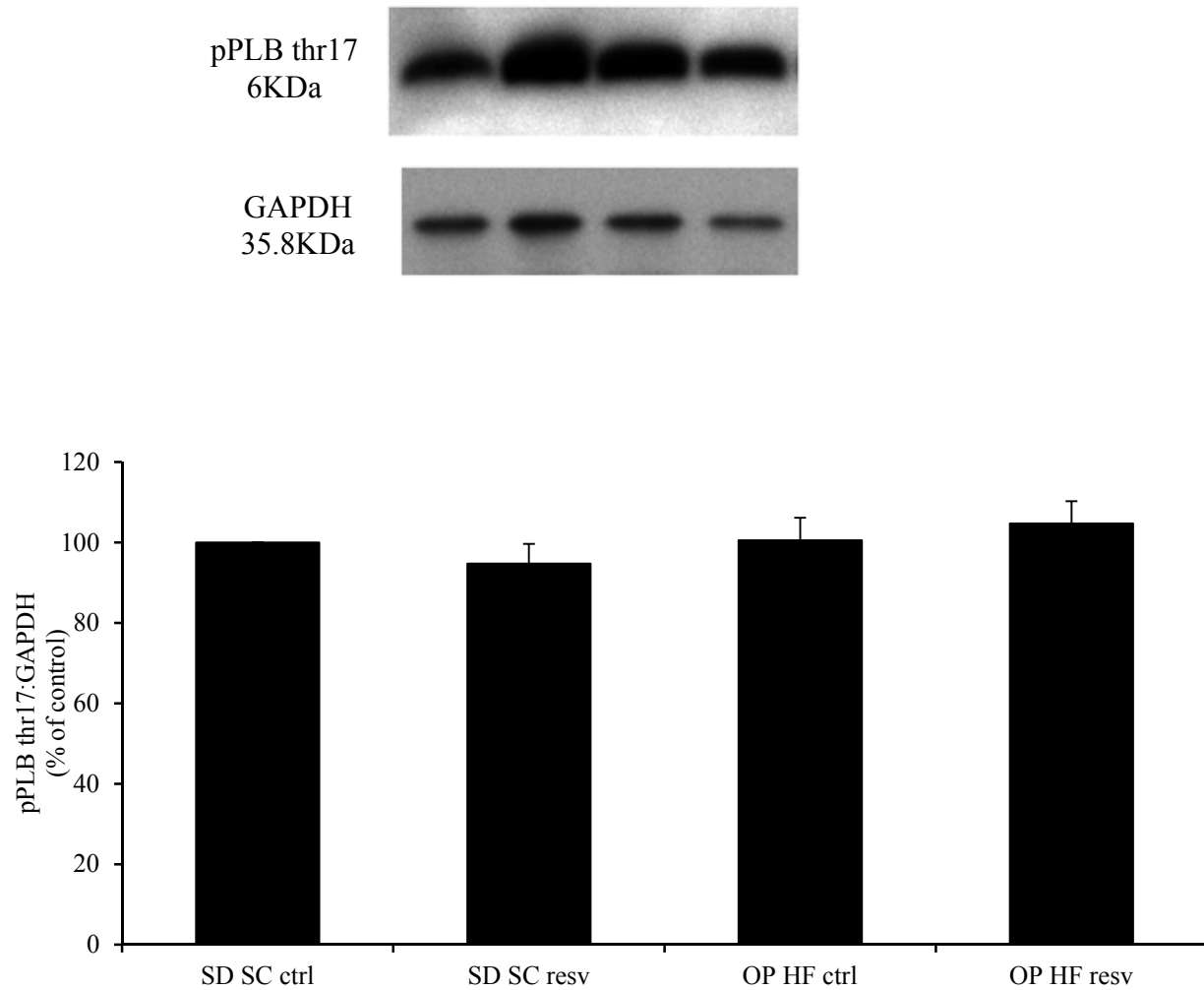


FIGURE 3.27: Phosphorylated phospholamban threonine17 (pPLBthr17) expression levels heart tissue of SD and OP rats at week 17. Data are means \pm S.E., $n=5-6$. SD, Sprague Dawley; OP, obese prone; SC, standard chow; HF, high fat; ctrl, control; resv, resveratrol treated.

3.3 Discussion

Diet, genetic predisposition and physical activity are major factors that influence obesity (Andreasen & Andersen, 2009). Due to differences in the genetic and metabolic makeup, a fraction of the population is resistant to diet-induced obesity and remains lean with increased caloric intake (D. Zhang et al., 2010). However, the leaner subgroup may still develop several comorbidities including cardiovascular disease as observed in the obese populations. Conversely, the obesity paradox is a real phenomenon, wherein obese individuals have a better prognosis as compared to their leaner counterparts with similar health issues (Lavie et al., 2009). This study used two animal models, OP and OR. These animals were fed a HF diet to mimic a population consuming a high energy diet on a daily basis. A set of normal SD rats were fed standard chow and represents the normal population consuming a regular diet with the recommended daily caloric intake. These models enabled us to compare the effect of a HF diet versus a regular diet in promoting the development of heart disease. It is important to highlight that the concentration of resveratrol used in this study (2.5 mg/kg/day in rat, which is approximately a human equivalent dose of 28 mg in a 70-kg person) is much lower than pharmacological levels (which is hundreds of milligrams) and more likely achievable through dietary means. This dosage has been reported in earlier studies from our laboratory which has shown significant cardioprotective benefits with resveratrol treatment (Juric, Wojciechowski, Das, & Netticadan, 2007a; Thandapilly et al., 2010b; Wojciechowski et al., 2010a). A number of other studies have also reported the cardioprotective properties of resveratrol in animals (Raj et al., 2014). More recently, human studies have also reported that resveratrol can protect from cardiovascular diseases (Tome-Carneiro, Gonzalez, et al., 2013b; Tome-Carneiro, Larrosa, et al., 2013). This animal study is one of the first to report that resveratrol is beneficial in reversing diastolic heart

dysfunction in obese rats. This study also reports that the OR rats develop cardiac dysfunction and have a higher mortality with HF feeding.

Echocardiographic data from this study shows that 12 weeks of HF feeding induces diastolic dysfunction as observed by marked prolongation in IVRt in the OP HF and OR HF rats. This observation is consistent with early stages of cardiac dysfunction in obese settings. In the majority of these cases, diastolic dysfunction precedes any systolic changes in the heart of an obese animal (Lavie et al., 2009). OR HF rats were also presented with significant changes in heart function and structure when compared with the SD SC rats. CO, LVIDd and LVPWd were significantly lower at the 12 week time point. However, these changes could be attributed to the fact that OR HF rats were significantly smaller than SD SC and had a smaller heart size. The changes in heart structure parameters LVIDd and LVPWd in OP HF rats were observed only at 6 and 12 week time points. Activation of compensatory remodeling in OP HF rat hearts in the late stages of the study could be a possible reason for this observation.

The changes in body weights were expected with OP HF having significantly higher body weight at the end of the study. The SD SC rats also gained significant body weight by the end of the study and were almost equal to that of OP HF rats. Visceral adiposity data, as measured by the weights of epididymal, perirenal and mesenteric fat pads confirmed that OP HF rats accumulated significantly higher visceral fat than SD SC and OR HF rats. Accordingly, most of the weight gain in OP HF rats could be due to the increase in fat deposits, whereas SD SC rats may have gained weight due to increased lean muscle mass. The increase in adiposity and subsequent adipose tissue dysfunction resulting in the unregulated release of detrimental adipokines is considered a major factor in the development of CVD associated with obesity (Mattu & Randeve, 2013). Compared to OP rats, OR rats have been reported to have higher fat oxidation

capacity (Chang et al., 1990), increased expression proteins involved in lipid metabolism in liver, skeletal muscles and decreased lipogenesis proteins in liver (Joo et al., 2011; Kim et al., 2011; X. Wang et al., 2011). Accordingly, CVD in OR HF rats may not be a result of adipose dysfunction and needs further studies to elucidate the mechanisms. Resveratrol has been reported to reduce adipose tissue accumulation and improve fat mobilization in animals (Baile et al., 2011; Macarulla et al., 2009). However, this study did not observe any reduction in adiposity in resveratrol treated OP HF rats. Compared to previous studies that reporting a reduction in adiposity, this study used a lower dose of resveratrol which may not be sufficient to elicit a reduction in adipose tissue deposition.

Hyperglycemia or high blood sugar is an independent risk factor for the development of cardiac dysfunction (Davidson & Parkin, 2009). In this study, HF feeding resulted in high blood glucose levels in both OP and OR rats and the levels were significantly lower in rats that received resveratrol treatment. This is consistent with other reports on the anti-hyperglycemic effects of resveratrol (Szkudelski & Szkudelska, 2011). Insulin sensitization is one possible mechanism speculated to be involved in the resveratrol-mediated reduction in blood glucose levels (Bagul et al., 2012). This study shows an increase in insulin levels with resveratrol treatment suggesting that increasing insulin secretion could also be a possible mode of action to improve glucose uptake by muscle tissues (Vetterli, Brun, Giovannoni, Bosco, & Maechler, 2011). However, many other studies have reported that resveratrol decrease insulin levels in animals and more recently human studies using resveratrol also reported reduction in insulin levels. Prolonged increase in insulin levels could be detrimental and insulin levels may need to be constantly monitored when administering resveratrol for long term.

High blood pressure is a major risk factor for the development of heart disease. Hypertension is also a common comorbidity observed in obese populations. Resveratrol has been shown to induce a reduction in blood pressure at higher doses ($>10\text{mg/kg}$) (Rivera et al., 2009). Consistent with our earlier reports (Thandapilly et al., 2010a; Wojciechowski et al., 2010b), a low dose of resveratrol (2.5mg/kg) did not reduce blood pressure in both OP and OR rats. However, similar to earlier reports from our laboratory (Juric et al., 2007b; Thandapilly et al., 2010a; Wojciechowski et al., 2010b), this study also reports an improvement in cardiac function independent of any reduction in blood pressure. This also suggests that resveratrol may be acting directly on the cardiac muscle tissues to improve heart function.

Oxidative stress and inflammation are major contributors in the development and progression of CVD. These factors are also involved in the pathogenesis of pathological cardiac remodeling (Tsutsui, Kinugawa, & Matsushima, 2011) which leads to an impairment in cardiac function. Obesity is associated with increased levels of oxidative stress and inflammatory markers in the blood and other tissues (Codoner-Franch, Valls-Belles, Arilla-Codoner, & Alonso-Iglesias, 2011; Fernandez-Sanchez et al., 2011). Adipose tissue dysfunction is one factor contributing to this increase (Crujeiras, Diaz-Lagares, Carreira, Amil, & Casanueva, 2013; Ramos, Shintani, Ikizler, & Himmelfarb, 2008). These circulating factors promote the development of an oxidative stress environment in the cells of the target organ tissues thereby disrupting normal cellular functions. Resveratrol structurally is a weak antioxidant but it is a strong inducer of intracellular antioxidant and anti-inflammatory mechanisms (Soufi, Vardiyani, Sheervalilou, Mohammadi, & Somi, 2012). Consistently, resveratrol treated rats had significantly lower levels of oxidative stress and inflammatory markers. Leptin and adiponectin are among other major adipokines released from adipose tissues. Leptin is a key hormone involved in the energy homeostasis in central and

peripheral tissues. Increased leptin levels are common in obese individuals (Yadav, Kataria, & Saini, 2013). Both excessive leptin levels and leptin deficiency has been found to promote cardiovascular disease (Hou & Luo, 2011). Leptin levels were higher in OR HF rats in this study and resveratrol treatment lowered leptin levels in OR rats. Most of the circulating leptin is secreted by adipocytes while, OR rats have low levels of adipose tissue in the body. Accordingly, other tissues such as the skeletal muscles, liver, pituitary etc. might be targeted by resveratrol to reduce leptin levels. However, since resveratrol treatment did not improve cardiovascular function in OR rats, it is hard to correlate a reduction in leptin levels as a mechanism of resveratrol-mediated cardioprotection in obese animals. Adiponectin has anti-inflammatory properties, improves lipid metabolism, insulin resistance and is also reported to be cardioprotective (Kawano & Arora, 2009). Low adiponectin levels are associated with an increased risk of the development of cardiovascular disease. Adiponectin levels are found to be higher in obese individuals and thought to be a compensatory mechanism to reduce inflammation and improve glucose homeostasis (Engeli et al., 2003; Hatziagelaki et al., 2013). Consistent with this, adiponectin levels were higher in OP HF rats. Resveratrol treatment has been shown to improve adiponectin levels (Tome-Carneiro, Gonzalvez, et al., 2013a). Despite earlier evidences on resveratrol affecting adiponectin levels (Jeon et al., 2012; Tome-Carneiro, Gonzalvez, et al., 2013a), the current study showed no effect of resveratrol on any of the groups.

Hyperlipidemia is another factor that significantly increases the risk of developing heart disease (Nelson, 2013). Usually, higher levels of circulating lipids promote their deposition in blood vessels disrupting the normal flow and creating an ischemic environment in the heart tissues (Kerenyi, Mihalka, Csiba, Bacso, & Bereczki, 2006). It is also possible that there is increased uptake of these circulating lipids by some organ tissues including heart (Schulze, 2009). High

levels of lipid deposition in tissues disrupt cellular function and malfunction ensues. This phenomenon is called lipotoxicity and considered as a possible mechanism for development of cardiac dysfunction in obesity (Schulze, 2009). Resveratrol has been reported to have antihyperlipidemic properties (Xie, Han, Chen, & He, 2014). This study also shows that resveratrol treated animals have significantly lower levels of TG and LDL.

Diastolic dysfunction of the heart involves defects in calcium handling and abnormalities in calcium regulating proteins (Asp, Martindale, Heinis, Wang, & Metzger, 2013). SERCA 2a is the major protein involved in the re-uptake of calcium resulting in cardiac relaxation. The function of SERCA 2a is inhibited by PLB and helps maintain the sequence of calcium cycling. PLB remains bound to SERCA during the systolic phase. Phosphorylation of PLB at either serine 16 or threonine 17 releases PLB enabling the uptake of calcium by SERCA and relaxation of the heart (Marks, 2013b). Diastolic dysfunction is usually accompanied by a decrease in the expression of SERCA, PLB or changes in PLB phosphorylation preventing the release of PLB from SERCA (Periasamy & Janssen, 2008). However, in this study there was no change in expression of any of these proteins detected and therefore, other mechanisms may be involved in the genesis of these contractile abnormalities. This may include oxidation (Balderas-Villalobos et al., 2013) and post-translational modification (Kho et al., 2011) of calcium cycling proteins.

In summary, this study reports for the first time that resveratrol reverses cardiac dysfunction in a model of diet-induced obesity. We also demonstrate for the first time that HF induces abnormalities in cardiac function in HF fed OR rats. The OR rats in this study had impaired cardiac function accompanied with higher mortality. Why the beneficial effects of resveratrol were specific to OP and not OR rats could be attributed to the discrepancy in the origin of cardiovascular abnormalities in these two models, as evident from the data presented here. The

ineffectiveness of resveratrol in reversing HF-induced cardiac dysfunction in OR rats is an important subject to be investigated in future studies. The reduction in hyperglycemia, hyperlipidemia, oxidative stress and inflammation could be mediating the cardioprotective effect of resveratrol in obese animals.

CHAPTER 4

RESVERATROL MEDIATED PREVENTION OF CONTRACTILE ABNORMALITY IN PALMITIC ACID EXPOSED ADULT RAT CARDIOMYOCYTES

4.1 RATIONALE AND HYPOTHESIS

Obesity is a condition associated with fat accumulation which is normally stored in the adipose tissues. However, in pathological conditions there is a dysregulation of fat storage which is associated with elevated blood lipid levels (Trayhurn, 2014). High levels of circulating FA will result in increased deposition of FA in organs including the heart, and disrupt the normal functioning of these organs (Drosatos & Schulze, 2013; Ussher, 2014). Increased consumption of fat, rich in saturated fatty acids has been shown to result in higher LDL levels and increase the risk for heart disease (Faghihnia, Mangravite, Chiu, Bergeron, & Krauss, 2012; Siri-Tarino, Sun, Hu, & Krauss, 2010). FA are a major source of energy for the heart, however, excess FA exert an adverse effect on cardiomyocytes (Sparagna, Hickson-Bick, Buja, & McMillin, 2001). Saturated long chain fatty acids causes more harm when compared to unsaturated fatty acids (de Vries et al., 1997; Van Bilsen, de Vries, & Van der Vusse, 1997). In the *in vivo* study discussed earlier, rats fed with a high fat diet developed cardiac diastolic abnormalities. Lard was the source of fat in the high fat diet used for the study. Palmitic acid (PA) is present in large quantities in lard and is also a long chain saturated fatty acid (Kouba & Mourot, 2011). It has been shown that PA increase cholesterol levels significantly and higher than other saturated fatty acids (Denke & Grundy, 1992). Arguably, palm oil which contains high levels of PA has shown to increase cardiovascular incidences in human populations (Fattore & Fanelli, 2013; Martinez-Ortiz, Fung, Baylin, Hu, & Campos, 2006). Based on these evidences it could be speculated that high levels of PA in lard based diet might have contributed to the cardiovascular complications seen in animal model of obesity. Also, resveratrol mediated cardioprotection in high fat fed animals might be associated with blocking PA induced adverse effect on the heart. We have earlier reported an *in vitro* model of adult cardiomyocytes to study the mechanism of resveratrol mediated cardioprotection in hypertension induced cardiac complications (Thandapilly et al.,

2011). Similarly, adult cardiomyocytes pretreated with resveratrol will be exposed to high levels of PA. The *in vivo* study has shown that resveratrol administration improved diastolic dysfunction in high fat fed rats. However, it is not certain if the effects of resveratrol were merely a result of improved oxidative stress and inflammation or, if resveratrol indeed can directly target cardiomyocytes via improving their function. Diastolic dysfunction observed in the *in vivo* study means the heart was not able to relax properly which is a result of improper cardiomyocyte contractile function (Fentzke et al., 1999). Therefore, contractile abnormalities in cardiomyocytes exposed to PA will be assessed in this study. The lipotoxic effects of FA could also result in cardiomyocyte cell death (Dyntar et al., 2001). **Our first hypothesis is that resveratrol treatment will prevent contractile dysfunction and cardiomyocyte cell death associated with *in vitro* exposure to PA.**

Contractile function of the heart is maintained through regulation of Ca^{2+} concentration inside the cell (Bers, 2008). This intracellular Ca^{2+} regulation involve a number of proteins located in the sarcoplasmic reticulum which is the calcium storage site inside the cell and in the myofibrils. SERCA2a, PLB, the phosphorylated form of PLB, pPLB Ser16 and pPLB Thr17 and the phosphorylated form of troponin (TnI) are the major proteins that help maintain Ca^{2+} concentration during the contraction-relaxation cycles (Bers, 2008). Any abnormal changes in the levels or state of these proteins will directly influence the contractile function of cardiomyocytes which in turn affects the function of the heart (Daniels, Naya, Rundell, & de Tombe, 2007). Changes in expression levels of SERCA2a, SERCA2a to PLB ratio, phosphorylation of PLB and TnI are known to affect cardiac function and these changes are also observed in diseased hearts (Marks, 2013a). Accordingly, it is important to examine whether PA exposure induces any changes to the Ca^{2+} cycling and regulatory proteins. Resveratrol has been

reported to affect Ca^{2+} cycling by altering the expression of Ca^{2+} proteins (Buluc & Demirel-Yilmaz, 2006; McCalley, Kaja, Payne, & Koulen, 2014; Sulaiman et al., 2010). Accordingly, **the second hypothesis is that treatment with resveratrol will prevent any changes in Ca^{2+} cycling and regulatory proteins, associated with cardiomyocyte exposure to PA.**

4.2 METHODS AND MATERIALS

All experimental protocols used in this study were approved by the University of Manitoba Animal Care Committee and are in agreement with the Canadian Council on Animal Care and Use of Experimental Animals (vol. 1, 2nd ed., 1993).

All chemicals used in this study were purchased from Sigma-Aldrich, Ontario, Canada.

4.2.1 Cardiomyocyte isolation

Ventricular myocytes were isolated from 12-week old male Sprague- Dawley rats (200-250 g) as described previously (Thandapilly et al., 2011). In brief, an intramuscular injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) was used to anesthetize the animal. Excised hearts were quickly transferred to a Langendorff perfusion apparatus and perfused with calcium (Ca^{2+}) free buffer containing (in mM); 90 NaCl, 10 KCl, 1.2 KH_2PO_4 , 5 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 NaHCO_3 , 30 taurine and 20 glucose for 5 min. The perfusion medium was then switched to Ca^{2+} free buffer containing collagenase (0.05%) and bovine serum albumin (0.2%). After 30min ventricles were cut into small pieces, incubated in a 37°C waterbath and separated into individual cardiomyocytes by slow pipetting. Cardiomyocytes were then suspended in buffer containing Ca^{2+} and the cells were allowed to settle. The supernatant was then replaced with Ca^{2+} buffers containing higher concentration of calcium (150 mM). This step was repeated twice to increase the extracellular Ca^{2+} concentration to 500 mM and then to 1.2 M. Cells were finally resuspended in Medium-199 (M199) containing 10% fetal bovine serum and transferred to laminin coated culture dishes. After 2 hours of incubation in a CO_2 incubator (5% CO_2 and 95% O_2), the existing medium was replaced with serum free M199 supplemented with 5 mM taurine,

2 mM carnitine, 1 mM creatine and 1 mM insulin. All cells were incubated for 24 hours at 37°C before starting any experimental procedure.

For treatment with resveratrol, 20 mM stock was prepared in 50% ethanol. 1.5 µl of resveratrol was added per milliliter of cell culture medium was used for the cell treatments, which gives a final resveratrol concentration of 30 µM in the medium. 10 mM PA stock was prepared by dissolving in 12.5 mM NaCl at 70°C. For conjugation with BSA, 5% BSA was mixed with PA in 1:3 ratio and incubated at 37°C for 30 min prior to addition into cardiomyocyte culture medium.

4.2.2 Cardiomyocyte contractility measurement

Cardiomyocytes were incubated with and without resveratrol (30µM) for 45 min and further exposed to PA (10,100 and 200 µM) for 4 hours. The contractile properties of cardiomyocytes were assessed using an Ionoptix Myocam system (Ionoptix Inc., Milton, MA, USA). Briefly, myocytes were placed on a mounting chamber attached to an inverted microscope and superfused with a buffer containing 1mM CaCl₂, 1 mM MgCl₂, 4 mM KCl and 131 mM NaCl (prepared as stock and stored at 4°C). On the day of experiment, 10mM HEPES and 10mM glucose were added and pH was adjusted to 7.4 before use. To measure contractility, myocytes were stimulated at a frequency of 1Hz, 10volt for 30 sec. Cell length, peak shortening, maximal velocities of shortening (+dL/dT) and relengthening (-dL/dT), time to 90% relengthening (TR90) and time to peak shortening (TPS) were calculated based on cell shortening and relengthening of the cardiomyocyte. Only, properly attached, long and rod shaped cells that are not touching other cardiomyocytes were selected for assessments. Cardiomyocytes with irregular beating upon stimulation were also excluded from measurement. At least n=38-50 cells from 5-6 isolations were assessed from each group.

4.2.3 Cell morphology measurements

Cardiomyocytes were incubated with and without resveratrol for 45 min and further exposed to 200 μ M PA for 24 hours. After 24 hours, phase contrast images were captured using an Olympus microscope. A total of 100 cells from three independent cardiomyocyte isolations were used for the analysis using ImageJ software. Cell death was analyzed by calculating the percentage of rod shaped cells.

4.2.4 Hoechst staining

Apoptosis was measured using a hoechst pentahydrate (bis-benzimide) stain (Invitrogen, ON, Canada). Cardiomyocytes were pretreated with resveratrol (30 μ M) for 45 min and then exposed to 200 μ M PA. After 24 hours of PA exposure, cells were then washed with warm 1X phosphate-buffered saline (PBS) and 2 μ M Hoechst stain was added. Cells were incubated with Hoechst stain for 10 min in the dark and cells were imaged using an Olympus fluorescent microscope. Cells with condensed nuclei were counted to measure apoptosis.

4.2.5 TBARS measurement

The measurement of TBARS was done after 4 hours of incubation with resveratrol and PA. Whole cell lysate was used for the assay. After incubation cells were scraped off the culture dish into microfuge tubes using 1mL ice cold 1X PBS. It was then centrifuged for 5 min at 3000 rpm at 4°C. 200 μ L lysis buffer (Sigma Aldrich, ON, Canada) was added and sonicated to lyse the cells.

4.2.6 Western blotting

Cardiomyocytes were extracted after 4 hours of incubation with 200 μ M PA and 30 μ M resveratrol. Cells were washed with 1X ice cold PBS and then scrapped of from the plates using 1mL of 1X PBS into eppendorf tubes. It was then centrifuged for 5 min at 3000rpm. The

supernatant was discarded and 150 μ L lysis buffer (Sigma Aldrich, ON, Canada) was added. Cells were then sonicated for 5sec three times with 10 sec intervals between sonication. Lysed cells were then centrifuged for 10 min at 6000 rpm and the supernatant was collected for western blotting. Protein concentration was estimated using Lowry method (Biorad, ON, Canada). Protein samples were then mixed with SDS-PAGE sample buffer and loaded onto gels at a concentration of 60 μ g/well. A 10% gel was prepared for SERCA2a and a 15% gel was used for PLB measurement. Western blotting was performed as explained earlier in the *in vivo* section. cTnI and its phosphorylated troponin I (pTnI) were measured by reprobng the SERCA2a membrane whereas phosphorylated PLB's were measured in the PLB membrane. GAPDH was used as the gel loading control.

4.2.7 Statistical analysis

Data were expressed as a mean \pm standard error (S.E.M). Statistical analysis of data was performed by applying one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test. A P value <0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 Effect of PA and resveratrol on cardiomyocyte contractile function

Cell length, +dL/dT, TPS and TR90 were unaffected by either exposure to resveratrol and/or PA (Figure : 4.1, 4.3, 4.5, and 4.6). Peak shortening was significantly lower in resveratrol treated control cardiomyocytes and resveratrol treated cardiomyocytes exposed to 10 μ M PA (Figure : 4.2). The peak shortening of all other groups remained unaffected by resveratrol and/or PA exposure. -dL/dT was significantly lower in cardiomyocytes exposed to the highest dose (200 μ M) of PA. Resveratrol treated cardiomyocytes exposed to 10 μ M PA were also significantly lower. Pretreatment with resveratrol prevented a PA (200 μ M) induced decrease in -dL/dT (Figure : 4.4).

4.3.2 Effect of PA and resveratrol on cardiomyocyte morphology

The percentage of rod shaped cells was significantly lower after exposure to 200 μ M PA. Pretreatment with resveratrol prevented this decrease in rod shaped cardiomyocytes (Figure : 4.7).

4.3.3 Effect of PA and resveratrol on cardiomyocyte apoptosis

The number of cardiomyocytes with condensed nuclei was significantly increased after exposure to 200 μ M PA. Pretreatment with resveratrol significantly reduced the number of apoptotic cells (Figure : 4.8 a and b).

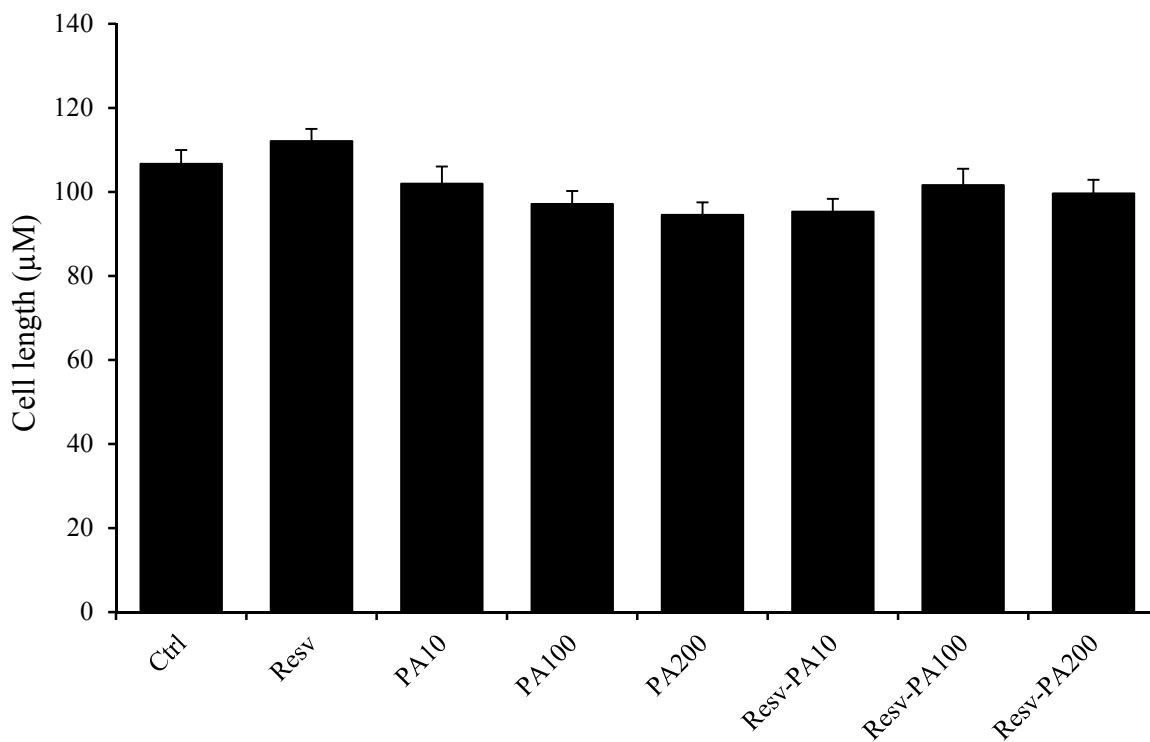


FIGURE 4.1: Effect of resveratrol on palmitic acid (PA) induced changes in cardiomyocyte cell length (resting cell length) at 4 hour time point. n=38-50 cells from four independent experiments. Ctrl, control; Resv, resveratrol.

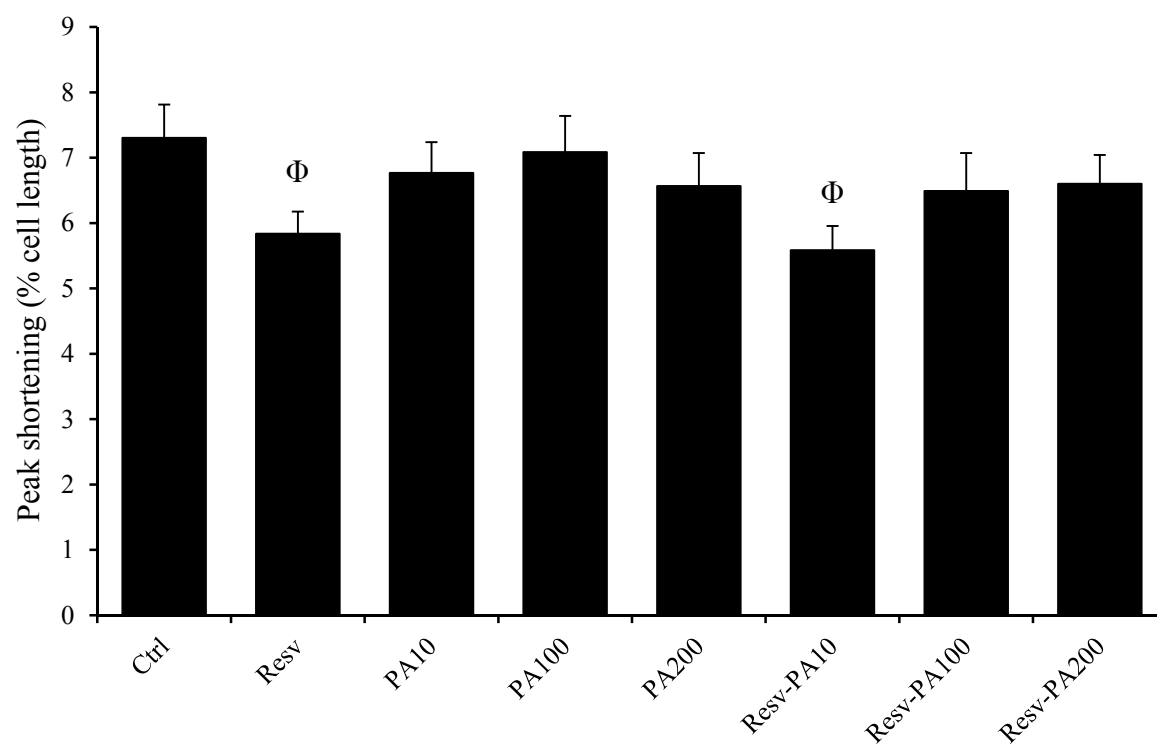


FIGURE 4.2: Effect of resveratrol on palmitic acid (PA) induced changes in cardiomyocyte peak shortening (PS) at 4 hour time point. n=38-50 cells from four independent experiments. Φ $P < 0.05$ vs control. Ctrl, control; Resv, resveratrol.

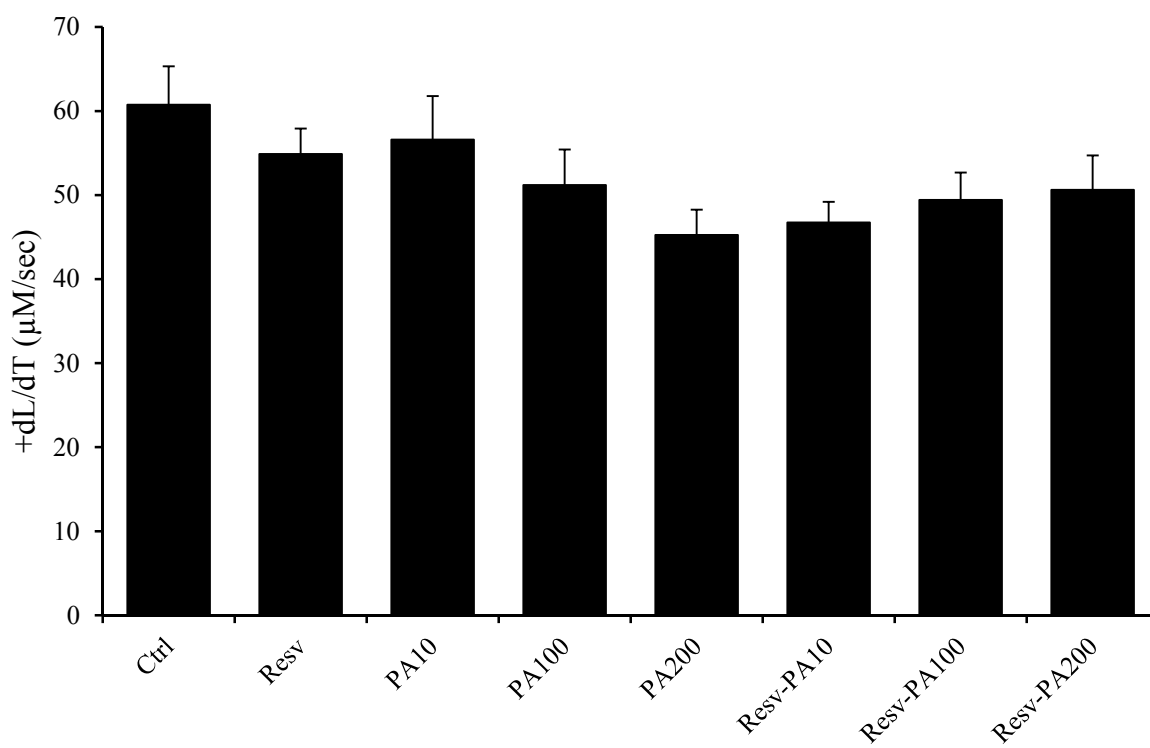


FIGURE 4.3: Effect of resveratrol on palmitic acid (PA) induced changes in cardiomyocyte maximal velocity of shortening (+dL/dT) at 4 hour time point. n=38-50 cells from four independent experiments. Ctrl, control; Resv, resveratrol.

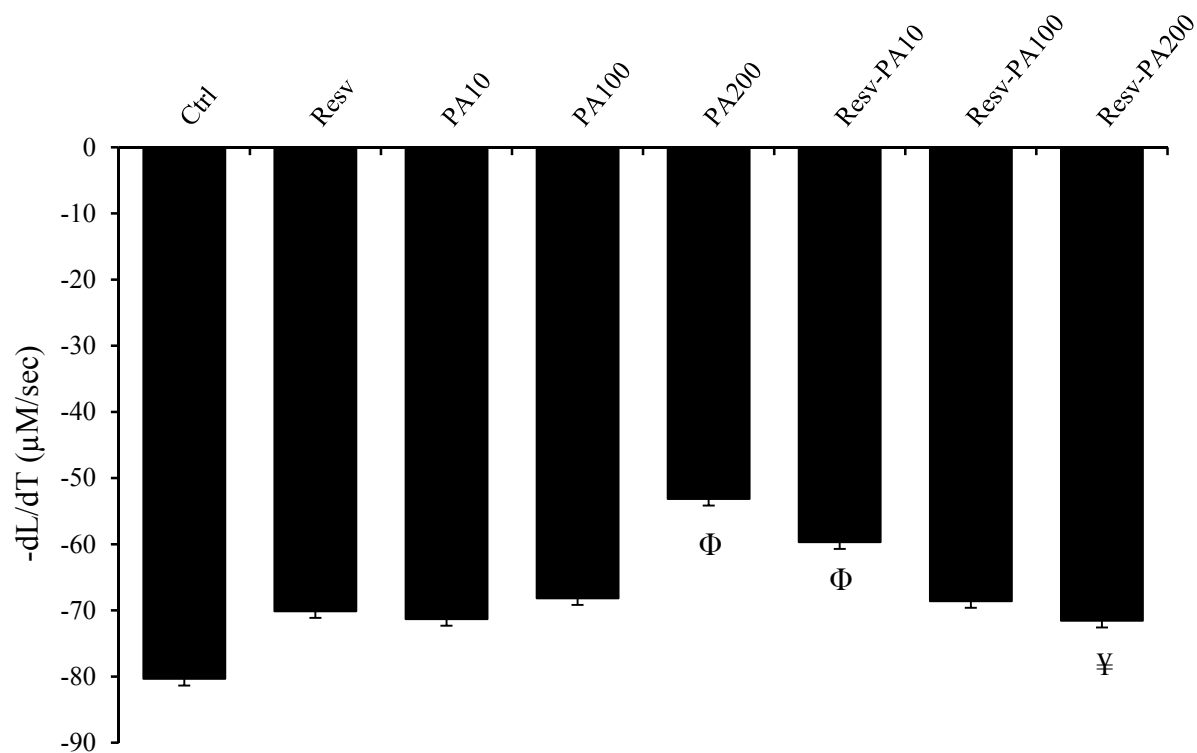


FIGURE 4.4: Effect of resveratrol on palmitic acid (PA) induced changes in cardiomyocyte maximal velocity of relengthening ($-dL/dT$) at 4 hour time point. $n=38-50$ cells from four independent experiments. Φ $P<0.05$ vs control; ¥ $P<0.05$ vs PA. Ctrl, control; Resv, resveratrol.

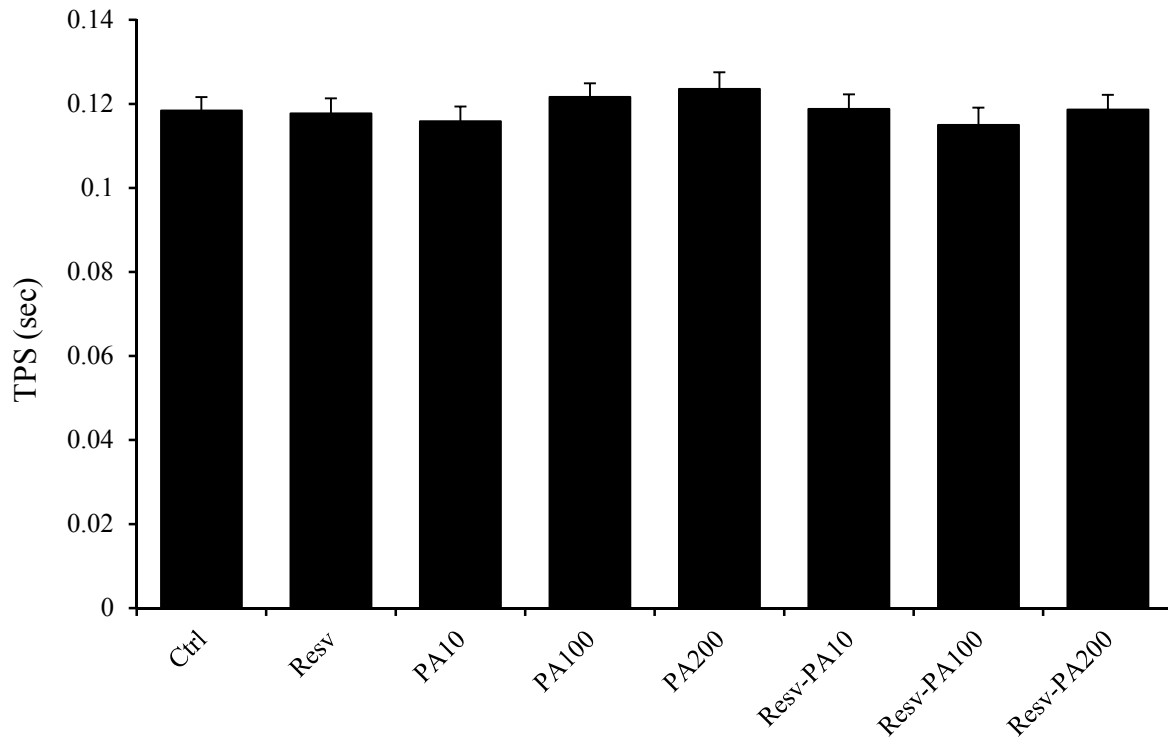


FIGURE 4.5: Effect of resveratrol on palmitic acid (PA) induced changes in cardiomyocyte time to peak shortening, TPS at 4 hour time point. n=38-50 cells from four independent experiments. Ctrl, control; Resv, resveratrol.

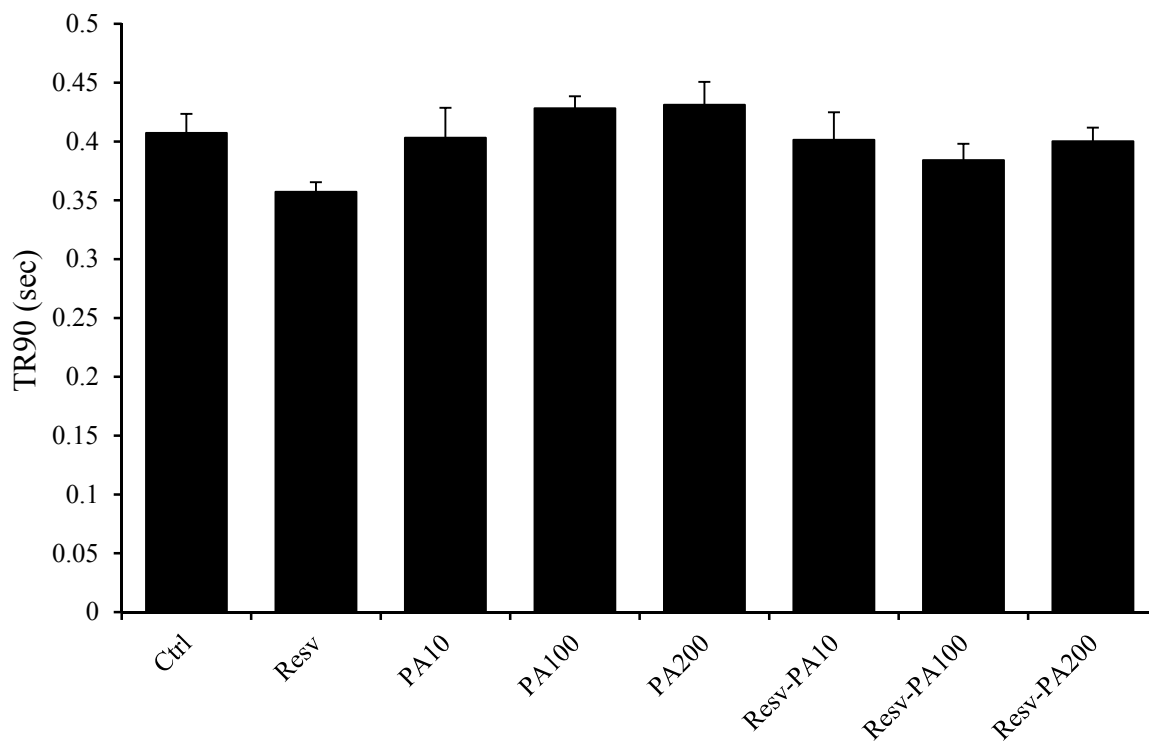


FIGURE 4.6: Effect of resveratrol on palmitic acid (PA) induced changes in cardiomyocyte time to 90% relengthening, TR90 at 4 hour time point. n=38-50 cells from four independent experiments. Ctrl, control; Resv, resveratrol.

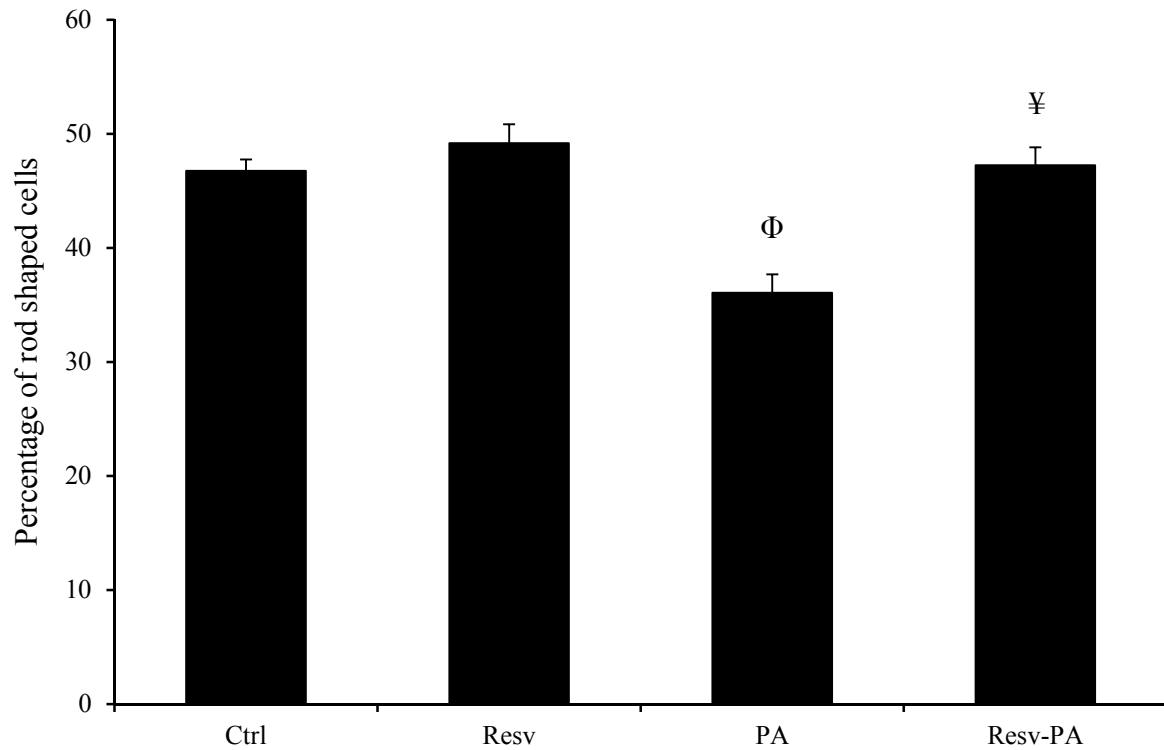


FIGURE 4.7: Effect of resveratrol on palmitic acid (PA) induced changes in cardiomyocyte morphology at 24 hour time point. n=100 cells from three independent experiments. Φ $P < 0.05$ vs control; ¥ $P < 0.05$ vs PA. Ctrl, control; Resv, resveratrol.

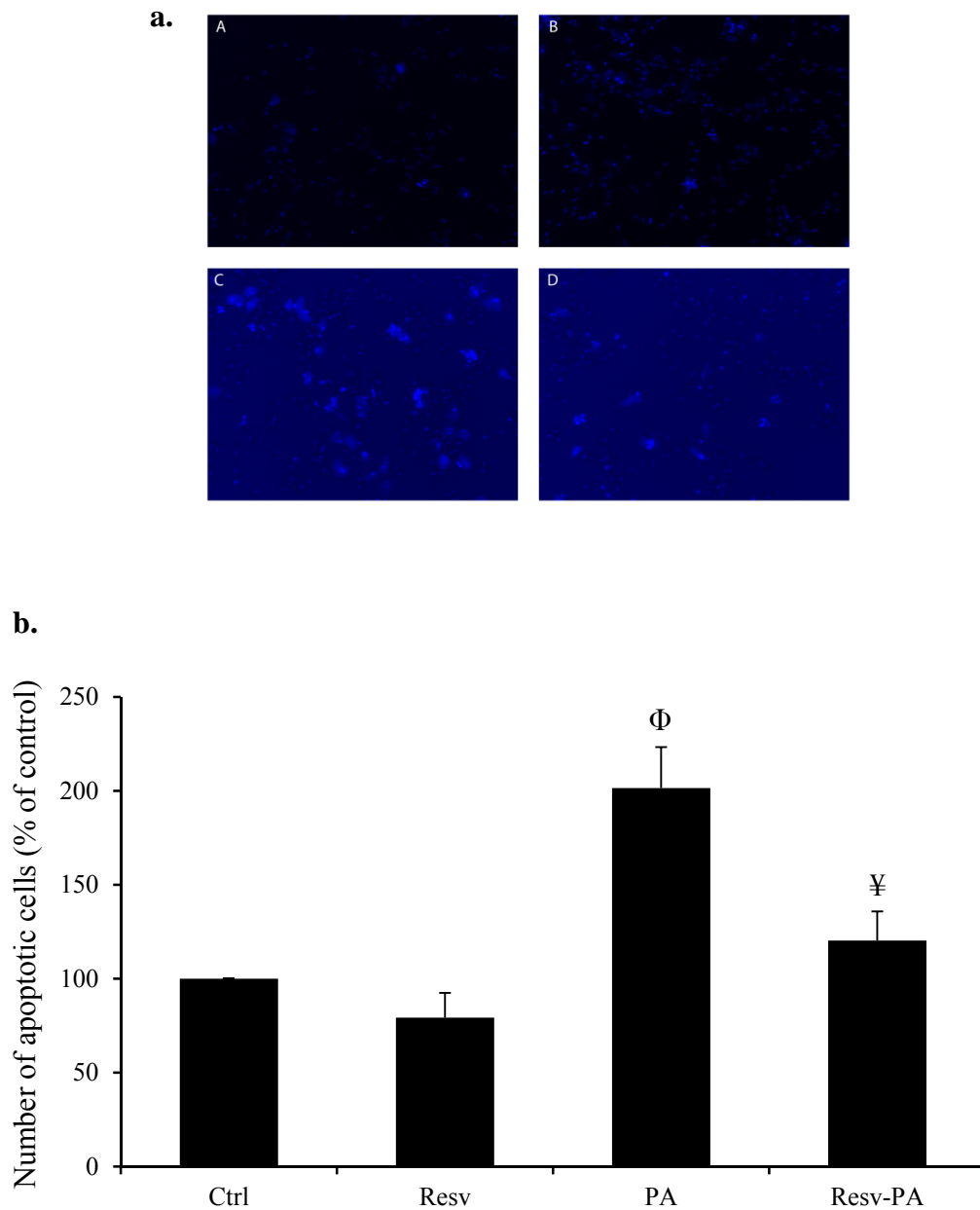


FIGURE 4.8: Effect of resveratrol on palmitic acid (PA) induced cardiomyocyte apoptosis. a. representative images showing condensed nuclei at 24 hour time point. A) ctrl; B) Resv; C) PA; D) Resv-PA. b. graphical representation of number of apoptotic cells in each group. n=3. Φ P<0.05 vs control; ¥ P<0.05 vs PA. Ctrl, control; Resv, resveratrol.

4.3.4 Effect of PA and resveratrol on oxidative stress in cardiomyocytes

PA exposure had no significant effect on the level of TBARS in cardiomyocytes. Resveratrol treated control and PA exposed cells had lower levels of TBARS but the decrease was not statistically significant (**Figure : 4.9**).

4.3.5 Effect of PA and resveratrol on expression levels of Ca²⁺ regulatory proteins

All protein expression levels were normalized using corresponding GAPDH levels. Expression of SERCA was suppressed by almost 17% after exposure to PA and pretreatment with resveratrol prevented this decrease (**Figure : 4.10**). However, these changes in SERCA2a expression levels did not reach statistical significance. PLB levels were unaffected after exposure of cardiomyocytes to resveratrol and/or PA (**Figure : 4.11**). The SERCA to PLB ratio showed a similar trend to that of SERCA2a expression levels however, these changes did not reach statistical significance (**Figure : 4.12**). The levels of phosphorylated PLB, pPLBSer16 and pPLBThr17 were unaffected by exposure to resveratrol and/or PA (**Figure : 4.13 and 4.14**).

4.3.6 Effect of PA and resveratrol on expression levels of cardiac troponin I

The expression level of cTnI was unchanged after exposure to resveratrol and/or PA (**Figure : 4.15**). Phosphorylated TnI was also unaffected by treatment with or without resveratrol and PA (**Figure : 4.16**). Similarly, the ratio of TnI to pTnI was unchanged in all groups (**Figure : 4.17**).

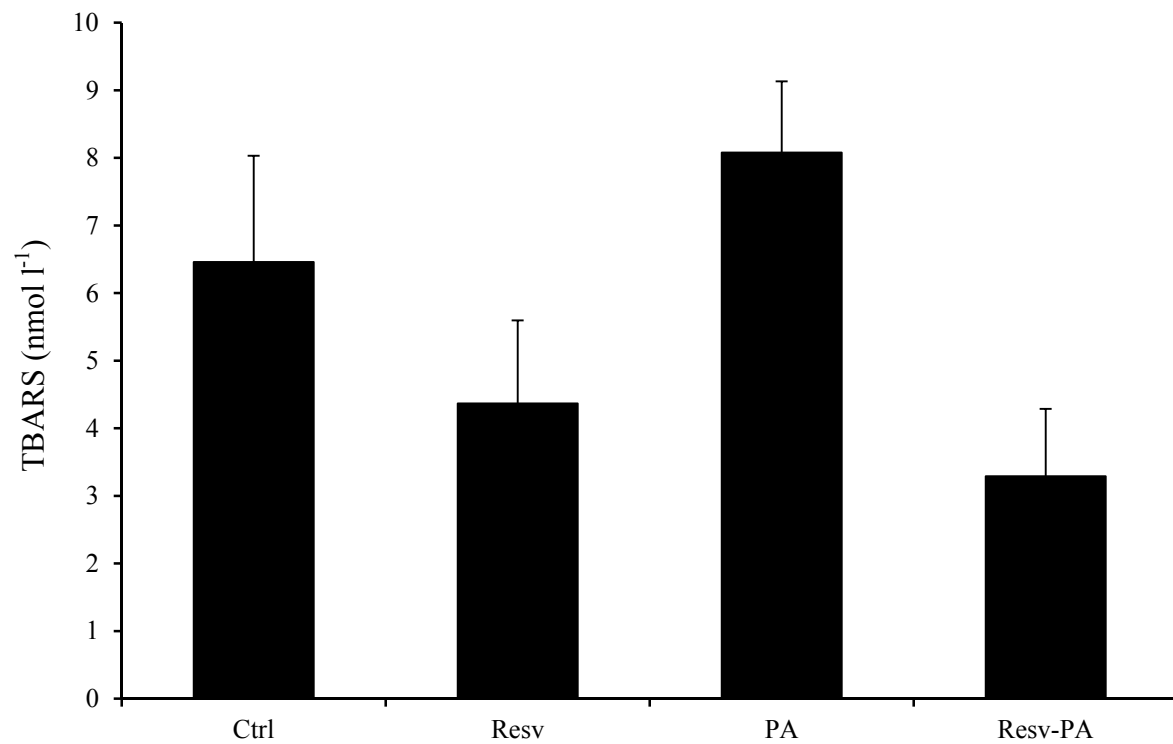


FIGURE 4.9: Effect of resveratrol on palmitic acid (PA) induced oxidative stress in cardiomyocytes at 4 hour time point. n=3. TBARS, thiobarbituric reactive acid substance; Ctrl, control; Resv, resveratrol.

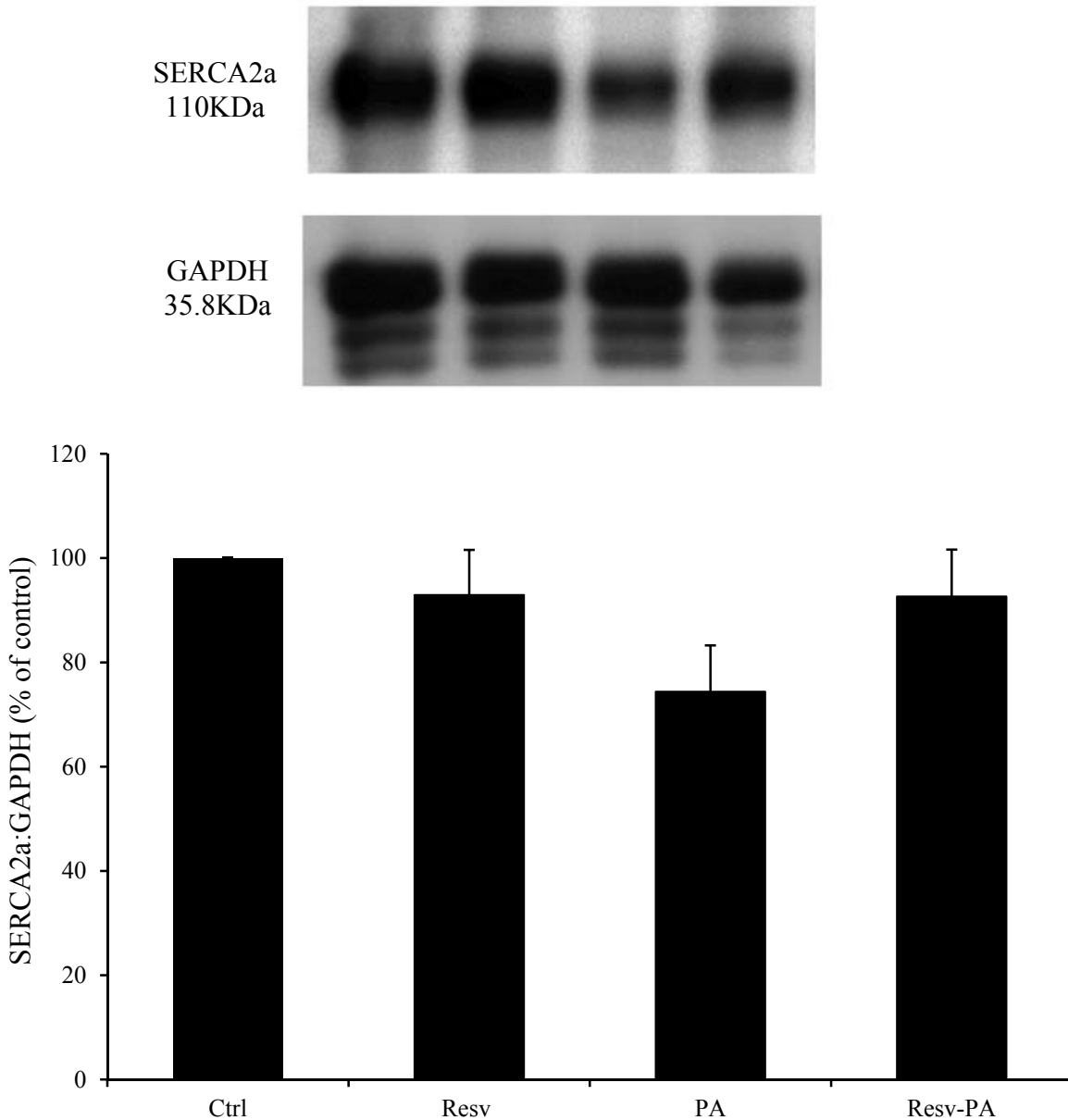


FIGURE 4.10: Effect of palmitic acid (PA) and resveratrol on expression of sarcoendoplasmic reticulum calcium ATPase 2a (SERCA2a) in cardiomyocytes at 4 hour time point. n=5. Representative protein bands presented here have been adjusted for brightness universally across the image. However, band density was measured using the original scans without any changes. Ctrl, control; Resv, resveratrol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

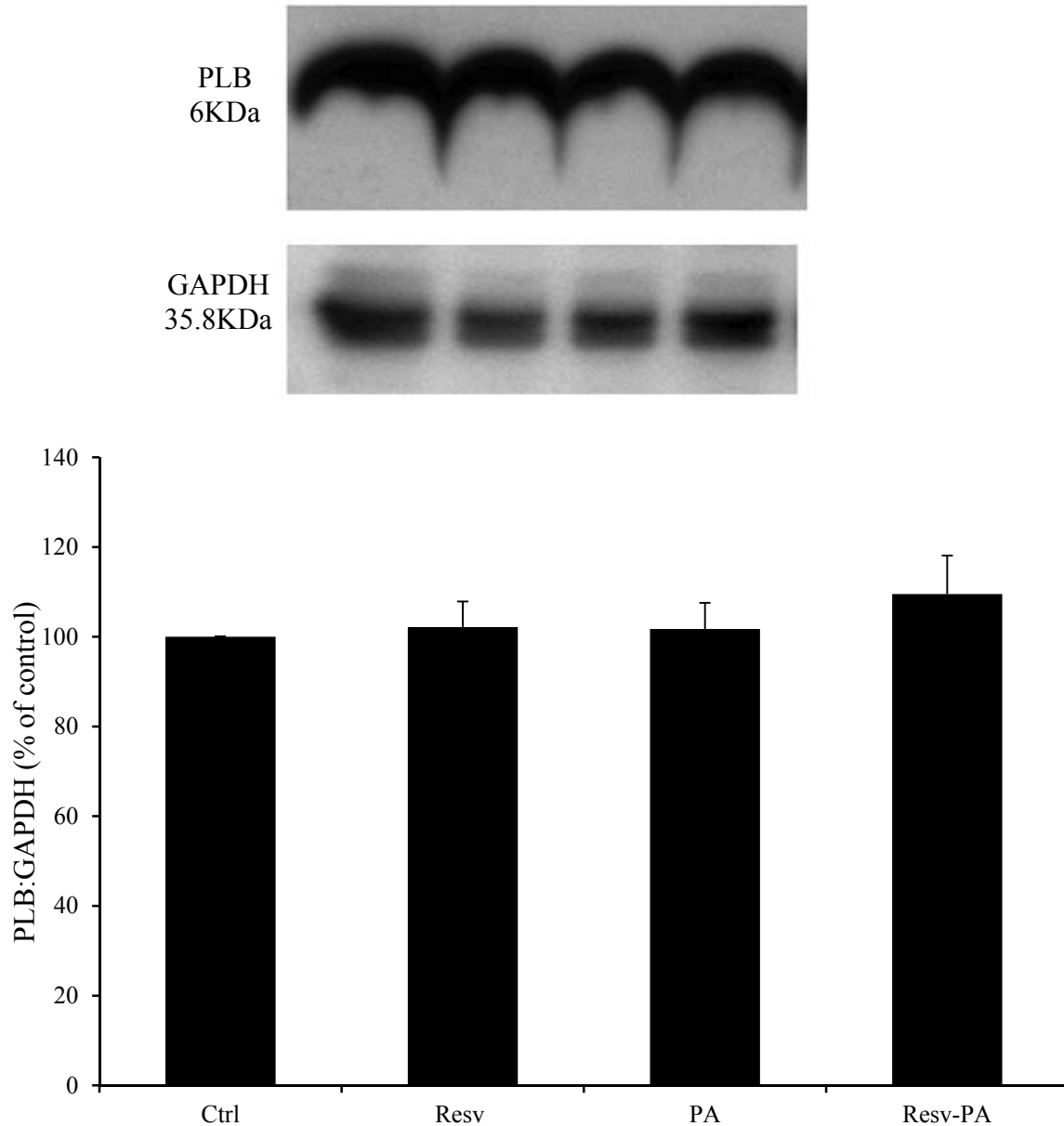


FIGURE 4.11: Effect of palmitic acid (PA) and resveratrol on expression of phospholamban (PLB) in cardiomyocytes at 4 hour time point. n=5. Representative protein bands presented here have been adjusted for brightness universally across the image. However, band density was measured using the original scans without any changes. Ctrl, control; Resv, resveratrol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

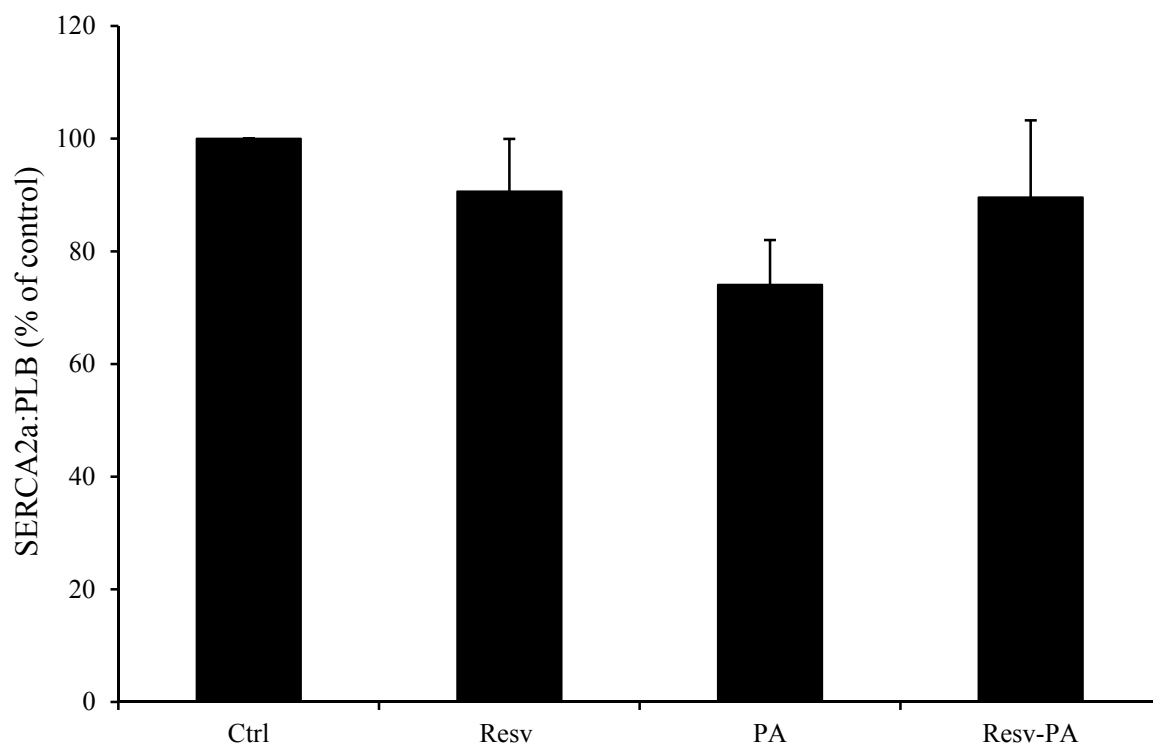


FIGURE 4.12: Effect of palmitic acid (PA) and resveratrol on sarcoendoplasmic reticulum calcium ATPase 2a (SERCA2a) to phospholamban (PLB) expression ratio in cardiomyocytes at 4 hour time point. n=5. Ctrl, control; Resv, resveratrol.

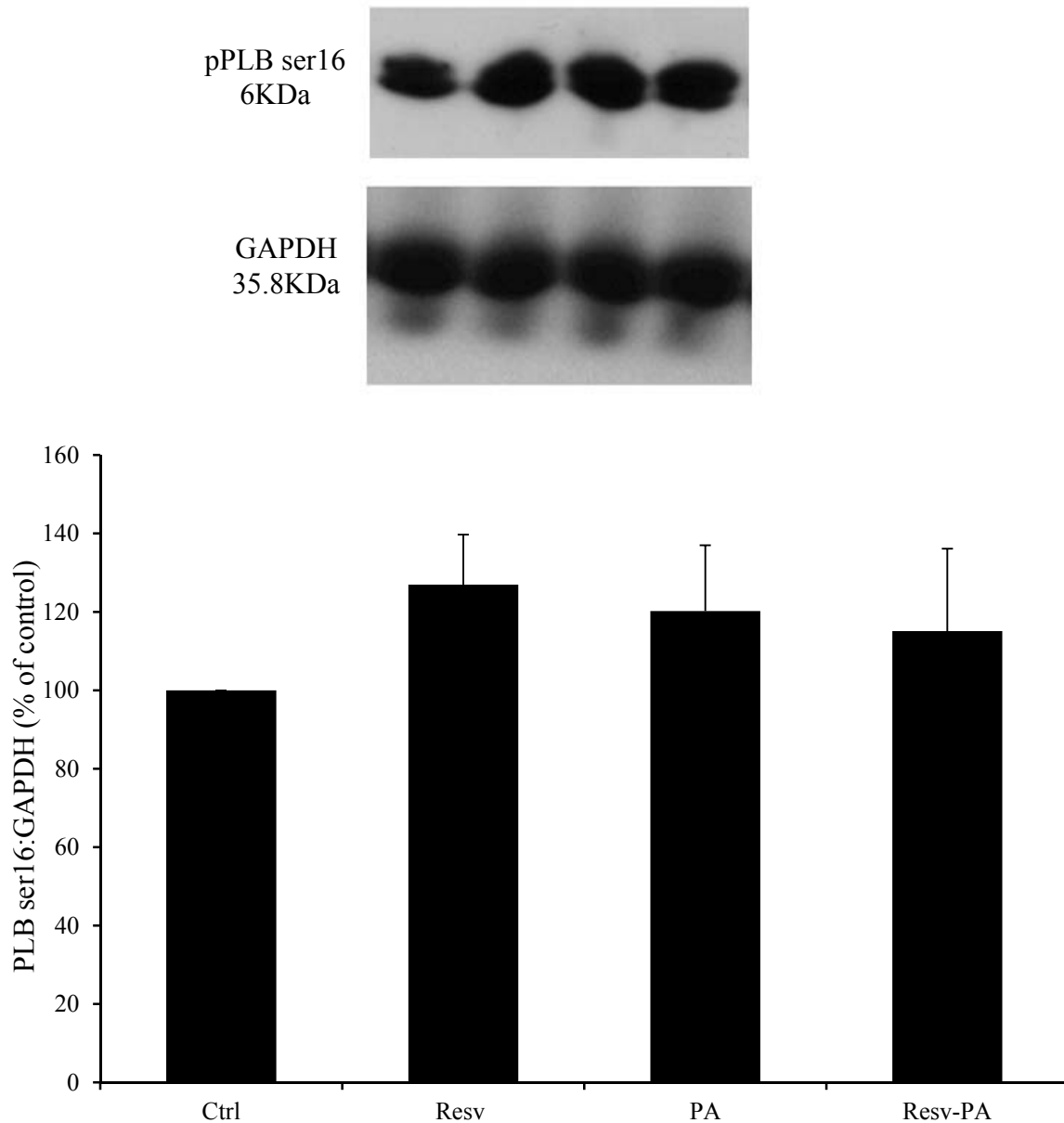


FIGURE 4.13: Effect of palmitic acid (PA) and resveratrol on serine phosphorylated phospholamban (PLB ser16) in cardiomyocytes at 4 hour time point. n=5. Representative protein bands presented here have been adjusted for brightness universally across the image. However, band density was measured using the original scans without any changes. Ctrl, control; Resv, resveratrol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

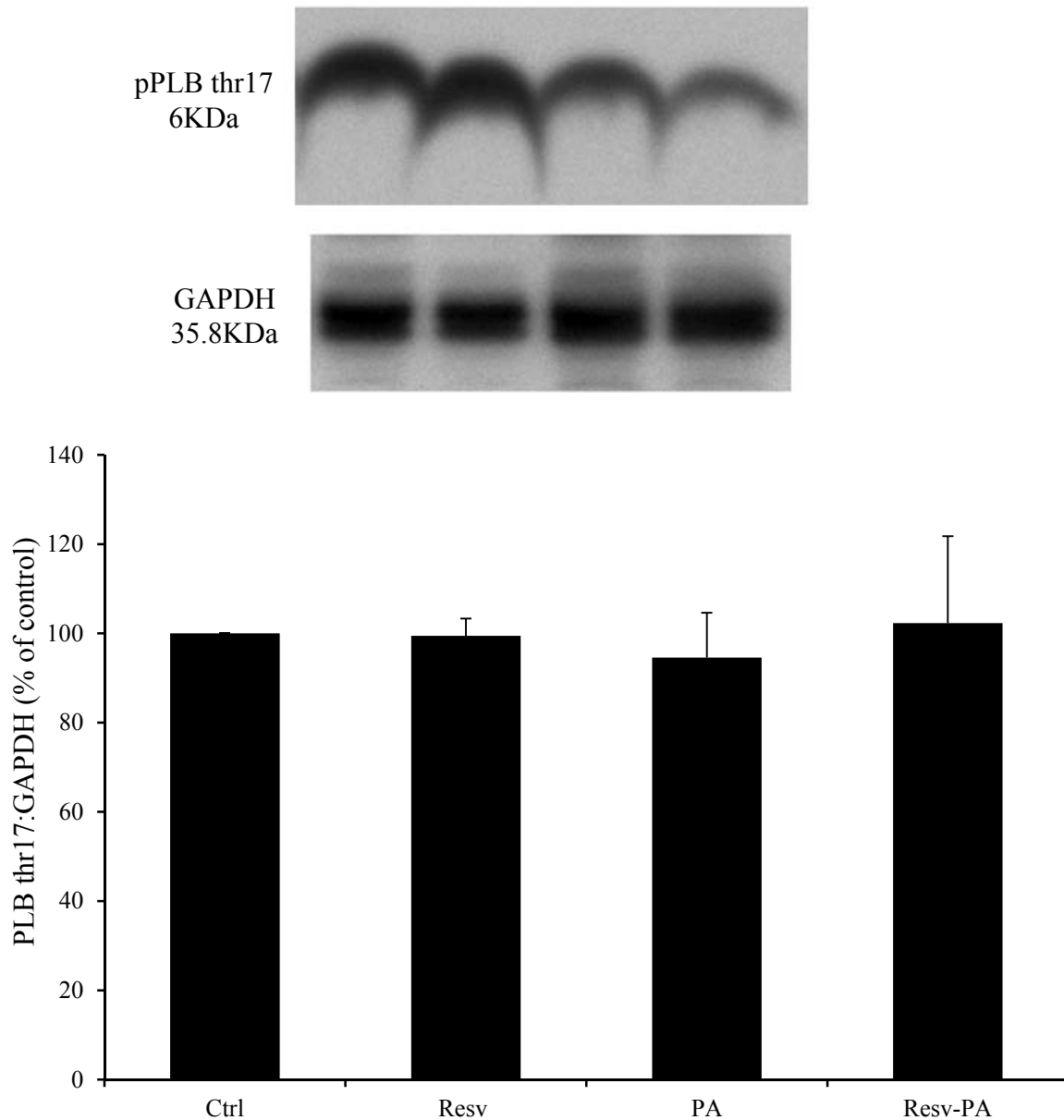


FIGURE 4.14: Effect of palmitic acid (PA) and resveratrol on threonine phosphorylated phospholamban (PLB thr 17) in cardiomyocytes at 4 hour time point. n=5. Representative protein bands presented here have been adjusted for brightness universally across the image. However, band density was measured using the original scans without any changes. Ctrl, control; Resv, resveratrol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

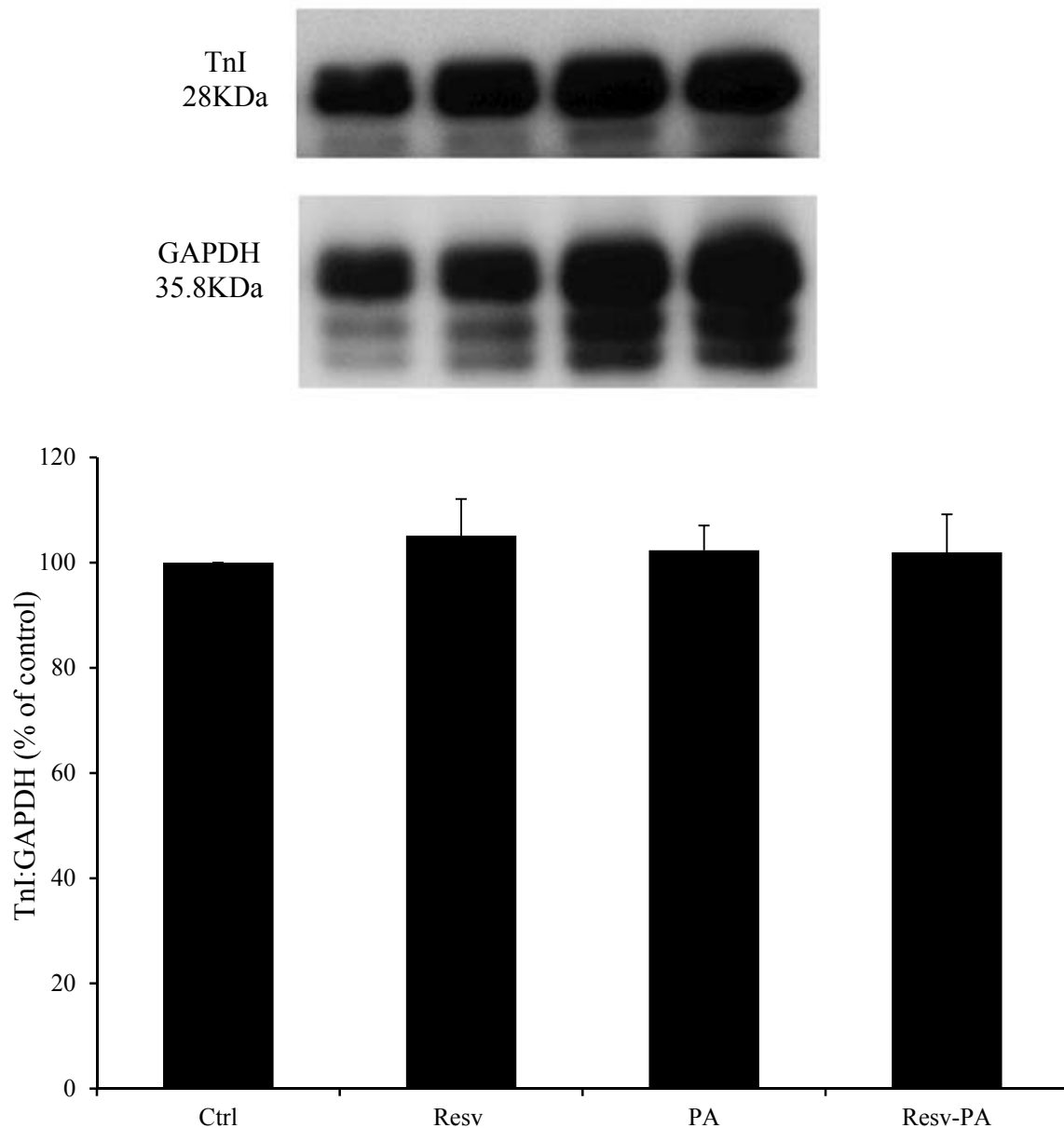


FIGURE 4.15: Effect of palmitic acid (PA) and resveratrol on troponin I (TnI) in cardiomyocytes at 4 hour time point. n=5. Representative protein bands presented here have been adjusted for brightness universally across the image. However, band density was measured using the original scans without any changes. Ctrl, control; Resv, resveratrol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

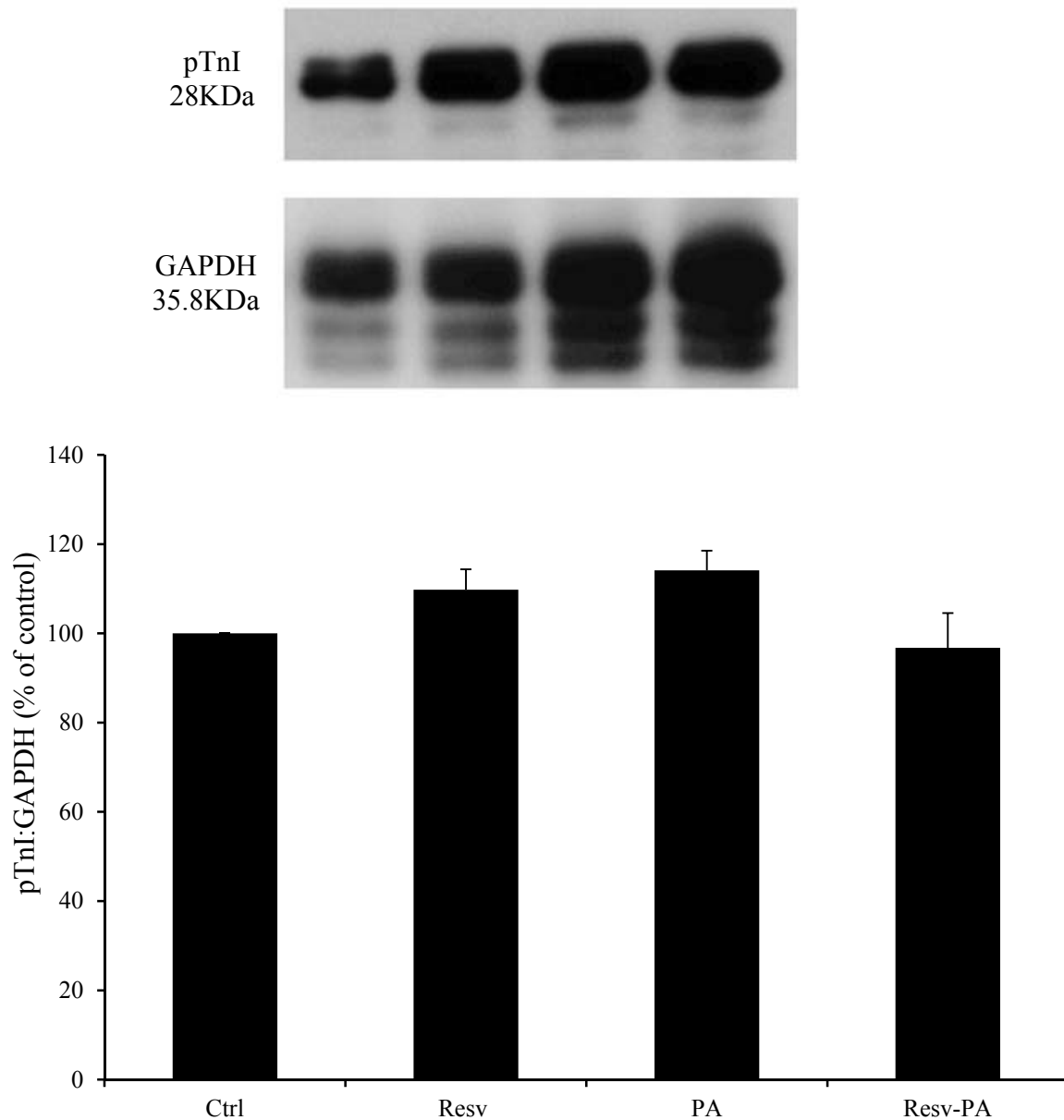


FIGURE 4.16: Effect of palmitic acid (PA) and resveratrol on phosphorylated troponin I (pTnI) in cardiomyocytes at 4 hour time point. n=5. Representative protein bands presented here have been adjusted for brightness universally across the image. However, band density was measured using the original scans without any changes. Ctrl, control; Resv, resveratrol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

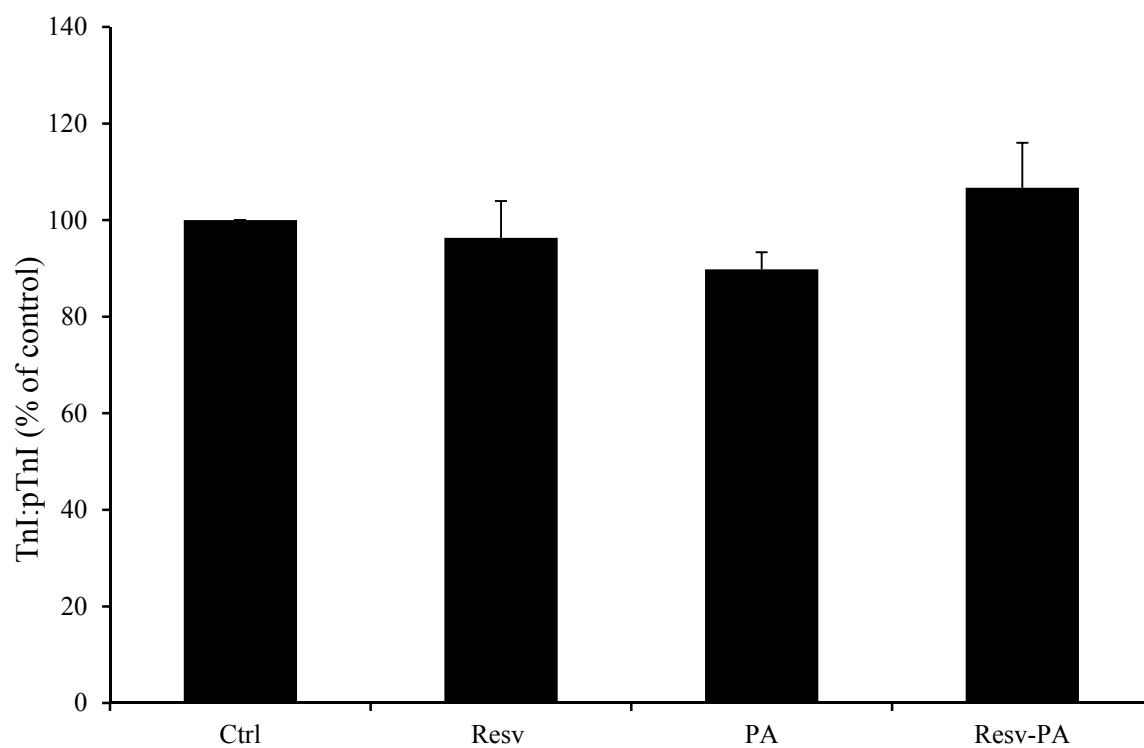


FIGURE 4.17: Effect of palmitic acid (PA) and resveratrol troponin I (TnI) to phosphorylated troponin I (pTnI) ratio in cardiomyocytes at 4 hour time point. n=5. Ctrl, control; Resv, resveratrol.

4.4 DISCUSSION

Obesity is a metabolic disease and circulating lipid levels are significantly higher in obese individuals (Pedersen, 2013). Excessive amounts of circulating lipids are an independent risk factor for the development of CVD (Poss et al., 2011). Deposition of lipids in the heart muscle and their lipotoxic effects have been associated with pathogenesis of heart failure (Schulze, 2009). Diet is a major source of these circulating lipids. In the *in vivo* study discussed earlier, lard was used as the dietary source of fat and lard is rich in both saturated and monounsaturated fatty acids. The cardiac muscle cells depend on FA for energy, however saturated fatty acids can also cause detrimental effects on cardiomyocytes (de Vries et al., 1997). PA is a long chain saturated fatty acid and is found in high amounts in lard. PA has been shown to cause apoptosis and contractile abnormalities in cardiomyocytes (Dyntar et al., 2001; Hickson-Bick, Sparagna, Buja, & McMillin, 2002). Accordingly, in this study adult rat cardiomyocytes were exposed to PA to mimic the hyperlipidemic condition observed in obesity. Another potential method to assess lipotoxic damage to cardiomyocytes is to use cardiomyocytes isolated from obese prone rats. Cardiomyocytes can be exposed to PA or animals could be fed with high fat diet for long term before isolating cardiomyocytes. However, the OP is originated from SD rats and cardiomyocytes could respond to lipid exposure in a similar manner. Accordingly, we expect that cardiomyocytes from SD would show pathophysiological characteristics similar to that of OP rat cardiomyocytes.

Consistent with earlier findings, this study also showed that PA induces cell death in cardiomyocytes (de Vries et al., 1997; Listenberger, Ory, & Schaffer, 2001; Ostrander, Sparagna, Amoscato, McMillin, & Dowhan, 2001). Morphological changes were measured after 24 hours exposure to PA as opposed to cardiac contractile measurements at 4 hours after PA addition

because there were no morphological changes observed at the 4 hour time point while, there were not sufficient numbers of rod shaped cells to measure contractility after 24 hours. The resveratrol dose used in this study is 30 μ M, which was used in our earlier studies that reported beneficial effects in cardiomyocytes (Movahed, Yu, Thandapilly, Louis, & Netticadan, 2012; Thandapilly et al., 2011). Three different doses (10, 100 and 200 μ M) were tested for effects on contractile performance of cardiomyocytes. The only dose (200 μ M) that induced significant cardiomyocyte contractile abnormality was used for all further experiments. The 200 μ M dose is considerably less than the doses reported in the literature which ranges from 0.5 to 1.2mM (Fauconnier et al., 2007; Kui et al., 2009; Ostrander et al., 2001). PA induced significant increase in apoptosis which confirms the lipotoxic effects of PA or other similar saturated long chain fatty acids on the heart tissue (Estadella et al., 2013). Therefore, diastolic cardiac dysfunction observed in the *in vivo* model of diet induced obesity could be partially attributed to a loss of cardiac muscle cells due to the lipotoxic effects of the hyperlipidemic environment. Consistent with earlier evidence of an anti-apoptotic property of resveratrol, cardiomyocytes were protected by resveratrol from PA-induced morphological changes and apoptosis (Movahed et al., 2012; Thandapilly et al., 2011).

The heart functions as a pump that contracts and relaxes in tandem to enable effective circulation of blood throughout the body. In pathological conditions there is an impairment of contraction and/or relaxation resulting in poor blood circulation in the body (Abraham et al., 2005; Mork, Sjaastad, Sejersted, & Louch, 2009). Shortening and re-lengthening of cardiomyocytes constitutes the contraction and relaxation or systolic and diastolic function of the heart. Diastolic dysfunction is a common phenomenon in cardiac abnormalities associated with obesity (Russo et al., 2011). It was found earlier in the *in vivo* study that fed a high fat diet, rats developed cardiac

diastolic dysfunction. An impaired cardiomyocyte relaxation could result in the observed cardiac dysfunction in animals fed a high fat diet. This was shown earlier in a murine model of obesity (Ceylan-Isik, Sreejayan, & Ren, 2011). This hypothesis was tested by measuring cardiomyocyte contractile function as a function of exposure to palmitate. The rate of cardiomyocyte re-lengthening (+dL/dT) was significantly altered after PA exposure and resveratrol treatment protected cardiomyocytes from PA-induced contractile abnormality. Accordingly, this data shows resveratrol may be beneficial against high fat diet-induced cardiac contractile disorders.

The contractile function of the heart is critical in normal pumping function and is controlled by a number of factors including neurohormonal molecules, electrical signals and Ca^{2+} homeostasis (Mohl et al., 2011; Solaro, 2011). Compensatory changes to these factors are normal as the heart tries to adapt to altered biological demands of the body. The heart responds to sympathetic or parasympathetic signaling by modulating the force and rate of the cardiac contraction-relaxation cycle. Ca^{2+} cycling and regulatory proteins are major players mediating these changes in contraction and relaxation (Bers, 2008). Pathological cardiac remodeling is associated with changes in the expression and activity of Ca^{2+} proteins. Changes to SERCA2A and PLB expression, phosphorylation of PLB relate to diastolic or relaxation abnormalities while, RYR2 and FKBP12 changes constitute systolic or contraction abnormalities of the heart (Marks, 2013a). In this study, PA induced changes in rate of relengthening or relaxation of the cardiomyocytes. Accordingly, dysfunction or altered SERCA2a and associated proteins might have a role in these pathological changes. Analysis of the expression of SERCA2a and PLB showed that SERCA2a expression was suppressed (17%) with PA exposure while PLB remained unaffected. Resveratrol has been shown to improve SERCA2a expression in other studies (Sulaiman et al., 2010) and similar to those reports treatment with resveratrol preserved

SERCA2a expression in PA exposed cardiomyocytes. Therefore, resveratrol may be protecting cardiomyocyte relaxation by preventing suppression of SERCA2a expression by PA. The lack of statistical significance makes it difficult to associate resveratrol mediated cardiomyocyte protection to changes in SERCA2a expression.

Cardiac TnI is the inhibitory subunit which is bound to actin and prevents the initiation of contraction in the absence of Ca^{2+} binding to troponin C (Parmacek & Solaro, 2004). TnI is used as a major marker for myocardial damage in patients with coronary disease (Inbar & Shoenfeld, 2009). In this study we measured TnI to detect myofibrillar damage. Also cTnI has two serine phosphorylation sites that are capable of interacting with protein kinase A. Increased phosphorylation of cTnI can accelerate the relaxation of cardiac myocytes (R. Zhang, Zhao, Mandveno, & Potter, 1995). However, there was no change in the levels of cTnI and pTnI in cardiac myocytes exposed to PA. This shows that the current experimental settings used in this study are not sufficient to elicit a change in the levels of cTnI and pTnI.

Oxidative stress is unequivocally associated with the development and progression of diseases (Pham-Huy, He, & Pham-Huy, 2008). In obesity, adipocyte dysfunction and organ damage are associated with oxidative stress (Crujeiras et al., 2013). Markers of oxidative stress are found to be elevated in the circulation in obesity. Oxidative stress is also a major factor in the development of cardiac disease and its progression into heart failure (Bhimaraj & Tang, 2012; Dai et al., 2012). Reactive oxygen species have been reported to regulate cardiac contractility (Perjes et al., 2012). High fat diets have been associated with a global increase in oxidative stress and may contribute to the development of comorbidities of obesity (Matsuzawa-Nagata et al., 2008). In this study, exposure to PA did not result in significantly higher oxidative stress in cardiomyocytes. Irrespective of exposure to PA or not, resveratrol treated cardiomyocytes had

lower oxidative stress levels. This result shows oxidative stress might not have a major role in the development of contractile abnormalities associated with PA exposure. Resveratrol has antioxidant properties and this property is considered to be a mediator of its pro-health effects (Raj et al., 2014). However, the antioxidant property of resveratrol may play only a minimal role in the prevention of cardiomyocyte contractile abnormalities in this model.

In summary, this study shows that exposure to high levels of saturated fatty acid induces contractile abnormalities and cell death in adult rat cardiomyocytes. The observed contractile abnormality was associated with suppression in SERCA2a levels and SERCA2a to PLB ratio. Treatment with resveratrol was able to prevent contractile abnormality with PA exposure. This protective effect of resveratrol might be associated with preserving SERCA2a expression levels. Resveratrol treatment also prevented changes in cardiomyocyte morphology and apoptosis due to PA exposure. Accordingly, resveratrol may be beneficial in preventing saturated fatty acid induced cardiomyocyte damage. These findings from the *in vitro* experiments could be associated with the findings from the *in vivo* study discussed earlier and protection extended with resveratrol treatment might be due to its direct effects on cardiac myocytes.

CHAPTER 5

OVERALL CONCLUSIONS, LIMITATIONS AND FUTURE DIRECTIONS

5.0 OVERALL CONCLUSION, LIMITATIONS AND FUTURE DIRECTIONS

Earlier studies report that resveratrol has potent cardioprotective properties and alleviates metabolic abnormalities. Consistent with this view, the current study shows that in a diet induced obese animal model, resveratrol treatment was beneficial in treating cardiac abnormalities and its associated metabolic imbalances. Furthermore, *in vitro* data from saturated fatty acid exposed cardiomyocytes showed resveratrol pretreatment was able to prevent contractile abnormality and cell death.

In the *in vivo* study, a high fat diet (55% kcal energy from fat) induced diastolic abnormalities in OP HF rats. Interestingly, OR HF rats also displayed diastolic dysfunction. Treatment with low dose resveratrol improved diastolic dysfunction associated with high fat diet in OP HF rats whereas treatment was not effective in OR HF rats. It is known that the genetic make-up of OP is different from OR. Therefore, it can be speculated that the mechanism of development of cardiac abnormalities due to high fat diet is also different in OP HF when compared to OR HF and resveratrol mediated cardioprotection might be effective only in OP HF like settings. However, more mechanistic studies will be necessary to prove this hypothesis. Oxidative stress is involved in the pathogenesis and progression of heart diseases. However, only OP HF rats showed increased lipid peroxidation levels. This further supports the speculation that development of cardiac abnormalities in OR and OP rats follow different routes. Anti-inflammatory, anti-hyperglycemic and anti-hyperlipidemic are established properties of resveratrol and the results from this study shows similar trends. Overall, the beneficial effect of resveratrol against cardiac dysfunction in OP HF rats might be associated with improvements in inflammation, oxidative stress, dyslipidemia and hyperglycemia while its ineffectiveness in OR HF rats despite decrease in inflammatory markers and hyperglycemia needs further investigation (**Figure: 5.1**).

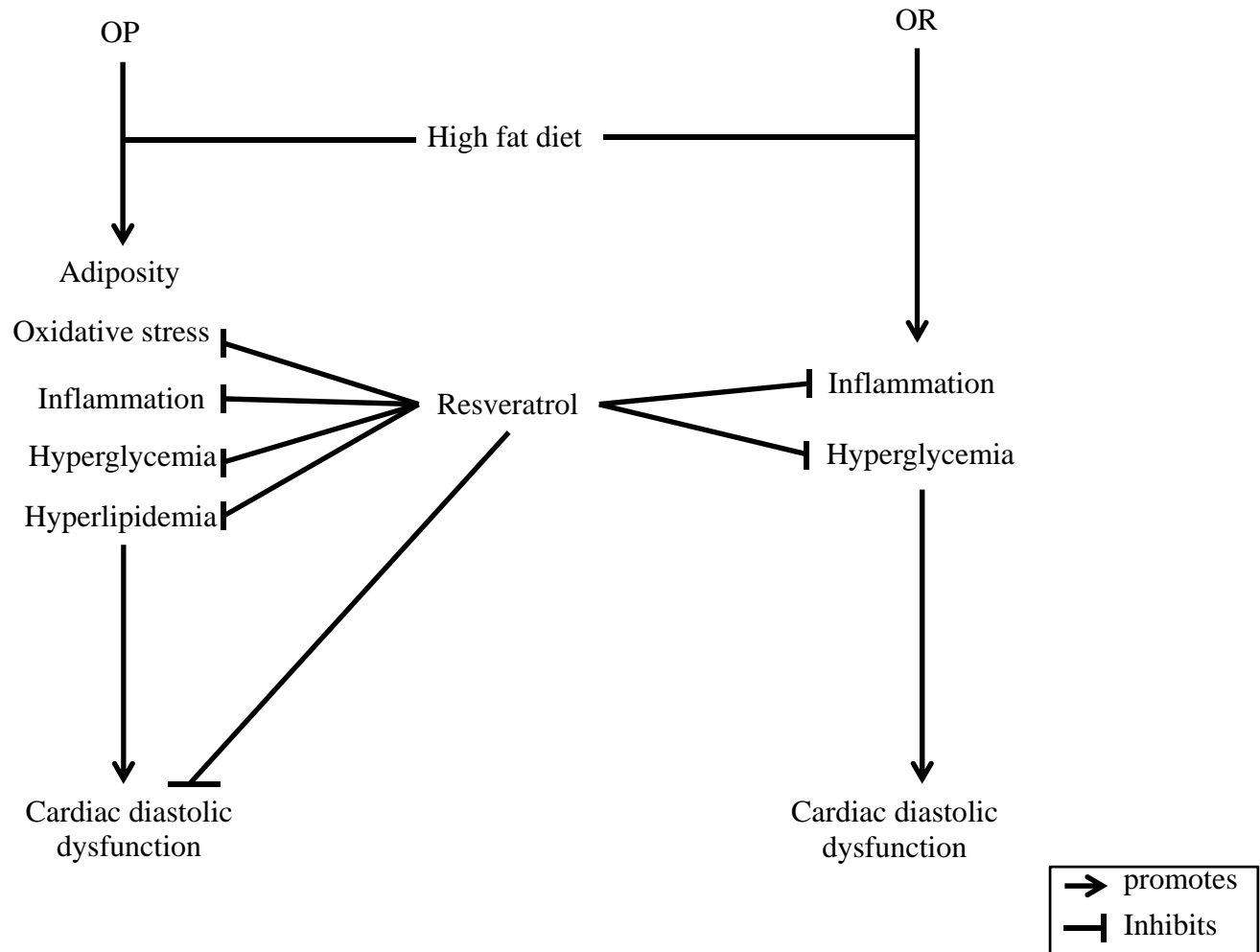


Figure 5.1: Summary of *in vivo* findings. The graphical summary shows metabolic cardiac abnormalities associated with high fat feeding and effects of resveratrol administration in obese prone (OP) and obese resistant (OR) rats.

Diastolic dysfunction observed in the *in vivo* study could be associated with a potential contractile abnormality in cardiac muscle cells or cardiomyocytes. Earlier evidences shows incubation with lipids induces contractile abnormalities in cardiomyocytes. Similarly, PA which is a long chain saturated fatty acid induces contractile abnormalities in adult rat cardiomyocytes. Resveratrol treatment prevented the suppression of contractile function of cardiomyocytes due to PA exposure. In *in vivo* study IVRT was prolonged while, velocity of relengthening was decreased with PA exposure in the *in vitro* experiment; both the parameters represent diastolic function. Accordingly, exposure to high levels of circulating lipids as in obesity or metabolic syndrome may effect cardiac diastolic function and based on the results presented here resveratrol might be beneficial in treating or preventing these abnormalities. Changes in the SERCA2a expression in the *in vitro* study suggests that resveratrol mediated protection against exposure to PA might be mediated by preserving gene expression of proteins involved in contractility. However, lack of statistical significance makes it difficult to correlate the SERCA2a changes to resveratrol mediated cardioprotection. Resveratrol also protected cardiomyocytes against PA-induced cell death and apoptosis which shows additional benefits of using resveratrol in obesity like conditions (**Figure: 5.2**).

This study presents several novel findings and complements existing knowledge in the area of diet induced obesity and potential of resveratrol as a cardioprotective agent. This is the first time high fat diet induced cardiac abnormalities in OP and OR is being compared. Also, this is the first time it has been showed that high fat diet induces cardiac and metabolic abnormalities in OR rats. Another novel finding is the effect of resveratrol on contractile performance of cardiomyocytes exposed to long chain saturated fatty acids. Overall, the *in vivo* and *in vitro* findings from this study highlight the potential of resveratrol in preventing cardiac abnormalities

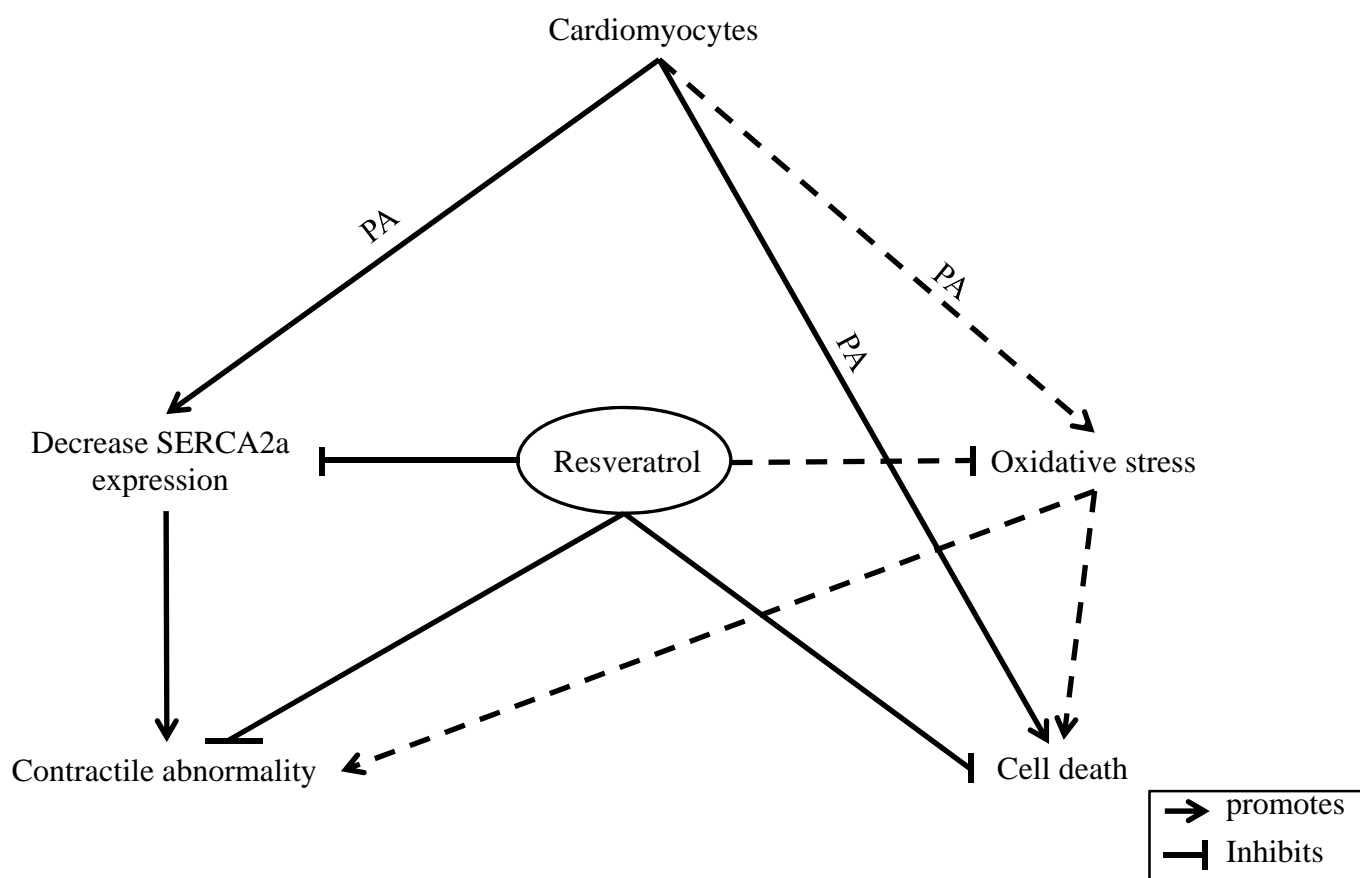


Figure 5.2: Summary of *in vitro* findings. The graphical summary shows abnormalities in cardiomyocytes exposed to palmitic acid (PA). It also shows the beneficial effects of resveratrol treatment. Solid line represents possible mechanism of PA induced pathology and resveratrol mediated rescue based on the data from this study. Dotted lines represent speculated mechanisms that might be involved in PA and resveratrol effects.

associated with obesity. The current findings will complement other reports on resveratrol mediated effects on improving adipocyte function, hypertension, type 2 diabetes, liver, kidney and other organ damages in obese animals. Obesity is a multifactorial disease and no single drug can be used to treat all the physiological anomalies. However, resveratrol has shown to modulate many different cellular targets, and effect metabolism, inflammation, oxidative stress and neurohormonal responses. Accordingly, resveratrol could be a potential molecule for treating obesity and associated disorders.

This study reports several novel findings, likewise there are few limitations. The animal model used in this study displayed mild to moderate level cardiac abnormalities and hence cannot fully assess the potential of resveratrol in established heart disease conditions. A standard chow fed OP would have been a better control for the study. The *in vitro* model of cardiomyocytes used in this study does not exactly mimic the *in vivo* conditions. Isolation of cardiomyocytes from high fat fed OP rats would have been a better *in vitro* model when compared to the cardiomyocytes from SD exposed PA. Therefore it is difficult to relate the observations from *in vitro* study to the *in vivo* conditions.

Compared to heart disease secondary to hypertension and ischemia, there are fewer animal studies that tested the efficacy of resveratrol against obesity induced cardiac impairment. Although, this study will help fill in some gaps, there is need to conduct more studies specifically testing potential of resveratrol in animal models of obesity with severe heart disease. Different doses of resveratrol need to be tested to find an optimal dose that could be effective against adiposity along with cardiac complications. The ineffectiveness of resveratrol in OR animals needs to be explored further. It needs to be tested if indeed the low dose of resveratrol used in this study is the reason for the lack of effect, or if molecular mechanisms of development of

cardiac complication in high fat fed OR rats cannot be targeted by resveratrol. Despite evidence for health benefits of resveratrol from animal studies, there is a long way to go to fully understand the potential and safety of resveratrol administration in obese population. While the current evidence from human studies shows promise, there are instances where resveratrol was ineffective. The positive effects of resveratrol in obesity range from improving metabolic parameters, flow mediated dilatation, decreasing oxidative stress markers and lipoprotein production. Long term studies are needed to identify the safest but also most effective dose that could be used in humans. Moreover, further studies are needed to understand potential cross talk between targets of other conventional anti-obesity, cardiovascular drugs or exercise. This will help prevent any antagonistic effects when used in combination with other drugs, as well as to identify potential synergies. More testing is also needed to know if the results on cardioprotection from animal studies can be translated to humans. To date, no study has examined whether resveratrol administration is beneficial against cardiac impairment in the obese. Accordingly, all existing data should be carefully analyzed, and properly designed human studies should be conducted to test the efficacy of resveratrol in obesity and associated cardiac abnormalities.

CHAPTER 6

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6.0 REFERENCES

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