Joel Filipe Garcia Guerra

Bachelor of Science in Biochemistry

Berries driven (poly)phenols-induced cytoprotection in cardiomyocytes

Dissertation submitted in fulfillment of
the requirements for the degree of
Master of Science in Biochemistry

Supervisor: Helena Vieira, Principal Investigator, CEDOC
Co-supervisor: Cláudia Santos, Principal Investigator, IBET

Master's committee:

President: Prof. Doutor Carlos Alberto Gomes Salgueiro
Jury: Prof. Doutor Henrique Manuel Paixão dos Santos Girão

FACULDADE DE
CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

September 2015

Joel Filipe Garcia Guerra

Bachelor of Science in Biochemistry

Berries driven (poly)phenols-induced cytoprotection in cardiomyocytes

Dissertation submitted in fulfillment of
the requirements for the degree of
Master of Science in Biochemistry

Supervisor: Helena Vieira, Principal Investigator, CEDOC
Co-supervisor: Cláudia Santos, Principal Investigator, IBET

Master's committee:

President: Prof. Doutor Carlos Alberto Gomes Salgueiro
Jury: Prof. Doutor Henrique Manuel Paixão dos Santos Girão

September 2015

Acknowledgments

Firstly, I would like to express my sincerest gratitude to my supervisors Dr. Helena Vieira, and Dr. Cláudia Santos. Without their continued support, guidance, and involvement throughout my thesis, I would not be able to accomplish this work. I would like to thank you both very much for being so kind and patient with me, and also for welcoming me into your teams this past year.

Besides my advisors, I would like to give a special thanks to Andreia Gomes, as she was a great teacher, colleague, and friend. Her teachings introduced me to the world of cardiovascular diseases and her help was indispensable throughout my thesis. Without any doubt, it was a great pleasure to be your "padawan" and work together with you.

I would like to thank all my DSB Lab. teammates, in special to Inês Figueira, Gonçalo Garcia, Carolina Jardim, and Inês Costa. All of them were of invaluable help during my thesis, not only they helped me master different methodologies, but also were exceptional friends that made every single day of work enjoyable even when experiments went wrong. Furthermore I would like to thank my fellow colleague Diana Macedo in for all the stimulating discussions, lively talks, proofreading, and continued incentive to always aim higher.

Getting through my thesis required more than academic support, and for that I have many people to thank and express my gratitude for their friendship. Afonso Carrêlo, Pedro Dias and Tiago Mestre have been unwavering in their personal and professional support since the time I start University until the end. For all the memorable evenings, pranks, rants, friendly competition, and support I must thank you.

Most importantly I would like to thank Mafalda Pereira who open her heart to me, gave me insightful helped in every major decision I toke, and was always the first one to point out my mistakes so that I could be a better me. Without her, I would not been able to be where I am now. Therefore from the bottom of my heart I would like to thank you for every time you made me laugh with your silliness, helped me with scientific work, argue with me from my bad decisions, and for all the joyful moments I am forever grateful.

Last but not the least, I would like to thank my family: my parents and to my brother for supporting me throughout writing this thesis and my life in general, without them nothing of this would ever be possible.

I would like to acknowledge both Fundação para a Ciência e a Tecnologia (FCT) and Agence Nationale de la Recherche (ANR) for funding this thesis in the ambit of the FCT project CardioBDP (FCT-ANR/BEX-BCM/0001/2013)

"Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth."

Jules Verne (1828-1905)

Abstract

Cardiovascular diseases (CVDs) are a prominent health problem, being the leading cause of death worldwide. Numerous studies have been focus on the beneficial effects of dietary (poly)phenols, as a way to prevent the onset of CVDs.

Recently, a link between CVDs, metabolism, mitochondria and diet has been evidenced. This link might involve a crosstalk between mitochondria functions, autophagy and cell death and would be regulated at the cellular level by various classes of sensors such as proteins as well as second messengers.

The purpose of this study is to evaluate the potential of novel berries driven (poly)phenols (BDP) metabolites in cardioprotection and unravel the BDP-targeted cytoprotective mechanisms namely in mitochondrial functionality. Therefore studies were conducted using H9c2 cells and neonatal rat cardiomyocytes treated with *tert*-butyl hydroperoxide or isoproterenol to promote cell death, modeling chronical cardiac diseases.

Analysis of mitochondrial population, in neonatal rat cardiomyocytes and H9c2 cells respectively, showed that BDP metabolites induce an increase of mitochondrial population. Moreover BDP metabolites appear to not protect against cell death induced by isoproterenol activated pathway. An important finding in this work was that BDP metabolites are capable of improving cardiac contractile functions, without decreasing isoproterenol induced cell death.

Although not completely conclusive, the obtained results support a need for future research, as these compounds can be promising therapeutic agents in CVDs prevention fostering an active and healthy ageing. Given the growing number of cardiovascular incidents, being able to possibly develop a prophylactic drug against CVDs, would be a great achievement. Creating a prophylactic drug from BDP metabolites, would help people with a propensity to CVDs keeping a higher quality of life and reduce the need for expensive cardiac treatments.

Keywords: Berries driven (poly)phenols, cardiovascular diseases, mitochondria, cardiomyocyte, oxidative stress.

Resumo

As doenças cardiovasculares são consideradas um dos mais importantes problemas de saúde, sendo apontadas como a principal causa de morte no mundo. Inúmeros estudos têm vindo a ser desenvolvidos no âmbito da prevenção deste tipo de doenças tendo como base os efeitos benéficos dos polifenóis provenientes da dieta.

Trabalhos recentes propõe que a ligação existente entre as doenças cardiovasculares e a dieta deve-se maioritariamente à regulação da autofagia e metabolismo celular realizado pelas mitocôndrias.

Este trabalho tem como finalidade avaliar o potencial cardioprotetor dos metabolitos de polifenóis de pequenos frutos, focando-se no estudo dos mecanismos citoprotetores e interação com a mitocôndria. Por forma a realizar estes estudos recorreu-se à linha celular H9c2 e cardiomiócitos de rato neonatal tratados com *terc*-butil-hidroperóxido ou isoproterenol de forma a mimetizar doenças cardiovasculares crónicas.

Resultados obtidos da análise à população mitocondrial e ao metabolismo celular evidenciaram alterações associadas ao pré-tratamento com os metabolitos de polifenóis de pequenos frutos. Contudo, resultados obtidos com isoproterenol apontam para uma ausência de interação entre os metabolitos de polifenóis de pequenos frutos e a morte celular induzida pelo isoproterenol. A descoberta mais importante deste trabalho, deve-se no entanto à capacidade dos metabolitos de polifenóis de pequenos frutos conseguirem melhorar a capacidade contrátil das células, quando estas estão expostas a stresse.

Este trabalho apresenta resultados que demonstram uma necessidade em continuar a explorar os metabolitos testados. Devido ao crescente número de incidentes cardiovasculares, o desenvolvimento de novos agentes terapêuticos capazes de prevenir o aparecimento de doenças cardiovasculares torna-se uma prioridade. A utilização de metabolitos de polifenóis de pequenos frutos podem apresentar-se como sendo uma solução capaz de reduzir a necessidade medicamentos dispendiosos.

Palavras-chave: Polifenóis de pequenos frutos, doenças cardiovasculares, mitocôndria, cardiomiócito, stresse oxidativo.

Table of Contents

Figures Index

Abbreviations

AIF – Apoptosis-inducing factor
Akt – Protein kinase B
β-AR – β-adrenergic receptors
BDP – Berries driven (poly)phenols
BSA – Bovine serum albumin
cAMP – cyclic Adenosine monophosphate
cDNA – complementary DNA
CVDs – Cardiovascular diseases
Cyt c – Cytochrome c
DAPI – 4',6-diamidino-2-phenylindole
DMEM – Dulbecco's modified eagle's medium
DNA – Deoxyribonucleic acid
EC_{50} – Half maximal effective concentration
ECM – Extracellular matrix
eNOS – Endothelium nitric oxide synthase
FACS – Fluorescence-activated cell sorting
FBS – Fetal bovine serum
FSC – Forward light scatter
GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
H_2O_2 – Hydrogen peroxide
HOCl – Hypochlorous acid
HOO· – Hydroperoxyl radical
HRP – Horseradish peroxidase
HS – Horse serum
ISO – Isoproterenol
MBA – Membrane blocking agent
MD – Mediterranean diet
mtCyt b – Mitochondrial cytochrome b
mtDNA – Mitochondrial DNA
MTDR – MitoTracker Deep Red FM
mtETC – Mitochondrial electron transport chain
NBCS – New born calf serum
NO· – Nitric oxide
NO_2· – Nitrogen dioxide
O_2^{-}· – Superoxide anion
OH· – Hydroxyl radical
$ONOO^-$ – Peroxynitrite
PARP-1 – poly(ADP-ribose) polymerase-1
PBS – Phosphate Buffered Saline
PCR – Polymerase chain reaction
PI – Propidium Iodide
PGC-1α – Peroxisome proliferator-activated receptor gamma coactivator 1 alfa
PKA – Protein kinase A
PVDF – Polyvinylidene fluoride
Q-PCR – Quantitative polymerase chain reaction
qRT-PC – Quantitative reverse transcription polymerase chain reaction
RIPA – Radio-immunoprecipitation assay
RNS – Reactive nitrogen species
ROO· – Peroxyl radical
ROS – Reactive oxygen species
RT – Room temperature
SD – Standard deviation
SDS-PAGE – Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
sGC-cGMP – Soluble guanylate cyclase cyclic guanosine monophosphate
SOD – Superoxide dismutase
SSC – Orthogonal light scatter
***t*-BHP** – *tert*-Butyl hydroperoxide
TBS – Tris-buffered saline
WHO – World Health Organization

1. Introduction

1.1. Cardiovascular diseases

Cardiovascular diseases (CVDs) are defined as a group of disorders that affect heart, blood vessels or both and encompass all of coronary artery disease, hypertension, congestive heart failure and stroke. CVDs have been seen as a prominent health problem as the main cause of death in developed countries, and account for 30% of the deaths worldwide according to World Health Organization (WHO) [1,2]. Firstly, CVDs were characterized as diseases of middle age adults, mainly men. Nevertheless, recent studies have proven that vascular injuries can accumulate throughout life making prevention necessary since childhood. Furthermore, there is an important relation between cardiovascular risk factors (smoking, arterial hypertension, hypercholesterolemia, hypertriglyceridemia, diabetes and obesity) and cardiovascular mortality, as the existence of cardiovascular risk factors are correlated with an increase in the possibility of CVDs and a greater probability of mortality after a cardiovascular event [1].

Heart is composed of multiple cell types such as cardiomyocytes (which constitute 30% of the total cell number but account for 70-80% of the hearts weight), fibroblasts, vascular smooth cells, endothelial cells and immune cells [3].

The ability to supply oxygenated blood to the body can be lost due to exposure to pressure or volume overload (e.g., hypertension, valvular heart disease), myocardial infarction (MI) or ischemia. Cardiac hypertrophy is the compensatory response to the diminished capability of the heart to supply oxygenated blood and consists in the increase in size and mass of the heart in order to normalize wall stress and allow normal cardiovascular function. Cardiomyocytes do not possess the ability to divide, thus compensatory response to cardiac hypertrophy is associated with cardiomyocyte enlargement. Cardiac hypertrophy compensatory response is also followed by alterations in calcium handling, metabolism, gene expression, cell death (e.g., apoptosis and autophagy), changes in extracellular matrix (ECM), development of fibrosis, and angiogenesis [3]. Although cardiac hypertrophy occurs in order to maintain cardiac function, continued stress will lead to ventricular dilatation and diminished contractile capability eventually progressing to heart failure [3,4].

1.2. Oxidative stress in cardiovascular health

Elevated levels of reactive oxygen species (ROS) or reactive nitrogen species (RNS) or both within the cells, leads to oxidative stress that has been implicated in heart failure, atherosclerosis and hypertension. Oxidative stress can be define as an imbalance between the production of reactive species and antioxidants defenses, which can result from the accumulation of highly reactive free radicals species or the decrease in the defense mechanisms capable of eliminating the free radicals [5, 6]. In a state of oxidative stress there is a change in the cell redox state that interferes with important physiological processes, which may cause mitochondrial dysfunction, DNA damage and apoptosis, triggering the initiation of several diseases [7,8].

ROS and RNS that are biologically produced from metabolic reactions and these processes can have or not enzymatic origin [5]. Some ROS are radical species, such as superoxide anion ($O_2^{-}\cdot$), hydroxyl radical (OH·), peroxyl radical (ROO·), and hydroperoxyl radical (HOO·). Hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) are also part of the ROS family; although they are not free radicals. The RNS include free radicals such as nitric oxide (NO·) and nitrogen dioxide ($NO_2\cdot$). Peroxynitrite ($ONOO^-$) is the non-free radical RNS [5].

As stated before, oxidative stress is tightly implicated with CVDs, as the major mechanism of endothelial dysfunction observed in CVDs comes from the reaction of $O_2^{-}\cdot$ with NO· resulting in $ONOO^-$ .This potent oxidant is capable of lipid peroxidation, activation of stress signaling and matrix

metalloproteinases, inactivation of enzymes and other proteins by oxidation and nitration such as α-actinin, myofibrillar creatine kinases and prostacyclin synthase damaging the contractile function of myofilaments [9]. The decomposition of $ONOO^-$ results in toxic products such as the highly reactive OH· and NO_2· [14,15,16]. Furthermore a continued production of $ONOO^-$ can trigger mitochondrial membrane permeabilization and release of proapoptotic factors such as cytochrome c (Cyt c) and apoptosis-inducing factor (AIF) from the mitochondria. These factors, mediate caspase-dependent and independent apoptotic death pathways. In addition to these reactions, $ONOO^-$ in association with other oxidants causes strand breaks in DNA which leads to the activation of the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) [10].

During a cardiovascular pathology, once there is extensive oxidative damage to the DNA, PARP-1 is overactivated resulting in a rapid depletion of cellular ATP and NAD^+, slowing the rate of glycolysis and mitochondrial respiration. Overactivation of PARP-1 can also result in an increased expression of a variety of inflammatory genes leading to increased inflammation and associated oxidative stress, therefore facilitating the progression of cardiovascular dysfunction and heart failure (Fig. 1-1) [10].

Although $ONOO^-$ is a very harmful RNS its precursor NO· is essential for a normal cardiovascular physiology. Nitric oxide is important in different cardiac functions such modulation of cardiac contractile function, inhibition of cardiac oxygen consumption and activation of the soluble guanylate cyclase cyclic guanosine monophosphate ((sGC)-cGMP). Moreover the sGC-cGMP signal pathway mediates vasodilation, inhibition of platelet aggregation and neutrophil adhesion and activation, anti-inflammatory, antiremodelling and antiapoptotic effects [10].

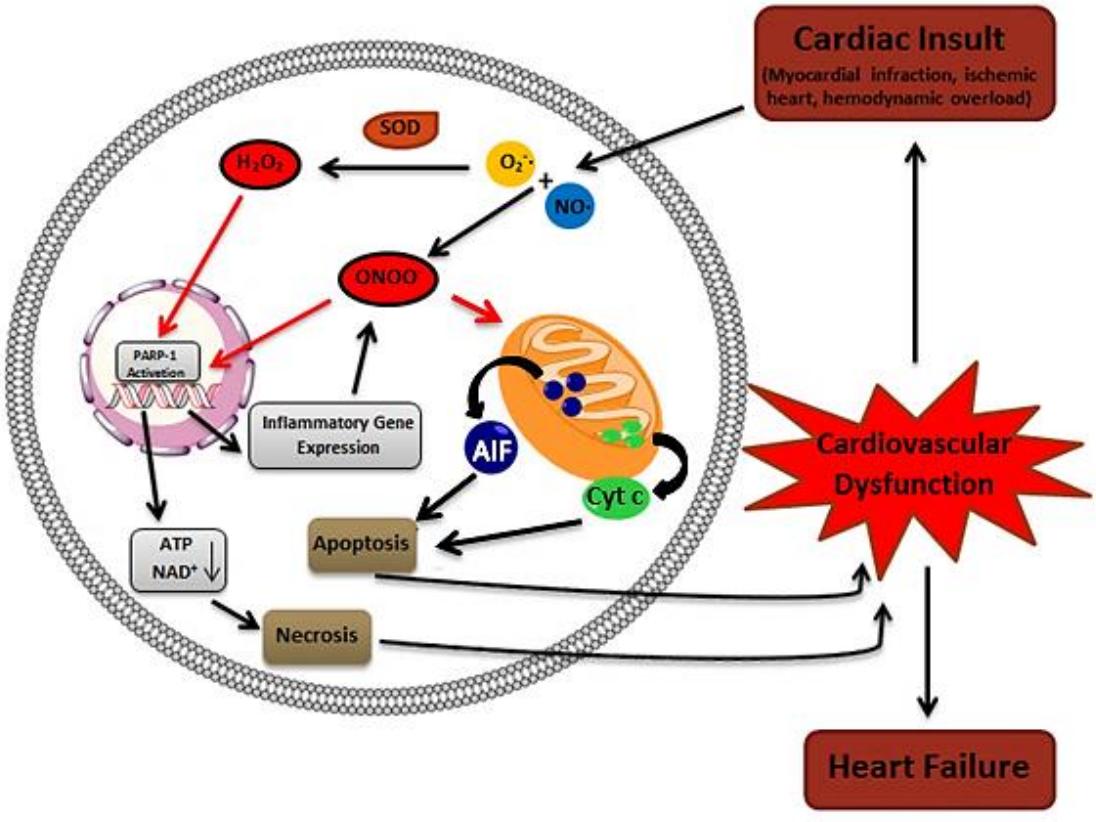

Figure 1-1 – Schematic representation of cardiac dysfunction development through oxidative stress (adapted from 10).

1.3. Mitochondrial oxidative stress

Mitochondrial respiration is the major source of ROS in mammals under physiological conditions, being the mitochondrial electron transport chain (mtETC) responsible for it. The mtETC is a sequence of the Complexes I-IV located in the mitochondria inner membrane and arrayed in a supramolecular organization with the purpose of producing ATP through a process of oxidative phosphorylation. Unpaired electrons are a natural consequence of mitochondrial respiration as electrons leak from several mtETC sites. As such, during mitochondrial respiration, closely to 1 % – 3 % of electrons leak, as a result there is formation of $O_2^{-}\cdot$ [7].

Complex I and III are the main sources of $O_2^{-}\cdot$ generation within all mtETC [6,11,12]. Complex I production of $O_2^{-}\cdot$ occurs *in vivo* when NADH levels are high, there is damage to the respiratory chain, slow respiration or ischemia. The production of $O_2^{-}\cdot$ in Complex I may possibly be from the iron sulfur center 2 or the mitochondrial NO synthase. Whereas Complex III-derived $O_2^{-}\cdot$ derives from an electron leak to oxygen at the ubiquinone site interface between Complexes II and III [11,12].

While the radical $O_2^{-}\cdot$ generated from Complex I is released into the mitochondrial matrix the Complex III-derived $O_2^{-}\cdot$ is released into both the mitochondrial matrix and the intermembrane space. Mitochondria possesses superoxide dismutase (SOD) enzymes in the mitochondrial matrix and in the intermembrane space, SOD2 and SOD1 respectively, with the purpose of dismutate $O_2^{-}\cdot$ in to H_2O_2 preventing therefore the formation of $ONOO^{-}$ [6]. Although the generation of unpaired electrons is a natural by-product of mitochondrial respiration ROS production can be regulate through the mitochondrial membrane potential.

Recent studies have suggested that at extremes of overall mitochondrial membrane potential would lead to an increase in pathophysiological ROS levels. This fact can be observed during hypoxia when mitochondrial membrane potential is significantly reduced or during an heart failure when an intensification in workload significantly increases mitochondrial membrane potential and there is also an increase in ROS production [11,12].

Even though ROS and RNS can produce extensive oxidative damage within the cell, this only happens in face of an endogenous antioxidant system dysfunction, otherwise cells during their physiological processes of energy generation produce a significant amount of ROS/RNS which is mainly storage within intact mitochondria. Furthermore the fact that ROS/RNS might possess a physiological role as been increasingly accepted. Namely in a functional system the production of ROS/RNS is in fact beneficial for the cell as it is involved as mediators of signal transduction, activation of proteins, expression of genes associated with the immune and inflammatory response, defense against infections agents, regulation of the G1 phase an also in the differentiation of embryonic cell lines into cardiomyocytes [7].

1.4. Mitochondrial dysfunction in CVDs

As aforementioned mitochondria are a very important organelles in cardiac physiology as they are the principal ATP generator and can influence cardiac development and remodeling into adult cells. Thus when mitochondria dysregulation happens, it influences the pathophysiological processes of CVDs [13].

Under a pathophysiological state where an increase in contractile functions is demanded, the best way to enhance energy production is to stimulate new mitochondrial production, termed mitochondrial biogenesis [14]. Nevertheless during a state of continued oxidative stress, cardiomyocyte mitochondria becomes dysfunctional presenting a reduced ATP synthesis, abnormal accumulation of metabolic intermediates, calcium dysregulation and increased ROS production [15].

Mitochondria are very dynamic organelles and can even within a single cell possess different sizes, morphology, and copy numbers of mitochondrial DNA (mtDNA). These differences exists as result of an important form of mitochondria quality control that occurs in a constant flux. This process of quality control mainly consists in mitochondria going through a perpetual fusing with each other

and dividing. Mitochondria dynamic system of continuous fission and fusion events, enables the control of their morphology, integrity and function [16]. Fission events are required for normal mitochondrial functions while fusion events allow the mixing of mitochondrial genomes as a way of diminishing the quantity of damaged DNA [15, 14].

Although quality control is important to keep a high percentage of functional mitochondria, when facing an increasing cardiac workload it is necessary to enhance mitochondrial biogenesis, as aforementioned. Mitochondrial biogenesis is mainly associated to an increase in activity from the nuclear encoded protein, peroxisome proliferator-activated receptor gamma coactivator alpha (PGC-1α) [14,17]. Through transgenic mouse models the role of PGC-1α in the heart have been extensively study [17]. However the role of PGC-1α during CVDs and heart failure in humans remains controversial as contradictory results have been reported [14,18]. On the other hand it has been demonstrated that estrogen-related receptor alpha, an orphan nuclear receptor required for PGC-1α dependent mitochondrial biogenesis, is downregulated during heart failure contributing to the decrease in transcription of some nuclear encoded mitochondrial proteins [18,19].

During CVDs, prolonged exposure of mitochondria to oxidative stress lead to the impairing of mitochondrial biogenesis through disruption of mitochondrial renewing mechanisms and induction of DNA damage [15]. Mitochondrial DNA is considerably more susceptible to oxidative stress compared to nuclear DNA, having a mutational rate 10-fold higher [13]. This high mutational rate is observed mainly due to the proximity of mtDNA with the mtETC , the lack of protective histones and chromatin and the dependence in a basic DNA repair process such as base excision repair [15].

1.5. Diet contribution to CVDs

Although CVDs are a global problem their incidence varies from country to country in part due to different nutritional habits as it has been observed from epidemiologic studies. The “Seven Countries Study” observed a lower incidence of CVDs in countries with a Mediterranean diet (MD), being this diet characterized by a high intake of olive oil, fruits, vegetables, cereals (mainly whole grain cereals), nuts, and seeds; a moderate consumption of fish, seafood, poultry, and eggs; and a low consumption of dairies, red meat, processed meat, and sweets [1,20]. Another study that showed the impact of the MD was the *PREDIMED* trial which included 7,447 high-risk participants initially free of CVDs that for 5 years followed one of three dietary interventions being one the control (advised in on a low-fat diet) and the others MD supplemented with extra virgin olive oil or nuts (walnuts, almonds, and hazelnuts) [21]. The diets supplemented with virgin oil or nuts brought in a reduction of 30 % of major cardiovascular events in comparison to the control diet [20,21].

A great number of studies aiming to prevent the appearance or to improve the outcome of CVDs have showed that the consumption of fruits and vegetables helps preventing the appearance of CVDs [22]. The preventive effect of fruits and vegetables has been linked to the presence of phenolic compounds [2,23]. These phenolic compounds have been designated as (poly)phenols and are characterized structurally by possessing one or more phenolic hydroxyl groups [24]. (Poly)phenols can be divided into two groups, flavonoids and non-flavonoids (Fig. 1-2). Flavonoids are characterized by a structural backbone of C6-C3-C6 and are sub divided according to their side chain into flavones, isoflavones, flavanones, flavonols, flavanols and anthocyanins. Non-flavonoids include molecules such as tannins, phenolic acids, stilbenes and lignans [25].

1.6. (Poly)phenols in cardioprotection

Recently (poly)phenols from dietary sources have started to take a central role in the search for bioactive compounds against CVDs and other diseases. There has been an increasing number of experimental studies using dietary (poly)phenols. However, almost all of them consists of *in vitro* studies with the direct application of purified (poly)phenols to the target cell culture. There was no regard for the reduced bioavailability of (poly)phenols reaching the target tissues *in vivo* [7]. Moreover, it must also be taken into account the molecular changes occurring after ingestion, such as changed bioactive properties [7]. Bioavailability is considered to be the fraction of a compound that reaches the systemic circulation and the specific sites of action and can exert its biological role. In the absorption of dietary (poly)phenols there are factors that can be a critical determinant for it, namely: the interaction of (poly)phenols with the gut microbiota, differential expression of efflux pump genes, and liver metabolism. Furthermore, some (poly)phenols can be reduced to metabolites with a different biological activity or with an increased rate of clearance [7,26].

Most dietary (poly)phenols exist within food as polymers or in a glycosylated form and cannot be directly absorbed. In order for (poly)phenols glycosides to be absorbed, they must be first hydrolyzed by the intestinal enzymes or by the colonic microflora [27,28,29]. After absorption, (poly)phenols undergo further metabolization within the liver through the conjugation process common to xenobiotics. This conjugation process consists in the phase II metabolism with methylation, sulfation and glucuronidation, but the type of conjugation change depending on the substrate [29,28]. As a consequence of these processes, generally there are not many parent compounds circulating in plasma. However, it is possible to identify several different molecules as metabolites with possibly distinct biological activity from their predecessor [28].

(Poly)phenols are well known for their antioxidant capacity *in vitro*, mainly due their aromatic structure with a highly conjugated system of pi electrons configuration and some hydroxylation patterns, that enables them to capture an electron thus neutralizing free radicals [27]. Although their antioxidant capacity derives from their structure, as previously mention the same is not fully conserved after digestion [29]. Furthermore, the concentration of (poly)phenols reaching tissues is much lower than the used ones for characterization as antioxidant molecules [30].

Thus far a numerous of studies have revealed a clear interaction between different types of dietary (poly)phenols and activation of the protein kinase B (Akt) signaling pathway that is responsible for inducing mitochondrial biogenesis, muscle hypertrophy, cardiac growth, survival metabolism, and contractility [31–35]. It has been also reported that metabolized (poly)phenols can inhibit some enzymes such as angiotensin-converting enzyme and xanthine oxidase or induce the expression of endothelium nitric oxide synthase (eNOS) and SOD enzymes, which are all linked to cellular regulation of oxidative stress [2,25].

Even though, the direct antioxidant capacity of (poly)phenols is not completely conserved after digestion, their metabolized intermediates can target a wide-ranging of different proteins, which in turn may trigger mechanisms leading to oxidative stress reduction and cardiovascular protection [2,23].

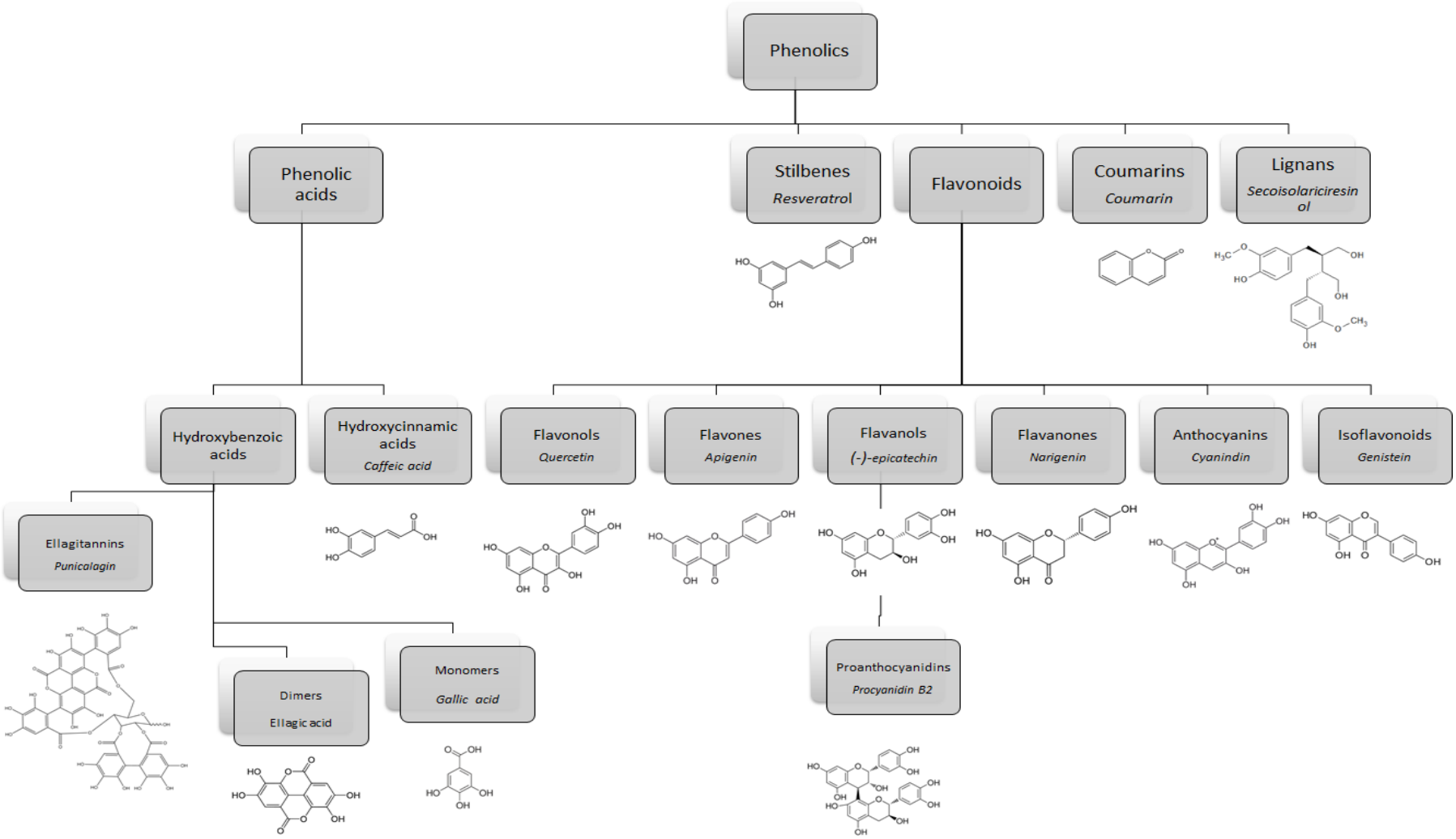

Figure 1-2 – Schematic representation of all the phenolic compound included within the term (poly)phenols.

1.7. Berries Driven (Poly)phenols

Most recent works that take into account dietary (poly)phenols metabolization are important to highlight [36,37], as little attention has been given to the actual (poly)phenols metabolites circulating and their physiologic concentration. As mentioned before, numerous epidemiologic and experimental studies suggest a vast number of health benefits from (poly)phenol ingestion, without taking into account which metabolites are truly active and if they are even able to reach the target tissue [28].

With the propose of shedding light into what can truly be found circulating within the human body, intervention studies have come to great lengths in helping the scientific community understanding the metabolic fate and bioavailability of ingested compounds [28,38]. Intervention studies are commonly used in order to evaluate the efficiency of a therapeutic or preventive measure, as followed subjects are compared to a control group. In epidemiologic studies, interventions studies are considered to be the ones that provide the most reliable evidence [39].

It has long been known that berries possess a high content of (poly)phenols,[40] and an elevated contribution to the overall ingestion of (poly)phenols in comparison to other food sources[41]. Notwithstanding all the existing studies with berries driven (poly)phenols there is still a gap needed to be filled, as little is known about which metabolites and conjugates produced after consumption are actually active. A recent intervention study performed in Dr Cláudia Santos laboratory using a berries mixture (blueberries, raspberries, blackberries, Portuguese crowberry, and strawberry tree fruits) as the only (poly)phenol source in the volunteers diet, identified, plasma levels and the excretion rate of (poly)phenols metabolites [36,37].

1.8. Mitochondria as a therapeutic target against CVDs

Playing mitochondria dysfunction an important role in CVDs, new therapeutic strategies based on mitochondria as target have been studied to prevent or to improve CVDs outcomes [14,42].

In search for novel cardioprotective therapies through mitochondrial regulation, (poly)phenols from dietary sources have acquired an important role, mainly due to all the extensive research done showing the cardioprotective effect of the different groups of (poly)phenols [42]. Majority of the beneficial effects showed by (poly)phenols may rest in their ability to interact with mitochondrial biogenesis and regulation of apoptotic pathways. For instance some (poly)phenols can upregulate PGC-1α, while others like flavanols can inhibit apoptotic signaling pathways. Anthocyanins another class of (poly)phenols can affect mitochondria from apoptosis modulation to the regulation of mitochondrial membrane potential, respiratory chain and mitochondrial permeability [42–47].

Although there is a vast amount of promising results, the pathways of these active compounds are still not completely known, thus a numerous of different aspects are yet to be study before there is a complete understanding of their full capability.

2. Objectives

Cardiovascular diseases constitute a prominent health problem, as it is account for 30% of the deaths worldwide. It has been evidenced a link between CVDs progression, mitochondria, and diet. The knowledge of these interactions opens many possibilities for development of new therapeutic agents in CVDs prevention.

In order to gain insight of the novel berries driven (poly)phenols (BDP) protective potential against CVDs, a set of specific aims were establish: (i) evaluation of BDP on cell death modulation; (ii) BDP role on mitochondrial biogenesis by assessment of mitochondrial population; and (iii) evaluation of BDP on cardiomyocyte beating activity.

To achieve these aims, rat primary cardiomyocytes culture and H9c2 cell line will be used in *in vitro* assays. Cell death will be induced using *tert*-butyl hydroperoxide (*t*-BHP) or isoproterenol (ISO), and viability will be followed by flow cytometry using propidium iodide. Cell metabolism will be assessed through resazurin based assay. Mitochondrial role will be study by assessing mitochondrial population through flow cytometry using a specific mitochondrial dye, and by mitochondrial DNA quantification through quantitative-PCR. Cardiomyocyte beating activity will be assessed through imaging recording.

3. Materials and Methods

3.1. Berries Driven (Poly)phenols

Recent work done by Disease and Stress Biology Laboratory (Dsb Lab.), within (poly)phenols bioavailability, identified and quantified a great array of (poly)phenols metabolites circulating in human plasma after consumption of a (poly)phenol rich juice. Furthermore it was also measured the circulating period of the different (poly)phenols metabolites [36].

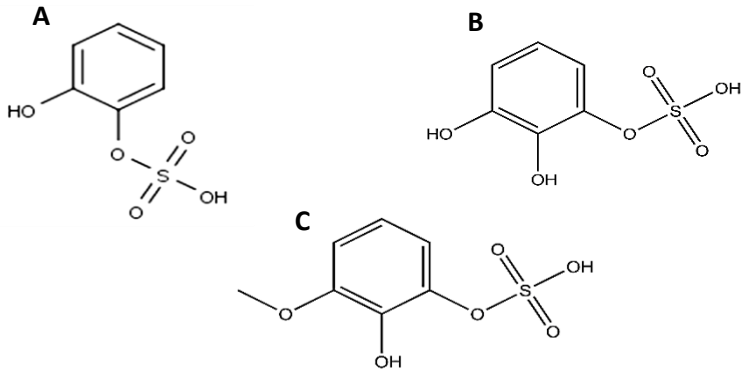

Figure 3-1 – Schematic representation of the different berry driven (poly)phenols used. (A) Catechol-*O*-Sulfate, (B) Pyrogallol-*O*-Sulfate, (C) 1-Methylpyrogallol-*O*-Sulfate.

Catechol-*O*-Sulfate, Pyrogallol-*O*-Sulfate, and 1-Methylpyrogallol-*O*-Sulfate were the (poly)phenols metabolites used in all experiments, they were used in the concentrations in which they were identified in plasma 12, 6, and 3 µM respectively [36]. These metabolites were identified in a previous work circulating in human plasma for a period of at least 2 h. Experimental conditions were chosen based on the human intervention studies, mimicking the concentrations found in plasma and resilience time. Synthesis of all (poly)phenols was performed previously within Disease and Stress Biology Laboratory as described elsewhere [36].

3.2. Cell Culture H9c2 differentiation

H9c2 cell line (provided by Dr. Catherin Brenner), was cultured in plating medium [89% (v/v) Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 4.5 g/L of glucose, 0.11 g/L of sodium pyruvate and L- glutamine (Sigma®), 10 % (v/v) Fetal Bovine Serum (FBS, Sigma®), and 1% (v/v) 50 U/mL of penicillin and 50 µg/mL of streptomycin (Sigma®)], in a 75 cm^2 tissue culture flasks (Starstedt), at 37 ^oC in a humidified atmosphere of 5 % CO_2. Cell medium where changed every 2-3 days, and cells were subculture when reached 70-80 % of confluence in order to prevent differentiation into smooth muscle cells. H9c2 cells where detached from the 75 cm^2 tissue culture flasks by removing the medium, then differentiation was induced by changing the medium serum from 10 % (v/v) FBS to 1 % (v/v) Horse Serum (HS, Gibco®) followed by the addition of 1 µM of all trans-retinoic acid. Firstly H9c2 cells were plated at a density of 3 $x10^4$ cell/mL and cultured for 1 day in medium with 10 % (v/v) FBS, in order to allow for cell attachment and growth. Secondly media serum was changed to 1% (v/v) HS with addition of 1 µM of all trans-retinoic acid, and cells were cultured for 5 more days with a daily medium change performed in a low light environment. Finally cell culture was maintained in differentiation medium throughout all experiments, never more than two weeks [48].

3.3. Isolation of neonatal rat cardiomyocytes

Cardiomyocytes were isolated from rat pups (Wistar rats) that were 2-3 days old, all animals were rapidly decapitated, hearts were removed and atria excised then the ventricles were minced in ADS buffer [0.12 M NaCl (Sigma®) , 0.018 M Hepes (Sigma®), $1.36x10^{-3}$ M NaH_2PO_2 (Applichem), $5.55x10^{-3}$ M glucose (Sigma®), $5.37x10^{-3}$ M KCl (Merck), $4.06x10^{-4}$ M $MgSO_4$ (Alfa Aesar)]. Ventricular tissue in ADS was transferred to a bottle and ADS solution was removed as much as possible. After ADS removal, it was added 3 mL of enzymatic solution made with 7 U Collagenase II (Roche) and 30 mg of Pancreatin (Sigma®) in 60 mL of ADS. Tissue homogenate was placed in a magnetic stirrer and spun at low speed for 20 min, at which point the solution is removed from the undigested tissue and set aside, while it is added 3 mL of the same enzymatic solution, this procedure was repeated until all tissue was digested. Collected fractions were added to 500 µL of New Born Calf Serum (NBCS, Gibco®) and centrifuged for 5 min at 524 *g* after which the supernatant was discarded and the pellet resuspended in 1 mL of NBCS, and stored at 37 ^{o}C. After complete digestion, all fractions collected were pooled together and centrifuged for 5 min at 524 *g*, the supernatant was discarded and pellet was resuspended in 4 mL of 1x ADS. In order to obtain a purified cardiomyocytes fraction the solution was added to a two phase discontinued Percoll (Sigma®) gradient, made from a bottom part with 65% Percoll and a top part with 45% Percoll. Percoll discontinued gradient was centrifuged for 30 min at 2095 *g*. Purified cardiomyocyte fraction was recollected and cells were plated in plating medium [68% (v/v) DMEM GlutaMAX supplemented with 1 g/L of glucose, 0.11 g/L of sodium pyruvate (Gibco®), 17% (v/v) Medium 199 (Sigma®) supplemented with 2.2 g/L of sodium bicarbonate and 0.1 g/L L-glutamine, 10% (v/v) HS, 5% (v/v) NBCS, 1% (v/v) L-glutamine (Sigma®)], at a concentration of $7.4x10^4$ cell/mL in multiwell plates coated with 0.1 % (w/v) gelatin (Sigma®) [49].

Throughout all experimental procedure, cells were maintained in plating medium with a medium change performed every three days until desirable confluence reached.

3.4. Cell treatment

In order to determine the cytoprotective capacity of the berries driven (poly)phenols (BDP), both neonatal rat primary cardiomyocytes and H9c2 cell line after reaching the desired confluence (50 % of confluence for H9c2 and neonatal cardiomyocytes) were primarily treated for 2 hours with the different BDP. After treatment, cells were washed with 1x Phosphate buffered saline (PBS pH 7.2, Gibco®) and incubated with the cell death inducers: *tert*-butyl hydroperoxide (*t*-BHP, Sigma®) for 24 h or with isoproterenol (ISO, Sigma®) for 48 h. *tert*-Butyl Hydroperoxide was used in order to produce extensive oxidative damage, while ISO was used to mimic the catecholamines release that occurs during heart failure.

3.5. CellTiter-Blue cell viability assay

CellTiter-Blue cell viability assay was performed on H9c2 cell line to measure cell metabolic capacity. H9c2 cell were plated in 96 well plates and after cell treatment described above (for cytoprotection evaluation), cells were washed with 1x PBS and incubated with CellTiter-Blue® reagent (Promega) for 4 h at 37 ^{o}C as manufacture instructions. The fluorescence signal from the reduction of resazurin to resorufin, was measured at 590 nm in a Synergy HT Biotek plate reader.

3.6. Flow cytometry for evaluation of viability and mitochondrial population

Flow cytometry analysis was performed on neonatal rat primary cardiomyocytes or H9c2 cell line that were plated in 24 wells plates. All samples received the same treatment, supernatant from wells was collected into eppendorfs and cells where washed one time with 100 µL of PBS that was collect in to the same eppendorf with the supernatant of each well. It was added to every well 100 µL of Accutase® (Gibco®) for 30 min in order to detach cells. After incubation, Accutase® activity was stopped by adding the previously stored supernatants. The resulting suspension was transferred in to eppendorfs and centrifuge at 350 *g* for 5 min, after which the supernatant was discarded and the pellet was resuspended in 300 µL of PBS with the fluorochrome Propidium Iodide (PI, Invitrogen) to evaluate cell viability or MitoTracker Deep Red FM (MTDR, Invitrogen) to evaluate mitochondrial population [50].

The staining for PI was performed on ice and in the dark for 15 min for MTDR the staining was performed at room temperature (RT) and in the dark for 30 min. All results were acquired using Becton Dickinson FACSCalibur equipped with a low-power air cooled 15 mW blue (488 nm) argon laser, from which 3 colours can be detected by the band-passes 530/30 (FL1), 585/42 (FL2), and the long-pass 670 (FL3). There is also a red (635 nm) diode laser, from which 1 colour can be detect by the band-pass 661/16 (FL4). From each sample at least 5,000 events were obtained, and all results were analyzed using FlowJoX software.

Analysis was performed using forward light scatter (FSC) vs orthogonal light scatter (SSC) for gating. Further analysis was performed using a plot of FSC area vs FSC height to distinguish doublets from single cells in order to choose the single cell subset. Within the single cell subset it was possible to acquire the percentage of cell viability in the PI experiment by using a FL3 plot to gate the percentage of PI negative and positive cells. In the experiment with MTDR it was possible to measure the variations in the mitochondrial population within the single cell subset by using a median fluorescence intensity vs FL4 plot analysis [50].

3.7. Immunofluorescence

Immunofluorescence staining was performed in fixed cells in 4% (w/v) of paraformaldehyde with 4% (v/v) sucrose for 10 min at room temperature. After fixation cells were washed three times with PBS and incubated with blocking buffer containing 0.1% (v/v) Triton (Merck) and 3% (w/v) BSA (Sigma®) in PBS for 1 h. For Troponin T, staining cells were incubated with anti-Troponin T, cardiac isoform ab-1 (mouse monoclonal, 1:100, Thermo Scientific) overnight at 4 ^{o}C. The cells were then washed three times with PBS, and were incubated with goat anti-mouse Alexa Fluor 488-conjugated antibody (1:500, Abcam) for 2 hours at RT. To visualize the nuclei, cells were counterstaining with a drop of ProLong®Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) for 10 min. All fluorescence images were captured using a fluorescence microscope (Leica DMRA2). Analysis and treatment of the captured images was performed using Fiji software.

3.8. Western blot

Cells cultured in 6 wells plates were submitted to protein extraction with Radio-Immunoprecipitation Assay (RIPA) buffer [50 mM Tris (CarlRoth® – Schoemperlenstr, Karlsruhe, Germany); 150 mM NaCl (Sigma®); 0.1% (w/v) Sodium dodecyl sulfate (SDS, Merck); 0.05% (w/v) sodium deoxycholate (Sigma®); 1% (v/v) NP-40 (Sigma®); 0.05% (v/v) cocktail protease inhibitors (AppliChem) ; and 0.4% (v/v) DNAse (Roche)] were added. Cells were lysed with the previous solution on ice for 5 min. following an overnight storage at -80 ^{o}C, after which the wells were scraped using a cold plastic cell scraper. Cell suspension was gently transfer into eppendorfs and stored at -80 ^{o}C until used. Protein quantification was then processed using the Bradford quantification kit from Bio-Rad.
It was used 50 µg of protein per well, and both the protein samples as well as the prestained protein marker VI (Applichem) were subjected to a sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) in 10% (w/v) and 1.5 mm thickness acrylamide gel, at 200 V for

approximately 40 min. The electrophoresis buffer was composed by 25 mM Tris-base, 190 mM glycine and 0.1% (w/v) SDS.

Protein electrophoretic transfer occurred after SDS-PAGE gel, and polyvinylidene fluoride (PVDF) membrane (Bio-RAD) were assembled in the transfer cell (Trans-Blot® Turbo™ Transfer System, BioRad), and transference was carried out at approximately 25 V for 7 min at RT according with manufacture instructions.

After transfer, membrane was rinse in methanol for 1 min. and then blocked with 5% (w/v) membrane blocking agent (MBA, GE Healthcare) in Tris-buffered saline (TBS) containing 0.01% (v/v) Tween20® (Sigma®) (TBST), for 1 hour at RT. Membrane was then incubated with primary antibody anti-Akt (rabbit monoclonal, 1:1000, Cell Signaling) or anti-phospho Akt Ser473 (rabbit monoclonal, 1:1000, Cell Signaling) and left overnight at 4 °C, after which it was washed three times in TBST for 5 min. each, and incubated with secondary antibody (goat anti-rabbit horseradish peroxidase (HRP) – conjugated, 1:2500, Thermo Fisher Scientific Inc.) for 2 h at RT. Rabbit anti-actin primary antibody (1:500, Santa Cruz Biotechnology) was used as loading control, along with secondary antibody goat anti-rabbit HRP – conjugated secondary antibody (1:2500, Thermo Fisher Scientific Inc.). All antibodies were diluted in 5% MBA. Antibody detection was performed with a chemiluminescent substrate (FemtoMax Super Sensitive Chemiluminescent HRP Substrate, Rockland Inc.) and membrane images were acquired in the Molecular Imager Chemidoc XRS (Quantity One© software 4.6.6, BioRad).

3.9. Quantitative-Polymerase chain reaction (Q-PCR)

For quantification of mitochondrial DNA, genomic DNA was extracted from neonatal rat primary cardiomyocytes using High Pure DNA isolation kit (Roche Diagnostics), according to the manufacturer's instructions. Assessment of DNA quality and yield, samples were analyzed with a NanoDrop 2000c spectrophotometer (Thermo Scientific) and DNA integrity and contamination were determined by DNA visualization in a 1% agarose gel, stained with ethidium bromide. PCR was performed using specific forward and reverse primers designed for the mitochondrial cytochrome b (mtCyt b) gene (forward: 5'-TTCATGTCGGACGAGGCTT-3', reverser: 5'-TCCTCATGGAAGGACGTAGC-3') and for the nuclear glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (5'-CCTTCATTGACCTCAACTACAT-3', 5'-CCAAAGTTGTCATGGATGACC-3'), respectively. Fast Start DNA Master Plus SYBR Green I (Roche Diagnostics) was used, according to the manufacturer's instructions, with the experimental run protocol: denaturation program was 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 10 seconds, 60°C for 10 seconds and 72 °C for 10 seconds.

3.10. Cell beating rate measurement

Cell beating rate was acquired by placing control and treated cells under the 10x/0.25NA dry objective lens on a Nikon Eclipse Ti-U inverted bright field light microscope. Images were recorded with a high speed FASTCAM MC2 camera (Photron Europe, Limited) and controlled with PFV (Photron FASTCAM Viewer) software. All measurements were acquired by Pedro Sampaio, from the Cilia Regulation and Disease laboratory from CEDOC. For each condition it was measured 20 different cells per condition by acquiring a total of 1500 frames at a rate of 60 frames per second. In order to quantify changes in cell beating background surface shape was removed, and images were converted to grayscale with 16 bits per sample pixel. This method permit to measure the difference in light intensity throughout time, as beating cardiomyocytes will diffract light differently during contraction. Analysis and treatment of captured images was performed using Fiji software, which calculates cell beating frequency by measuring light intensity variation through a specific time period.

3.11. Statistical analysis

Reported results were statistically evaluated using SigmaPlot 11 software (Systat Software Inc.). Box-and-Whiskers plots were analyzed by Kruskal-Wallis one way analysis of Variance on ranks and compared by Dunnetts Method with a confidence level of 95%. Remaining data were analyzed by one way analysis of variance (one way ANOVA) and compared by Tukey honest significant difference test with a confidence level of 95%.

4. Results

(Poly)phenols intake as long been associated with a reduced risk of CVD development, however their mechanisms of action continues to elude the scientific community. This study aims to evaluate the effects in cardiac cells induced by BDP metabolites capable of reaching the human circulatory system. A previous human intervention study for identification of circulating BDP metabolites was the base for choosing the metabolites tested herein[36]. The metabolites used were Catechol-*O*-Sulfate, Pyrogallol-*O*-Sulfate, and 1-Methylpyrogallol-*O*-Sulfate, as their concentration was the highest found in human plasma [36].

4.1. H9c2 cells differentiation into cardiomyocytes

With the purpose of studying *in vitro* the cytoprotective effect and the associated mechanisms involved with the (poly)phenols, it was chosen the H9c2 cell line. This cell line is isolated from the ventricular part of a thirteenth-day rat heart embryo and presents a myoblastic proliferative phenotype while maintained in 10 % (v/v) FBS containing culture media [48]. H9c2 cells have been applied in several *in vitro* studies, as a model for cardiac diseases, namely hypertrophy, cardiotoxicology, ischemia/ reperfusion injury and oxidative stress as well as in studies of mechanisms for cardiac differentiation [32,48,51–54]. Most studies are performed with undifferentiated H9c2 cell line which can rise questioning into the applicability of the results, as it is described to have a less physiological response [54]. H9c2 cell line has the capacity to differentiate into either skeletal or cardiac muscle-like cells depending on whether or not the serum reduction to 1 % is accompanied by a daily treatment with 1 µM of all-trans-retinoic acid [48,54].

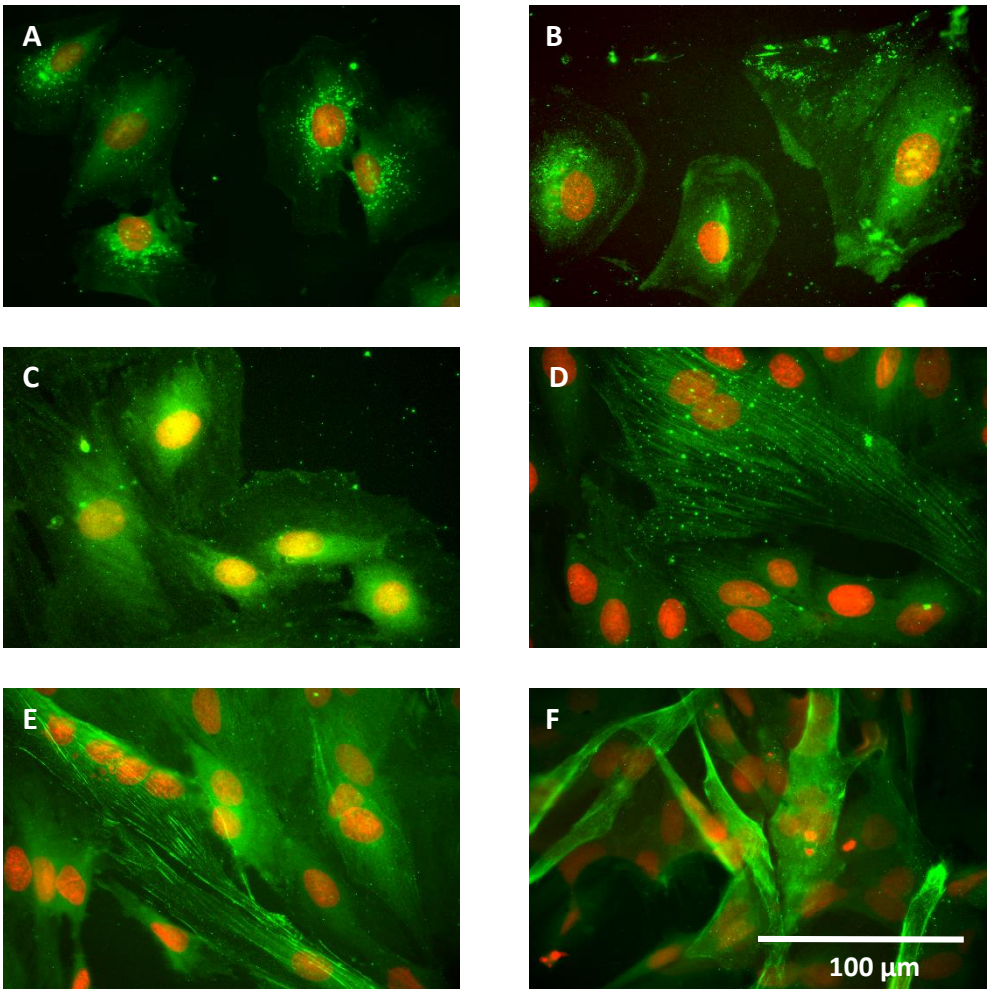

Figure 4-1 – Morphology change of H9c2 cells after induced differentiation into cardiac muscle-like cells, showed by immunofluorescence staining. Cardiac troponin T isoform stained in green fluorescence, nucleus stained with DAPI in red. H9c2 cells with differentiation treatment: (A) 0 days, (B) 1 day, (C) 2 days, (D) 3 days, (E) 4 days, (F) 5 days.

Troponin T is a thin filament protein involved in the regulation of muscle contraction. Although cardiac troponin T isoform is expressed transiently in immature skeletal and cardiac muscle, in adult cells is expressed exclusively by cardiac muscle cells [55]. Therefore troponin T expression may be used as a marker for cardiac cells [56].

Following cardiomyocyte differentiation protocol into cardiac muscle-like cells, it was possible to verify an increase in cardiac troponin T isoform expression throughout the first two days (Fig.4-1). At the third day, cells appear to have lost the ability to undergo mitosis, organized structures containing cardiac troponin T isoform start to be formed, and cells acquire a different morphology. After five days of differentiation H9c2 cells had acquired a cardiac-like cell shape. During differentiation process it was possible to confirm that H9c2 cells express specific cardiac markers, making it a more representative model.

In conclusion the differentiation protocol was successful, allowing for this *in vitro* cell model to be used in the study of the cytoprotective effects of BDP against CVDs.

4.2. Differentiated H9c2 cell line viability with *t*-BHP

As oxidative stress is the mainly cause of CVDs, oxidative stress induced cell death was chosen as a model to study the potential cytoprotective effects of BDP. As it is a well-known membrane permeant pro-oxidant agent and inhibitor of mitochondrial function, *t*-BHP was used as oxidative agent. Exposition of H9c2 cells to *t*-BHP promotes nucleotide degradation and adenosine formation, increases ROS production, alterations in mitochondrial network, chromatin condensation and nuclear shrinkage [51,57].

Cytoprotective ability of BDP metabolites was tested against an oxidative stress model with *t*-BHP [51]. Differentiated H9c2 cells were treated with 80 and 120 µM *t*-BHP for 24 h, not to give an acute damage but instead a mild and chronic stress.

Concentrations of *t*-BHP were chosen through measurement of the half maximal effective concentration (EC_{50}), from metabolic capacity. After treatment for 24h with 80 µM of *t*-BHP reduced in 50 % metabolic capacity, while treatment with 120 µM of *t*-BHP reduced cell metabolic capacity in 80 % (results not shown).

Although 80 µM of *t*-BHP for 24 h was the EC_{50} for metabolic capacity, it was not enough to diminish in 50 % viability of differentiated H9c2 cells, monitored by flow cytometry. However this concentration was enough to cause significant cell death.

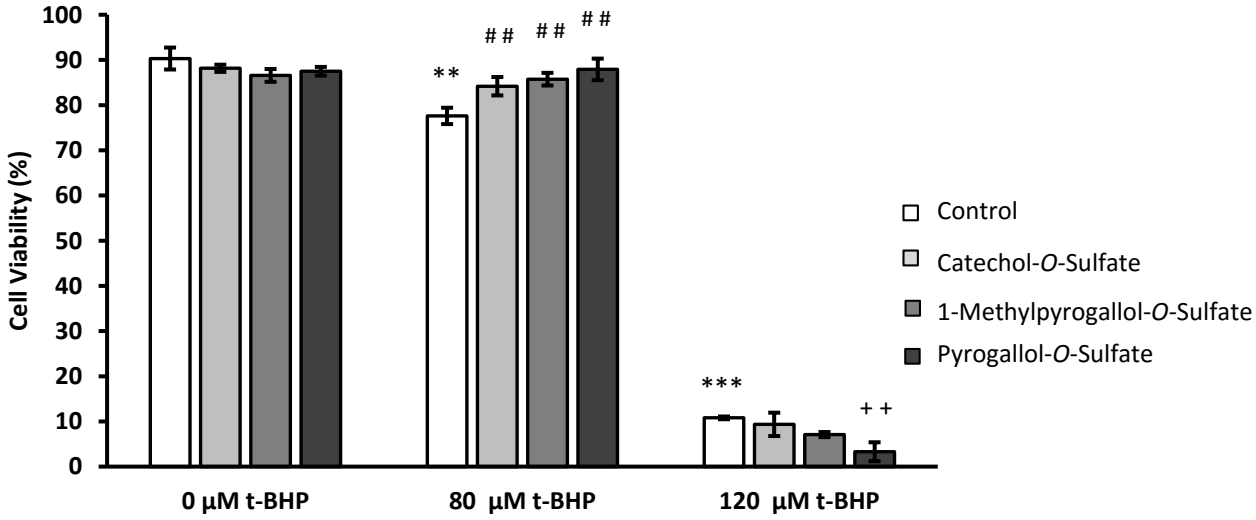

Figure 4-2 – Differentiated H9c2 cells viability after being pre-treated for 2 h with the different BDP metabolites and treated for 24 h with two concentrations of *t*-BHP.BDP concentrations used where: Catechol-*O*-Sulfate 12 µM, 1-Methylpyrogallol-*O*-Sulfate 3 µM, and Pyrogallol-*O*-Sulfate 6 µM. Cell viability was determined through cell permeability to PI by flow cytometry. Data are mean ± SD from three independent experiences. *** $P < 0.001$, ** $P < 0.05$ versus Control; $^{\#\#}P < 0.05$ versus Control with 80 µM *t*-BHP; $^{++}P < 0.05$ versus Control with 120 µM *t*-BHP.

Differentiated H9c2 cells treated with 80 µM of *t*-BHP presented a 10 % diminish in viability comparatively to control. This viability reduction was significantly prevented when cells were pre-treated with BDP metabolites, suggesting a cytoprotection (Fig.4-2). However treatment with 120 µM *t*-BHP induced an excessive death, making it impossible to obtain conclusive results from the BDP metabolites effect other than the incapacity to protect against this high stimulus. Nevertheless, evaluation through flow cytometry using PI only allows to distinguish viable cells from late apoptotic ones, leaving early apoptotic unstained and possibly giving some inaccuracy in the viability measured.

4.3. Differentiated H9c2 cell line viability with ISO

Although incubation with *t*-BHP is a frequently utilized oxidative stress model, it is not a cardiac specific toxicant as ISO, which is a β-adrenergic agonist. β-adrenergic receptors (β-AR) are essential for cardiac functions as regulators of cardiac performance. Adrenergic amines released by the sympathetic nervous bind to β-AR, resulting in an increase in cardiac contractility, frequency, and rate of relaxation [58]. During a heart failure episode there is an increased release of catecholamines in an attempt to restore homeostasis. Nevertheless an extensive and excessive signaling of catecholamine on β-AR leads to loss of adrenergic pathways, decreased contractility, changes in gene expression, calcium overload, and ultimately cell death [7,53].

Concentration and time period for ISO treatment were chosen through measurement of EC_{50} from metabolic capacity. After treatment for 48 h with 200 µM ISO, metabolic capacity was reduced by 50 % (data not shown).

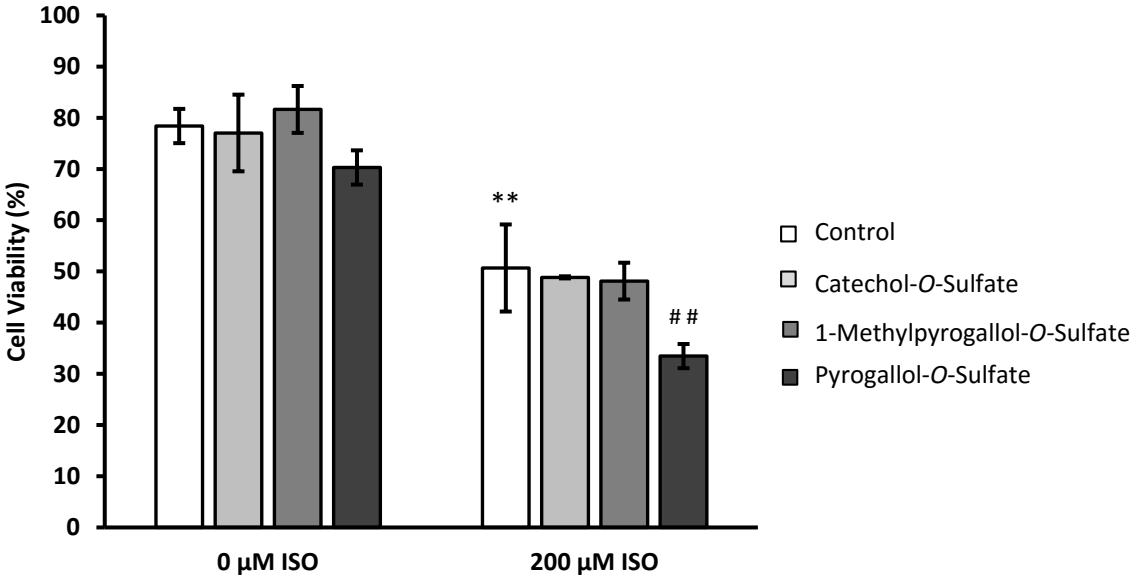

Figure 4-3 – Differentiated H9c2 cells viability after being pre-treated for 2 h with the different BDP metabolites and treated for 48 h with ISO. BDP concentrations used where: Catechol-*O*-Sulfate 12 µM, 1-Methylpyrogallol-*O*-Sulfate 3 µM, and Pyrogallol-*O*-Sulfate 6 µM. Cell viability was determined through cell permeability to PI by flow cytometry. Data are mean ± SD from three independent experiences, ** $P < 0.05$ versus Control; ## $P < 0.05$ versus Control with 200 µM ISO.

Treatment with 200 µM ISO for 48 h reduced cell viability in 50 %. These results were obtained through cell permeability to PI by flow cytometry, and showed a similar EC_{50} to the one obtained when measuring metabolic capacity.

Differently from the results obtained with *t*-BHP (Fig.4-2), pre-treatment with BDP metabolites did not possess cytoprotective effects against ISO treatment. Additionally, Pyrogallol-*O*-Sulfate appears to significantly increase ISO toxicity, while the remaining BDP do not affect cell viability (Fig.4-3). Catechol-*O*-Sulfate and 1-Methylpyrogallol-*O*-Sulfate seem to protect against oxidative injury, but do not protect against ISO induced damage suggesting that BDP targets may not be directly connected to ISO cell death activated pathway.

4.4. Cell metabolic capacity

In order to evaluate (poly)phenols effect in overall metabolic capacity, differentiated H9c2 cells were pre-treated with BDP metabolites for 2h. Cell metabolic capacity was assessed through CellTiter-Blue viability assay, in which reduction of resazurin to resorufin by mitochondrial, cytosolic, and microsomal enzymes was measured [59,60].

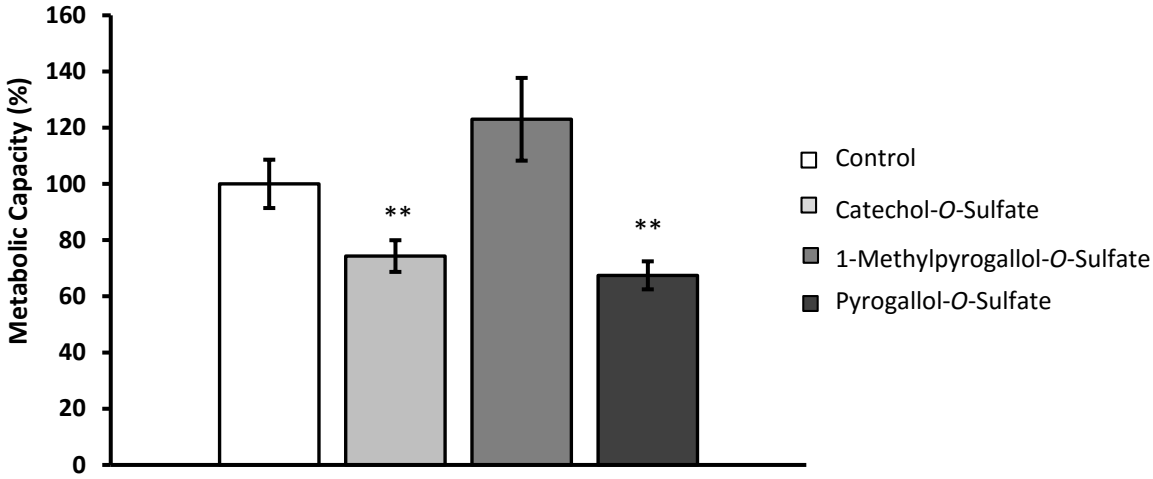

Figure 4-4 – Differentiated H9c2 cells metabolic capacity measured after being pre-treated for 2 h with the different BDP metabolites. BDP concentrations used where: Catechol-*O*-Sulfate 12 µM, 1-Methylpyrogallol-*O*-Sulfate 3 µM, and Pyrogallol-*O*-Sulfate 6 µM. Data are mean ± SD from three independent experiences. ** P < 0.05 versus Control.

Berries driven (poly)phenols metabolites appear to influence cell metabolic capacity, as shown in Fig.4-4. Through the obtained data it appears 1-Methylpyrogallol-*O*-Sulfate increases metabolic capacity, while the remaining tested metabolites diminish it. However, PI permeability results have shown that all BDP metabolites in the concentrations tested were not cytotoxic. These results suggest that, BDP metabolites possess two different mechanisms of action. Catechol-*O*-Sulfate and Pyrogallol-*O*-Sulfate are capable to reduce metabolic capacity as reflected by the reduction in enzymatic activity responsible for resazurin reduction to resorufin. Moreover, 1-Methylpyrogallol-O-Sulfate promotes an increase of resazurin reduction. Nevertheless, given these results it would be interesting to further investigate cell metabolic changes, in order to comprehend the fully extend of BDP metabolites influence over cell metabolism.

4.5. Neonatal rat cardiomyocytes viability with *t*-BHP

Primary cultures of neonatal rat cardiomyocytes are as H9c2 cultures, a well-established model for studying CVDs, but contrary to H9c2, these cells possess intrinsic cardiac beating activity, and allow for a metabolic response closer to one observable *in vivo* [61].

Treatment of cells with 80 µM *t*-BHP showed excessive death, making it impossible to conclude if there was any real influence from pre-treatment with (poly)phenols metabolites (Fig.4-5). One unanticipated result was the slight cytotoxicity presented by Catechol-*O*-Sulfate. This one-off cytotoxicity presented is still not fully understand.

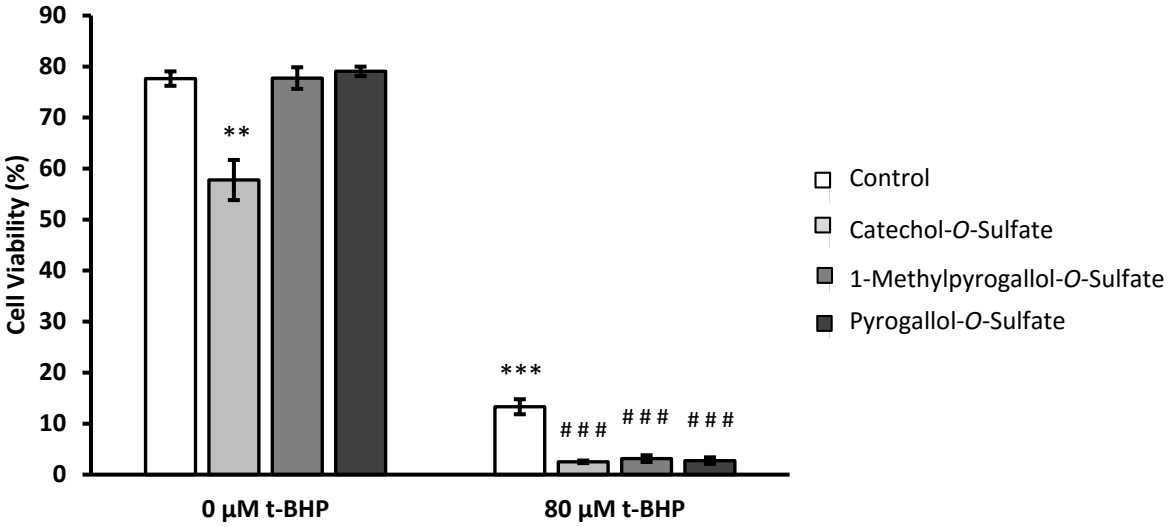

Figure 4-5 – Neonatal rat cardiomyocytes viability after being pre-treated for 2 h with the different BDP metabolites and treated for 24 h with 80 µM *t*-BHP. BDP concentrations used where: Catechol-*O*-Sulfate 12 µM, 1-Methylpyrogallol-*O*-Sulfate 3 µM, and Pyrogallol-*O*-Sulfate 6 µM. Cell viability was determined through cell permeability to PI by flow cytometry. Data are mean ± SD from three independent experiences. *** $P < 0.001$, ** $P < 0.05$ versus Control; ### $P < 0.001$ versus Control with 80 µM *t*-BHP.

Neonatal rat cardiomyocyte cells were substantially more susceptible to treatment with 80 µM *t*-BHP than differentiated H9c2 cells. These results imply that even though differentiating H9c2 cells to cardiac muscle like cells helps obtaining a more physiologic response, H9c2 cells are still an immortalized cell line making them much more resilient to oxidative stress than a primary cell culture. These differences could explain the discrepancy in the obtained viability results from the different cell models.

Using 80 µM *t*-BHP for 24 h did not make it possible to study the BDP effects in neonatal rat cardiomyocytes, given the excessive cell death. Other concentrations of *t*-BHP were tested, such as 20 and 40 µM for 24 h of incubation (Fig.8-1 in appendix), which revealed 40 µM *t*-BHP to be the concentration closer to EC_{50}. Further experiments will be performed using 40 µM *t*-BHP and BDP to confirm the protective ability previously detected when using differentiated H9c2 cells (Fig.4-2)

Although not having observed cytoprotection with neonatal rat cardiomyocytes, comparing both models, this model is closer to *in vivo* than the H9c2 model, and thus it was decided to use only neonatal model for the remaining experiments.

4.6. Neonatal rat cardiomyocytes viability with ISO

As neonatal rat cardiomyocytes are a closer to *in vivo* model than H9c2 cells, it was decided to also test ISO, in order to measure a more *in vivo* like response. This treatment produce a similar response in cells to the one applied to the differentiated H9c2 cells. It was applied 200 µM ISO for 48 h, which is described to lead to impairment of cardiac cell functions, such as a reduced contractility and apoptosis [54,62].

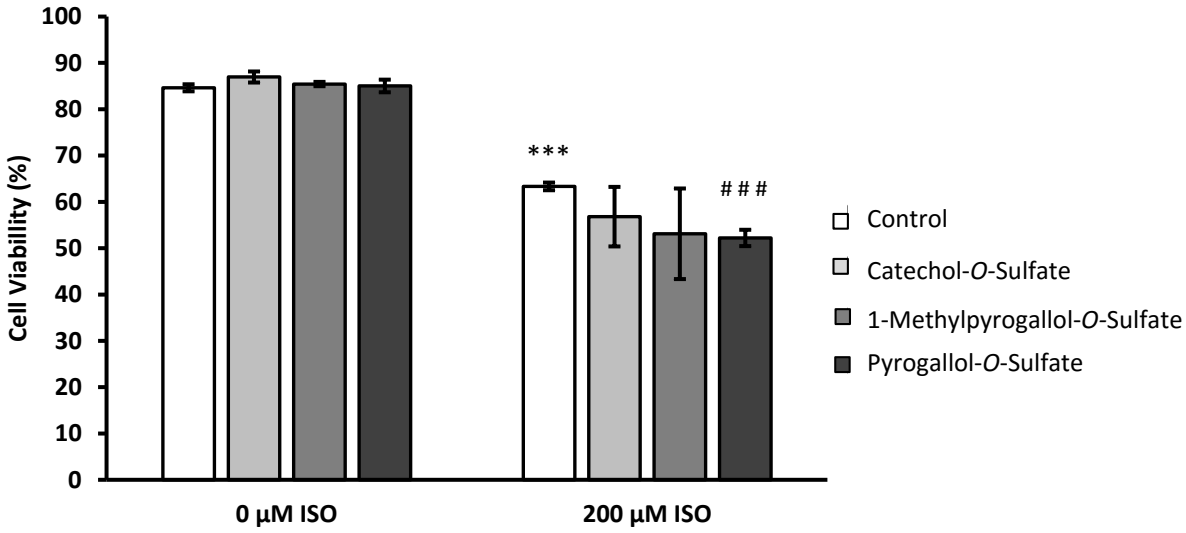

Figure 4-6 – Neonatal rat cardiomyocytes viability after being pre-treated for 2 h with the different BDP metabolites and treated for 48 h with ISO. BDP concentrations used where: Catechol-*O*-Sulfate 12 µM, 1-Methylpyrogallol-*O*-Sulfate 3 µM, and Pyrogallol-*O*-Sulfate 6 µM. Cell viability was determined through cell permeability to PI by flow cytometry. Data are mean ± SD from three independent experiences, *** $P < 0.001$ versus Control; ### $P < 0.001$ versus Control with 200 µM ISO.

When exposed to 200 µM ISO, primary cultures of neonatal rat cardiomyocytes showed an attenuated decrease in viability comparatively to the results obtained with *t*-BHP treatment. Even so BDP metabolites were not efficient in preventing a diminishing in viability against 200 µM of ISO (Fig. 4-6). Additionally, with the exception of Pyrogallol-*O*-Sulfate that significantly increased ISO induced death, the remaining BDP metabolites do not increasing ISO toxicity. These results go into accordance with the ones previously obtained by differentiated H9c2 cells and ISO treatment (Fig.4-3), further supporting that BDP metabolites cannot prevent ISO induced cell death in the conditions tested.

4.7. Cell beating activity

Despite none of the BDP metabolites showed cytoprotective effects in neonatal rat cardiomyocytes, it is still hypothesised a beneficial influence in physiological heart hypertrophy. An animal study conducted by Dr. Catherine Brenner (Paris-Sud University, France), in collaboration with our group, was performed using Dahl-salt sensitive rats (confidential data not published), where the berries mixture used was equal to the one used in the human intervention study [36].

Dahl-salt sensitive rats are a well-accepted animal model for hypertensive and diastolic heart failure studies, as they can also be preconditioned by diet. They exhibit many phenotypic traits common with hypertensive disease observed in humans, such as high blood pressure, salt sensitivity, insulin resistance and hyperlipidaemia.

The results obtained from the animal study performed by our collaborators showed that hypertension-induced by a high salt diet (HS diet) was associated with cardiac hypertrophy and a decrease in cardiac functions. Interestingly, HS diet supplemented with the berries mixture (HSB diet) decrease in mortality comparatively to rats fed only with HS diet. Moreover, HSB diet prevented cardiac damage independently of changes in systolic pressure, also increase in heart weight index was attenuated in rats fed with HSB diet comparatively to the ones fed with HS diet (confidential results).

Direct extrapolation from induced responses in rat to human are not possible, as both species possess different metabolic rates and it has been proven that findings in rat tissue do not always reproduce the metabolism observed in human.[63] However it is plausible to assume that even though in different concentrations from the ones tested in this work, the BDP metabolites tested could also

be present in the Dahl-salt sensitive rat plasma. Presently, the berries metabolites bioavailability is being evaluated in plasma, urine, heart, liver and kidneys collected from the animal study performed by Dr. Catherine Brenner. When new metabolites are detected, they will be chemically synthesized and include in future *in vivo* assays.

Taking into consideration Dr. Catherine Brenner results and the fact that pathologic heart growth from hypertension leads to reduced cardiac contractility, exploiting this BDP effects on cell beating activity could shed some light on the extent of their effects.

Prolonged exposure to ISO leads to cell beating impairment, making it possible to use this model to evaluate BDP effects in beating rate of ISO treated neonatal rat cardiomyocytes after 24 and 48 h (Fig. 4-7, 4-8). As cell death becomes evident after 48 h of ISO treatment, time points were chosen in order to determine if contractile dysfunction occurred prior or simultaneously to induced cell death.

Through the box-whiskers plot it is possible to directly evaluate overall cell synchronization by comparing box-whiskers plot dispersion between different conditions.

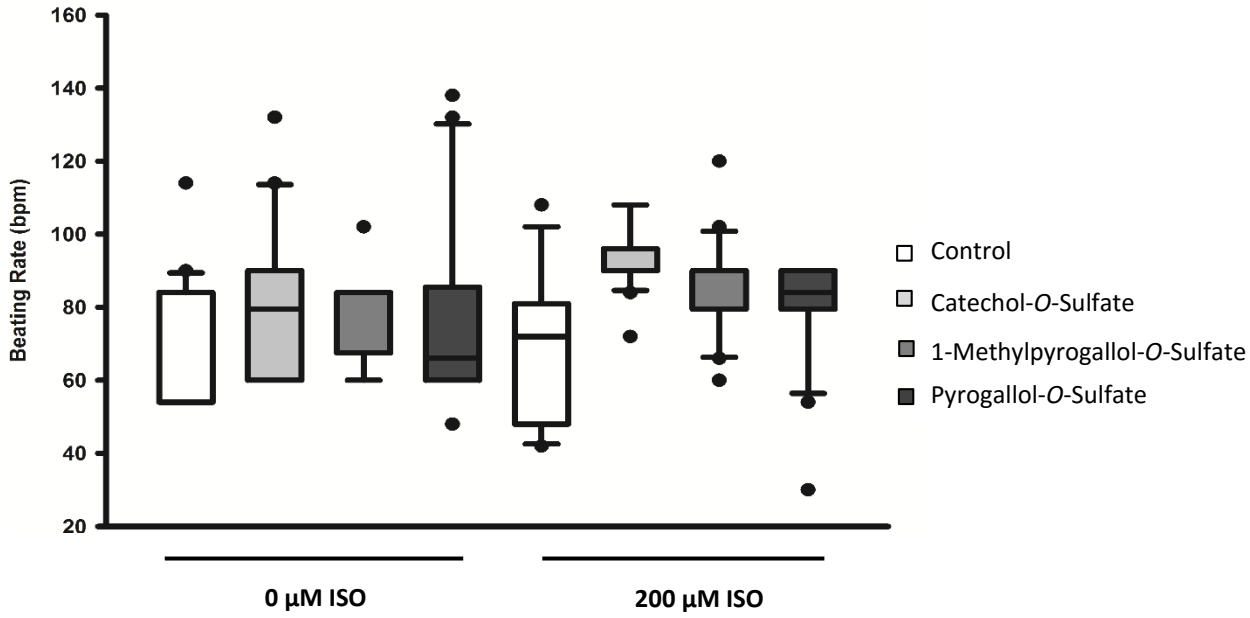

Figure 4-7 – Neonatal rat cardiomyocytes cell beating measurement, after a 2h pre-treatment with the different BDP metabolites and treated for 24 h with ISO. BDP concentrations used where: Catechol-*O*-Sulfate 12 µM, 1-Methylpyrogallol-*O*-Sulfate 3 µM, and Pyrogallol-*O*-Sulfate 6 µM. Outliers are identified as ●.

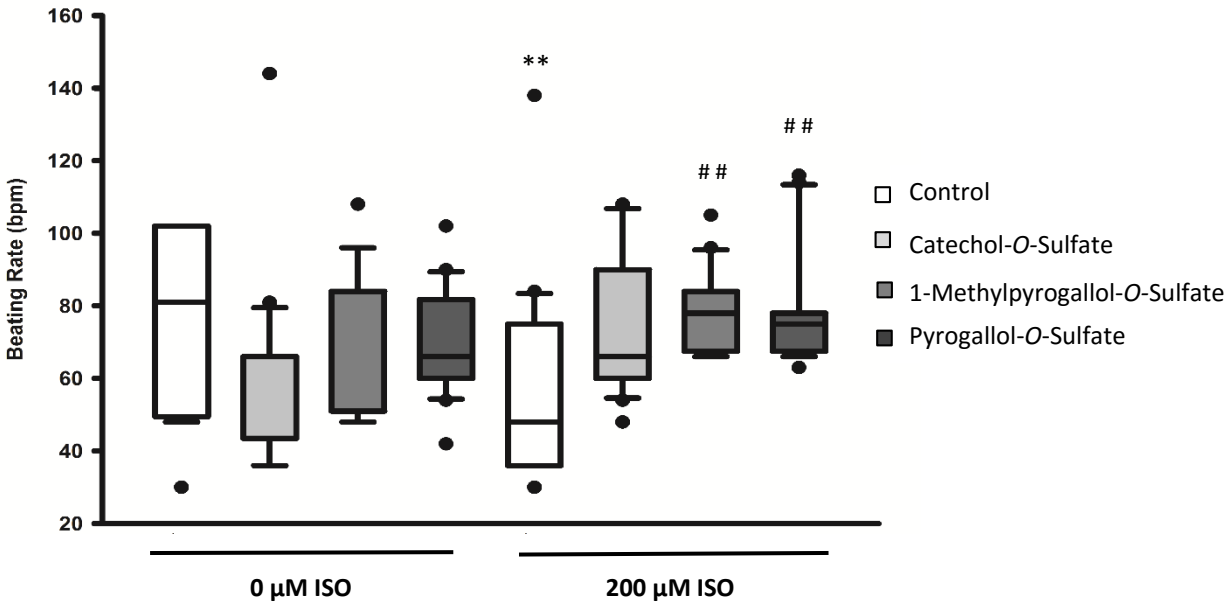

Figure 4-8 – Neonatal rat cardiomyocytes cell beating measurement, after a 2h pre-treatment with the different BDP metabolites and treated for 48 h with ISO. BDP concentrations used where: Catechol-*O*-Sulfate 12 µM, 1-Methylpyrogallol-*O*-Sulfate 3 µM, and Pyrogallol-*O*-Sulfate 6 µM. Outliers are identified as ●. ** $P < 0.05$ versus Control; $^{\#\#}$ $P < 0.05$ versus Control with 200 µM ISO.

It was verified that treatment with 200 µM ISO for 24 h does not induce significant decrease in cardiac contractile function (Fig.4-7), contrary to the 48 h time point where significant damage to cardiac contractile function can be observed (Fig.4-8).

1-Methylpyrogallol-*O*-Sulfate and Pyrogallol-*O*-Sulfate appear to after 48 h of ISO treatment, prevent loss of contractile functionality and increase beating synchronization relatively to the remaining conditions.

Pre-treatment with BDP metabolites maintained a normal beating rate comparatively to control without ISO treatment (Fig.4-7), which indicates that they do not possess chronotropic effects. Nevertheless cells that were pre-treated with BDP metabolites and treated with ISO showed a better synchronization and elevated beating rate compared to control treated with ISO (Fig.4-8).

These results are very promising, as they suggest an interaction between BDP metabolites and pathways responsible for contractile regulation. It appears that BDP metabolites *per se* do not influence cardiac beating, only when cells are exposed to ISO. After 24 h of ISO treatment, cells pre-treated with BDP metabolites do not show difference in beating rate. Nevertheless they are the most synchronized ones, as reflected by the lower dispersion between measurements (Fig.4-7). However after 48 h of ISO treatment this effect is no longer evident, their synchronization becomes very similar to the controls, while maintaining a normal beating rate.

There is still need for future experiments to also measure cell length and evaluate the expression of specific cardiac hypertrophy protein markers, in order to associate this changes with a possible physiological cardiac cell growth.

4.8. Protein phosphorylation induced by BDP

Recently Akt has been gaining attention from the scientific community, as it has been demonstrated its importance in cardiac development, suppressing fetal genes expression, and release of cytochrome c and AIF from mitochondria [13,64].

In order to study a possible interactions between BDP metabolites and Akt activation, efforts were made to measure Akt phosphorylation levels by western blot. Despite numerous attempts, probably due to technical problems (data not shown), it was not possible to visualize phosphorylated Akt. Measuring Akt phosphorylated turned into a real challenge in which the reason for failure is still unknown.

4.9. Mitochondrial population

Mitochondria are very important organelles, as they are the cells main source of energy and also control apoptosis[14,15]. During pathological events there is an increase in energy demand, which leads to a necessity in increasing total mitochondrial population, in order to keep up with the energy demand [13].

Therefore, to test BDP metabolites influence in mitochondrial population, it was evaluated total mitochondrial population regardless of the mitochondrial membrane potential, in neonatal rat cardiomyocytes through the MTDR staining method [50].

Recent studies have shown that excessive oxidative stress can induce an increase in mitochondrial population by damaging the autophagic capacity of the cells[57]. In order to prevent false positives all data referent to mitochondrial population was acquired without any toxicant treatment.

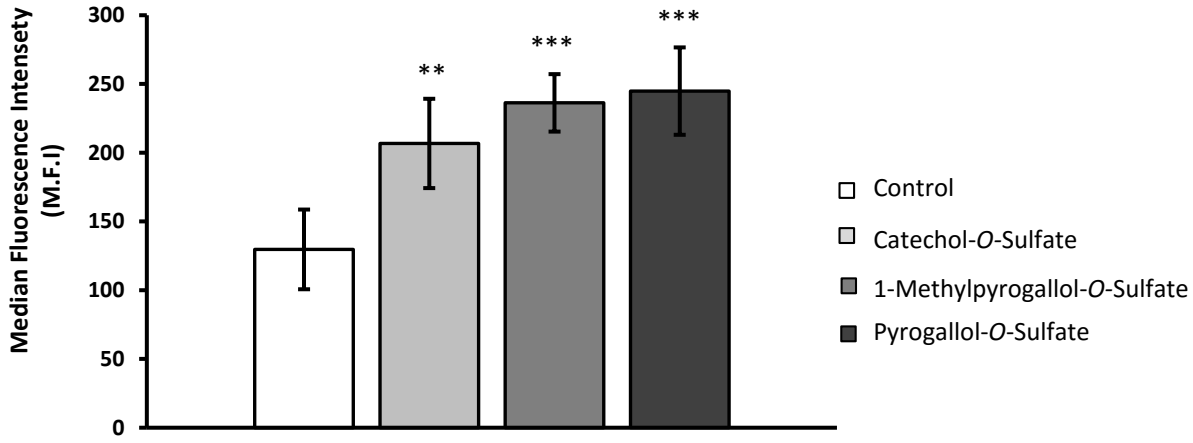

Figure 4-9 – Effects of BDP metabolites on the mitochondrial population of neonatal rat cardiomyocytes cells pre-treated with different BDP metabolites for 2h and cells harvest after 24 h. BDP concentrations used where: Catechol-*O*-Sulfate 12 µM, 1-Methylpyrogallol-*O*-Sulfate 3 µM, and Pyrogallol-*O*-Sulfate 6 µM. Data are mean ± SD from three independent experiences. . *** P < 0.001, ** P < 0.05 versus Control.

After cell pre-treatment with the BDP metabolites, it was possible to observe that all compounds are able to significantly increase mitochondrial population under physiologic conditions (Fig. 4-9). These results demonstrate that BDP (poly)phenols increase mitochondrial population, which can implicate an improvement on mitochondrial metabolism. Further studies where perform to confirm these data.

4.10. Mitochondrial DNA

In order to increase reliability from the MTDR method measurements, it was evaluated the mtDNA through Q-PCR using GAPDH gene as an endogenous reference gene and mtCyt b as target gene.

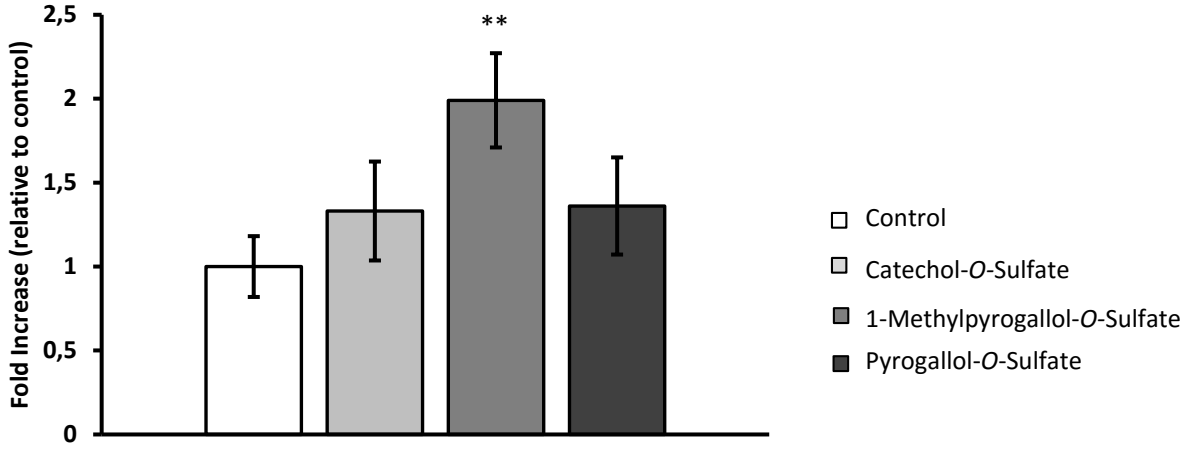

Figure 4-10 – Effects of BDP metabolites on the mtCyt b gene quantity. Neonatal rat cardiomyocytes were pre-treated with different BDP metabolites for 2h and cells harvest after 24 h. BDP concentrations used where: Catechol-*O*-Sulfate 12 µM, 1-Methylpyrogallol-*O*-Sulfate 3 µM, and Pyrogallol-*O*-Sulfate 6 µM. Data are mean ± SD. ** $P < 0.05$ versus Control.

It was observe that of all BDP metabolites tested, only pre-treatment with 1-Methylpyrogallol-*O*-Sulfate seems to increase mtDNA content relatively to control (Fig. 4-10). It was only possible to perform one biological replicate so it is not possible to assume definitive conclusions from these results. Even so it can be observed a clear tendency in the increase of mtDNA, resulting from the BDP metabolites pre-treatment.

It has been shown that in presence of autophagic dysfunction, there is mitochondrial accumulation but a decrease in total mtDNA [18,57]. However, the data obtain supports the hypothesis that BDP metabolites induce increase in mitochondrial population probably due to mitochondrial biogenesis. Further experimental data are necessary to clarify whether or not this increase is due to mitophagy dysfunction

Nonetheless further assays are necessary, such as autophagosome colocalization techniques or western blot analysis of specific transcription factors associated with mitochondrial biogenesis. Only after all these assays it will be possible to affirm that BDP metabolites induce mitochondrial biogenesis.

5. Discussion

The aim of this study was to evaluated cytoprotection and associated mechanisms in cardiomyocytes by different (poly)phenols metabolites, against two distinct cell death inducers, *t*-BHP and ISO. Both inducers were used in concentrations and time points to mimic progressive cardiovascular diseases, like congestive heart failure or hypertensive heart disease that can be attenuated or even prevented through diet.

For the first time, it was possible to demonstrate compelling evidence of possible health benefits against CVDs, derived from BDP metabolites pre-treatment. Berries driven (poly)phenols metabolites have shown to be able to protect cells against an unspecific insult produced by *t*-BHP (Fig.4-2). However, with a prolonged insult of ISO, cells were not protected. Of the BDP metabolites tested, Pyrogallol-*O*-Sulfate aggravated induced cell death, while the remaining BDP metabolites did not present the same response (Fig.4-3; 4-6). This further demonstrates how complex the functional interactions between BDP metabolites and cardiac cells pathways are during a chronicle insult.

It was also observed a clear influence of BDP metabolites over mitochondria, the most important organelle in cell metabolism. Pre-treatment with BDP metabolites induced a significant increase in mitochondrial population (Fig. 4-9), that can be supported by the tendency in mtDNA raise, observable from Q-PCR data (Fig.4-10). Confirmation of these results will be very relevant, as during CVDs there is an incremental need for ATP, and mitochondria are the principal ATP generator. These results suggest a possible increase in ATP production, associated with the mitochondrial population increase, which could bridge the energy gap felt by cells during CVDs.

Although it was observed an increase in mitochondrial population through two different methods, it could be argued that mitochondria are accumulating instead of mitogenesis, and even if there is an increase in mitochondria population there is no indication that majority is correctly functioning. Previews work have shown the ability of different (poly)phenols to either increase mitochondrial biogenesis or impair autophagy [65–67]. In order to debunk these possibilities it would be necessary to perform a quantitative reverse transcription polymerase chain reaction (qRT-PCR) to measure the expression level of a mitochondrial biogenesis related gene, such as PGC-1α gene. Furthermore it would also be necessary to evaluate mitochondrial membrane potential of the mitochondrial population, in order to prove their functionality.

An important finding in this work was the ability of BDP metabolites to maintain normal cardiac contractile functions without affecting ISO induced cell death (Fig.4-8) which to my knowledge was never observed before. These results were most intriguing, as pre-treatment with BDP metabolites *per se* did not influence neither cell beating rate nor cell synchronization. However, when cells were pre-treated with BDP metabolites and treated with ISO, it is observable a clear improvement in cell synchronization at 24 h (Fig.4-7). It was also shown that after extensive damage caused by ISO exposition (48 h), cells pre-treated with BDP metabolites presented a cell beating rate similar to the control even though there was a diminish in 40 % of viability.

Continued stimulation by ISO, induces an increased production of cyclic adenosine monophosphate (cAMP) which in turn activates protein kinase A (PKA) [68,69]. Overactivation of cAMP-PKA that will phosphorylate an extensive number of proteins, including L-type calcium channels, proteins from the contractile apparatus such as troponin I , β-AR, and other G protein-coupled receptors, causing a desensitization of the β-AR to further stimulation [69]. Regardless of all the activation caused by cAMP-PKA, recent studies have shown that apoptosis driven from β-AR overactivation, originates from the activation of Ca_2^+/calmodulin-dependent protein kinase II (CaMKII), as changes in L-type calcium channels phosphorylation state will lead to an intercellular calcium accumulation. This impairment in calcium handling will result in a dysfunction in the contractile mechanism leading to arrhythmias, and finally an excessive accumulation of calcium within mitochondrial matrix will lead to apoptosis [69].

Taking into consideration the ISO overactivated pathway and the results obtained from cell viability and cell beating activity, it becomes evident the complexity of the functional interaction

between the BDP metabolites and cardiac cells under a pathological stress such as the one induced by ISO over exposition.

Summarizing the results obtain through this thesis, it was hypothesized that BDP metabolites may not affect the cAMP-PKA signaling pathway, which is the principal cause for apoptosis from β-AR stimulation, but may in fact interact with proteins related to cell contractile functions. Further studies are still needed to better understand the complex interactions observed between the BDP metabolites and cardiac cells.

6. Conclusion and Future work

Working with human bioavailable (poly)phenols metabolites in physiological concentrations is a step further into unraveling the true beneficial effects from dietary (poly)phenols ingestion. The underlying mechanism behind BDP metabolites effects still alludes us. However data from cell beating capacity and mitochondrial population corroborates the possibility of an interaction between BDP metabolites and with proteins related to cardiomyocyte contractile functions.

Despite these promising results, this research has thrown up many questions in need of further investigation. It would be important to optimize the western blot procedure for measuring Akt phosphorylation. Considering that cardiac contractility, metabolism, mitochondrial population and physiological cardiac hypertrophy are influenced by Akt signaling pathway, it would be possible for Akt to be a target for the BDP metabolites [64]. Taking into consideration contractibility improvement and mitochondrial population increase, it would be plausible to assume such interaction.

Assuming that BDP metabolites can increase Akt phosphorylation levels, it would be necessary to treat cells with Akt pathway inhibitor (LY294002) and repeat all experiments to prove the possible link between Akt pathway and the obtained data. Despite the results obtained from cells beating rate, it is necessary to complement this data with, patch clamp experiments to measure variations in calcium intercellular concentration and analysis of proteins from the contractile apparatus such as troponin I. Measuring these factors will bring us to a closer understanding of the fully metabolic modulation abilities possessed by BDP metabolites.

All insults tested so far were to mimic chronic diseases, such has congestive heart failure. To develop a full picture of the preventive potentialities of BDP metabolites, additional studies will be needed to cover other types of CVDs, such as myocardial ischemia, hypertension, and cardiomyopathy. Pre-treatment with BDP metabolites could possibly diminish the damage caused during a one-off cardiovascular incident.

There is *in vivo* data showing a positive influence of BDP metabolites in hypertensive rats by limiting animal death and decreasing heart hypertrophy (confidential data). Future work using hypertrophic cell models with angiotensin-II [70], will permit to study the influence of BDP metabolites in preventing hypertensive heart disease.

A further study with more focus on using BDP metabolites as possible therapeutic agents, would be worthwhile. It would be interesting to assess the of BDP metabolites against different animal models of CVDs, such as ischemic heart disease, cardiomyopathy, hypertensive heart disease, and congestive heart failure . These animal studies would permit to evaluate against which disease, BDP metabolites possess a better preventive effect. Subsequently to identifying which disease has a better chance to be prevented through BDP metabolites pre-treatment, human trails would be the next step in order to fully validate one if not all BDP metabolites tested as novel prophylactic drugs against CVDs.

Given the growing number of cardiovascular incidents, being able to possibly develop a prophylactic drug against CVDs, would be a great achievement. Creating a prophylactic drug from BDP metabolites, would help people with a propensity to CVDs keeping a higher quality of life and reduce the need for expensive cardiac treatments.

7. References

1. Roquer, J., Ois, A., Panagiotakos, D., Pitsavos, C. & Mikhailidis, D. *Handbook of Disease Burdens and Quality of Life Measures*. (Springer, 2010).

2. Quiñones, M., Miguel, M. & Aleixandre, A. Beneficial effects of polyphenols on cardiovascular disease. *Pharmacol. Res.* **68,** 125–31 (2013).

3. Tham, Y. K., Bernardo, B. C., Ooi, J. Y. Y., Weeks, K. L. & McMullen, J. R. Pathophysiology of cardiac hypertrophy and heart failure: signaling pathways and novel therapeutic targets. *Arch. Toxicol.* (2015).

4. Ong, S.-B., Hall, A. R. & Hausenloy, D. J. Mitochondrial dynamics in cardiovascular health and disease. *Antioxid. Redox Signal.* **19,** 400–14 (2013).

5. Wang, S., Melnyk, J. P., Tsao, R. & Marcone, M. F. How natural dietary antioxidants in fruits, vegetables and legumes promote vascular health. *Food Res. Int.* **44,** 14–22 (2011).

6. Li, H., Horke, S. & Förstermann, U. Oxidative stress in vascular disease and its pharmacological prevention. *Trends Pharmacol. Sci.* **34,** 313–9 (2013).

7. Khurana, S., Piche, M., Hollingsworth, A., Venkataraman, K. & Tai, T. C. Oxidative stress and cardiovascular health : therapeutic potential. **212,** 198–212 (2013).

8. Khurana, S., Hollingsworth, A., Piche, M., Venkataraman, K., Kumar, A., Ross, G. M. & Tai, T. C. Antiapoptotic actions of methyl gallate on neonatal rat cardiac myocytes exposed to H2O2. *Oxid. Med. Cell. Longev.* **2014,** 9 (2014).

9. Fogli, S., Nieri, P. & Breschi, M. C. The role of nitric oxide in anthracycline toxicity and prospects for pharmacologic prevention of cardiac damage. *Fed. Am. Soc. Exp. Biol.* **18,** 664–75 (2004).

10. Pacher, P., Beckman, J. S. & Liaudet, L. Nitric oxide and peroxynitrite in health and disease. *Physiol. Rev.* **87,** 315–424 (2007).

11. Madamanchi, N. R. & Runge, M. S. Free Radical Biology and Medicine Redox signaling in cardiovascular health and disease. **61,** 473–501 (2013).

12. Levine, T. B. & Levine, A. B. *Metabolic Syndrome and Cardiovascular Disease*. (Blackwell Publishing Ltd., 2012).

13. Dominic, E. a, Ramezani, A., Anker, S. D., Verma, M., Mehta, N. & Rao, M. Mitochondrial cytopathies and cardiovascular disease. *Heart* **100,** 611–8 (2014).

14. Bayeva, M., Gheorghiade, M. & Ardehali, H. Mitochondria as a therapeutic target in heart failure. *J. Am. Coll. Cardiol.* **61,** 599–610 (2013).

15. Yu, E., Mercer, J. & Bennett, M. Mitochondria in vascular disease. *Cardiovasc. Res.* **95,** 173–82 (2012).

16. Milane, L., Trivedi, M., Singh, A., Talekar, M. & Amiji, M. *Mitochondrial Biology, Targets, and Drug Delivery*. *J. Control. Release* (Elsevier B.V., 2015).

17. Riehle, C. & Abel, E. D. PGC-1 Proteins and Heart Failure. *Trends Cardiovasc Med.* **22,** 98–105 (2013).

18. Karamanlidis, G., Nascimben, L., Couper, G. S., Shekar, P. S., del Monte, F. & Tian, R. Defective DNA replication impairs mitochondrial biogenesis in human failing hearts. *Circ. Res.* **106,** 1541–8 (2010).

19. Schreiber, S. N., Emter, R., Hock, M. B., Knutti, D., Cardenas, J., Podvinec, M., Oakeley, E. J. & Kralli, A. The estrogen-related receptor α (ERRα) functions in PPARγ coactivator 1α (PGC-1α)-induced mitochondrial biogenesis. *Proc. Natl. Acad. Sci.* **101,** (2004).

20. Chiva-Blanch, G., Badimon, L. & Estruch, R. Latest evidence of the effects of the Mediterranean diet in prevention of cardiovascular disease. *Curr. Atheroscler. Rep.* **16,** 446 (2014).

21. Estruch, R., Ros, E., Salas-Salvadó, J., Covas, M.-I., Corella, D., Arós, F., Gómez-Gracia, E., Ruiz-Gutiérrez, V., Fiol, M., Lapetra, J., Lamuela-Raventos, R. M., Serra-Majem, L., Pintó, X., Basora, J., Muñoz, M. A., Sorlí, J. V, Martínez, J. A. & Martínez-González, M. A. Primary prevention of cardiovascular disease with a Mediterranean diet. *N. Engl. J. Med.* **368,** 1279–90 (2013).

22. Hollman, P. C. H., Cassidy, A., Comte, B., Heinonen, M., Richelle, M., Richling, E., Serafini, M., Scalbert, A., Sies, H. & Vidry, S. The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. *J. Nutr.* **141,** 989S–1009S (2011).

23. Nicholson, S. K., Tucker, G. a & Brameld, J. M. Effects of dietary polyphenols on gene expression in human vascular endothelial cells. *Proc. Nutr. Soc.* **67,** 42–7 (2008).

24. Sandoval-Acuña, C., Ferreira, J. & Speisky, H. Polyphenols and mitochondria: An update on their increasingly emerging ROS-scavenging independent actions. *Arch. Biochem. Biophys.* **559C,** 75–90 (2014).

25. Yamagata, K., Tagami, M. & Yamori, Y. Dietary polyphenols regulate endothelial function and prevent cardiovascular disease. *Nutrition* (2014).

26. Farooqui, A. A. *Phytochemicals, signal transduction,and neurological disorders*. *Zhurnal Eksp. i Teor. Fiz.* (Springer, 2012).

27. Tsao, R. Chemistry and biochemistry of dietary polyphenols. *Nutrients* **2,** 1231–46 (2010).

28. D'Archivio, M., Filesi, C., Varì, R., Scazzocchio, B. & Masella, R. Bioavailability of the polyphenols: status and controversies. *Int. J. Mol. Sci.* **11,** 1321–42 (2010).

29. Pimpão, R. C., Dew, T., Oliveira, P. B., Williamson, G., Ferreira, R. B. & Santos, C. N. Analysis of phenolic compounds in Portuguese wild and commercial berries after multienzyme hydrolysis. *J. Agric. Food Chem.* **61,** 4053–62 (2013).

30. Walle, T., Hsieh, F., Delegge, M. H., Oatis, J. E. & Walle, U. K. High absortion but very low bioavaibility of oral resveratrol in humans. *Drug Metab. isposition* **32,** 1377–1382 (2004).

31. Cui, G., Shan, L., Hung, M., Lei, S., Choi, I., Zhang, Z., Yu, P., Hoi, P., Wang, Y. & Lee, S. M. Y. A novel Danshensu derivative confers cardioprotection via PI3K/Akt and Nrf2 pathways. *Int. J. Cardiol.* **168,** 1349–1359 (2013).

32. Kim, D.-E., Kim, B., Shin, H.-S., Jeong Kwon, H. & Park, E.-S. The protective effect of hispidin against Hydrogen peroxide-induced apoptosis in H9c2 cardiomyoblast cells through Akt/GSK-3β and ERK1/2 signaling pathway. *Exp. Cell Res.* **327,** 264–275 (2014).

33. Anter, E., Thomas, S. R., Schulz, E., Shapira, O. M., Vita, J. a & Keaney, J. F. Activation of endothelial nitric-oxide synthase by the p38 MAPK in response to black tea polyphenols. *J. Biol. Chem.* **279,** 46637–46643 (2004).

34. Penumatsa, K., Abualkhair, S., Wei, L., Warburton, R., Preston, I., Hill, N. S., Watts, S. W., Fanburg, B. L. & Toksoz, D. Tissue transglutaminase promotes serotonin-induced AKT signaling and mitogenesis in pulmonary vascular smooth muscle cells. *Cell. Signal.* **26,** 2818–2825 (2014).

35. Lee, H.-C. & Wei, Y.-H. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. *Int. J. Biochem. Cell Biol.* **37,** 822–834 (2005).

36. Pimpão, R. C., Ventura, M. R., Ferreira, R. B., Williamson, G. & Santos, C. N. Phenolic sulfates as new and highly abundant metabolites in human plasma after ingestion of a mixed berry fruit purée. *Br. J. Nutr.* **113,** 454–463 (2015).

37. Pimpão, R. C., Dew, T., Figueira, M. E., Mcdougall, G. J., Stewart, D., Ferreira, R. B., Santos, C. N. & Williamson, G. Urinary metabolite profiling identifies novel colonic metabolites and conjugates of phenolics in healthy volunteers. *Mol. Nutr. Food Res.* **58,** 1414–1425 (2014).

38. Williamson, G. & Manach, C. Bioavailability and bioefficacy of polyphenols in humans.II.Review of 93 intervention studies. *Am J Clin Nutr* **81,** 243S–255S (2005).

39. Kendall, J. M. Designing a research project: randomised controlled trials and their principles. *Emerg. Med. J.* **20,** 164–168 (2003).

40. Paredes-López, O., Cervantes-Ceja, M. L., Vigna-Pérez, M. & Hernández-Pérez, T. Berries: Improving Human Health and Healthy Aging, and Promoting Quality Life-A Review. *Plant Foods Hum. Nutr.* **65,** 299–308 (2010).

41. Pinto, P., Cardoso, S., Pimpão, R. C., Tavares, L., Ferreira, R. B. & Santos, C. N. Daily polyphenol intake from fresh fruits in Portugal: contribution from berry fruits. *Int. J. Food Sci. Nutr.* **64,** 1022–9 (2013).

42. Forbes-Hernández, T. Y., Giampieri, F., Gasparrini, M., Mazzoni, L., Quiles, J. L., Alvarez-Suarez, J. M. & Battino, M. The effects of bioactive compounds from plant foods on mitochondrial function: a focus on apoptotic mechanisms. *Food Chem. Toxicol.* **68,** 154–82 (2014).

43. Dorta, D. J., Pigoso, A. a., Mingatto, F. E., Rodrigues, T., Prado, I. M. R., Helena, A. F. C., Uyemura, S. a., Santos, A. C. & Curti, C. The interaction of flavonoids with mitochondria: effects on energetic processes. *Chem. Biol. Interact.* **152,** 67–78 (2005).

44. Rasbach, K. a & Schnellmann, R. G. Isoflavones promote mitochondrial biogenesis. *J. Pharmacol. Exp. Ther.* **325,** 536–43 (2008).

45. Skemiene, K., Rakauskaite, G., Trumbeckaite, S., Liobikas, J., Brown, G. C. & Borutaite, V. Anthocyanins block ischemia-induced apoptosis in the perfused heart and support mitochondrial respiration potentially by reducing cytosolic cytochrome c. *Int. J. Biochem. Cell Biol.* **45,** 23–29 (2013).

46. Youn, H., Jeong, J.-C., Jeong, Y. S., Kim, E.-J. & Um, S.-J. Quercetin Potentiates Apoptosis by Inhibiting Nuclear Factor-kappaB Signaling in H460 Lung Cancer Cells. *Biol. Pharm. Bull.* **36,** 944–51 (2013).

47. Trumbeckaite, S., Bernatoniene, J., Majiene, D., Jakštas, V., Savickas, a. & Toleikis, a. The effect of flavonoids on rat heart mitochondrial function. *Biomed. Pharmacother.* **60,** 245–248 (2006).

48. Pereira, S. L., Ramalho-Santos, J., Branco, A. F., Sardão, V. a, Oliveira, P. J. & Carvalho, R. a. Metabolic remodeling during H9c2 myoblast differentiation: relevance for in vitro toxicity studies. *Cardiovasc. Toxicol.* **11,** 180–90 (2011).

49. Louch, W. E., Sheehan, K. a. & Wolska, B. M. Methods in cardiomyocyte isolation, culture, and gene transfer. *J. Mol. Cell. Cardiol.* **51,** 288–298 (2011).

50. Mauro-Lizcano, M., Esteban-Martínez, L., Seco, E., Serrano-Puebla, A., Garcia-Ledo, L., Figueiredo-Pereira, C., Vieira, H. L. a & Boya, P. New method to assess mitophagy flux by flow cytometry. *Autophagy* **11,** 833–843 (2015).

51. Sardão, V. a, Oliveira, P. J., Holy, J., Oliveira, C. R. & Wallace, K. B. Vital imaging of H9c2 myoblasts exposed to tert-butylhydroperoxide--characterization of morphological features of cell death. *BMC Cell Biol.* **8,** 11 (2007).

52. Yin, Y., Guan, Y., Duan, J., Wei, G., Zhu, Y., Quan, W., Guo, C., Zhou, D., Wang, Y., Xi, M. & Wen, A. Cardioprotective effect of Danshensu against myocardial ischemia/reperfusion injury and inhibits apoptosis of H9c2 cardiomyocytes via Akt and ERK1/2 phosphorylation. *Eur. J. Pharmacol.* **699,** 219–226 (2013).

53. Branco, A. F., Sampaio, S. F., Wieckowski, M. R., Sardão, V. a & Oliveira, P. J. Mitochondrial disruption occurs downstream from β-adrenergic overactivation by isoproterenol in differentiated, but not undifferentiated H9c2 cardiomyoblasts: differential activation of stress and survival pathways. *Int. J. Biochem. Cell Biol.* **45,** 2379–91 (2013).

54. Branco, A. F., Pereira, S. L., Moreira, A. C., Holy, J., Sardão, V. a & Oliveira, P. J. Isoproterenol cytotoxicity is dependent on the differentiation state of the cardiomyoblast H9c2 cell line. *Cardiovasc. Toxicol.* **11,** 191–203 (2011).

55. Malouf, N. N., McMahon, D., Oakeley, a. E. & Anderson, P. a W. A cardiac troponin T epitope conserved across phyla. *J. Biol. Chem.* **267,** 9269–9274 (1992).

56. Mueller, M., Vafaie, M., Biener, M., Giannitsis, E. & Katus, H. a. Cardiac Troponin T – From Diagnosis of Myocardial Infarction to Cardiovascular Risk Prediction –. *Circ. J.* **77,** 1653–1661 (2013).

57. Luo, C., Li, Y., Wang, H., Feng, Z., Li, Y., Long, J. & Liu, J. Mitochondrial accumulation under oxidative stress is due to defects in autophagy. *J. Cell. Biochem.* **114,** 212–219 (2013).

58. El-Armouche, A. & Eschenhagen, T. B-Adrenergic stimulation and myocardial function in the failing heart. *Heart Fail. Rev.* **14,** 225–241 (2009).

59. Hamid, R., Rotshteyn, Y., Rabadi, L., Parikh, R. & Bullock, P. Comparison of alamar blue and MTT assays for high through-put screening. *Toxicol. Vitr.* **18,** 703–710 (2004).

60. Gonzalez, R. J. & Tarloff, J. B. Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity. *Toxicol. Vitr.* **15,** 257–259 (2001).

61. Natarajan, A. R., Rong, Q., Katchman, A. N. & Ebert, S. N. Intrinsic cardiac catecholamines help maintain beating activity in neonatal rat cardiomyocyte cultures. *Pediatr. Res.* **56,** 411–417 (2004).

62. Suzuki, M., Ohte, N., Wang, Z. M., Williams, D. L., Little, W. C. & Cheng, C. P. Altered inotropic response of endothelin-1 in cardiomyocytes from rats with isoproterenol-induced cardiomyopathy. *Cardiovasc. Res.* **39,** 589–599 (1998).

63. Kerkhof, E. Van De. Drug metabolism in human and rat intestine: An in vitro approach. (2007). at <http://dissertations.ub.rug.nl/FILES/faculties/science/2007/e.g.van.de.kerkhof/thesis.pdf>

64. Reinartz, M., Raupach, A., Kaisers, W. & Go, A. AKT1 and AKT2 Induce Distinct Phosphorylation Patterns in HL - 1 Cardiac Myocytes. (2014).

65. Laurent, C., Chabi, B., Fouret, G., Py, G., Sairafi, B., Elong, C., Gaillet, S., Cristol, J. P., Coudray, C. & Feillet-Coudray, C. Polyphenols decreased liver NADPH oxidase activity, increased muscle mitochondrial biogenesis and decreased gastrocnemius age-dependent autophagy in aged rats. *Free Radic. Res.* **46,** 1140–1149 (2012).

66. Mohan, K., Gunasekaran, P., Varalakshmi, E., Hara, Y. & Nagini, S. In vitro evaluation of the anticancer effect of lactoferrin and tea polyphenol combination on oral carcinoma cells. *Cell Biol. Int.* **31,** 599–608 (2007).

67. Larrosa, M., Tomás-Barberán, F. a. & Espín, J. C. The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. *J. Nutr. Biochem.* **17,** 611–625 (2006).

68. Iwai-Kanai, E., Hasegawa, K., Araki, M., Kakita, T., Morimoto, T. & Sasayama, S. Alpha- and Beta-Adrenergic Pathways Differentially Regulate Cell Type-Specific Apoptosis in Rat Cardiac Myocytes. *Circulation* **100,** 305–311 (1999).

69. Mann, D. L. *Heart Failure: A Companion to Braunwald's Heart Disease*. (Elsevier Inc., 2011).

70. Watkins, S. J., Borthwick, G. M., Oakenfull, R., Robson, A. & Arthur, H. M. Angiotensin II-induced cardiomyocyte hypertrophy in vitro is TAK1-dependent and Smad2/3-independent. *Hypertens. Res.* **35,** 393–398 (2012).

8. Appendix

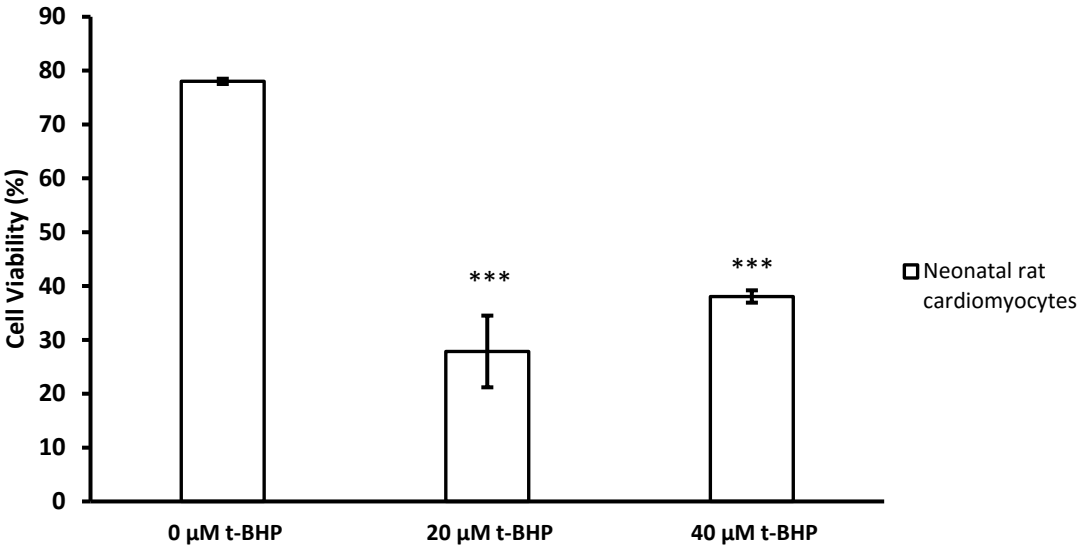

Figure 8-1 – Neonatal rat cardiomyocytes cell viability determined through cell permeability to PI by flow cytometry, after treatment with 20 and 40 µM *t*-BHP . Data are mean ± SD from one biological replicate. *** P < 0.001, versus Control with 0 µM *t*-BHP.