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Protection Provided by Phytoestrogens against Cardiac Lipotoxicity induced by Palmitic Acid

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Paulo Oliveira (Centro de Neurociências e Biologia Celular) e da Professora Doutora Paula Veríssimo (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologias, Universidade de Coimbra)

Renata Lopes Familiar Couto

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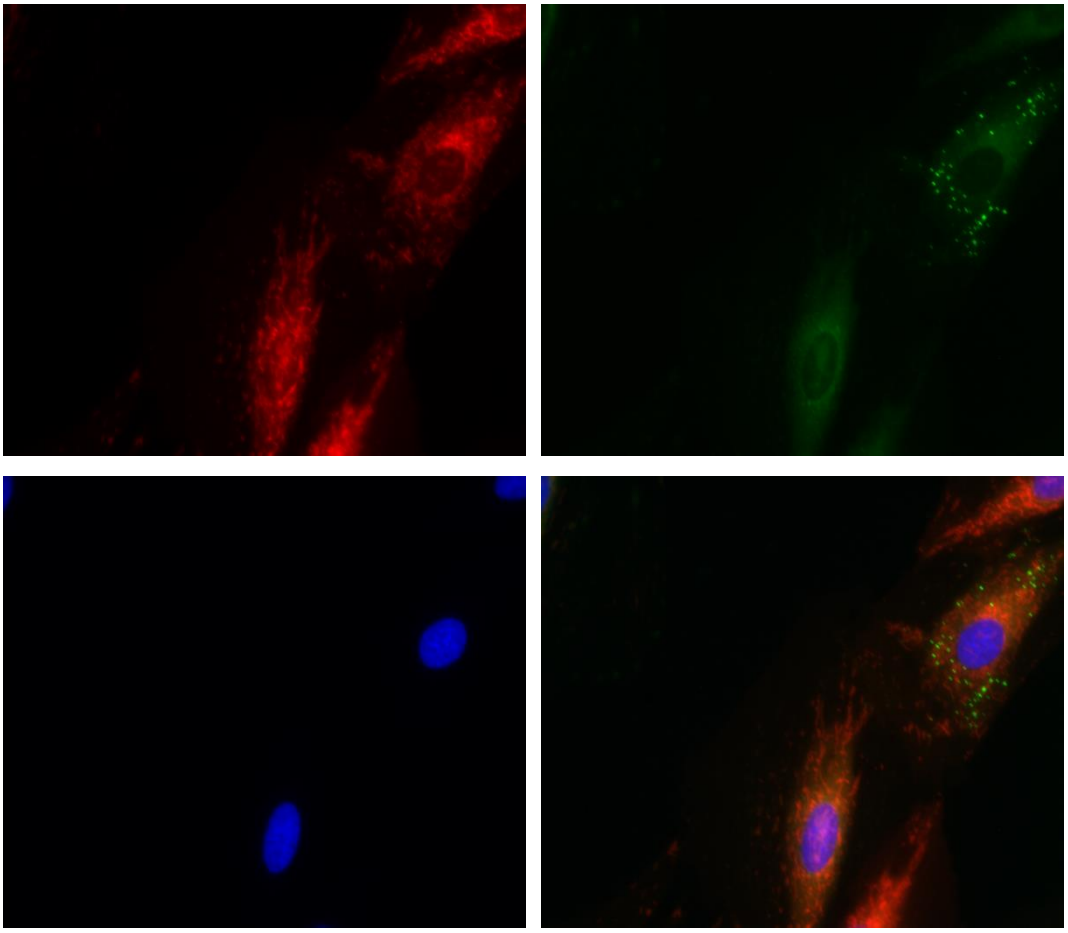

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Fluorescence microscopy images of H9c2 myoblasts treated with bovine serum albumin for 24 hours. Mitochondrial network appears in red, labeled with TMRM, the nucleus appears in blue, stained with Hoechst 33342 and neutral lipids were probed with HCS LipidTOX™ probe.

Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra com vista à obtenção do grau de Mestre em Bioquímica.

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This work was performed at the MitoXT - MITOCHONDRIAL TOXICOLOGY AND EXPERIMENTAL THERAPEUTICS laboratory at the Center for Neuroscience and Cell Biology, University of Coimbra, under the guidance of Doctor Paulo J. Oliveira, (Center for Neuroscience and Cell Biology, University of Coimbra) and Doctor Vilma Sardão (Center for Neuroscience and Cell Biology, University of Coimbra).

Institutional supervision was provided by Doctor Paula Veríssimo (Department of Life Sciences, DCV, University of Coimbra).

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I declare that this is a true copy of my thesis, including any final revisions, as approved by my supervisors, and that this thesis has not been submitted for a master degree to any other University or Institution.

Renata Couto

August, 2014

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Abbreviations

AMP- adenosine monophosphate

APS - Ammonium persulfate

ATG – Autophagy related gene

ATP - Adenosine triphosphate

BSA – Bovine serum albumin

Ca^{2+} - Calcium

$CaCl_2$- Calcium chloride

CAT - Carnitine acylcarnitine translocase

CM-H2DCFDA - 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate

CPT I - Carnitine palmitoyl transferase I

CPT II - Carnitine palmitoyl transferase II

CVD - Cardiovascular disease

DAG - Diacylglycerol

DIC - Differential interference contrast

DMEM - Dulbecco's modified Eagle's medium

DMEM-HG - Dulbecco's modified Eagle's medium high glucose

DMSO – Dimethylsulphoxide

Dox – Doxorubicin

E1 – Estrone

E2 – Estradiol

E3 – Estriol

ECF – Enhanced chemi-fluorescence

ER - Estrogen receptors

ERE – Estrogen-responsive elements

FA - Fatty acid

FACS - Fatty acyl CoA synthase

FATP - Fatty acid transport protein

FBS – Fetal bovine serum

FFA – Free fatty acid

GPx - Glutathione peroxidase

H2O2 - Hydrogen peroxide

HRT - Hormone replacement therapy

ITS – Insulin-transferrin-sodium selenite

KCl – Potassium chloride

KH_2PO_4 – Potassium hydrogen phosphate

LC3 – Light chain 3

LPL – Lipoprotein lipase

$MgCl_2$ – Magnesium chloride

MLC2V - Ventricular myosin light chain

Mn-SOD – Mitochondrial superoxide dismutase

MPTP – Mitochondrial permeability transition pore

mTOR – mechanistic target of rapamycin

Na_2HPO_4 – Sodium hydrogenophosphate

NaCl – Sodium chloride

NaOH – Sodium hydroxide

$NaSO_4$ – Sodium sulfate

NHS – Nurses' Health Study

PA – Palmitic acid

PBS – Phosphate buffered saline

PE - Phosphatidyl ethanolamine

PEs - Phytoestrogens

PI3K – Phosphatidylinositol 3-kinase

PI3P - Phosphatidylinositol-3-phosphate

PMSF - Phenylmethylsulfonyl fluoride

POF- Premature ovarian failure

PVDF – Polyvinylidene difluoride

RA – Retinoic acid

SDS – Sodium dodecyl sulfate

SDS-PAGE – SDS-polyacrylamide gels

SRB - Sulforhodamine B

TAG - Triacylglycerol

TBST – Tris-buffered saline supplemented with 0.1% tween-20

TEMED – Tetramethylethylenediamine

TMRM – Tetramethylrhodamine methyl ester

ULK1 – UNC-51–like kinase 1

VLDL – Very low-density lipoprotein

WHI – Women’s Health Initiative

$\Delta\psi$ – Mitochondrial transmembrane electric potential

ABSTRACT

Menopause is a natural biological process characterized by reduction of sex hormone levels, namely estrogen and progesterone. The loss of estrogens appears to trigger a cascade of downstream events, leading to increased risk for cardiovascular diseases (CVD). The conjugation of the aging process and loss of estrogen during menopause negatively impacts lipid metabolism in the heart, resulting in increased lipotoxicity and the risk for cardiovascular diseases.

Structurally similar to 17β-Estradiol, phytoestrogens (PEs), plant-derived compounds displaying estrogenic and/or antiestrogenic activity, demonstrate a positive impact on CVD in several studies. The main objective of our study was to investigate if coumestrol and enterodiol, two promising PEs, are able to protect the cardiac tissue against lipotoxicity induced by free fatty acids. Estradiol was also used, as a positive control. The cell line H9c2 was used as an *in vitro* model for cardiac cells to investigate palmitic acid (PA)-induced lipotoxicity and the possible protection by coumestrol and enterodiol. However, a new approach was used in this study, based on cell culture in a serum-free medium. In this context a preliminary characterization of the cells was performed, showing that culture in serum-free medium leads to differentiation to a cardiac phenotype, with the activation of autophagy possibly having a role in the differentiation process.

Regarding the main hypothesis of the present work, PA-induced cardiac lipotoxicity was used to mimic the accumulation of free fatty acids (FFA) in cardiomyocytes. The protective effect of 17β-estradiol, coumestrol and enterodiol, was determined by cell proliferation using the Sulforhodamine (SRB) assay, mitochondrial membrane potential alteration by flow cytometry and finally by detecting markers for autophagy signaling by Western Blotting.

The results shows a dose- and time-dependent decrease in H9c2 cell mass when incubated with PA, with a maximal effect obtained for 100μM. For the PEs analyzed, we observed that non-toxic concentrations have reduced toxicity on H9c2 cardiomyoblasts. Moreover, we found that treatment with coumestrol significantly prevented the effect of PA on H9c2 cells, increasing the mitochondrial membrane potential when compared with cells treated with PA-treated cells. Altogether, we can conclude that PA is an effector of lipotoxicity and that coumestrol and enterodiol have

reduced toxicity on H9c2 cells. Furthermore, coumestrol protects against the PA toxicity. However, the mechanisms underlying this protection are still largely unknown. Since women have elevated serum fatty acids and increased risk for CVD during menopause, it is possible that coumestrol can be used to prevent lipotoxicity and reduce the risk of CVD in the menopausal women.

Keywords: Menopause; Cardiac Lipotoxicity; Estrogens; Phytoestrogens.

SUMÁRIO

A menopausa é um processo biológico natural caracterizado pela redução dos níveis hormonais, nomeadamente, o estrogénio e progesterona. A perda de estrogénios parece desencadear uma cascata de eventos, levando ao aumento do risco para doenças cardiovasculares. A conjugação do processo de envelhecimento e perda de estrogénio durante a menopausa tem um impacto negativo no metabolismo dos lípidos no coração, o que resulta no aumento da lipotoxicidade e portanto do risco de doenças cardiovasculares.

Estruturalmente semelhantes ao 17β-estradiol, os fitoestrogénios, são compostos derivados de plantas que podem exibir actividade estrogénica ou anti-estrogénica, demostrando em vários estudos um impacto positivo nas doenças cardiovasculares. Assim, o principal objetivo do nosso estudo foi investigar se coumestrol e enterodiol, dois promissores fitoestrogénios, são eficientes em proteger o tecido cardíaco contra lipotoxicidade induzida por ácidos gordos livres. O Estradiol também foi usado, como um controlo positivo.

A linha celular H9c2 foi usada como um modelo *in vitro* para células cardíacas, de modo a investigar a lipotoxicidade induzida pelo ácido palmítico, bem como a possível protecção do coumestrol e enterodiol. No entanto, uma nova abordagem foi usada no presente trabalho, tendo como base a cultura de células em meio isento de soro. Neste contexto uma caracterização preliminar das células foi realizada, evidenciando que a cultura em meio sem soro conduz à diferenciação de um fenótipo cardíaco, com a activação da autofagia possivelmente tendo um papel no processo de diferenciação.

Em relação à principal hipótese do trabalho, a lipotoxicidade cardíaca foi induzida pelo, de modo a mimetizar a acumulação de ácidos gordos nos cardiomiócitos. O efeito protector do 17β-estradiol, coumestrol e enterodiol, foi determinado pela proliferação de células utilizando o ensaio de SRB, alterações no potencial mitocondrial membranar por citometria de fluxo e, finalmente, pela detecção de marcadores de sinalização de autofagia usando Western Blot.

Os resultados mostram de uma forma dependente da dose e tempo uma diminuição no número de células quando incubadas com PA, com um máximo efeito máximo para 100μM. Para os fitoestrogénios analisados, observou-se que concentrações não tóxicas apresentam toxicidade reduzida em cardiomioblastos. Além disso, mostrámos que o tratamento com coumestrol preveniu significativamente o efeito do PA nas células

H9c2, aumentando o potencial de membrana mitocondrial, quando comparado com células tratadas com PA. Em conjunto, podemos concluir que o PA é um eficaz na lipotoxicidade e que o coumestrol e enterodiol apresentam toxicidade reduzida em células H9c2. Além disso, o coumestrol protege contra a toxicidade exercida pelo PA. No entanto, os mecanismos subjacentes a esta proteção são ainda desconhecidos.
Uma vez que as mulheres apresentam elevados níveis de ácidos gordos no soro e um aumento do risco de doenças cardiovasculares durante a menopausa, é possível que o coumestrol possa ser utilizado para prevenir lipotoxicidade e reduzir o risco de doenças cardiovasculares em mulheres na menopausa.

Palavras-chave: Menopausa; Lipotoxicidade cardíaca; Estrogénios;

1. GENERAL INTRODUCTION

1.1. The role of estrogen in woman's life

The menstrual cycle is generated and regulated by estrogens, steroid hormones mainly produced in ovaries and adrenal glands (Moreira *et al.*, 2014; Rettberg *et al.*, 2014). In the female body, three forms of estrogens exist, namely estrone (E_1), estradiol (E_2) and estriol (E_3) (Rettberg *et al.*, 2014).

Estrogens synthesis results from the conversion of cholesterol to androstenedione or testosterone and subsequently aromatization to estrone and estradiol in granulosa cells **(Figure 1)** (Cui *et al.*, 2013; Porter *et al.*, 1991).

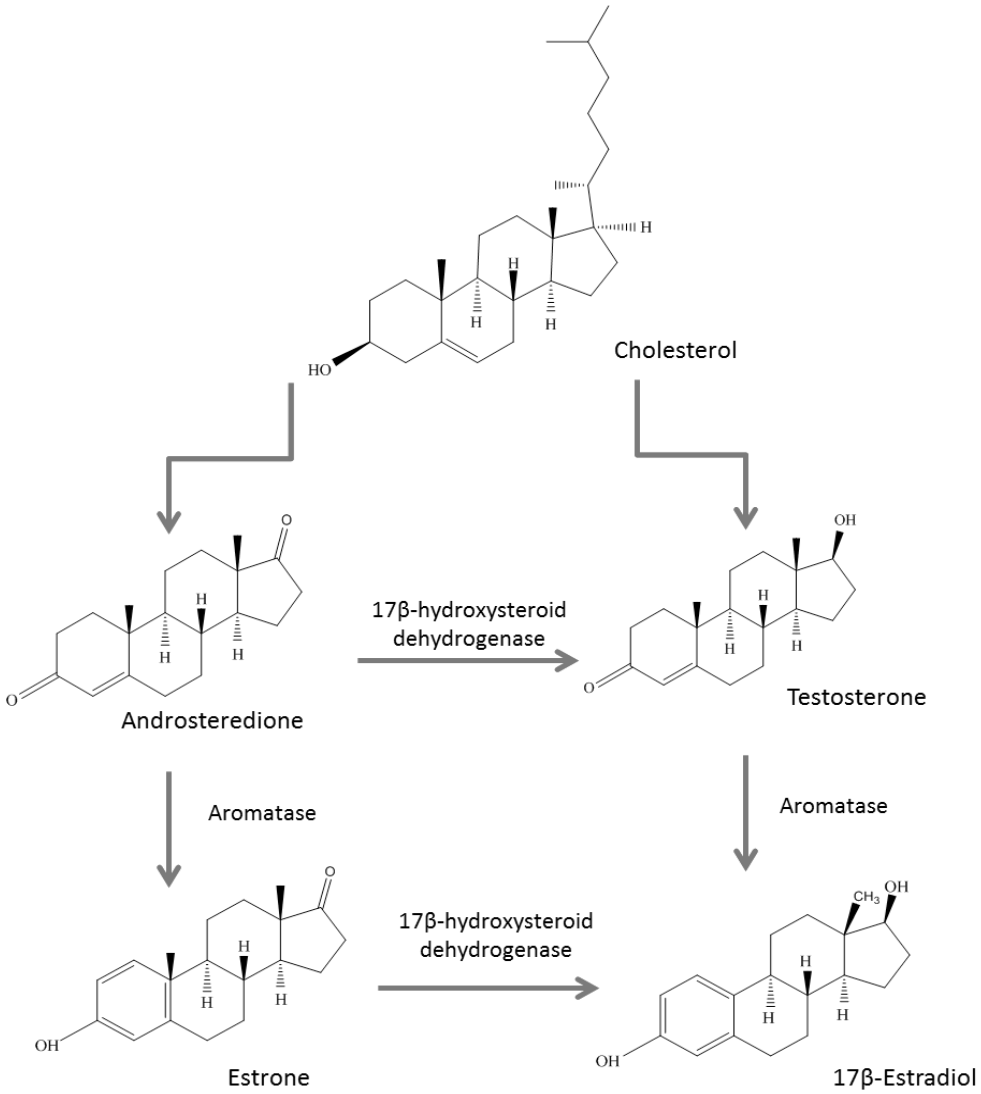

Figure 1 - Estradiol production in granulosa cells, adapted from (Cui *et al.*, 2013).

The role of estrogens in the promotion of female sexual characteristics and the maintenance of the reproductive system has been described (Rettberg *et al.*, 2014). In non-pregnant and non-menopausal women, the main circulating estrogen is 17β-estradiol, being the most potent form of estrogen (Rettberg *et al.*, 2014). Estrogens have a fundamental role in the development and functioning of several systems, including cardiovascular system, brain, reproductive system, skin and bone (Gibson and Saunders, 2012; Kuiper *et al.*, 1997; Murphy, 2011). The effects of estrogens are mediated by nuclear estrogens receptors (ER), namely ER-α and ER-β, localized in many cell types but with different distribution in whole body (Cui *et al.*, 2013; Gibson and Saunders, 2012). The binding of estrogens to these receptors in the cytoplasm results in their translocation to the nucleus, stimulating their action as transcription factors (Murphy, 2011). Estrogen-Responsive Elements (ERE) are the specific DNA target sequences for the complex estrogens-ER (Moreira *et al.*, 2014; Welboren *et al.*, 2007). Moreover, ER can act through a non-genomic pathway that is based on the binding of estrogens to the classical and non-classical ERs present in plasma membrane, being mediated by second messengers (Cui *et al.*, 2013; Moreira *et al.*, 2014).

1.1.1. Menopause

In women's life, menopause is one of the most critical periods, although being natural biological process resulting from aging, caused by reduction of ovarian hormones (Nelson, 2008; Oliveira *et al.*, 2012). Menopause involves the end of menstrual cycle, and female fertility (Oliveira *et al.*, 2012; Wylie-Rosett, 2007).

Natural or spontaneous menopause occurs around the age of 50 and is confirmed by the absence of menstrual periods for 12 months, being considered non-pathological amenorrhea **(Figure 2)** (Nelson, 2008; Wylie-Rosett, 2007).

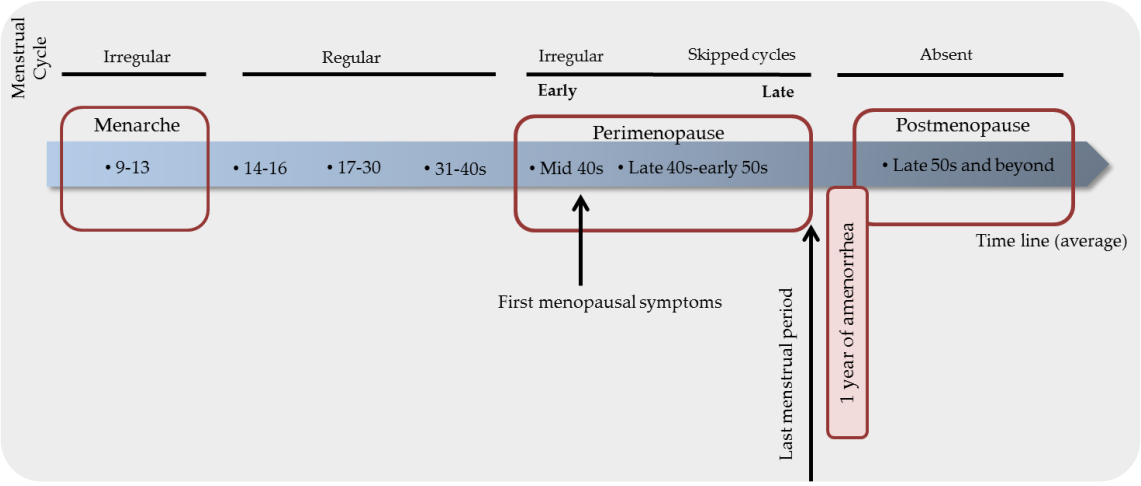

Figure 2 - Reproductive stages in the women during aging: from menarche to postmenopausal adapted from (Nelson, 2008; Oliveira *et al.*, 2012).

The phases of menopause are dynamic periods of time because these do not occur at any specific age (Blake, 2006; Soules *et al.*, 2001). Normally, perimenopause starts around mid-to-late 40s and persist several years before the last menstrual period, for 4-5 years (Nelson, 2008). During this phase, alterations in hormonal levels, with estrogen and progesterone decreasing and menstrual periods become irregular. Additionally, several menopausal symptoms appear (Blake, 2006; Greendale *et al.*, 1999). The menopausal transition ends after 12 months of amenorrhea, and is followed by postmenopause (Blake, 2006; Soules *et al.*, 2001). This phase can be divided into two periods: early and the late menopause. Early postmenopause usually last for 5 years and includes 12 months associated with final menstrual period and the following 4 years (Soules *et al.*, 2001). This phase is clinically important because of the physiological changes and the prevalence of the symptoms experience in the early postmenopausal years. The late postmenopausal years extend throughout the latter years of a woman's life, a period of drastic changes related with age (Blake, 2006).

Although menopause is a process related with aging process, early or premature ovarian failure (POF) can occur before the age of 40 years, being disconnected from the aging process (Nelson, 2009; Oliveira *et al.*, 2012). The early menopause can be due induced during medical interventions or surgical interventions or spontaneous (Shuster *et al.*, 2010). There are several causes of POF spontaneous, including autoimmune disorders, genetic causes, infections or inflammatory conditions, enzyme deficiencies,

metabolic syndromes and idiopathic (Beck-Peccoz and Persani, 2006; Shuster *et al.*, 2010).

All women undergo menopause, but their experience may be different, depending on their lifestyle and/or genetic information. Menopause symptoms usually include: vasomotor symptoms (hot flashes), depressed mood, sleep disturbances, sexual concerns or problems, cognitive symptoms, vaginal dryness and urinary incontinence (Blake, 2006; Moreira *et al.*, 2014).

Regarding hot flashes, they are thought to be related with the withdrawal of estrogens and the resulting vasomotor instability, in which leads to a sensation of warmth in the face, neck and chest and perspiration (Greendale *et al.*, 1999; Samsioe *et al.*, 2006). These symptoms are usually associated with anxiety, irritability, sweating, palpitations and panic. If all these symptoms continue for months, chronically disturbed sleep may occur, which may lead to a cognitive deficits (Samsioe *et al.*, 2006). The cause for vasomotor symptoms is not completely understood.

Besides hot flashes, dyspareunia, vaginal dryness, itching, and irritation resulting from vaginal atrophy, can be experienced by women during menopause (Greendale *et al.*, 1999; Samsioe *et al.*, 2006). Also, due to loss of estrogens, the vaginal pH changes from acid to neutral and vaginal flow blood become reduced (Greendale *et al.*, 1999).

1.1.2. Menopause and Health Problems

The menopause condition is also accompanied with more severe complications, including increased cardiovascular risk, osteoporosis and body weight gain due of changes in hormone levels and aging (Carr, 2003; Oliveira *et al.*, 2012). During aging, the metabolic rate decreases, which justifies that after menopause an increase in body weight and visceral adipose tissue accumulation occurs because of the ovarian hormone withdrawal (Babaei *et al.*, 2010; Oliveira *et al.*, 2012). An increase in visceral adipose tissue is thought to be related with various metabolic syndromes such as insulin resistance, dyslipidemia, hypertension, prothrombotic, and proinflammatory states (Barros and Gustafsson, 2011; Carr, 2003). The risk of cardiovascular disease (CVD) associated to the metabolic syndrome appears to be high in women, and it is probable that the most cardiovascular events in women are related to the metabolic syndrome (Carr, 2003).

1.1.3. Cardiovascular disease in women during menopause

Cardiovascular disease is a multifactorial disease and is influenced by different factors, such as bad lifestyle that includes inappropriate diet, sedentary life, smoking and drinking as well as aging, menopause and genotype.

Cardiovascular diseases include coronary heart disease, myocardial infarction, heart failure, and coronary death (Stevenson, 2009). Premenopausal women appear to be protected from CVD compared with men of similar age. After menopause, CVD risk increases and postmenopausal women have the same risk for this disease as do men which may be related to the substantial metabolic changes that occur in women transition from premenopause to postmenopause (Barros and Gustafsson, 2011; Carr, 2003; Prelevic and Jacobs, 1997). Still, women who experience a premature menopause are more susceptible to certain health problems, such as osteoporosis and heart diseases because these women spend more time in their lives without the benefits of estrogens (Nelson, 2009; Oliveira *et al.*, 2012; Shuster *et al.*, 2010).

The decline in estrogen associated with menopause leads to an increase in visceral fat mass, lipid accumulation and a reduced use of lipids, which increases the risk for developing CVDs and insulin resistance (Barros and Gustafsson, 2011; Stevenson, 2009). In conclusion, the removal of estrogens during induced or natural menopause leads to several complications, which may involve alterations of lipid metabolism that sometimes results in increased cardiovascular risk (Chen, 2006).

Another factor that can promote CVD is increased oxidative stress. The classical definition of oxidative stress involves an imbalance between reactants and antioxidants. Increases in reactive oxygen species causes damage to lipoproteins, lipids, DNA, proteins and oxidative stress-induced modifications of these molecules have been implicated in many disease pathways such as CVD (Strobel *et al.*, 2011; Wattanapitayakula and Bauerb, 2001). Oxidative stress is believed to play a significant role in the initiation and progression of atherosclerosis. Lipid peroxidation occurs mostly in polyunsaturated fatty acids because they of the greater number of hydrogen chains, being more susceptible to oxidation. The process of lipid peroxidation can affect membrane fluidity, functioning, permeability, electron potential and the transport of metabolites across membranes (Strobel *et al.*, 2011). As mentioned above, premenopausal women have decreased risk of heart disease compared with men but after menopause the risk of CVD in women increases. This increase of CVD may be

caused by increased levels of lipid peroxidation. In agreement, post-menopausal women exhibit higher levels of lipid peroxidation and therefore increased risk of CVD (Castelao and Gago-Dominguez, 2008).

It still remains unclear if the type of menopause (surgical or spontaneous) influences cardiovascular risk in women. However, the Nurses' Health Study (NHS) demonstrated that women with a natural menopause and who had never taken postmenopausal estrogen had no increased risk of coronary heart disease compared to premenopausal women. Also, women with a natural menopause who were being treated with estrogens also showed no difference in terms of CVD risk. In contrast, participants reporting a bilateral oophorectomy had a 2-fold increased risk if they did not use postmenopausal estrogen (Barrett-Connor, 2013; Ventura-Clapier *et al.*, 2011). However, it is important to mention that aging *per se* has more impact than menopause for CVD risk factors.

1.1.4. Cardiac Mitochondrial Fatty Acid Beta-Oxidation during Menopause

The heart is one of the organs of the body with the highest energy requirement, necessary for muscle contraction. The heart is considered an omnivorous organ, using several substrates for energy generation, such fatty acids, carbohydrates, ketone bodies, lactate and even amino acids. However, fatty acids are the preferred substrate (Ventura-Clapier *et al.*, 2011). One fundamental characteristic of the heart is that this organ is metabolic flexible which is the ability to respond to changing substrate availability, circulating hormones, coronary flow, and fuel metabolism by choosing the right substrate at the right moment. So, impaired substrate metabolism leads to contractile dysfunction and cardiac remodeling characteristic of heart failure (Ventura-Clapier *et al.*, 2011).

Mitochondrial oxidation of fatty acids is the major source of energy in the adult healthy heart (Kienesberger *et al.*, 2013). Fatty acids (FA) can be transported in the plasma as free fatty acids (FFAs), including saturated and unsaturated FFAs. These are the major energy source in human body during periods of starvation or prolonged endurance exercise and are released mainly from adipose tissue by lipolysis of triglycerides (Rial *et al.*, 2010; Yuan *et al.*, 2013).

Under physiological conditions, fatty acids are largely bound to albumin or as a part of triacylglycerol (TAG) contained in chylomicrons or very-low-density lipoproteins (VLDLs) which are hydrolyzed by lipoprotein lipase (LPL) (Kienesberger *et al.*, 2013).

Fatty acid beta-oxidation is very important in the context of energy, adenosine triphosphate (ATP) production in the heart and skeletal muscle (Eaton *et al.*, 1996). Beta-oxidation is different for short-chain, medium-chain fatty acids, and long-chain fatty acids because the mitochondrial membrane is not permeable to long-chain fatty acids. Therefore, short-chain and medium-chain fatty acids of less than 10 carbon atoms can cross both the outer and inner mitochondrial membranes and enter the mitochondrial matrix after activation into their CoA esters, to which beta-oxidation follows (Darras and Friedman, 2000). Long-chain fatty acids, after entering cardiomyocytes, are converted to CoA esters, through the action of fatty acyl CoA synthase (FACS). The inner mitochondrial membrane is also impermeable to the CoA thioesters, and so these are subsequently bound to carnitine by the enzyme carnitine palmitoyl transferase I (CPT I), located on the inner side of the outer mitochondrial membrane, forming acylcarnitine. This molecule is then transferred across the inner mitochondrial membrane by carnitine acylcarnitine translocase (CAT). In the mitochondrial matrix, acylcarnitine is converted back to the acyl-CoA derivative and carnitine by CPT II, which is localized on the inner side of the inner mitochondrial membrane. Finally, the long-chain acyl-CoA derivative enters the beta-oxidation pathway **(Figure 3)** (Darras and Friedman, 2000).

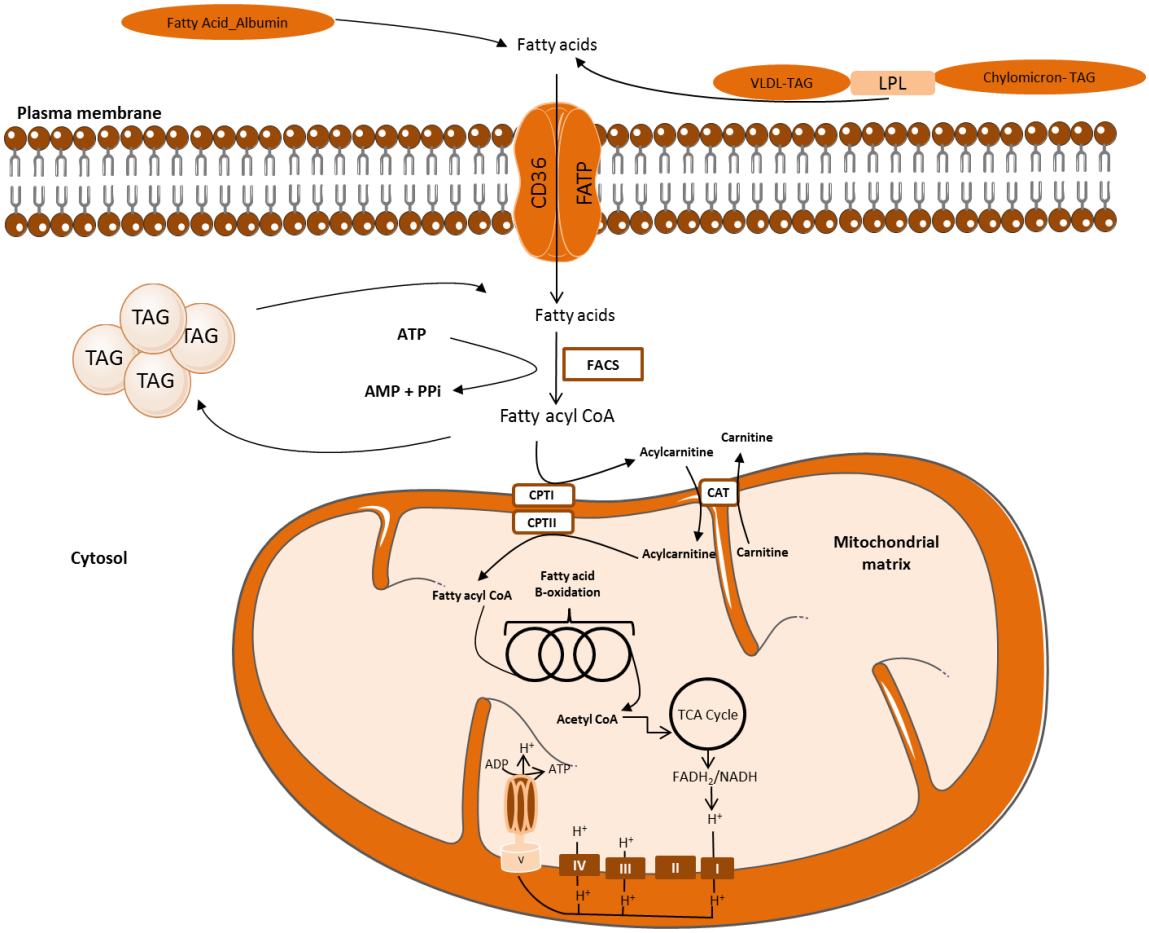

Figure 3 - Transport of fatty acids from the cytoplasm to the mitochondrial matrix for oxidation, adapted from (Darras and Friedman, 2000; Lopaschuk *et al.*, 2010; Oliveira *et al.*, 2012). Abbreviations: VLDL - very-low-density lipoproteins; TAG – triacylglycerol; LPL - lipoprotein lipase; FATP - fatty acid transport protein; FACS - fatty acyl CoA synthase; ATP - adenosine triphosphate; AMP - adenosine monophosphate; CPTI - carnitine palmitoyl transferase I*;* CPTII - carnitine palmitoyl transferase II; CAT - carnitine acylcarnitine translocase.

Long-chain fatty acyl CoA can be used for the synthesis of intermediates, such as TAG, diacylglycerol (DAG) and ceramide. These intermediates are stored and/or channeled to different biosynthetic pathways, namely biomembrane synthesis (Li *et al.*, 2010; Lopaschuk *et al.*, 2010). When alterations of normal fatty acid homeostasis occur, for example, during high FFA levels in the plasma, these intermediates can accumulate in cells. Depending on the tissue, this accumulation can have different effects. Excessive accumulation of TAG in non-adipocyte tissues can result on impairment of insulin signaling in the liver and skeletal muscle (van Herpen and Schrauwen-Hinderling, 2008), as well as apoptosis in the heart (Borradaile and

Schaffer, 2005; Li *et al.*, 2010). Diacylglycerol is also thought to be involved in apoptosis and in triggering insulin resistance (Wende and Abel, 2010). Moreover, ceramide increases apoptotic signaling in several tissues, being involved in the triggering of the mitochondrial permeability transition pore (MPTP) and outer-membrane permeabilization. Furthermore, FA *per se* causes the release of apoptogenic factors from mitochondria, consequently causing mitochondrial dysfunction and cell death (Rial *et al.*, 2010). The failure of mitochondrial bioenergetics destabilizes fatty acid metabolism that may result in the accumulation of fatty acyl-CoA esters in the cytosol of cardiomyocytes. This, in turn, leads to increased synthesis of the intermediates previously mentioned, TAG, DAG and ceramide, leading to cell death, thus working in a positive feedback cycle.

Furthermore, exposure to saturated fatty acid but not to unsaturated fatty acid triggers apoptosis in cell culture. Palmitic acid (C16:0) (PA) induces apoptosis via *de novo* ceramide formation and activation of the mitochondrial apoptotic pathway (Dyntar *et al.*, 2001). Incubation of adult rat cardiomyocytes with PA damages contractile elements and cytoskeleton (Dyntar *et al.*, 2001). It is apparent that the saturated FFAs in the human body may be involved in the development of many chronic diseases, such as obesity, diabetes and cardiovascular diseases (Yuan *et al.*, 2013). As apoptosis of cardiomyocytes plays a central role in heart failure (Dyntar *et al.*, 2001), a balance between FFA cell uptake and metabolism is necessary. Moreover, there must be an efficient transport of fatty acyl-CoA for to beta-oxidation and subsequent energy production.

Aging is a biological process characterized by a general and progressive decline in physiological functions that affects many tissues. For example, mitochondrial function in the heart decreases with aging (Paradies *et al.*, 2010; Petrosillo *et al.*, 2009). Decreased mitochondrial function occurs with aging, affecting both the bioenergetics and several signaling pathways to and from mitochondria.

Figure 4 shows the rationale proposed by Oliveira and colleagues (Oliveira *et al.*, 2012), for the conjugation of aging process and hormonal alterations resulting from menopause, leading to a degradation of mitochondrial capacity in the heart. These alterations are associated with a progressive decrease in lipid oxidation in mitochondria and increased lipid storage in adipocytes. Moreover, the formation of fatty acyl intermediates, such as DAG, TAG and ceramide in the cytosol of cardiomyocytes may causes myocardial lipotoxicity and result in the activation of apoptotic signaling.

Therefore, cardiovascular risk increases under these circumstances. Estradiol increases fatty acid oxidation by mitochondria preventing the potential accumulation of deleterious metabolites and increasing fatty acid-derived mitochondrial ATP production **(Figure 4)**.

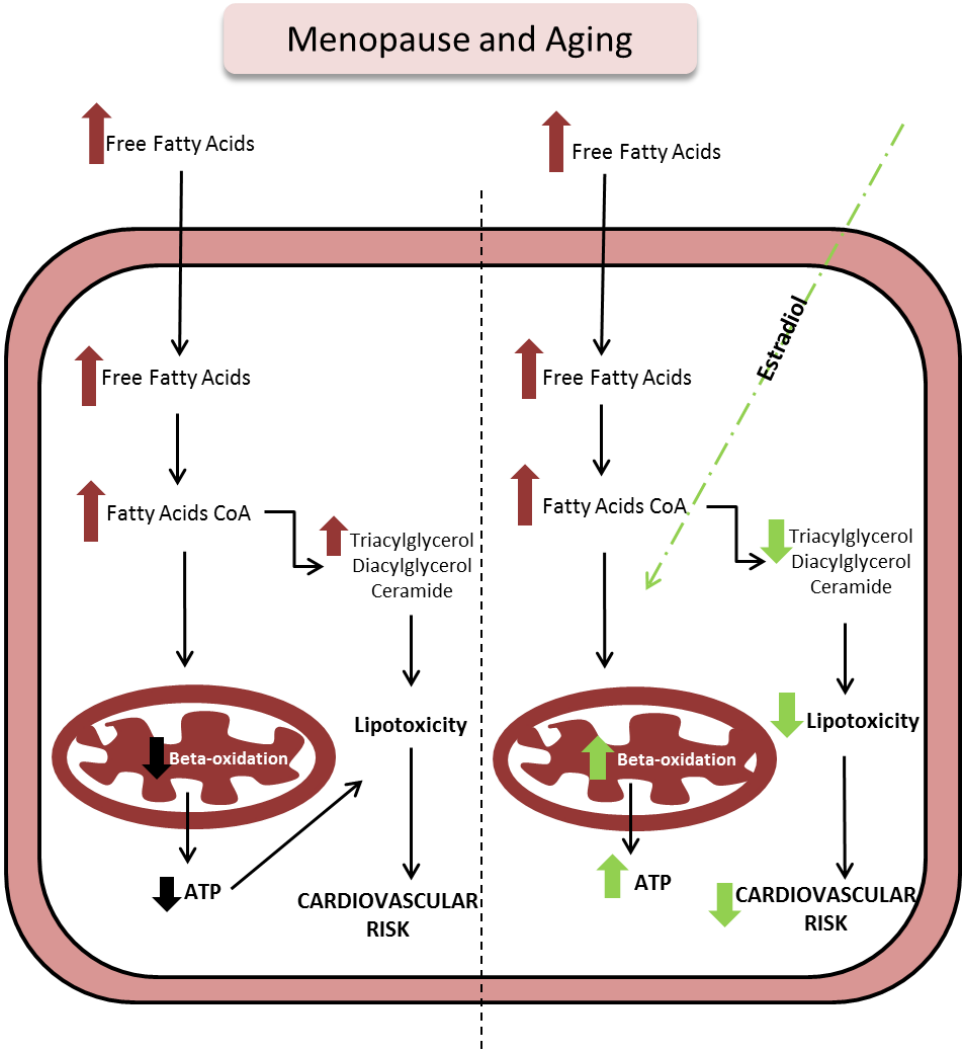

Figure 4 -Specific mitochondrial alterations in the menopausal women. **(A)** The conjugation of aging process and hormonal alterations caused by menopause leads to an impaired fatty acids metabolism contributing to increased fatty acids CoA and also fatty acyl intermediates, such as DAG, TAG and ceramide in the cytosol of cardiomyocytes, which in turn causes lipotoxicity. Under these conditions, cardiovascular risk increases. **(B)** Estradiol (green arrows) may act on improvement of fatty acid oxidation in the heart, decreasing the levels of the fatty acyl intermediates and consequently lipotoxicity, preventing cardiovascular diseases. (Adapted from (Oliveira *et al*., 2012)). Abbreviations: DAG- diacylglycerol; TAG - triacylglycerol; ATP - adenosine triphosphate.

Pelzer and collaborators (Pelzer *et al.*, 2000) demonstrated that apoptosis in cardiomyocytes can be prevented by physiological levels of 17β-estradiol, preventing the loss of cardiomyocytes in heart failure. Other reports demonstrated that estrogen reduces cardiac hypertrophy and also reduces infarct size, improve contractile function after ischemia/reperfusion (Murphy, 2011). Also Viña and colleagues (Viña *et al.*, 2005) demonstrated that estrogen reduces mitochondrial hydrogen peroxide (H_2O_2) production by up-regulating the expression of antioxidant enzymes, such as mitochondrial superoxide dismutase (Mn-SOD) and glutathione peroxidase (GPX), which are longevity-associated enzymes.

Thus, it is evident that sex hormones have an important role in the regulation of lipid metabolism and consequently in the protection against CVD mediated by different mechanisms.

1.1.5. Hormone Replacement Therapy

The menopausal women present alterations in metabolism and cardiovascular function. Currently, hormone replacement therapy (HRT) is the most used approach to prevent menopausal symptoms, being based on the administration exogenous hormones. The most commonly used hormones are estrogen and progestin used together, but estrogen can also be used alone in women without uterus. Estrogen-progestin therapy is prescribed for women who have not had hysterectomies and thus still have uterus because the progestin protects the endometrium from the harmful effects of estrogen (Nelson, 2008).

The effects of HRT are believed to be mostly beneficial, owing to a reduction in risk of cardiovascular disease, osteoporosis, and colon cancer (Schierbeck *et al.*, 2012). Schierbeck and colleagues (Schierbeck *et al.*, 2012) showed that when HRT was started early in postmenopause, a significantly decreased risk of composed endpoint of death, heart failure, or myocardial infarction was observed. These results suggest that HRT, when initiated in recent menopausal women and continued for a prolonged duration does not increase or provoke adverse cardiovascular events. A previous study showed that HRT modulates lipid homeostasis; postmenopausal women that received HRT presented decreased levels of PA and increased levels of oleic acid (Piperi *et al.*, 2005). However, HRT has been subject to much discussion and speculation. In 2002, the

Women's Health Initiative (WHI) showed that the use of Estrogen plus Progestin does not present cardiovascular benefit and suggested in fact an increased risk in breast cancer. Furthermore, the treatment with estrogen alone had no effect on CVD risk (Writing Group for the Women's Health Initiative Investigators, 2002). Because of this study, women started searching for safer alternatives to HRT.

Despite the controversy surrounding HRT, it is true that the current therapy is still the folder standard therapy to decrease the pathophysiology of menopause. Nevertheless, the use of phytoestrogens as alternatives to HRT has been increasing.

1.1.6. Phytoestrogens

An alternative to the classical HRT involves phytoestrogens (PEs), natural, plant-derived, estradiol mimetics. Phytoestrogens are plant-derived compounds that are structurally or functionally similar to 17β-estradiol and therefore exhibit some estrogenic and/or antiestrogenic activity, although being less potent than natural hormones (Borrelli and Ernst, 2010; Eden, 2012). There are four main classes of phytoestrogens: isoflavones, lignans, coumestans and stilbenoids **(Figure 5)** (Borrelli and Ernst, 2010).

The main type that has been therapeutically given to menopausal women involves isoflavones because are the most abundant and studied. In the human diet, phytoestrogens are mostly found in legumes such as soybean, chickpeas, alfalfa and red clover (Eden, 2012). In plants, isoflavones are mainly present as inactive glycosides: genistin and daidzin (Lissin and Cooke, 2000). The majority of phytoestrogens are introduced into the diet as inactive compounds. After consumption, a complex enzymatic conversion occurs, with the sugar residue being removed in the gastrointestinal tract, resulting in the formation of compounds with a steroidal structure similar to estrogens (Albertazzi and Purdie, 2008; Fitzpatrick, 2008).

The class of coumestans and stilbenoids are less abundant in diet and so these compounds have been less studied (Dixon, 2004). Coumestrol belongs to the class coumestans and has higher affinity for estrogens receptors, exhibiting stronger estrogenic activity (Tinwell *et al.*, 2000). Furthermore, coumestrol has direct influence on liver metabolism, through an increase in lipid synthesis and glycogen metabolism in an independent mechanism from its estrogenicity (Nogowski, 1999). The main source

of coumestrol is *Medicago sativa* (alfalfa), *Trifolium pratense* (red clover) and soybeans. The most well-known stilbenoid is resveratrol that has been demonstrated to have antioxidant properties, with its main source of resveratrol being the skin of red grapes and red wine (Khan *et al.*, 2008).

Lignans are biotransformed by intestinal microflora and converted to hormone-like compounds with weak estrogenic activity (enterodiol and enterolactone). Lignans exist in plants as glycosides stored in vacuoles (Adlercreutz, 2007).

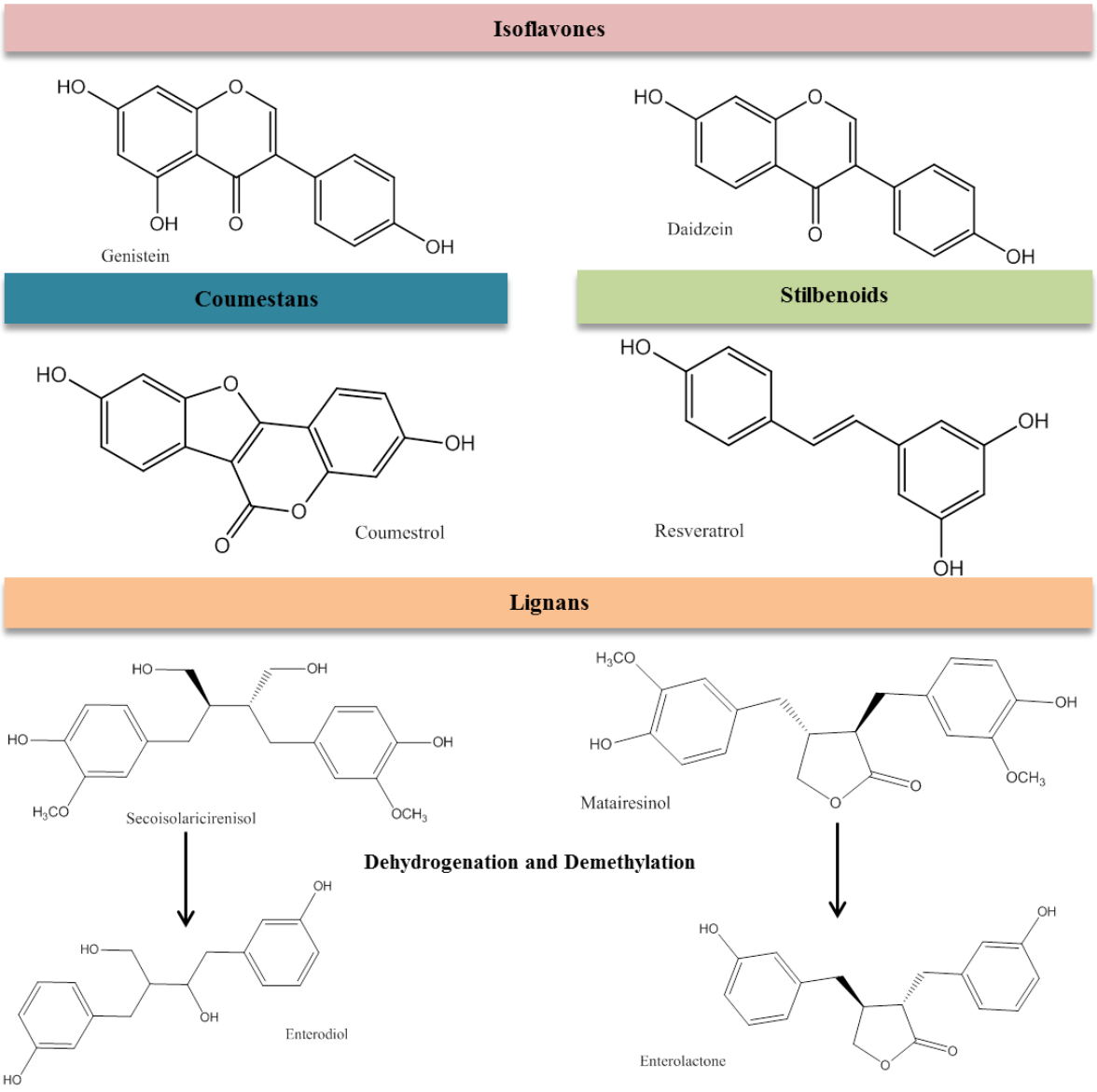

Figure 5 - The main classes of phytoestrogens.

The phenolic ring present in phytoestrogens is one of the prerequisites for the bonding to ER and therefore can work as agonists or antagonists of ERs. Several factors influences the cellular effects of PEs, such as presence or absence of endogenous estrogens, the target tissue and concentration (Setchel, 1998).

Genistein is the most popular PE and is effective as an agonist of both ER at concentrations lower that 10μM (Miyazaki, 2004; Sun *et al.*, 2012). The efficacy of these substances can be explained by their specific binding to the estrogen receptor. There are two types of ER: ER-α and ER-β, with PEs binding with higher affinity to ER-β than to Er-α (Terzic *et al.*, 2012). Thus, PEs show positive estrogenic effects mainly in tissues expressing basically ER-β, such as the bone or the cardiovascular system.

Relatively to symptoms PEs have shown satisfactory results (Jacobs *et al.*, 2009). It has been reported that several PEs can be cardioprotective during the transition to menopause (Song *et al.*, 2007). After treatment with 35 mg isoflavones, osteoprotection, decreased CVD risk, attenuation of hot flushes and night sweats in treated women were already reported (Guojun *et al.*, 2007). One study with lyophilized grape powder in postmenopausal women showed alterations in lipoprotein metabolism, oxidative stress, and inflammatory markers (Zern *et al.*, 2005). Phytoestrogens derived from both, soy and red clover, were shown to have a positive metabolic effect on serum lipids of postmenopausal women. So both sources of PEs can be equally used (Terzic *et al.*, 2012).

1.2. H9c2 cell line as a model for cardiac cells

The H9c2 cell line was derived from embryonic rat heart and isolated by the method of selective serial passage (Kimes and Brandt, 1976). This cell line has been used as *in vitro* model for both skeletal and cardiac muscle due their morphological features and electrophysiological characteristics (Menard *et al.*, 1999).

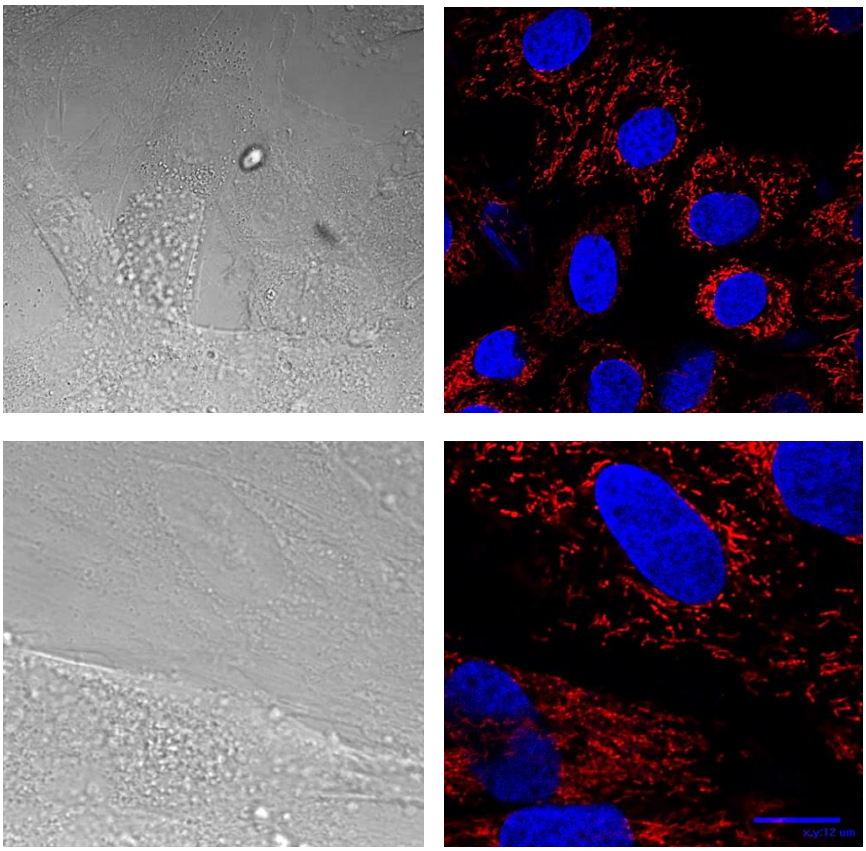

Figure 6 – An example of H9c2 cells incubated with tetramethylrhodamine, methyl ester (TMRM) and Hoechst 33342. The images were obtained using a Nikon C-1 laser scanning confocal microscope equipped with 60x Plan Apo 1.4 NA oil immersion DIC objective. TMRM signal was acquired using a green He-Ne laser and the Hoechst 33342 signal was obtained by using a violet diode laser. Differential interference contrast (DIC) images using the confocal microscope were collected using the air-cooled argon laser and the appropriate detector. Images were captured using the Nikon EZ-C1 software (version 2.01).

One advantage of this cell line is the ability to differentiate and acquire both a phenotype of skeletal muscle such as the heart muscle, depending on the stimulus (Menard *et al.*, 1999). Upon reduction of media serum concentration, H9c2 cell line can be differentiated from mono-nucleated myoblasts to myotubes (Hescheler *et al.*, 1991).

Menard and colleagues (Menard *et al.*, 1999) demonstrated that the treatment of these cells with all-trans retinoic acid (RA) leads to cell differentiation to an adult cardiac muscle phenotype, as seen by specific differentiation markers. Thus, these findings support the possibility that RA may inhibit myogenic differentiation of these cells providing the cardiac phenotype **(Figure 7)** (Menard *et al.*, 1999).

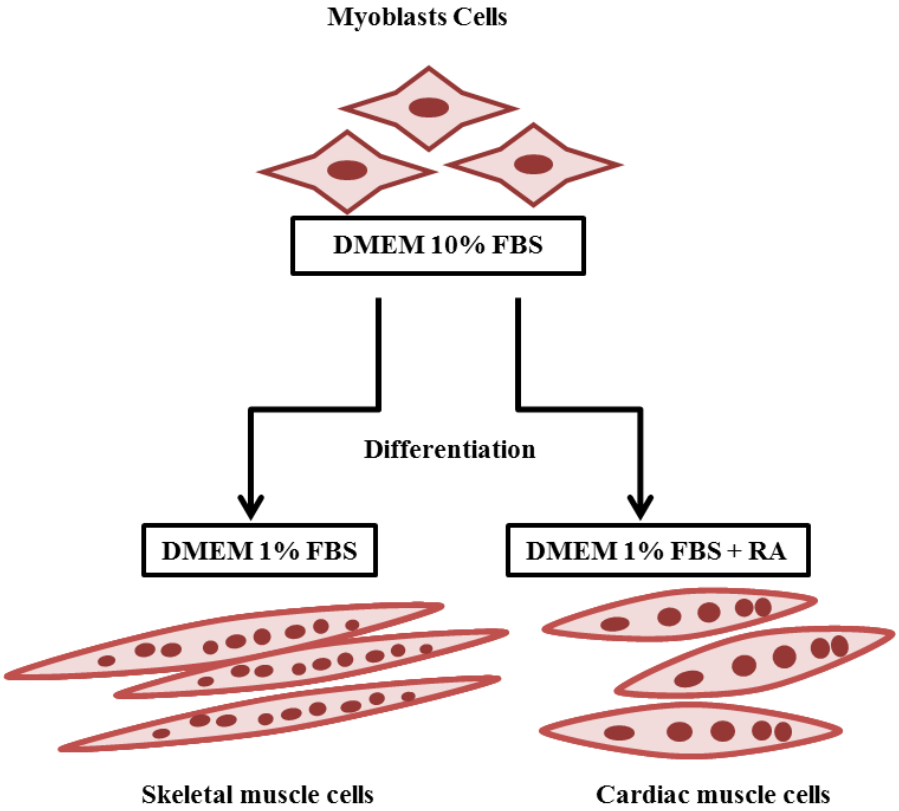

Figure 7 - Schematic representation of the differentiation process of H9c2 myoblasts. After reduction of media serum to 1%, cells differentiate in skeletal muscle cells. However, supplementing with RA induces differentiation into cardiac muscle cells. Abbreviations: DMEM - Dulbecco's modified Eagle's medium; FBS- fetal bovine serum; RA - retinoic acid.

During muscle differentiation some typical features appears, including fusion of myoblast to myotubes and expression of myogenic transcription factors (Chun *et al.*, 2000), calcium channels proteins (Menard *et al.*, 1999).

In the same line of investigation, other authors also reported that the reduction of serum to 1% supplemented with RA, leads into a long and multinucleated cardiomyocytes with higher content in troponin T, phosphorylated troponin I, and ventricular myosin light chain (MLC2V), all markers of cardiac differentiation, in H9c2 differentiated cells (Branco *et al.*, 2011; Pereira *et al.*, 2011). On the other hand, only the reduction of serum to 1% conducts to predominantly skeletal muscle phenotype, presenting multinucleated skeletal myotubes and a reduced content in cardiac markers (Apostolova *et al.*, 1999; Branco *et al.*, 2011; Pereira *et al.*, 2011).

Thus, H9c2 cardiomyoblasts are a good cellular model and an attractive tool to a cardiac surrogate, due to it differentiation potential. Several studies have been used in this cell line to investigate the cardiac toxicity or even to the protection of several compounds, including resveratrol, doxorubicin (DOX) (Branco *et al*., 2012; Sardao *et al.*, 2009b) and isoproterenol (Branco *et al*., 2011; Branco *et al*., 2013). Nevertheless, several studies gave little importance to the differentiation state of the H9c2 cells, but it is known that the susceptibility to different agents is different between undifferentiated and differentiated cells (Branco *et al.*, 2011). During differentiation process the H9c2 metabolism is also altered, involving different effects on both cell populations when compounds interacts and modulate cell metabolism (Pereira *et al.*, 2011). Another characteristic of H9c2 cardiomyoblasts is the expression of ERs, more concretely ER-β (Urata *et al.*, 2006), which is a feature essential for the main goal of the present thesis.

Due to all the reasons refereed above, we consider the H9c2 cell line a good cellular model to study the protection of PEs against cardiac lipotoxicity, although the *in vitro* differentiation can cause stress to the cells activating different signaling pathways that can lead at autophagic processes.

1.2.1. Autophagy Pathway

The autophagy ("to eat oneself") is a process that involves the cellular degradative pathways, being the major regulated mechanism an also the only one degradative pathway of the organelles (Levine and Yuan, 2005). There are three different autophagic pathways identified, namely macroautophagy, microautophagy and chaperone mediated autophagy (Levine and Kroemer, 2008), but in the present thesis we will focus only on macroautophagy (hereafter referred to as autophagy).

In normal conditions, autophagy occurs at low basal levels in order to maintain cell homeostasis, namely, cytoplasmic and organelle turnover (Klionsky, 2000; Levine and Kroemer, 2008; Levine and Yuan, 2005). However, cell starvation or deprivation of growth nutrients can lead to an induction of autophagy, providing the energy required for cell survival (Ichimura and Komatsu, 2010; Klionsky, 2000).

During autophagy, the formation of an isolation membrane (vesicle nucleation), which sequesters cytoplasmic material including mitochondria, endoplasmic reticulum,

and ribosomes (vesicle elongation) occurs. Afterwards, the edges of the membrane fuse and form a structure with a double membrane, designed by autophagosome. Then, the autophagosome fuses with lysosome leading to autolysosome formation, where the sequestered material is degraded and recycled. The degradation of the sequestered material generates fatty acids or aminoacids, which in turn can be used by mitochondria for the ATP production, provided cell survival **(Figure 8)** (Ichimura and Komatsu, 2010; Levine and Kroemer, 2008; Levine and Yuan, 2005).

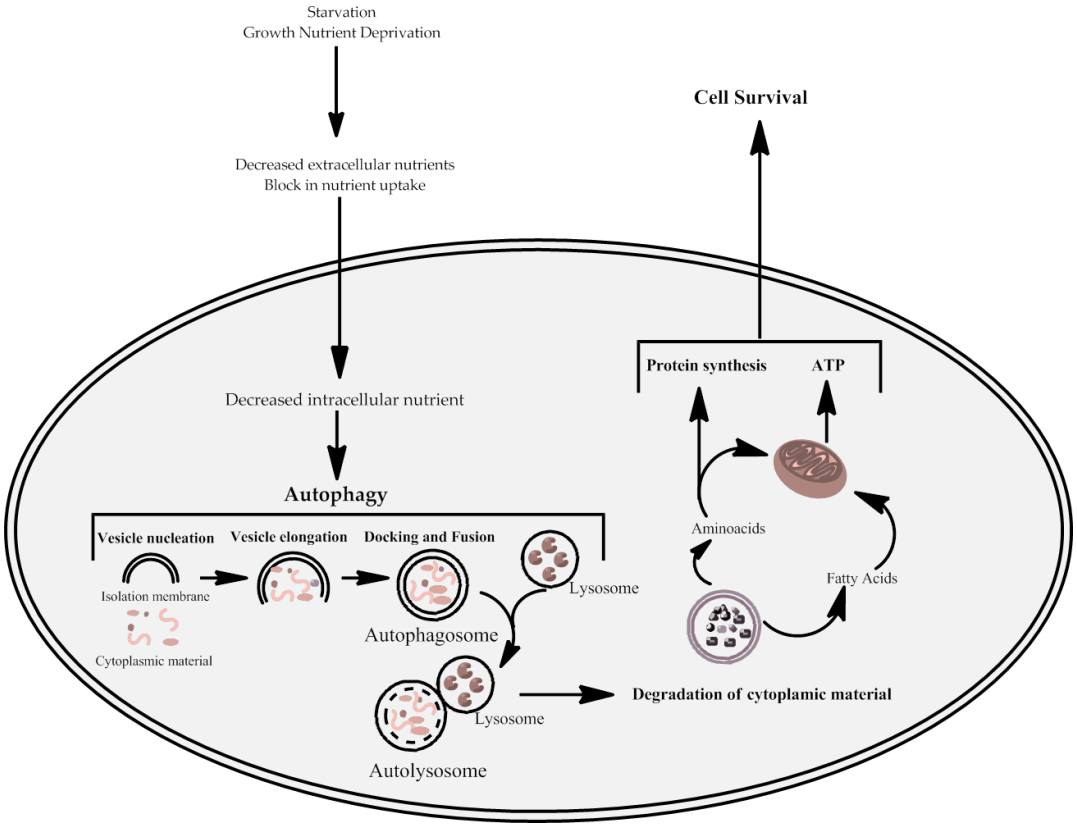

Figure 8 - The macroautophagy pathway, during a starvation or in growth nutrient deprivation condition, adapted from (Levine and Kroemer, 2008; Levine and Yuan, 2005).

As mentioned above, the initiation of the autophagic pathway is characterized by the presence of an induction phase, which is triggered by cellular stresses. Considering the starvation condition, autophagy can be induced by inhibition of mechanistic target of rapamycin (mTOR), the major autophagy inhibitory signal (Kang *et al.*, 2011; Klionsky, 2000; Levine and Kroemer, 2008). The complex UNC-51–like kinase 1 (ULK1), autophagy related gene (ATG13), FIP200, and ATG101 can be activated due

to mTOR inhibition, allowing the initiation of autophagy signaling. After activation of the ULK1 complex, it can regulate the class III phosphatidylinositol 3-kinase (PI3K) complex, which includes Beclin-1 (Mizushima and Komatsu, 2011). Moreover, the stimulation of this complex leads to phosphatidylinositol-3-phosphate (PI3P) generation, contributing to vesicle nucleation and serves as signal to the recruitment of proteins required for vesicle elongation (Sinha and Levine, 2008). There are two ubiquitin-like conjugation systems, such as the ATG5-ATG12 and the light chain 3 (LC3), which are required for the autophagosomal elongation (Choi *et al.*, 2013).

Regarding the LC3 pathway, firstly it is proteolyzed in its C-terminus by the cysteine protease ATG4 and afterwards it is activated by the E1 ubiquitin-like enzyme ATG7, being further transferred to the E2 ubiquitin-like enzyme ATG3 allowing phosphatidyl ethanolamine (PE) connection to LC3-I that origin LC3-II that associates to the autophagosomal membrane (Choi *et al.*, 2013; Levine and Kroemer, 2008). This conversion, LC3-I into LC3-II is a key regulatory step in autophagosome formation.

In the final step, the degradation of autophagosomal contents is carried out by lysosomal acid hydrolases, being released for metabolic recycling (Choi *et al.*, 2013). Notwithstanding, selective mechanisms were described (Ichimura and Komatsu, 2010; Komatsu and Ichimura, 2010), taking advantage of p62/SQSTM1 (sequestosome 1). This interacts with LC3-II, being transported into the autophagosome (Ichimura and Komatsu, 2010), acting as selective autophagy substrate and cargo receptors for degradation of ubiquitinated substrates.

2. HYPOTHESIS AND OBJECTIVE

Menopausal women have increased incidence of CVD because estrogen loss negatively impacts lipid metabolism and cardiovascular function (Rosano *et al.*, 2007). Some studies with PEs have demonstrated some effects on the symptoms and complications of menopause, such as osteoporosis and cardiovascular disease (Beck *et al.*, 2005). For that reason, the main hypothesis of the present work is that coumestrol and enterodiol, two promising PEs, are able to protect cardiac tissue against cardiac lipotoxicity induced by free fatty acids.

In this context, the H9c2 cell line, an *in vitro* model for cardiac cells and PA as a induced-lipotoxicity agent were used in the present work. Nevertheless, for this study an alternative protocol for cell culture was used, namely, the use of serum-free medium without phenol-red, a pH indicator present in culture media. Phenol-red can mimic the biological effects of estradiol in estrogen-sensitive cells because it binds to estrogen receptors (Berthois *et al.*, 1986; Welshons *et al.*, 1988), masking the effect of estradiol or even of PEs and leading to underestimation of the response potential of cells. On the other hand, serum, specifically Fetal Bovine Serum (FBS), contains hormonal factors and other growth factors which stimulate cell proliferation; further we are generally not aware of the real composition of FBS. So, this is disadvantage because it may lead to underestimation of the results.

Concerning these evidences, the first step of the present work was the characterization of H9c2 cells in absence of serum. Given the literature (Branco *et al.*, 2011; Menard *et al.*, 1999), we hypothesize that H9c2 cardiomyoblasts cultured in absence of serum can acquire a cardiac phenotype. In order to characterize the cell line, cardiac markers were evaluated. Furthermore, autophagy signaling was assessed due to its occurrence during cell starving and also due to their role in cell differentiation (McMillan and Quadrilatero, 2014; Mizushima and Levine, 2010).

Regarding the main aim of this project, it can be divided in different experimental objectives:

1. The cytotoxicity evaluation of PA using different concentrations and cytotoxicity of the selected PEs;
2. The evaluation of the protective effects of PEs against PA by several parameters including cell proliferation, mitochondrial alterations and autophagy signaling markers.

The study of the protective effects of PEs in cardiac cells is important because it may allow design new strategies to minimize the pathologies associated with menopause, including CVD.

3. MATERIAL AND METHODS

3.1. Reagents

The reagents for cell culture, such as Dulbecco's modified Eagle's medium - high glucose (DMEM-HG) (D5648), Dulbecco's modified Eagle's medium (D5030), L-glutamine, glucose, sodium bicarbonate, taurine (Catalog #T8691), creatine (Catalog #C0780), L- carnitine, insulin-transferrin-sodium selenite (ITS) (I3146), bovine serum albumin (BSA) (Catalog #A1595), hydrocortisone (Catalog #H0135), dimethylsulphoxide (DMSO), 17beta-estradiol, enterodiol, coumestrol, sodium palmitate, BSA free fatty acid and phenylmethylsulfonyl fluoride (PMSF), sulforhodamine B (SRB) were obtained by Sigma (St. Louis, MO, USA). FBS and 0.05% Trypsin–EDTA were purchased from Gibco-Invitrogen (Grand Island, NY). Cell lysis buffer (Catalog #9803) was purchased from Cell Signaling (Danvers, MA, USA). Additionally, the reagents used for Western Blot, including, nonfat dry milk (Catalog #170-6404), Tris-HCl, 0.5M pH 6.8, 1.5 M Tris pH 8.8, sodium dodecyl sulfate (SDS) and acrylamide were purchased from BioRad (Hercules, California, USA) Also, tetramethylethylenediamine (TEMED) and protein marker (Catalog #MB17601) were obtained by Nzytech (Lisboa, Portugal).

3.2. Preparation of palmitic acid/BSA complexes

Palmitate has a lower solubility in aqueous solutions and thus its careful preparation is a critical step for cell studies that involves the utilization of this compound. Accordingly, BSA has been used as a vehicle, and stabilizing agent, for insoluble fatty acids. The conjugation of palmitate to BSA allows cells to absorb and use palmitate.

A 6:1 molar ratio palmitate:BSA was used, with concentrations of 5mM palmitate and 0.83mM BSA solution. Firstly, a 1.66mM BSA solution in 150mM sodium chloride (NaCl) was prepared. In this step BSA was added to pre-warmed NaCl solution while stirring constantly allowing to dissolve BSA. Then, 10mM PA solution was prepared in 150mM NaCl solution at 70 °C while stirring, to help in the solubilization. Finally, the conjugation of palmitate and BSA was performed by transferring 50ml of 70 °C palmitate solution to 50ml of BSA solution while stirring at 37 °C during 1 hour. After this, the pH of the solution was checked and adjusted to 7.4 with 1N sodium hydroxide (NaOH) and the solution was filtered. Aliquots were kept frozen at -80 °C. The

remaining BSA solution was diluted with 50ml of 150mM NaCl solution to make a 0.83mM stock and was filtered and aliquots were freeze at -80 °C.

3.3. Cell line and culture conditions

The H9c2 cardiomyoblasts, derived from embryonic rat heart were purchased from ATCC (Manassas, VA; catalog # CRL – 1446), stored frozen in liquid nitrogen vapor phase and expanded following manufacturer's instructions.

For the present study, cells were cultured in DMEM-HG supplemented with 1.5g/L sodium bicarbonate, 10% of FBS at 37 °C in a humidified atmosphere of 5% CO_2. Cells were fed every 2-3 days and subcultured once they reached 70-80% confluence in order to prevent the loss of differentiation potential. For this routine, subculturing cells were first rinsed with 1x phosphate buffered saline (PBS) (0.137M NaCl, 2.7mM potassium chloride (KCl), 1.4mM potassium hydrogen phosphate (KH_2PO_4), 0.01M sodium hydrogenophosphate (Na_2HPO_4)) and then incubated with 1 volume of trypsin-EDTA for 5 min at 37 °C. Trypsin activity was inhibited by the addition of 1 volume of complete growth medium and the final volume was centrifuged at 115 x*g* for 5 minutes at room temperature. An appropriate aliquot of the cell suspension was added to new culture flasks. Cells were used between passages 4 and 21.

In order to answer our question, H9c2 cells were seeded and cultured for 24 hours in high-serum that promoted proliferation. After this time, the medium was changed to DMEM serum and phenol-red free media supplemented with L-glutamine (4mM), glucose (250mM), sodium bicarbonate (17.8mM), taurine (60mM), creatine (20mM), L-carnitine (1mM), insulin (1mg/ml)-transferrin (0.55mg/ml)-sodium selenite (0.5µg/ml) (ITS), BSA 10% and hydrocortisone (50µg/ml) **(Table 1)**. Cells were cultured for 3 more days to allow the adaptation to new conditions.

Table 1 - Differences between the media composition used in this work.

Medium composition	
Serum-free	10% Serum
DMEM BASE D5030 (Sigma, St. Louis, MO, USA) Supplemented with: **L-glutamine (4mM), glucose (250mM), sodium bicarbonate (17.8mM), taurine (60mM), creatine (20mM), L- carnitine (1mM), insulin (1mg/ml)-transferrin (0.55 mg/ml)-sodium selenite (0.5 µg/ml) (ITS), 10% of BSA and hydrocortisone (50µg/ml).**	**DMEM D5648** (Sigma, St. Louis, MO, USA) Supplemented with: **1.5 g/ml of sodium bicarbonate and 10% of FBS**

Experiments were thus conducted in two experimental groups: **(a)** undifferentiated myoblasts in 10% serum **(b)** differentiated muscle cells in serum-free medium.

For Western Blot assays, cells were seeded in 150mm bottom diameter dishes with the density of 5,000 cells/cm^2 for the 10% serum condition and 8,000 cells/cm^2 for serum-free condition.

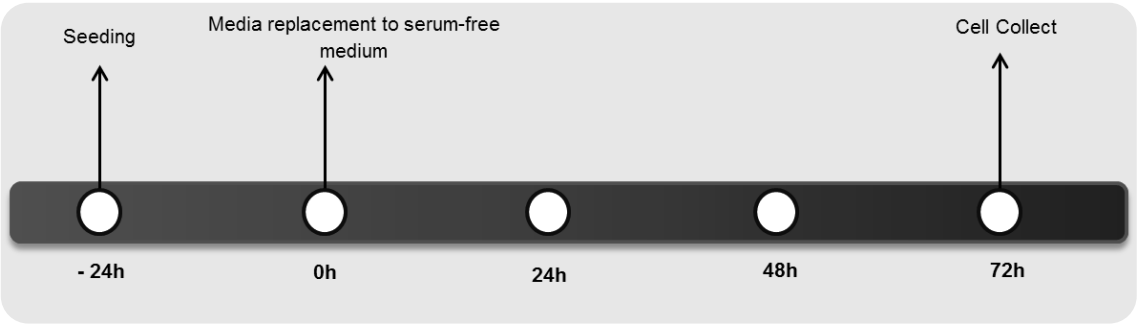

Figure 9 - Experimental design of cell culture and collection. Cells were seeded in high serum medium and after 24 hours, corresponding 0 hours time point, the medium was replaced by a serum-free modified medium. After that, cells were maintained in the same medium during 72 hours, being collected for western blot after this time in culture.

In order to answer our second objective, H9c2 cells were seeded and cultured for 24 hours in high-serum media. After this time the medium was changed to serum-free medium and cells were cultured for 3 more days to allow the adaptation to new conditions. In the third day, cells were treated as desired and experiments were performed in five experimental conditions: **(a)** Control BSA, **(b)** Control PA, **(c**) Estradiol plus PA, **(d)** Enterodiol plus PA, **(e)** coumestrol plus PA.

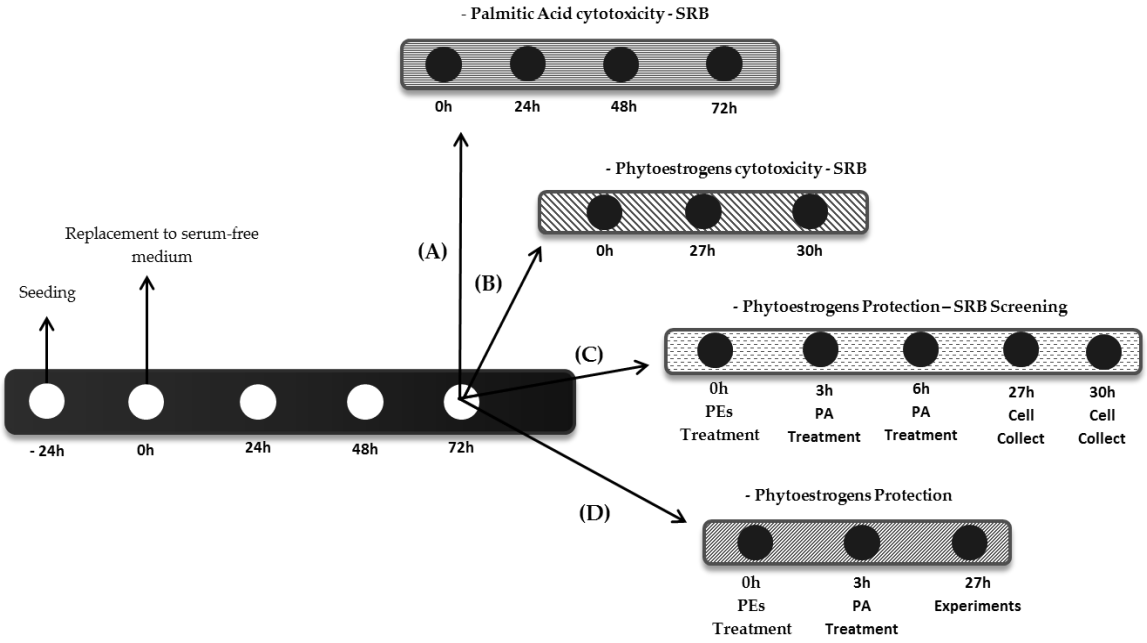

Figure 10 - Experimental design of cell culture and treatment. Cells were seeded in high serum medium and after 24 hours, corresponding to time 0 hours, the medium was replaced by serum-free medium. After that, cells were maintained in the same medium during 72 hours, being **(A)** treated with PA (up 500µM) during 24h, 48 and 72h in order to evaluate the cytotoxicity by SRB assay; **(B)** treated with PEs (coumestrol, enterodiol) and 17β-estradiol (up to 10µM) during 27h and 30h in order to evaluate the cytotoxicity by SRB assay; **(C)** treated with PEs during 3h or 6h (up to 10µM) and after with PA (50µM) during 24h and cell mass was evaluate by SRB assay; **(D)** treated with 50µM of PA 3h after treatment with PEs (1µM) and 17β-estradiol (100nM) and was maintained for 24h. Several parameters were evaluated, including mitochondrial membrane potential by flow cytometer and protein content by Western Blot.

For the present thesis, the cytotoxic effect of PA on H9c2 cells was evaluated to determine the exposure time and concentration of PA to use in the following experiments. The cell line was cultured according as described above and was seeded with the density of 8,000 cells/cm^2 in 48 well-plates. The cell treatment was performed

with different PA concentrations (0, 50, 100, 250 and 500μM) for 24, 48 and 72h. The cytotoxic effect of PA on H9c2 cells was assessed by the SRB assay. Control with BSA, the vehicle for palmitic acid was also performed.

In order to evaluate PEs cytotoxicity, H9c2 cells were treated with PEs (enterodiol and coumestrol) and 17β-estradiol with concentrations of 0.1μM, 1μM and 10μM during 27 hours or 30 hours of incubation, the total time that cardiomyoblasts were incubated with PEs (3 and 6 hours of pre-incubation plus 24 hours with PA). Control with 0.1% of DMSO (the vehicle for PEs) was also used. After treatment, H9c2 cell density was assessed by the SRB assay.

3.4. Samples

3.4.1. Total Protein isolation

In order to obtain cellular extracts, H9c2 cells were harvested by trypsinization and washed once with 1x PBS with two centrifugation steps performed at 1,000 xg for 5 minutes at 4 °C, cells were lysed in cell lysis buffer (Cell Signaling, Danvers, MA, USA) supplemented with 1 mM PMSF. The cell suspension was then rapidly frozen and kept at -80 °C until used. For protein analysis by western blot, the protein contents were determined by the Bradford method, using BSA as a protein standard.

3.5. Protein quantification

3.5.1. Bradford Assay

The Bradford method is based on the binding of Coomassie Brilliant Blue G-250 dye to proteins, being converted to a stable unprotonated blue form with maximum absorbance of the 595 nm (Bradford, 1976). Compto and Jones (Compton and Jones, 1985) demonstrated that this dye binds primarily to basic (especially arginine) and aromatic amino acid residues.

The Bradford Reagent 2x was prepared by adding 0.02% (w/v) Brilliant Blue G, 5% (v/v) methanol and 8.5% (w/v) phosphoric Acid. BSA was used as a standard with protein solutions ranging from 0.125mg/ml to 2mg/ml. The reaction was initiated by adding 100 μL of working reagent diluted 1:2 in ultrapure water to 5μL of sample diluted 1:100 in ultrapure water and collection buffer, in a 96 microplate **(Table 2)**.

After 20 minutes incubation at room temperature, the absorbance of the wells was read in a VICTOR X3 plate reader (Perkin Elmer Inc.) at 595 nm. Standards and unknown samples were performed in triplicates.

Table 2 - Quantification table for Bradford assay with standards and samples preparation

Tube #	[Standard] (mg/ml)	Standard Volume (µl)	Collecting buffer (µl)	dH_2O (µL)	$[Protein]_{final}$ (µg/ml)
1	2	5	10	485	20
2	1	5	10	485	10
3	0.5	5	10	485	5
4	0.25	5	10	485	2.5
5	0.125	5	10	485	1.25
6 (blank)	-----	----	10	490	0
Sample (µl)					
Unknown	-----	5	5	490	-----

3.6. Protein analysis by Western Blot

After protein quantification, samples were prepared in 6x concentrated Laemmli Buffer (5µL of sample to 1µL of Laemmli buffer 6x) to achieve a working concentration of 1.25 µg/mL. When the Laemmli buffer contains detergent (SDS in the present case), all of the proteins obtained a uniform negative charge that provides a separation according to their molecular size (Gallagher, 2012).

In order to denaturate protein, samples were heated at 95 °C for 5 minutes, allowing the loss of quaternary, tertiary and secondary protein structure. Subsequently equivalent amounts of proteins (10 to 25 µg) were separated by electrophoresis on 10% or 14% SDS-polyacrylamide gels (SDS-PAGE), depending on the molecular size of the proteins of interest, allowing for a better separation resolution. Acrylamide and bis-acrylamide are two compounds that after polymerization allow the formation of polyacrylamide gels. The polymerization was initiated by the addition of ammonium persulfate (APS) and catalyzed by TEMED. Separation was carried out at room temperature and until the front of the run reached the bottom end of the gel. When

protein separation was complete, proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) (Millipore, Billerica, MA, USA) membrane pre-activated (5 sec in 100% methanol followed by 15 min in 25mM Tris, 190mM glycine and 20% methanol). For this purpose, gels were placed in a 'transfer sandwich' (filter paper-gel-membrane-filter paper) and protein transfer was performed at a constant voltage (100V) during 90 min at 4 °C.

Once protein transfer was complete, membranes were stained with Ponceau reagent before blocking to confirm equal protein loading in each lane (Colella *et al.*, 2012; Gilda and Gomes, 2013). Afterwards, membranes were incubated with blocking solution, 10 % non-fat dry milk or 5 % BSA in Tris-buffered saline Tween-20 (TBS-T) (154mM NaCl, 50mM Tris pH 8.0 HCl) and 0.1% Tween-20, depending of the protein of interest, overnight at 4 °C with agitation to block non-specific binding. Membranes were then incubated with a primary antibody directed against the respective protein overnight at 4 °C **(Table 3)**. Primary antibodies were prepared in 1% non-fat dry milk or 1% BSA solution in TBS-T supplemented with sodium azide to a final volume of 5 ml.

Membranes were further washed with TBS-T and incubated with alkaline phosphatase conjugated secondary antibodies (1:2,500 dilution) for 1 hour at room temperature under continuous stirring. Finally, membranes were washed again and incubated with the Enhanced Chemi-Fluorescence (ECF) detection system (from Amersham, Little Chalfont, Buckinghamshire, United Kingdom) and read with a Versa Doc imaging system (Bio-Rad). The ECF substrate is dephosphorylated by alkaline phosphates that are present in secondary antibody, leading to the formation of a fluorescent product which emits at 540 to 560 nm when excited. Densities of each band were calculated with Quantity One Software (Bio-Rad).

Table 3 - List of primary antibodies used in Western Blot protein analysis.

Primary Antibody	Molecular weight	Dilution	Host specie	Company	Catalog Number
GATA4	54kDa	1:1,000	Rabbit	abcam	ab134057
Troponin T	40kDa	1:500	Rabbit	Cell Signaling	5593
MLC 2v	19kDa	1:1,000	Mouse	Synaptic Systems	310.111
Beclin-1	60kDa	1:2,000	Rabbit	Cell Signaling	3595
p62	62kDa	1:1,000	Rabbit	MBL	PM045

3.7. Cell density evaluation by Sulforhodamine B (SRB) assay

Sulforhodamine B is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino-acid residues under mild acidic conditions (Vichai and Kirtikara, 2006). This colorimetric method is based on measurement of cellular protein content and depends on the ability of SRB to bind to protein components of cells that have been fixed by methanol (Vichai and Kirtikara, 2006).

This assay was performed in order to evaluate the cytotoxic effects of PA, the selected PEs and the protective effects of PEs against PA-induced lipotoxicity. After treatment, cells were washed in PBS and fixed in ice-cold methanol supplemented with 1% acetic acid for at least one hour at -20 °C and allowed to dry. After that, 0.05% SRB dissolved in 1% of acetic acid was added to each well and incubated for 1h at 37 °C. The unbound dye was removed with 1% acetic acid solution and allowed to dry. The dye bound to cell protein was solubilized in 10mM Tris pH 10 solution and the optical density of the solution was determined at 530 nm in a Victor X3 plate reader (Perkin Elmer Waltham, USA).

3.8. Evaluation of mitochondrial membrane electric potential

The mitochondrial membrane electric potential ($\Delta\Psi_m$) was evaluated using tetramethylrhodamine methyl ester (TMRM) probe by distinct methods, flow cytometer and microscopy. This probe is a lipophilic cation that accumulates in polarized mitochondria according to their $\Delta\psi_m$ value because of their charge and solubility in both

the inner mitochondrial membrane and matrix space (Scaduto and Grotyohann, 1999). The extent of its accumulation, as measured by intensity of cellular fluorescence, is proportional to cellular $\Delta\psi_m$ (Wlodkowic *et al.*, 2009). The decreased intensity of TMRM fluorescence is associated to cells with collapsed mitochondrial transmembrane potential (Wlodkowic *et al.*, 2009).

3.8.1. Flow cytometer

On the day of the assay, treated cells were harvested by trypsin treatment and ressuspended in the following buffer: 120mM NaCl, 3.5mM KCl, 0.4mM KH_2PO_4, 20mM HEPES, 5mM sodium bicarbonate, 1.2mM sodium sulfate ($NaSO_4$), 10mM sodium pyruvate at pH 7.4 supplemented with 1.2mM magnesium chloride ($MgCl_2$), 1.3mM calcium chloride ($CaCl_2$). Incubation with 100nM TMRM (Invitrogen, Molecular Probes) for 30 min at 37 °C was performed. A sample of non-labeled cells was also analyzed in order to calibrate the system, taking cell self-fluorescence into account. Cells (10,000 cells) were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, California, USA), using the FL2 filter sets. During preliminary tests, all cell groups without TMRM were analyzed to ascertain the sensitivity, reliability and best analysis to perform. Data was analyzed using BD CellQuest Pro software package.

3.9. Statistics analysis

Data analysis was performed by using GraphPad Prism 6.0 program (GraphPad Software, Inc.) and data were expressed as mean ± SEM for the number of experiments indicated in the legends of the figures. For statistical analysis of two means, including for comparison of control groups (10% Serum) and serum-free medium, the Student's t test was performed. Multiple comparisons were performed using one-way analysis of variance (ANOVA) or two-way ANOVA, depending on the variables under study. For one- and two-way ANOVA, corrections for multiple comparisons were made using Bonferroni post-hoc test. Significance was accepted with p value < 0.05.

4. RESULTS

4.1. Characterization of H9c2 cardiomyoblasts

4.1.1. H9c2 cells can differentiate into cardiomyocytes in absence of serum

In this chapter, we aim to characterize the H9c2 differentiation process when cultured in serum-free medium. During the differentiation process, alterations are observed in H9c2 cells, including myoblasts fusion to form multinucleated and larger cells, reduced proliferation, and increased content in specific markers for adult cardiomyocytes (Menard *et al.*, 1999). In this study, cells were maintained in serum-free medium in order to avoid interference with serum's estrogens, as described before. Thus, we investigated whether serum-free medium-driven cell differentiation leads to increase of specific markers for adult cardiac cells. In this regard, GATA4, Mlc2v and Troponin T were selected because they are recognized of markers of cardiac differentiation (Molkentin *et al.*, 1997; Ng *et al.*, 2010; York *et al.*, 2007). GATA4 is a cardiac-specific marker that is implicated in heart development (Molkentin *et al.*, 1997). In the contractile system, cardiac troponin T has a fundamental role, and is also present in a confined stage of fetal skeletal muscle development (Swiderski and Solursh, 1990). The degradation of cardiac troponin T is also associated with dysfunction in the contractile structure during cardiac pathologies (Beatty *et al.*, 2013). Although no differences were observed between cells grown in 10% serum and serum-free medium, it is possible to observe that all cardiac markers were similar in both tested groups **(Figure 11)**.

Additionally, is important refer that the results were normalized to Ponceau labeling. This experimental strategy was preferred over the use of housekeeping proteins because they can be affected by the differentiation process, since that H9c2 cells morphology alters in this condition.

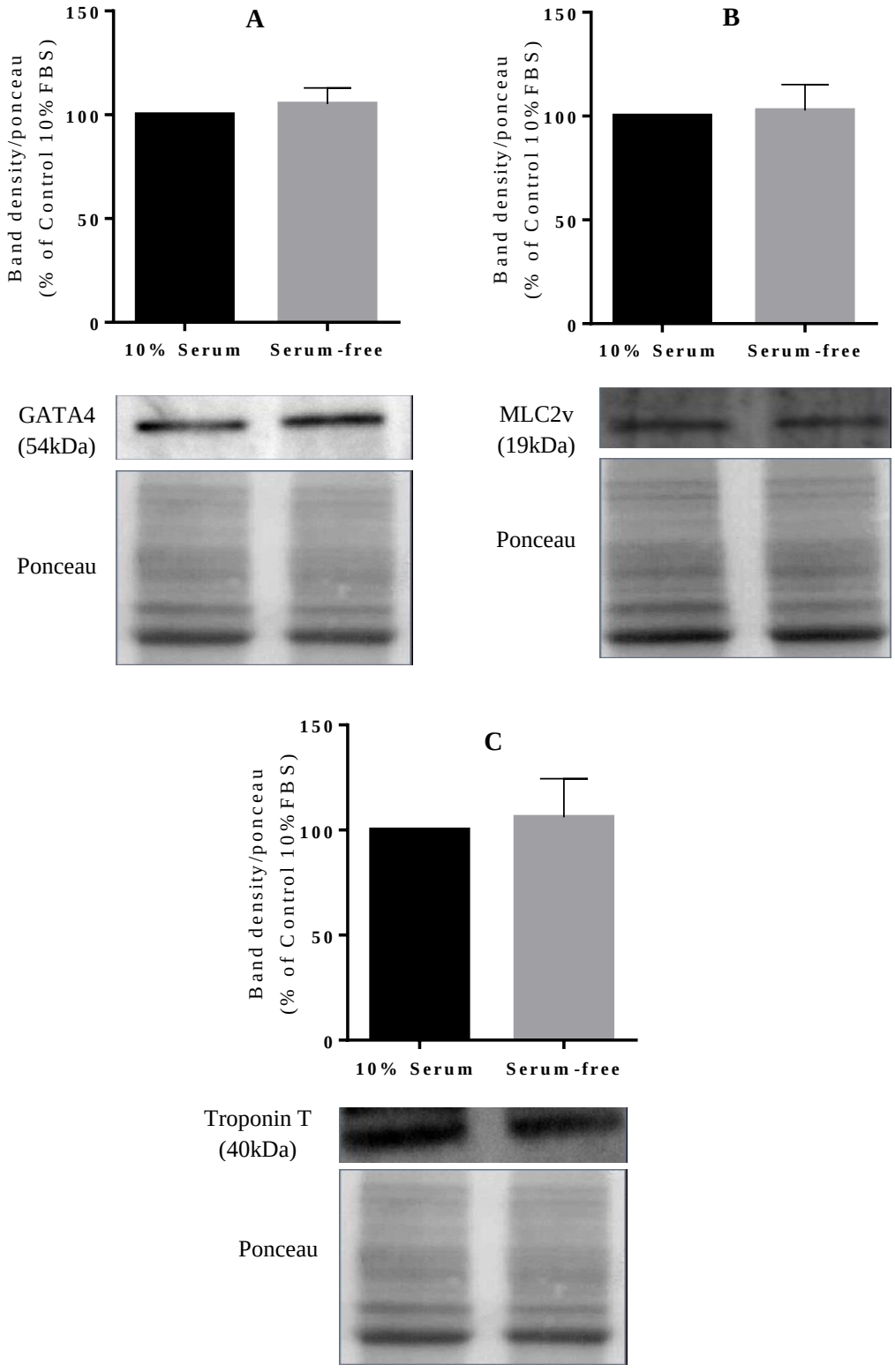

Figure 11 - Western Blot analysis of specific cardiac markers in cells grown in 10% serum and serum-free medium. **(A)** GATA4 **(B)** Mlc2v **(C)** Troponin T protein content, three recognized cardiac m markers. The images and quantification are representative of 4 separate experiments from independent cell cultures and bars show mean±SEM. Control loading was performed by using Ponceau labeling. Protein levels were normalized to Ponceau.

4.1.2. Autophagy pathways is activated when H9c2 cells were cultured in serum-free media

Taking into account that serum starvation can lead to activation of autophagy pathways, the next question was how serum absence affects these processes of quality control in cells. Moreover, alterations in autophagy can occur during cell differentiation, in which morphological and biochemical transformation are required. In this regard, several autophagy markers were evaluated through Western Blot in H9c2 cells. First, we evaluated Beclin-1 protein content, after culture of H9c2 cells during 72h in serum-free medium. This protein is required for the promotion of autophagossome formation in cytoplasm, initiating macroautophagy (Levine and Kroemer, 2008). The results showed an increase of Beclin-1 protein content in myoblasts cultured in medium without serum, when compared to control (10% of serum) **(Figure 12)**.

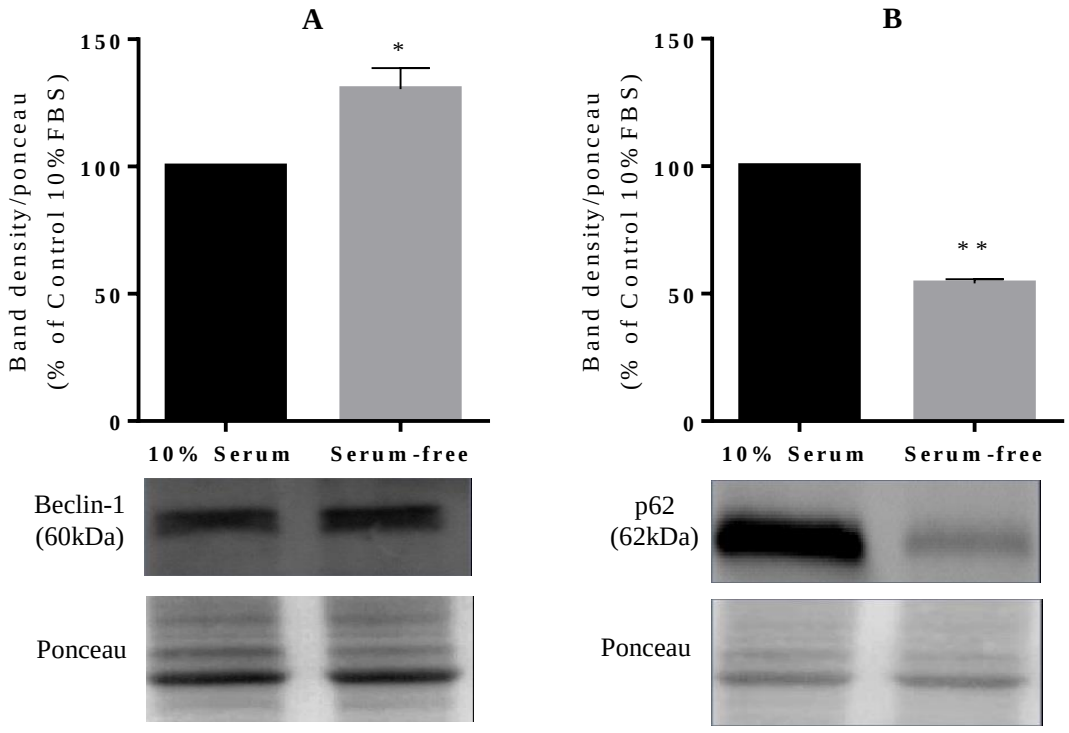

Figure 12 - Alterations in autophagy pathways during H9c2 myoblast differentiation. **(A)** Beclin-1, associated with autophagosome formation, was evaluated by Western Blot. Beclin-1 protein content in H9c2 cells was increased when compared to 10% serum control. **(B)** The p62 protein content was decreased in cells cultured in serum free medium, when compared to 10% of serum control. The images and quantification are representative of 4 separate experiments (for Beclin-1) and 3 separate experiments (for p62) from independent cell cultures and bars show mean ±SEM (*$p<0.05$ and **$p<0.01$, two-tailed t-students test). Control loading was performed by using Ponceau labeling. Protein levels were normalized to Ponceau.

Altogether, cell culture in serum-free medium results in an increase of Beclin-1. These results suggest that autophagy signaling may be activated when H9c2 cells are cultured in serum-free medium.

Moreover, to confirm that autophagy signaling was activated, autophagy flux was evaluated by analysis of p62 protein content by western blot. The p62 protein is involved in the recognition of toxic cellular waste by interaction with polyubiquitinated cargo (Komatsu and Ichimura, 2010). Additionally, p62 interacts with LC3 during autophagosome formation (Mizushima and Komatsu, 2011). Taking into account its role in autophagy, p62 is considered a marker for autophagy flux in cells (Komatsu and Ichimura, 2010; Komatsu *et al.*, 2012). Thus, suppression in autophagy leads to an accumulation of p62 (Komatsu and Ichimura, 2010). As can be seen in Figure 12, a significant decrease in levels of p62 protein content was observed in cells cultured in serum-free medium in comparison with control group (10% of serum).

In summary, the presence of main autophagy markers as well as the decreased levels of p62, suggest an augmented autophagy flux in H9c2 cells cultured in serum-free medium.

4.2. Protection provided by phytoestrogens against lipotoxicity

4.2.1. Palmitic Acid decrease cell proliferation of cardiomyoblasts

The cytotoxicity associated with PA was assessed on H9c2 cardiomyoblasts in the concentration range of 50 - 500µM using SRB assay, after 24h, 48h and 72h. Cells were exposed to palmitic acid-BSA conjugate or to BSA only.

Figure 13A shows cell density in relation to time zero, which is the time that the treatment begins, while Figure 13B shows cell density in comparison to control with vehicle, BSA.

The results in both graphs show decreased cell density of cardiomyoblasts in a dose-dependent manner for each incubation time. The toxicity of palmitic acid was statistically significant starting from 50µM PA in all incubation time points (24h, 48h and 72h). However, for the higher PA concentrations (from 100µM) cell mass did not change. Based on toxicity assays, 50µM concentration of PA during 24 hours was chosen for further experiments, aimed at measuring the protection afforded by PEs. Taking into account the literature, this concentration of palmitic acid is physiologically

appropriate. Total plasma FAs can be as high as 1mM or even higher under some pathologies but including obesity or metabolic syndrome (Belfort *et al.*, 2005).

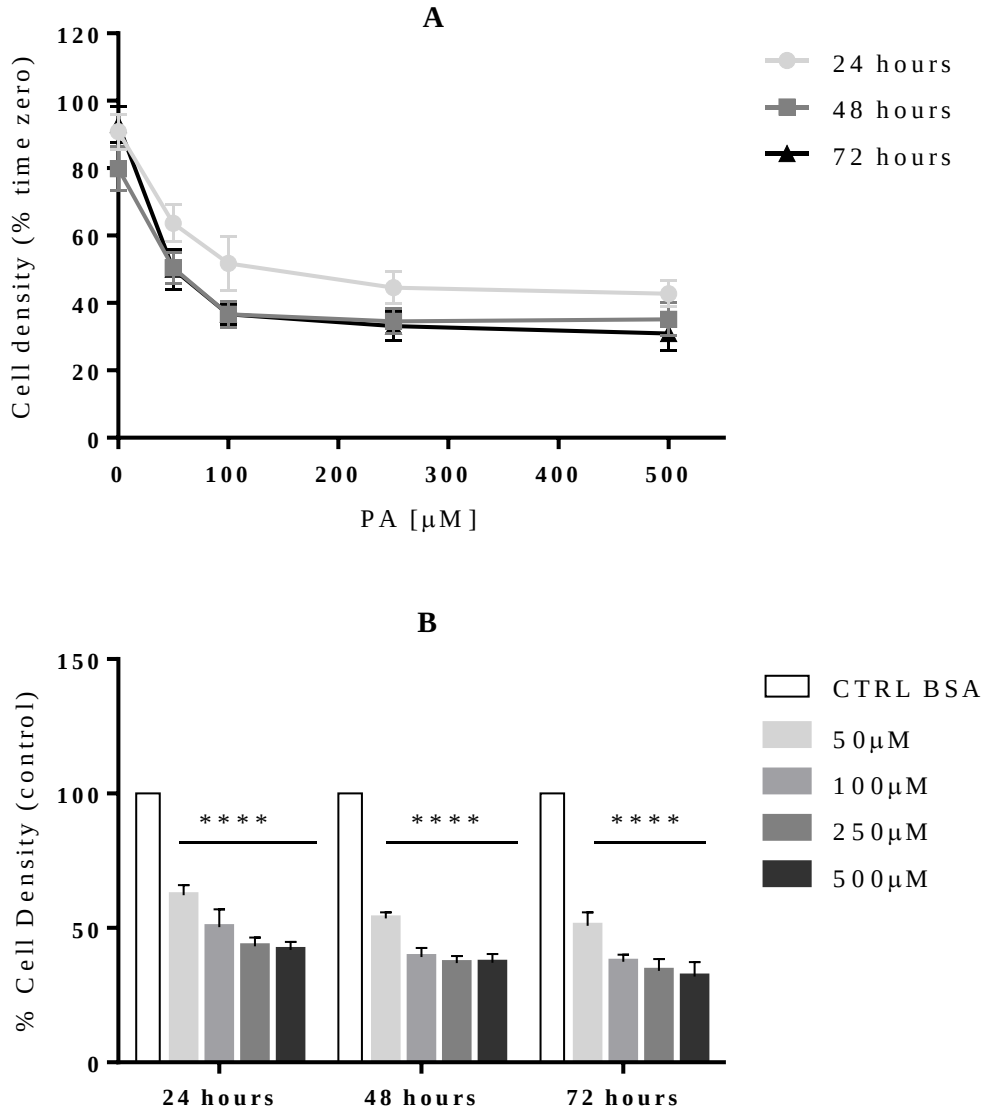

Figure 13 - Palmitic acid (PA) decreases cell proliferation of H9c2 cardiomyoblasts **(A)** The % of cell density in relation to time zero and **(B)** the % of cell density in comparison to control BSA only. Data represent mean ±SEM from 6 independent experiments and were analyzed by two-way ANOVA following Bonferroni's multiple comparison test, using GraphPad Prism software. *p<0.05 and ****p<0.0001 when compared to control with BSA.

4.2.2. Phytoestrogens tested showed a reduced toxicity in H9c2 cardiomyoblasts

The evaluation of cytotoxicity of PEs in H9c2 cells was assessed by the SRB assay, after treatment with 0.1, 1 and 10µM during 27 and 30 hours. These two times

correspond to, 3 and 6 hours of pre-incubation, respectively. This evaluation is important in order to ensure that doses used are not toxic to H9c2 cells. The obtained results demonstrate that all compounds in study do not have toxicity for different concentrations during 3 or 6 hours of pre-incubation **(Figure 14)**. Since no statistically differences were observed, coumestrol and enterodiol showed little or no cytotoxicity. Estradiol also did no present any toxicity in the time points and concentrations tested **(Figure 15)**.

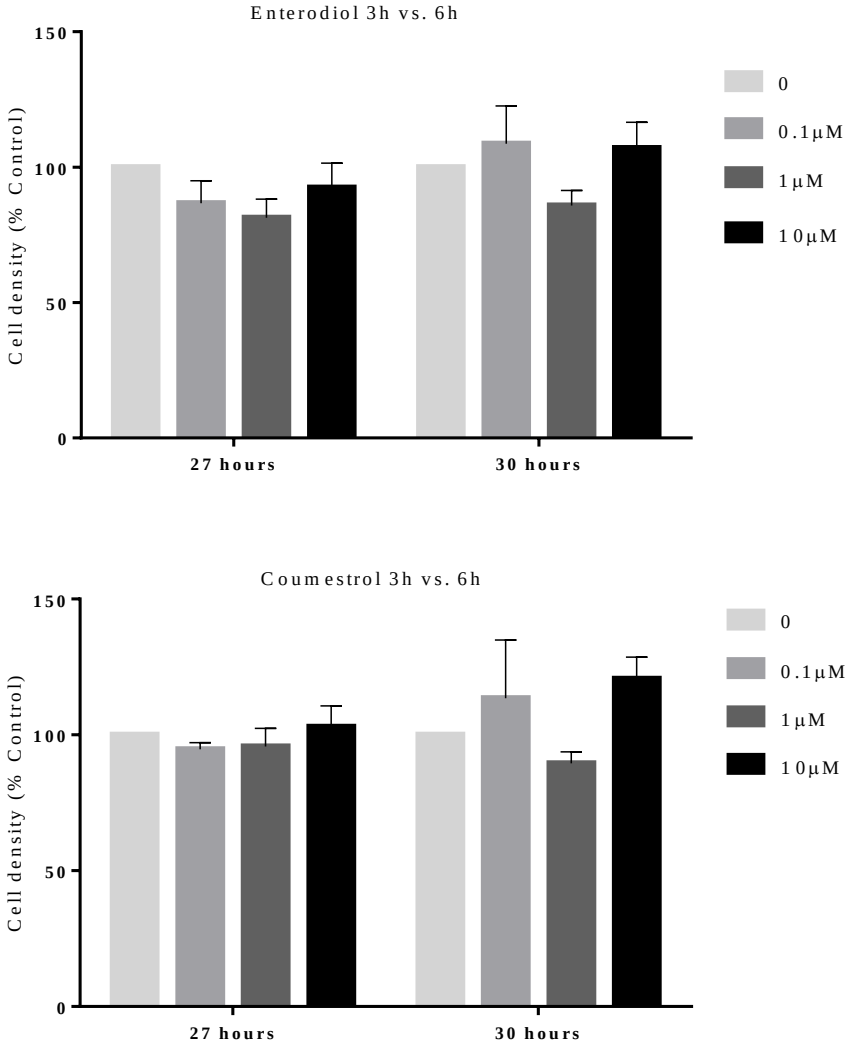

Figure 14 - Cytotoxicity of PEs (enterodiol and coumestrol) in H9c2 cardiomyoblasts during 27 and 30 hours with different concentrations (up to 10µM). Data represent mean ±SEM from 6 independent experiments and were analyzed by two-way ANOVA, using the GraphPad Prism program.

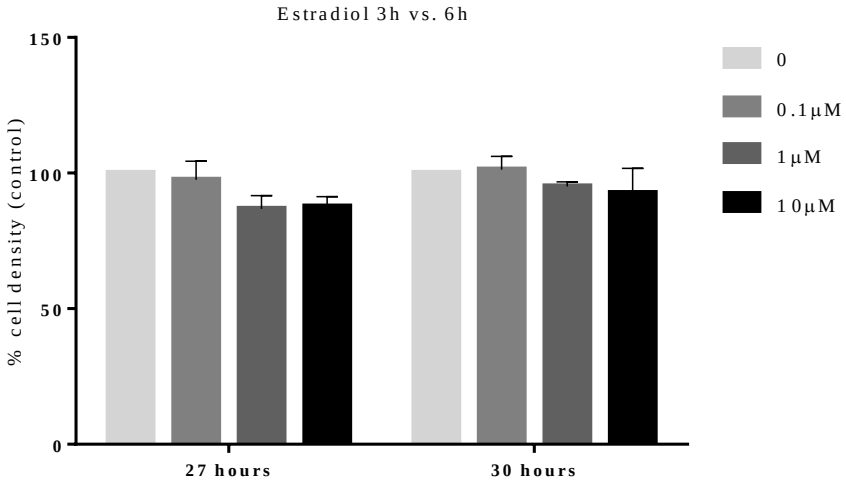

Figure 15 - Cytotoxicity of 17β-estradiol in H9c2 cardiomyoblasts during 27 and 30 hours with different concentrations (up to 10µM). Data represent mean ±SEM from 6 independent experiments and were analyzed by two-way ANOVA, using the GraphPad Prism program.

4.2.3. The effect of phytoestrogens against palmitic acid toxicity

4.2.3.1. Cell Density Evaluation

Based on the previous results, we pre-treated the cells with different concentrations of coumestrol and enterodiol and 17β-estradiol during 3 and 6 hours and then incubated cells with PA (50µM) for 24 hours. By measuring cell mass with the SRB dye method, the protective effect of PEs and 17β-estradiol was evaluated against PA lipotoxicity **(Figure 16)**. The results showed a significant decrease in H9c2 cell density after 24 hours of treatment with 50µM PA, compared to control BSA, which is consistent with previous results **(Figure 13B)**. Regarding the protective effects of 17β-estradiol against PA, no statistically significant differences were observed in comparison with 50µM of PA, for both pre-incubation times. Relatively to coumestrol and enterodiol no protective effect on PA-induced loss of cell mass was observed, with results being similar for both incubation times. Despite the lack of protective effects, concentrations of 0.1µM and 1µM were chosen for 17β-estradiol and PEs, respectively. The pre-treatment time selected for further experiments was set for 3 hours.

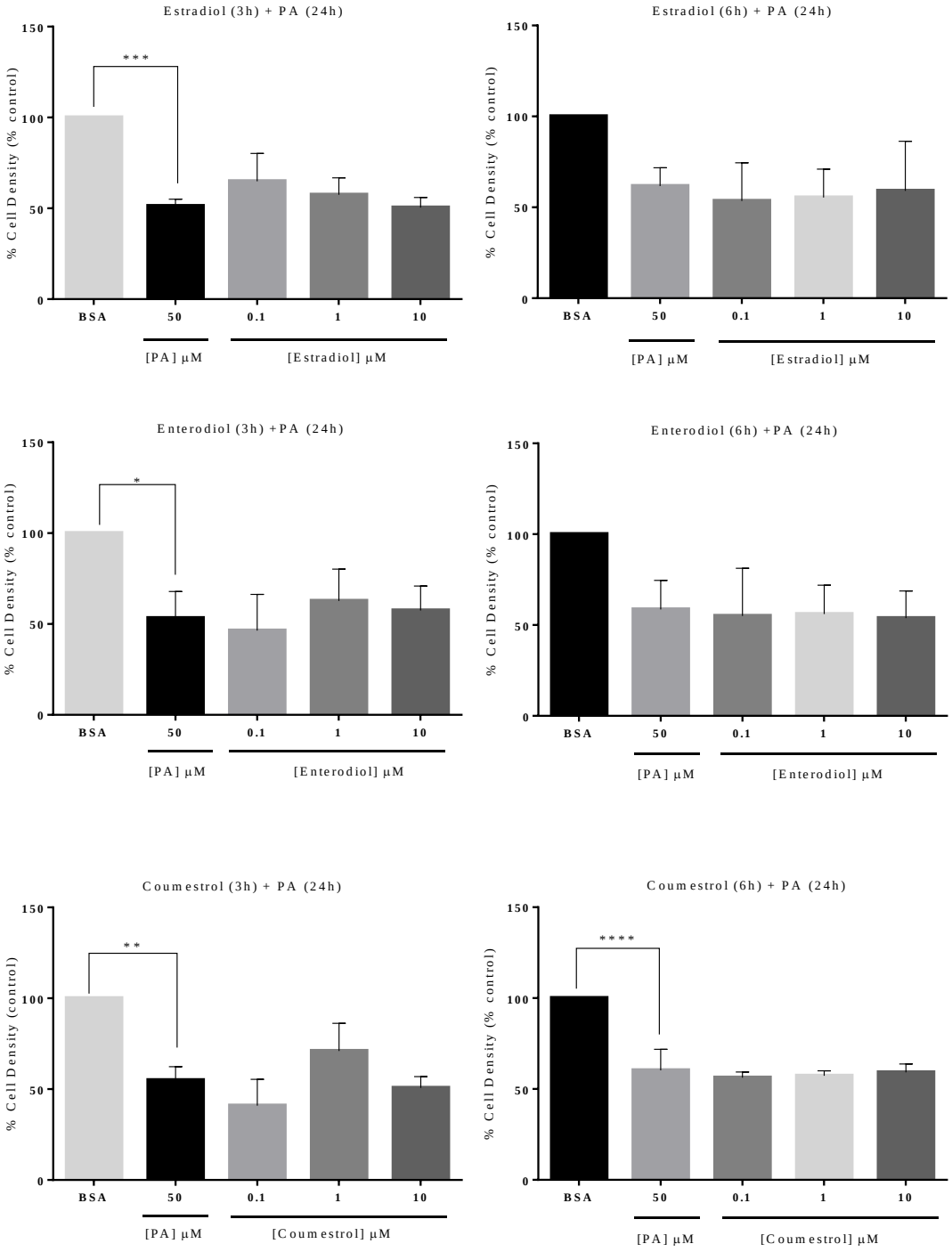

Figure 16 - Cell density evaluation. H9c2 cells were treated with different concentrations of 17β-estradiol and PEs (0.1µM, 1µM and 10µM) while treatment with 50µM of PA was performed 3 hours after treatment with PEs and was maintained for 24h. Control with 0.83mM of BSA without PA was also performed. Data are expressed as mean ± SEM of 3 independent experiments and were analyzed by one-way ANOVA following Bonferroni's multiple comparison test, using the GraphPad Prism program; * $p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$when compared to PA.

4.2.3.2. The evaluation of mitochondrial membrane electric potential

In order to assess the possible impact of PA on mitochondrial function and also the protective effects of PEs against PA, alterations in $\Delta\Psi_m$ were evaluated in all experimental groups through flow cytometry using the fluorimetric probe TMRM.

For this purpose and based on previous results, H9c2 cells were treated with 17β-estradiol (100nM), enterodiol (1µM) and coumestrol (1µM) during 3 hours and incubated with PA (50µM) for 24 hours more. No alterations on TMRM fluorescence were found based on flow cytometer results. PA-treated cells appeared to have lower TMRM fluorescence, suggesting a decrease in $\Delta\psi_m$ when compared with control (BSA-treated cells), although the difference was not significant. However, when cells were incubated with coumestrol plus PA, increased TMRM fluorescence was measured which may result from increased mitochondria transmembrane electric potential when comparing to cells treated with PA alone **(Figure 17)**.

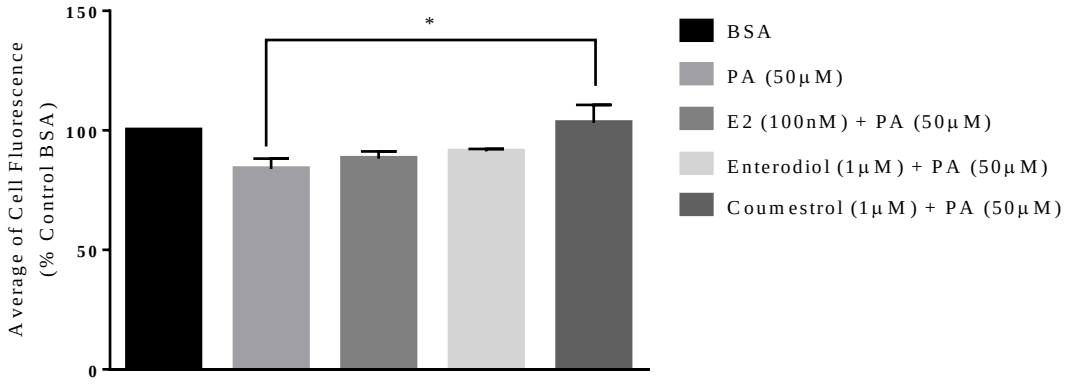

Figure 17 – TMRM fluorescence in H9c2 cells treated with 17β-estradiol (0.1µM), enterodiol (1µM) and coumestrol (1µM) during 3 hours and with 50µM of PA for 24 hours more. Control with 0.83mM of BSA without PA was also performed. Mitochondrial membrane potential alterations were evaluated using TMRM probe and analyzed through flow cytometry Data are means of 3 independent experiments, plus SEM. * $p < 0.05$ vs. PA (50µM).

4.2.3.3. Autophagy markers

Finally, we intended to demonstrate if cell treatment with PA leads to alteration in autophagy markers and if the incubation with PEs was able to reverse those alterations. In this regard, protein content of autophagy markers, Beclin-1 and p62, was evaluated by Western Blot in all H9c2 cell groups. After 72 hours in serum-free medium culture, cells were treated with PEs during 3 hours and after this time were incubated with 50μM of PA and in the end were collect and western blot was performed.

Alterations of Beclin-1 protein content in all cell groups was not statistically significant; however the results showed a slight decrease in protein content in cells treated with PA when compared with control BSA as well as a small increase in cells treated with PEs and 17β-estradiol in comparison to cell group treated with PA **(Figure 18A)**. The p62 protein content was also assessed allowing exploring the autophagy flux in all cell groups. As can be seen in figure 18B no differences existed in p62 protein content between all cell groups, suggesting that autophagy flux is not altered with cell treatment, although a larger number of samples must be tested.

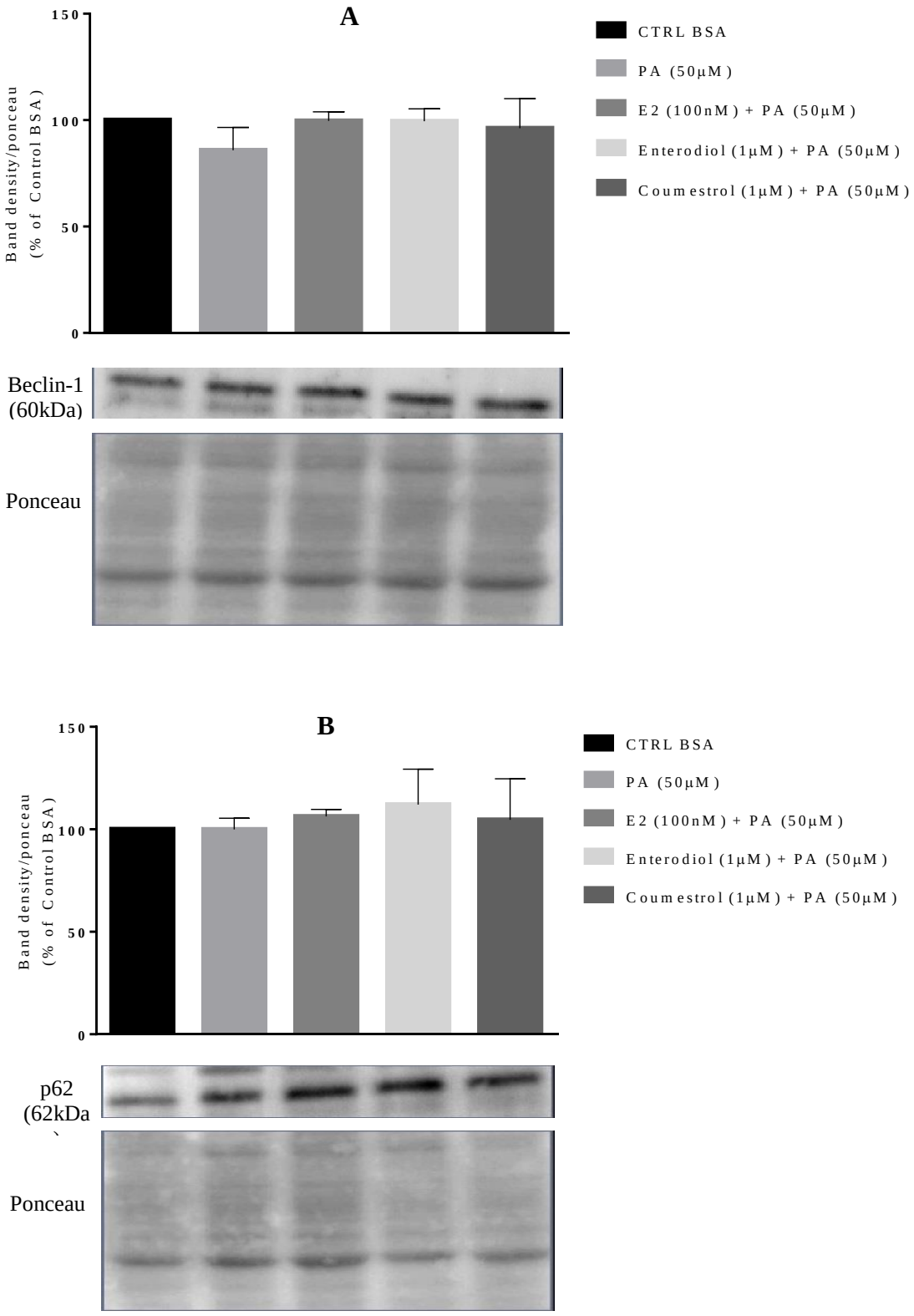

Figure 18- Alterations in Beclin-1 and p62 during H9c2 myoblasts treatment with PE/PA. **(A)** Beclin-1, total protein was evaluated by Western Blot **(B)** p62, measured by Western Blot and no differences was observed between all cell groups. Protein levels were normalized to Ponceau labeling (N=2), bars show mean ±SEM.

5. DISCUSSION

H9c2 cardiomyoblasts are a recognized *in vitro* cellular model for cardiac cells due their morphological features and electrophysiological characteristics (Menard *et al.*, 1999). In a wide variety of experimental designs, this cell line is usually used in an undifferentiated state (Sardao *et al.*, 2007, 2009a, b). Nevertheless, H9c2 myoblasts can fuse and differentiate into skeletal muscle cells when cultured in a low-serum medium. Likewise, a cardiac-phenotype can be obtained after differentiation in a low serum and supplemented with all-trans RA (Menard *et al.*, 1999). Cardiac specific markers, including expression, troponin T, MLC2v and phosphorylated troponin I have been demonstrated to occur after cardiac differentiation (Branco *et al.*, 2011; Pereira *et al.*, 2011). This differentiation capacity of H9c2 myoblasts allows using this model for toxicology studies, since the use of undifferentiated H9c2 cells may not be the best approach to use as a cardiac cell surrogate. Additionally, the adult heart tissue is composed by differentiated cardiomyocytes and therefore toxicological studies can have different effects depending on the developmental state of the biological model used (Branco *et al.*, 2011; Branco *et al.*, 2012; Branco *et al.*, 2013).

Taking into account all the considerations previously mentioned, and also gearing towards our main study, a question is created: does cells cultured in the absence of serum differentiate in cardiac muscle cells? In this regard, one of the objectives in the first part of the present thesis was to characterize H9c2 cardiomyoblasts in the absence of serum in culture medium, since FBS, contains hormonal factors and other growth factors which stimulate cell proliferation. So, a cell culture system lacking hormone-surrogates must be developed.

We observed by Western Blot that cells cultured in the absence of serum have the same protein content in GATA4, MLC2v and Troponin T **(Figure 11)**, markers for cardiac differentiation, when compared with cell cultured in 10% of serum. The transcription factor GATA4 is considered one of the early cardiac genes required for the initiation of cardiac differentiation (Yilbas *et al.*, 2014) being involved in cardiogenesis during the development (Morin *et al.*, 2000; Turbendian *et al.*, 2013; Yilbas *et al.*, 2014). Therefore, the results presented in Figure 11(A) are in agreement with these reports, since that cells cultured in 10% of serum are derived from embryonic rat heart (Kimes and Brandt, 1976) and thus can express this transcription factor.

The cardiac troponin T although it is a widely used cardiac marker, it can also be present in a confined stage of fetal skeletal muscle development (Swiderski and Solursh, 1990). Moreover, these results are in agreement with the fact that differentiation

protocols produce heterogeneous populations (Menard *et al.*, 1999). Furthermore is important to mention that the initial cell confluence and how cells were maintained in terms of confluence influence the differentiation process and the expression of cardiac markers in a specific undifferentiated population is very dependent of these particular features. Moreover, the use of the present cell culture model raises the question whether nutrient deprivation and serum withdrawal can activate autophagy signaling. Several reports revealed that autophagy can be stimulated during starvation or even growth factor nutrient deprivation (Ichimura and Komatsu, 2010; Levine and Yuan, 2005).

Concerning the results presented in Figure 12(A), in general, cells cultured in the absence of serum showed an increase in proteins involved in the autophagic pathway, such as Beclin-1, which has an important role in the initiation of autophagy allowing vesicle nucleation (Choi *et al.*, 2013). This suggests that autophagy pathways are stimulated, although more data and end-points are necessary to investigate this. One important autophagic marker is p62, a selective substrate for autophagy because it is incorporated in the autophagosome by interaction with LC3 (Komatsu *et al.*, 2012). During autophagic stimulation, p62 decreases, the opposite occurring when autophagy is suppressed. In this context, the data presented in Figure 12(B) demonstrated that cells cultured in serum-free medium showed decreased p62 protein content, indicating increased autophagic flux. The conjugation of both experiments is a good experimental approach because the evaluation of p62 by itself is not sufficient to estimate autophagic flux, since the expression of this protein can be changed independently of autophagy (Kuusisto *et al.*, 2001). Likewise, the measurement of autophagic markers, such as Beclin-1, is also not sufficient to confirm the stimulation of autophagy pathways. Overall, the results suggest activation of autophagy pathway when H9c2 cells are cultured in serum-free medium.

Autophagy has fundamental roles in physiology and pathology, including adaptive responses due to starvation, even growth factors deprivation, cellular quality control, aging and others (Choi *et al.*, 2013; Klionsky, 2000). Furthermore, autophagy can be required for cellular differentiation, which is associated with alteration on cell morphology, signaling pathways and function (McMillan and Quadrilatero, 2014; Mizushima and Levine, 2010). During differentiation, alterations in autophagy occur in several types of cells, including myoblasts (McMillan and Quadrilatero, 2014; Pantovic *et al.*, 2013; Zeng and Zhou, 2008; Zhuang *et al.*, 2011).

During myoblast differentiation and mature myotube formation, degradative processes are important. However, myoblasts differentiation involves a significant remodeling which requires the induction of autophagy (McMillan and Quadrilatero, 2014). Regarding these facts, a recent study using mouse C2C12 skeletal myoblasts demonstrated that during myoblast differentiation an alteration in autophagy markers occur, showing increased Beclin-1 and decreased p62 (McMillan and Quadrilatero, 2014). These findings are consistent with results obtained in the present thesis, concerning the differentiation of H9c2 cells in serum-free medium. Therefore, our explanation for these observations relates to the fact that the stimulation of autophagy due to an external signal, such as absence of cell growth of the cells, promotes differentiation and remodeling, facilitating the elimination of pre-existing structures and proteins (McMillan and Quadrilatero, 2014). Additionally, autophagy activation may act as a mechanism of cell survival, allowing the cell to reuse free fatty acids and amino acids generated by degradation of membrane lipids and proteins by the autolysosome. This mechanism promotes the maintenance of mitochondrial ATP energy generation and protein synthesis for cell survival (Levine and Yuan, 2005). Altogether, our findings, together with literature reports, allow us to confirm that autophagy activation is important in the context of cell survival or even cell differentiation, being related to its ability to sustain life during growth nutrients deprivation. Notwithstanding, it is crucial a controlled regulation of autophagy during myoblast differentiation, since excessive autophagy can be harmful for cells (Iovino *et al.*, 2012).

Despite the benefic effects of PEs in health, and potential role in menopause, several questions are still unanswered, including their toxicity or the real extent of their benefic effects.

The role of PEs in the protection against cardiac lipotoxicity has not yet been explored, specially focusing on the inhibition of mitochondrial dysfunction through fatty acid overload. In this sense, we investigated the protective effects of PEs against lipotoxicity in H9c2 cells, using PA as a lipotoxicity-inducing agent. It has been demonstrated that cell treatment with saturated FA, including PA, triggers apoptosis in cell cultures (Listenberger *et al.*, 2003). Also PA toxicity occurs via *de novo* ceramide formation and induces damages on contractile elements and cytoskeleton in adult rat cardiomyocytes (Dyntar *et al.*, 2001). Therefore, this is a relevant model since during menopause women have elevated serum FAs and increased risk of CVD. The initial experimental approach regarding the second part of present study was to evaluate only

the effect of PA on the proliferation of H9c2 cells and afterwards the assessment of PEs alone on H9c2 cell mass.

Palmitic acid is cytotoxic for the cell line in study in a dose - and time-dependent manner but with a maximal effect obtained for 100μM. A recent study also evaluated the effect of palmitate in C2C12 myoblasts and myotubes, demonstrating that palmitate decreased the viability of the cells in study in a dose-dependent manner with results being significant starting from 0.05mM PA in myoblasts (Patkova *et al.*, 2014). The same effect was observed with neuronal stem cells, where their viability is suppressed when cells are treated with PA (Wang *et al.*, 2014). Altogether, our data and previous reports show that PA-induced toxicity is useful for clarifying the role of FAs, since that PA has important impact on proliferation of cardiomyoblast *in vitro*.

Although there are studies demonstrating positive effects of PEs treatment in several diseases related with menopause, it is important to take into account that its excessive consumption can trigger harmful effects on health. Not to mention that not all PEs provided protection or even increased women's quality life during menopause. For this purpose, is critical to perform the screening of PEs in our experimental model in order to test the toxicity of the compounds in study (coumestrol and enterodiol) and also to roughly estimate safe concentrations. Thus, assessment of PEs toxicity on myoblast proliferation was also performed with different concentrations and two different incubation times. The results presented in Figure 14 showed that no toxicity occurs with any of the concentrations tested. As well as, no differences was observed between both time points (27 and 30 hours). These results are in agreement with several studies showing that some PEs, including genistein may have beneficial effect in the heart (Fan *et al.*, 2013) and resveratrol (Zhao *et al.*, 2008). Despite the reduced toxicity in this cell model, or in the whole tissue it is crucial to take into account that PEs, including genistein, coumestrol and resveratrol can exert genotoxic effects in several *in vitro* models (Stopper *et al.*, 2005).

As stated before, PEs are recognized to be cardioprotective. Thus, one of the main goals of the present thesis was to evaluate the protective effects of coumestrol and enterodiol against PA-induced lipotoxicity. Considering the results present in Figure 16, we can conclude that at least by using this method, there was no evident protection from PEs against PA lipotoxicity. A non-statistically tendency for protection observed for 1μM of coumestrol and enterodiol during 3 hours of pre-incubation, may suggest that extra work may be needed in this regard. 17β-estradiol did not show any significant

effect, this despite the results showing a small, non-significant protection for the lowest concentration.

The charge imbalance that results from the generation of an electrochemical gradient across the inner mitochondrial membrane forms the basis of the $\Delta\psi_m$ (Kroemer *et al.,* 2007). Therefore, the maintenance of the proton gradient is crucial for the survival the cell, since it leads to the synthesis of ATP and maintains oxidative phosphorylation (Ly *et al.*, 2003). In this context, $\Delta\psi_m$ was evaluated in all cell groups in study. Our data demonstrated that PA caused a small $\Delta\Psi_m$ decrease, which was not significant, most likely because of the low number of replicates. In the other hand, pre-treatment with coumestrol during 3 hours increased $\Delta\psi_m$ in the presence of PA, although the mechanism is not known.

Recently, Cai and colleagues (Cai *et al.*, 2014) reported evidences of autophagy activation induced by PA in several cell lines, including hepatocytes. In this regard, we assessed autophagy markers in our cell line model, and investigated whether PEs may be protective in this context. However, the results no alterations in autophagy markers, suggesting unchanged autophagy fluxes. Taken together, the results show modest protection by the PEs used, with coumestrol showing the more promising effects. One important issue concern the cell model used. The present work should be replicated in primary cardiomyocytes, a more closely related model and in which the PEs studied may present more robust effects.

6. CONCLUSION

Several complications during menopause exist, namely lipotoxicity associated with the accumulation of FFA in the cell, contributing to the risk of CVD. Since estradiol has a cardioprotective effect the main hypothesis of the present study was that PEs protect cardiac tissue against cardiac lipotoxicity induced by FFA (Oliveira *et al.*, 2012). The H9c2 cell line, a model for cardiac cells was used in the present study. Notwithstanding, the problems raised due to the serum used, led us to develop a new cell culture approach which may have disturbed the results obtained.

Since during menopause women have elevated serum fatty acids and increased risk for CVD, it is possible that some PEs can be used to ameliorate the cardiovascular performance of menopausal women, without further side effects to health. Inclusion of PEs in specific foods or dairy products can be a possible innovative strategy in the future for therapeutic of problems during menopause. Thus, this is clearly of relevance for geriatric studies and commercially important for the food industry.

Nevertheless the present results were inconclusive since no protection was observed from the used PEs in PA-induced lipotoxicity. A better cell model or treatment protocols may help confirming our hypothesis in the future.

7. FUTURE EXPERIMENTS

Although PEs are widely studied in the context of several diseases, their effectiveness and safety is still under debate. This is particularly important in the context of cardiovascular diseases. In this regard, and taking into account the data here presented, further experiments in the same or alternative cell models can be performed, namely:

- Evaluation of apoptosis (intrinsic pathway) by measuring caspases 3 and 9-like activity by a colorimetric assay. The objective is to investigate the anti-apoptotic activity of PEs studied here against PA toxicity. Also, this allows providing the underlying molecular mechanisms of PEs on PA-induced cardiomyocytes apoptosis, since we used here a technique (SRB labelling) that does not distinguish between cell death or arrest of cell cycle.
- Detection of intracellular oxidative stress by flow cytometer and fluorescence microscopy using the probes 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) or MitoSox Red in order to assess the potential antioxidant effect of PEs per se and against the toxicity induced by PA.
- Measurement of intracellular calcium (Ca^{2+}) fluorescence by microscopy. This evaluation allows demonstrating if Ca^{2+} alterations occur in cells treated with PEs and PA. Since several reports show that Ca^{2+} alterations can lead to $\Delta\Psi_m$ collapse and mitochondrial dysfunction (Fan *et al.*, 2013; Hajnoczky *et al.*, 2006; Yokoshiki *et al.*, 1996), one possibility is that the selected PEs decreased Ca^{2+} dysregulation caused by PA.

Moreover, it is necessary to increase the number of independent experiments in all experiments if using this model, because n=3 is clearly not enough for this type of study.

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