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EVALUATION OF ANTIOXIDANT EFFECTS OF MITOCHONDRIA-TARGETED POLYPHENOLIC AGENTS IN HUMAN SKIN FIBROBLASTS

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To Dani

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Abstract

Mitochondria are the core of several cellular processes, being the redox balance fundamental to cellular life and death pathways. Despite the well-known healthy benefits of dietary antioxidants and their *in vitro* satisfactory results to neutralize the impaired redox network, their therapeutic success is limited due to pharmacokinetic drawbacks. In that patch, a great effort has been done to develop bioactive molecules based on polyphenolic dietary antioxidants with mitochondria-targeted properties, to improve mitochondrial function. Phenolic acids such as hydroxybenzoic (HBA) and hydroxycinnamic acids (HCA) are natural regulators of the cellular redox status and have pharmacological interest due to their intrinsic antioxidant properties. In this context, we previously developed novel mitochondria-targeted agents based on HBA (MitoBENs) and on HCA (MitoCINs). The objective of the present work is investigating the hormetic-like mechanism of action of the novel mitochondria-targeted cinnamic and gallic antioxidants.

We studied the effects of MitoBENs and MitoCINs on human dermal fibroblasts, cells that in their natural environment are constantly subjected to stress, although their essential role in modulating the dermal extracellular matrix.

Mitochondria-targeted antioxidants revealed two distinct profiles. An increase in proliferative capacity and an alteration in mitochondrial function without compromising the cellular energetic production was measured. The cells kept the mitochondrial balance between biogenesis and self-removal by mitophagy, in addition the mitochondria-targeted antioxidants prevented *ter*t-butyl hydroperoxide-induced cytotoxicity. Thus, it is likely that mitochondria-targeted antioxidants up-regulated the intracellular antioxidant defence system as a result of an adaptative response of cells, a process that can protect them against subsequent stress-inducing events.

In summary, phenolic acids derivatives attached to mitochondria-targeting moieties can stimulate stress responses and contribute to cells and/or tissue protection, inhibiting directly or indirectly an excessive mitochondrial ROS production. Thus, mitochondria-targeted antioxidants can be considered putative drug candidates to improve mitochondrial health in primary and/or secondary mitochondrial diseases.

Keywords: Mitochondria, mitochondrial antioxidants, hormesis, redox signalling, antioxidant system

Resumo

A mitocôndria é o centro de vários processos celulares, incluindo o balanço redox que é fundamental para o controle de vias relacionadas com as decisões de vida ou morte celular. Apesar dos conhecidos efeitos de antioxidantes e seus resultados satisfatórios em neutralizar a prejudicial desregulação na interação de vias redox *in vitro*, o seu sucesso terapêutico é limitado devido a limitações farmacocinéticas. Neste sentido, foram feitos esforços para desenvolver moléculas bioativas baseadas em antioxidantes provenientes da dieta direcionados para a mitocôndria, a fim de melhorar a função mitocondrial. Os ácidos fenólicos, como os ácidos hidroxibenzóico (HBA) e hidroxicinâmico (HCA) são reguladores naturais do estado redox da célula e têm interesse farmacológico devido às suas propriedades antioxidantes intrínsecas. Neste contexto, nós previamente desenvolvemos novos agentes direcionados para as mitocôndrias, desenvolvidos a partir de HBA (MitoBENs) e HCA (MitoCINs). O objetivo do presente trabalho é investigar o mecanismo de ação do tipo hormético dos novos antioxidantes derivados dos ácidos fenólicos direcionados para as mitocôndrias.

Estudamos os efeitos de MitoBENs e MitoCINs em fibroblastos humanos da derme, células que no seu ambiente natural estão constantemente sujeitas a condições adversas, além de que têm um papel essencial na síntese e manutenção da matriz extracelular.

Os antioxidantes direcionados para a mitocôndria revelam dois perfis distintos. Um aumento na capacidade proliferativa e uma alteração na função mitocondrial sem comprometer a produção energética celular. As células mantiveram o equilíbrio mitocondrial entre biogênese e auto remoção através da mitofagia, além de os antioxidantes direcionados para a mitocôndria preveniram a citotoxicidade induzida pelo hidroperóxido de *tert*-butilo. Assim, é provável que os antioxidantes direcionados para a mitocôndria regulem positivamente o sistema de defesa antioxidante intracelular como resultado de uma resposta adaptativa das células, um processo que pode protegê-las contra eventos subsequentes de indução de estresse.

Em resumo, os derivados de ácidos fenólicos ligados a moléculas transportadoras direcionadas para a mitocôndria podem estimular uma resposta ao estresse e contribuir para a proteção de células e/ou tecidos, inibindo direta ou indiretamente uma produção excessiva de ROS mitocondriais. Assim, os antioxidantes direcionados para a mitocôndria podem ser considerados candidatos putativos para melhorar a saúde mitocondrial em doenças mitocondriais primárias e / ou secundárias.

Palavras chave: Mitocôndria, antioxidantes mitocondriais, hormese, sinalização redox, defesa antioxidante

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Abbreviation List

$^{\cdot-}O_2$	Superoxide anion
$^{\cdot}O_2$	Singlet oxygen
$^{\cdot}OH$	Hydroxyl radical
2DG	2-deoxy-D-glucose
4-HNE	4-hydroxy-2-nonenal
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
AMPK	AMP-dependent kinase
ANT	Adenine Nucleotide Translocase
AP-1	Activator protein 1
ARE	Antioxidant response elements
ATP	Adenosine 5'-triphosphate
Bcl-2	B cell lymphoma protein-2
Bcl-x_L	B cell lymphoma-extra-large
CAT	Catalase
$CoQH_2$	Ubiquinol
CR	Caloric restriction
Cu/ZnSOD	Cupper/zinc superoxide dismutase
cyt *c*	Cytochrome *c*
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenol
DRP-1	Dynamin related protein-1
ECAR	Extracellular acidification rate
EGF-R	Epidermal growth factor receptor
EpRE	Electrophile response element
ER	Endoplasmic reticulum
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide (oxidised form)
$FADH_2$	Flavin adenine dinucleotide (reduced form)
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

FeS	Iron sulphide
FMN	Flavin mononucleotide
FOX	Forkhead transcription factors
FOXA	Forkhead transcription factors A
FOXO	Forkhead transcription factors O
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidised form)
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HBA	Hydroxybenzoic acid
HCA	Hydroxycinnamic acid
HIF-1	Hypoxia-inducible factor 1
HSF	Human skin fibroblasts
HSF-1	Heat shock factor
I/R	Ischemia /reperfusion
IGFR	Insulin like growth factor receptor
IMS	Intermembrane space
IR	Insulin receptor
Keap 1	Kelch-like ECH-associated protein 1
MAPK	Mitogen activated protein kinase
MDA	malondialdehyde
MIM	Mitochondrial inner membrane
MMPs	Matrix metalloproteinases
MnSOD	Manganese superoxide dismutase
MOM	Mitochondrial outer membrane
mtDNA	Mitochondrial DNA
mTOR	1-Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex
mtROS	Mitochondrial reactive oxygen species
mtUPR	Mitochondrial unfolded protein response
mΔΨ	Mitochondrial membrane potential

NAC	N-acetylcysteine
NAD^+	Nicotinamide adenine nucleotide (oxidised form)
NADH	Nicotinamide adenine nucleotide (reduced form)
NADPH	Nicotinamide adenine nucleotide phosphate (reduced form)
NF-κB	Nuclear factor-κB
NHDF	Normal human dermal fibroblasts
$NO^{\cdot}$	Nitric oxide
NRF	Nuclear respiratory factor
Nrf2	Nuclear factor erythroid 2-related factor
OCR	Oxygen consumption rate
$ONOO^-$	Peroxynitrite
OPA 1	mitochondrial dynamin like GTPase
OXPHOS	Oxidative phosphorylation
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PGC-1α	Peroxisome proliferated-activated receptor-γ co-activator 1α
Pi	Inorganic phosphate
PINK 1	PTEN-induced putative kinase protein 1
PMF	Proton motive force
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PTP	Permeability transition pore
qPCR	Quantitative polymerase chain reaction
RET	Reverse electron transport
RNA	Ribonucleotide acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Sirt	Sir2
SKN-1	Skinhead-1 protein
SOD	Superoxide dismutase
SRB	Sulforhodamine B assay
SS-peptides	Szeto-Schiller peptides
***t*-BHP**	*tert*-butyl hydroperoxide
TCA	Tricarboxylic acid

TFAM	Transcription factor A mitochondrial
TIMP	Tissues inhibitors of metalloproteinases
TMRM	Tetramethylrhodamine, methyl ester
TNF-α	Tumour necrosis factor alpha
TPP^+	Triphenilphosphonium cation
TRx	Thioredoxin
TRxR	Thioredoxin reductase
UV	Ultra violet
VDAC	Voltage dependent anion channel
Δp	Proton motive force
ΔpH	pH component of proton motive force

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Introduction

1 Mitochondria structure and function

Mitochondria are intracellular organelles mainly responsible for energy production, but which also play a crucial role in other intracellular functions such as tricarboxylic acid (TCA) cycle, fatty acid β-oxidation, apoptosis signalling, calcium homeostasis and redox signalling (Smith *et al*., 2012). Mitochondria are organized in a dynamic network, which depends on the energetic state of a specific cell type and tissue (Benard *et al*., 2007; Koopman *et al*., 2010). This mitochondrial network is dynamic and constantly in fusion or fission processes (De Vos *et al*., 2005) varying from a punctuate shape to tubular networks (Bereiter-Hahn and Vöth, 1994). For instance, mitochondrial network may assume a filamentous shape in fibroblasts or mitochondrial clusters in cardiomyocytes (Amchenkova *et al*., 1988). The mitochondrial network is extended to the endoplasmic reticulum (ER), with which has close contacts (Szabadkai *et al*., 2003), being associated with mitochondrial division (Friedman and Nunnari, 2014). The mitochondrial network also moves along the cell carried by cytoskeleton elements in order to supply energy demand whenever is necessary (Anesti and Scorrano, 2006).

Mitochondria are composed by four compartments each with quite different composition, and biological activities: a porous ion permeable mitochondrial outer membrane (MOM); a convoluted, invaginated and highly impermeable mitochondrial inner membrane (MIM) containing enzymes responsible for energy provision and a series of metabolic carrier proteins; an intermembrane space (IMS) containing a number of specialized proteins; and the mitochondrial protein-rich matrix responsible for many different metabolic pathways (Mannella, 2008). The MOM permeability is controlled by the voltage dependent anion channel (VDAC) that is permeable to uncharged molecules in its open configuration (Colombini, 2012). Disruption of this permeability phenomena affects mitochondrial homeostasis leading to a release of cytochrome *c* and induction of apoptotic pathway. The MIM is very impermeable to ions and hydrophilic solutes creating a barrier between the matrix and the IMS, an essential feature to chemiosmotic machinery (Mannella, 2008). The inner membrane is formed by two distinct structures, the inner boundary membrane and the cristae, which is responsible for increasing the surface area four times when compared to the outer membrane (Mannella *et al*., 1994; Ikon and Ryan, 2017). Imbedded in the MIM are enzymes responsible for energy

provision and a series of metabolic carrier proteins composing the oxidative phosphorylation (OXPHOS) system. This process is carried out by a series of five large and multi-subunit complexes (I-V): complex I – NADH: ubiquinone oxidoreductase; complex II – succinate: ubiquinone oxidoreductase; complex III – ubiquinol: cytochrome *c* oxidoreductase; complex IV – cytochrome *c* oxidase; and complex V – F_1F_o-ATP synthase. The electron transport chain (ETC) is composed by complexes I to IV, responsible for the generation of an electrochemical gradient, while complex V (ATP synthase) is responsible for the conversion of ADP to ATP (while the opposite reaction is also possible).

The IMS is compartmentalised in peripheral IMS and intracristae space, which is limited by the intracristae junction. The most abundant protein in IMS is cytochrome *c*, which is normally found bound to the MIM and which is responsible for shuttle electrons from complex III to complex IV (Ott *et al*., 2002; Garrido *et al*., 2006; Herrmann and Riemer, 2010). Moreover, the IMS possess an important antioxidant defence system such as glutathione redox buffer, responsible for redox homeostasis. Metallothionein, a protein that play an important role in metal homeostasis, such as zinc and copper, that act as cofactor of many enzymes present in MIM and mitochondrial matrix, is also present in the IMS (Sturtz *et al*., 2001; Herrmann and Riemer, 2010).

The mitochondrial matrix is very dense in proteins and enzymes related with mitochondrial metabolic pathways such as lipid β-oxidation and TCA cycle (Kennedy and Leninger, 1949; Scheffler, 2011). An important enzymatic complex found in mitochondrial matrix is the pyruvate dehydrogenase (PDH) that converts pyruvate, mainly provided from glycolysis, in acetyl-CoA to be utilized as fuel in TCA cycle (Modak *et al*., 2002; Scheffler, 2011). Enzymes related with oxidative stress response, such as manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPx) can also be found in mitochondrial matrix (Jackson *et al*., 2002). Other enzymes related with metabolic pathways, such as urea cycle enzyme carbamoyl phosphate synthetase I, heme biosynthesis enzyme δ-aminolaevulinic acid synthase, cardiolipin and lipid biosynthesis and ubiquinol biosynthesis; calcium signalling, such as TCA cycle dehydrogenases regulated by calcium for instance pyruvate, α-ketoglutarate and isocitrate dehydrogenases; and iron homeostasis, such as frataxin which promotes heme biosynthesis, assembly and repair iron-sulphur clusters as well as may be able to store large amounts of iron in ferrihydrite mineral form by oligomerization, are also found in mitochondrial matrix (Rouault and Tong, 2005; Scheffler, 2011; Rizzuto *et al*., 2012).

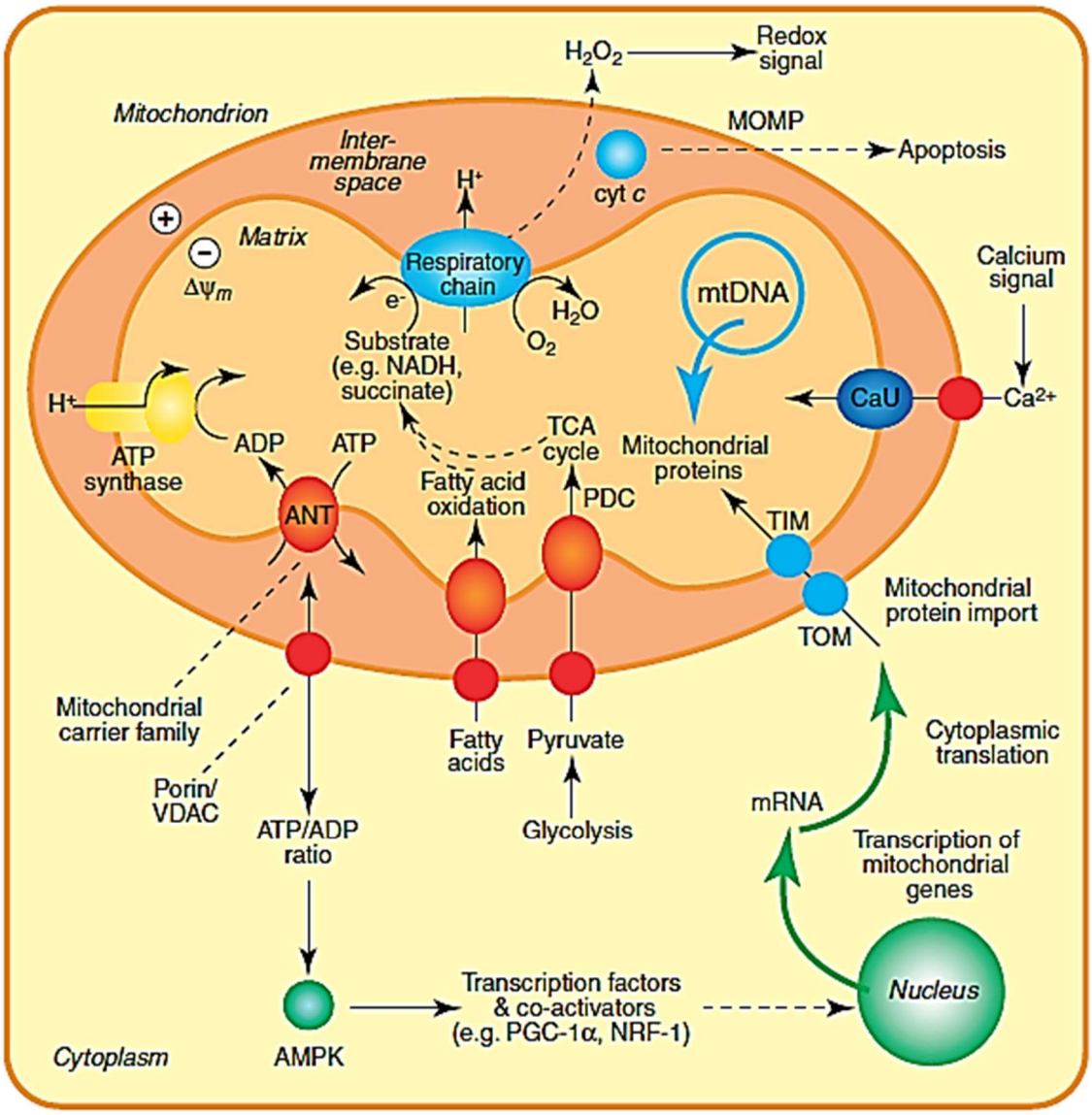


Figure 1. Mitochondrial structure and function. The main mitochondrial function is the ATP production by oxidative phosphorylation (OXPHOS). Another metabolic event also take place in mitochondria such as tricarboxylic acid (TCA) cycle, fatty acid oxidation, apoptosis signalling, and calcium homeostasis. Mitochondria are formed by mitochondrial outer membrane, which has voltage-dependent anion channels (VDACs) as large channels transporters, mitochondrial inner membrane is crossed through by specific carriers such as adenine nucleotide translocase (ANT). Mitochondrial respiratory chain feed equivalent reducers, such as NADH and succinate, take place in mitochondrial inner membrane and pumps protons to intermembrane space, which return to matrix through ATP-synthase yielding ATP. Respiratory chain also may generate H_2O_2 that will trigger redox signalling. From intermembrane space may be released some signalling proteins such as cytochrome *c* (cyt *c*), triggering apoptotic pathways. Mitochondria also mediate the calcium signalling taking up into the matrix from the cytosol through the calcium uniporter channel (CaU). AMP-dependent kinase (AMPK) regulated by ATP/ADP ratio, regulate the activity of some transcription factors and transcription co-activators (e.g. NRF-1, PGC-1α) of mitochondrial biogenesis, being the most mitochondrial proteins encoded by nuclear genome. Mitochondrial proteins are imported through specific transporters: translocase of outer membrane (TOM) and translocase of inner membrane (TIM). Mitochondria also has their own genome in a circular molecule the mitochondrial DNA (mtDNA) that encode some sub-units of oxidative phosphorylation complexes and their replicative and transcriptional machinery, as well as their own transfer-RNAs (Smith *et al*., 2012). Image used under permission [343] (See annex).

Mitochondria are organelles having their own genome, a circular DNA molecule (mtDNA) (Van Bruggen *et al*., 1966; Wallace, 1992; Wolstenholme, 1992), that replicates in an independent manner of nuclear genome. The mtDNA molecule has two strands, a heavy strand rich in guanine residues and a light strand rich in cytosines residues. The mtDNA genome encode 13 polypeptides of OXPHOS subunits, two ribosomal RNA and 22 transfer RNA, plus some small peptides including humanin and MOTS-c (Kim *et al*., 2017). The other polypeptides of OXPHOS subunits and enzymes of mitochondrial metabolic pathways are encoded by nuclear DNA, including all subunits for Complex II, the mitochondrial biogenesis co-activators such as PGC-1α and transcription factors such as NRF 1 and 2, and mtDNA polymerase, mtRNA polymerase, mtDNA transcription factors and ribosomal proteins (Wallace, 1992; Wolstenholme, 1992; Scarpulla, 2008; Wallace *et al*., 2010).

Mitochondria has their own quality control mechanism so called mitophagy that allows cells to repair and remove damaged mitochondria or part of mitochondrial network. The loss of mitochondrial membrane potential (mΔΨ) is major cause that trigger mitophagy, but the opening of permeability transition pore (PTP) may also induce the autophagic process in mitochondria. In mammalian cells, PTEN-induced putative kinase protein 1 (PINK1) a mitochondrial kinase is rapidly degraded in heathy mitochondria, whereas it accumulates on the surface of the damaged or depolarized organelles, which induce the translocation of Parkin from the cytosol to damaged mitochondria and promote the degradation of mitochondria through mitophagy. Mitochondrial autophagic process is dependent of mitochondrial fission segregating the damaged organelles from the mitochondrial network. It is thought that upon depolarisation, Parkin also induces the degradation of PGC-1α repressor promoting mitochondrial biogenesis, which highlights the PINK1/Parkin pathway of mitophagy as an important regulator of mitochondrial homeostasis (Ashrafi and Schwarz 2013). In the maturated staged of mitophagy the autophagosome fuse with a lysosome, leading to the formation of autolysosome. The lysosome has a highly acidic lumen (pH 4.5 – 5.0), which contains more than 50 acidic hydrolases that degrades the inner membrane of autophagosome and the mitochondrial components (Nishida *et al*., 2015).

2 Mitochondrial bioenergetics

The human cells have two main pathways to supply energy demands, the glycolytic pathway and OXPHOS. Energy production processed in mitochondria use an intricate system that interplay fatty acid oxidation, glycolysis, TCA cycle and OXPHOS, being the

later responsible for more than 96 % of the ATP requirement within the cell. Mitochondrial fatty acid metabolism occurs through the β-oxidation process. Although fatty acids synthesis occurs in the cytosol, their oxidation takes place in mitochondrial matrix. As end products, fatty acid β-oxidation leads to a production of acetyl-CoA, that enters in TCA cycle, and reducing equivalents (NADH) and reduced enzymatic co-factors ($FADH_2$), which feed electrons to the mitochondrial respiratory chain (Fillmore *et al*., 2014). The TCA cycle, which is fed from fatty acid β-oxidation and glycolysis are another source of energy production. Glucose undergoes glycolysis with production of pyruvate, which is metabolized by mitochondria and converted to acetyl-CoA and NADH by the rate-limiting enzyme of glucose oxidation, pyruvate dehydrogenase (PDH) (Fillmore *et al*., 2014). The TCA cycle leads to the production of NADH and succinate, which enter in the ETC displayed along the MIM. The electrons carried by NADH entering in ETC through NADH: ubiquinone oxidoreductase (Complex I) which catalyses the NADH oxidation. First the electrons are transferred from NADH to a noncovalently bounded flavin mononucleotide (FMN), and then one electron at a time is transduced through the iron-sulphur clusters redox centres to the final acceptor coenzyme Q. Ubiquinone reduction reaction release free energy that is used for translocate protons (4 H^+) through complex I membrane harm channels to IMS (Jansson *et al*., 2006; Koopman *et al*., 2010; Sazanov, 2015). The succinate: ubiquinone oxidoreductase (Complex II) also called succinate dehydrogenase intervene in both TCA cycle by oxidizing succinate to fumarate, and ETC by transferring electrons from succinate to coenzyme Q. First electrons are transferred to prosthetic group FAD, and then transferred one at time by the tree iron-sulphur clusters to coenzyme Q, a system that does not pump protons (Cecchini, 2003; Bezawork-Geleta *et al*., 2017). Coenzyme Q is a lipophilic benzoquinone that act as electron mobile carrier imbedded in MIM (Rauchova *et al*., 1995). The next step is catalysed by ubiquinol: cytochrome *c* oxidoreductase (Complex III). The coenzyme Q transport the electrons for complex III where is catalysed the cytochrome *c* reduction and ubiquinol oxidation. This redox reaction is coupled to generation of a proton gradient across the membrane. In complex III, the ubiquinol transfer electrons in two steps, one electron is transferred through the high-potential chain Rieske iron-sulphur centre and then to cytochrome c_1 that deliver it to hydrophilic carrier cytochrome *c*. The other electron is transferred along the low-potential chain, the two *b*-type hemes in the cytochrome b subunit, and then to ubiquinone leading the formation of the radical semi-ubiquinone. For each electron that is transferred to cytochrome *c* two protons are translocated to IMS (Mitchell, 1975; Saraste, 1999; Sazanov, 2015). The final step of ETC is the transfer of electrons to its final acceptor molecular oxygen. This reaction is catalysed by cytochrome *c* oxidase (Complex IV), in

which molecular oxygen is reduced to water. The electrons provided by cytochrome *c* protein are transferred one at time for intermembrane side of MIM to a cupper centre of subunit II of cytochrome *c* oxidase, and then transferred through hemes centres to another cupper centre that reduce oxygen. For the reduction of molecular oxygen atom two electrons are necessary, while two protons are consumed from the matrix to form water. The protons enter in the complex IV through a proton channel that also pumps two protons across the MIM to IMS (Saraste, 1999; Capaldi, 1990; Schultz and Chan, 2001; Wikström, 2004). The transference of electrons from NADH to oxygen forming H_2O results in the pumping of four protons by complex I, four protons by complex III and two protons by complex IV creating a proton gradient through the MIM, the so called proton-motive force (PMF), that might be utilized by the ATP-synthase (Complex V or F_1F_o ATPase) to synthetize ATP (Kanabus *et al*., 2014; Smith *et al*., 2012).

3 Mitochondrial reactive oxygen species production

Mitochondria are constantly metabolizing oxygen and thereby producing small amounts of mitochondrial reactive oxygen species (mtROS) as by-products. As consequence of oxygen molecule being the final electron destination in ETC, some electrons leak out and may form superoxide anion ($^{\bullet-}O_2$). The one-electron reduction reaction of O_2 to $^{\bullet-}O_2$ is thermodynamically favoured when electrons are available. A wide range of electron donors in mitochondria could potentially carry out this reaction, though only a small proportion of mitochondrial carriers have the thermodynamic potential to reduce O_2 to $^{\bullet-}O_2$ (Murphy, 2009).

The mainly sources of reactive oxygen species (ROS) in mitochondria are complex I and complex III of the ETC. At complex I level, there are essentially two main ways by which mitochondria can produce ROS; first, a high NADH/NAD^+ ratio lead to a high proportion of reduced FMN that accept the electrons from NADH, and then transfer them from the coenzyme Q through the iron-sulphur clusters. Hence high levels of reduced FMN can generate $^{\bullet-}O_2$, which is enhanced in the presence of inhibitor rotenone that binds to the binding site of coenzyme Q and electrons return to FMN increasing it reduced form. Complex I can also increase ROS production through reverse electron transport (RET), occurring when are a high proton motive force (Δp). With high proton gradient (ΔpH) and mitochondrial membrane potential (mΔΨ) combined with a low production of ATP, the electrons of the reduced coenzyme Q ($CoQH_2$) revert to complex I where they reduce NAD^+ to NADH at FMN site. (Andreyev *et al*., 2005; Murphy, 2009). At complex III, $^{\bullet-}O_2$ is produced when ubiquinone reducing site (Q_i) is inhibited by

Antimycin A, preventing the transference of semiquinone electron at Q_o site to Q_i site, and the O_2 bounds to semiquinone at Q_o site generating $^{\cdot-}O_2$. which is quickly dismutated to hydrogen peroxide (H_2O_2) (Turrens *et al*., 1985; Andreyev *et al*., 2005; Murphy, 2009).

The $^{\cdot-}O_2$ is the precursor for the most of free radicals and the main mediator of the oxidative damage. A free radical can be defined as a specie, capable of independent existence, which contains an unpaired electron in an atomic orbital (Halliwell *et al*., 1996). Radicals are in general highly reactive and the majority has a very short life-span (10-6 seconds or less) in biological systems. There are two major types of free radical species: reactive oxygen species (ROS) and reactive nitrogen species (RNS). Free radicals containing oxygen are highly reactive molecules that include numerous partially reduced oxygen species such as hydroxyl radical ($^{\cdot}OH$) and $^{\cdot-}O_2$, while RNS includes nitric oxide ($NO^{\cdot}$), that can react with $^{\cdot-}O_2$ yielding an also very powerful oxidant, peroxynitrite ($ONOO^-$) (Beckman and Koppenol, 1996; Liochev and Fridovich 1999; Turrens, 2003). On the other hand, hydrogen peroxide (H_2O_2) is a ROS which is not a free radical.

4 Mitochondrial hormesis (mitohormesis)

Hormesis can be defined as the response of the cell or organism to low dose of toxin, which can be considered an adaptive compensatory process following an initial disruption in homeostasis. Consequently, a toxin which in high doses induce damage in cell or organism causing toxicity, when administrated in low doses can trigger adaptative beneficial effects, that is a kind of biphasic or non-linear response to potentially harmful substances. Hormesis biphasic dose-response can be activated by endogenous and exogenous agents such as hormones, peptides, or numerous drugs. The hormetic response is mediated by cellular signalling which induce molecular response that usually is activated by enzymes such as kinases and deacetylases, and transcription factors such as Nfr2 and NF-κB that activate the expression of genes that encode cytoprotective proteins. The hormetic response confer increased stress resistance and involved several proteins such as chaperones as well as heat-chock proteins, antioxidant enzymes such as superoxide dismutases and glutathione peroxidase, and growth factors such as insulin-like growth factors (Calabrese *et al*., 2007; Mattson, 2008).

In mitochondrial hormesis (mitohormesis), mitochondria are subjected to a mild stress that can be triggered by a variety of insults, resulting in a broad and diverse cytosolic and/or nuclear response. This response appears to induce a wide-ranging of cytoprotective pathways resulting in long-lasting metabolic and biochemical changes,

and hence, rather than being harmful, these changes may reduce the susceptibility for disease (Yun and Finkel, 2014).

Recently, several mechanisms have been described to be capable of inducing mild stress triggering mitohormetic response. Aerobic metabolism autonomously produces oxidant molecules as by-products, such as ROS, which can often act as intracellular pathways regulators (Finkel, 2012). The balance between ROS generation and their removal by endogenous antioxidant system determine their amount in cell. ROS can act as signalling pathways modulators depending of their levels as well as their localization and the cellular antioxidant activity (Rhee *et al*., 2000). When in low levels, ROS can act as second messengers, which may regulate the cells (redox) state. In addition, ROS can activate antioxidant response and drug detoxification enzymes (Lichtenberg *et al*., 2015).

Caloric restriction (CR) is also described to trigger mitohormetic response by slightly increase ROS levels paralleled by an increased respiration rate and elevated antioxidant enzyme activity, which increases lifespan (Ristow and Schmeisser, 2014). The biochemical mechanism underlying caloric restriction is mediated by the antioxidant response element (ARE), also called electrophile response element (EpRE), that induces thioredoxin (TrX) gene expression following the Nrf2 activation. Under unstressed situations, Nrf2 is sequestered in cytosol by Keap 1, while when subjected to ROS activates the Keap 1 redox sensitive cysteine residues that release Nrf2. Nrf2 is then translocate into nucleus to execute its transcriptional function (Itoh *et al*., 1997; Zhang, 2006; Ma, 2013). Lifespan also is extended by others transcription factors that are involved in stress response, such as members of forkhead transcription factors (FOX) and heat shock factor (HSF-1), which is thought to upregulate oxidative stress-eliminating enzymes.

Glucose deprivation and muscle contraction in mammals trigger AMP-dependent kinase (AMPK) pathway that is thought to provide positive benefits for cells. The AMPK pathway is activated by an increase in AMP/ATP ratio that indicates a cellular lack of energy caused by metabolic stress. This pathway leads to mitochondrial biogenesis, which leads to a compensation of the energy deficit and likely to additional health promoting effects. Moreover, this pathway also activates catabolic process and represses anabolic process (Hardie and Carling, 1997; Hardie *et al*., 2006). FOXO3 activation, which is triggered by AMPK phosphorylation, participates in cell cycle arrest and DNA damage repair (Greer *et al*., 2007).

Sirtuins (Sirt) activation, a NAD^+-dependent deacetylase that catalyses the removal of acetyl groups from lysine residues of histones and other specific proteins, is also thought to provide positive outcomes for the organism. There is evidence that the

mammalian Sirt1 is involved in mediating oxidative stress and caloric restriction response, as it directly deacetylates several FOXO members, and also regulate cell metabolism and survival (Zschoernig and Mahlknecht, 2008). Similarly, Sirt3 is a mitochondrial sirtuin that participate in the regulation of ATP production, metabolism, apoptosis, and cell signalling (Verdin *et al*., 2010). Sirt3 function is related with cell response to oxidative stress, mitochondrial biogenesis, and metabolic adaptation, also is necessary to mitigate oxidative stress during CR. In muscle cells contraction, Sirt2 induce the overexpression of antioxidant genes of ROS-eliminating enzymes, to reduce the ROS levels and trigger PGC-1α-mediated mitochondrial biogenesis (Ristow and Schmeisser, 2014).

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth, proliferation, and survival and other cellular functions. Reduction of mTOR signalling may lead to an increase in lifespan (Afanas'ev, 2010), mediated by an increase in mtROS generation, which rather than being harmful, this mtROS production are required to the observed increased lifespan (Pan *et al*., 2011; Yun and Finkel, 2014).

Mitochondrial unfolded protein response (mtUPR) is another mechanism by which hormetic response may be triggered. Under stress, mitochondria matrix accumulates misfolded proteins, and mtUPR pathway induce a nuclear transcriptional response that results in the induction of several mitochondrial-specific protein chaperones, as well as, genes involved in mitochondrial protein import and ROS metabolism (Yun and Finkel, 2014).

Mitohormesis has been linked to the beneficial effects of regular physical exercise. Intermittent elevation of tissue and serum l-lactate levels occurring during exercise causes a mild inhibition of mitochondrial respiratory chain and increases production of mitochondrial H_2O_2. Consequently, increased ROS levels induce the activation of AMPK pathway and increase the transcriptional activity of PGC-1α. This can prevent the onset mitochondrial dysfunction in skin-aged fibroblasts associated with the lower activity of mTORC1, and higher activity of the intracellular quality control mechanisms by autophagy (Zelenka *et al*., 2015).

Indeed, the increase of lifespan expectance induced by hormetic response, may also induce neuroprotection in age related diseases such as Parkinson disease, Huntington disease and stroke. This protection is mediated by the activation of chaperons' proteins like heat-shock proteins and glucose-regulated proteins. In response to hypoglycaemia, ischemia and oxidative stress, the expression of neuroprotector factors is increased and activate receptors coupled to kinase cascades that mediate the expression of antioxidant

enzymes, antiapoptotic proteins and proteins that regulate ions homeostasis. Neuroprotector factors such as basic fibroblast growth factor, brain-derived growth factor and insulin like growth factor induce the protection of neurons against pathologies such as Alzheimer disease, Parkinson disease and stroke (Arumugam *et al.*, 2006).

5 Mitochondrial dysfunction, oxidative stress, and disease

The correct cell function depends of metabolic events, that are primarily regulated by mitochondrial function, since these organelles, as described above, are involved in many metabolic functions (Smith *et al.*, 2012). Mitochondrial dysfunction, which can at the end generate a disease condition, can be caused by two tips of dysfunctions: primary mitochondrial dysfunctions characterized by mutations in mitochondrial or nuclear genes which encode for mitochondrial proteins or toxins (Smith *et al.*, 2012); and secondary mitochondrial dysfunctions caused by external events that impaired mitochondrial function, such as ischemia/reperfusion, sepsis, neurodegeneration, metabolic syndrome, organ transplantation, cancer, autoimmune diseases and diabetes (Smith *et al.*, 2012). Notwithstanding the origin of mitochondrial dysfunction, its consequences cause a common pattern on cell function, that coincide with three aspects of mitochondrial damage: oxidative damage, calcium dyshomeostasis and ATP production deficit (Smith *et al.*, 2012).

Oxidative stress is multifactorial process, the impact of which in the organism depends on the type of oxidant, the site and intensity of its production, the composition and activities of various antioxidants, and the ability of repair systems (Ďuračková, 2010). Under normal circumstances, mitochondrial $^{\cdot-}O_2$ production is rather low and does little damage, simply because it is efficiently removed by an efficient antioxidant defence system. However, its levels can arise for a variety of reasons, mainly related with an increased $^{\cdot-}O_2$ production (e.g. chemicals that act as radical amplifiers, medically applied high concentrations of oxygen, or during periods of reperfusion of tissues with oxygen following ischaemia), decrease in antioxidant defence activity or both.

Mitochondria are one of the most relevant targets of oxidative damage within the cells as oxidative damage is mostly provoked by $^{\cdot-}O_2$ and derived ROS, since these species have the ability to react with iron sulphur centres in mitochondria, mtDNA and proteins,as well as inner membrane unsaturated lipids particularly vulnerable to peroxidation. mtDNA represents a critical cellular target for oxidative damage that could lead to lethal cell injury through the loss of ETC activity, $m\Delta\Psi$, and ATP generation. mtDNA is especially susceptible to ROS attack owing to its proximity to the ETC, the major locus

for free-radical production, and the lack of protective histones. Oxidative damage induced by ROS could be the major source of mitochondrial genomic instability, leading to respiratory disfunction (Orrenius *et al*., 2007). In fact, the accumulation of mtDNA mutations is 10-fold greater than in nuclear DNA (Cottrell *et al*., 2000). The $^{\cdot-}O_2$ toxicity is directly related with oxidation and inactivation of proteins. Carbonylation is a consequence of an irreversible and non-enzymatic modification of proteins induced by ROS. The oxidation of iron-sulphur clusters in mitochondria causes its inactivation hence iron is reduced. In addition, Fe^{2+} in the presence of H_2O_2, catalizes the formation of the very active, yet short lived $^{\cdot}OH$, through Fenton reaction, thereby amplifying $^{\cdot-}O_2$ damage (Fridovich, 1997; Orrenius *et al*., 2007). Lipid peroxidation is another consequence of oxidative stress. End products of lipid peroxidation, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) can be accumulated in biological systems under oxidative stress conditions. Lipid peroxides alter mitochondrial membrane fluidity and vital functions, such as OXPHOS, MIM permeability, preservation of mΔΨ, and mitochondrial Ca^{2+} buffering capacity (Orrenius *et al*., 2007).

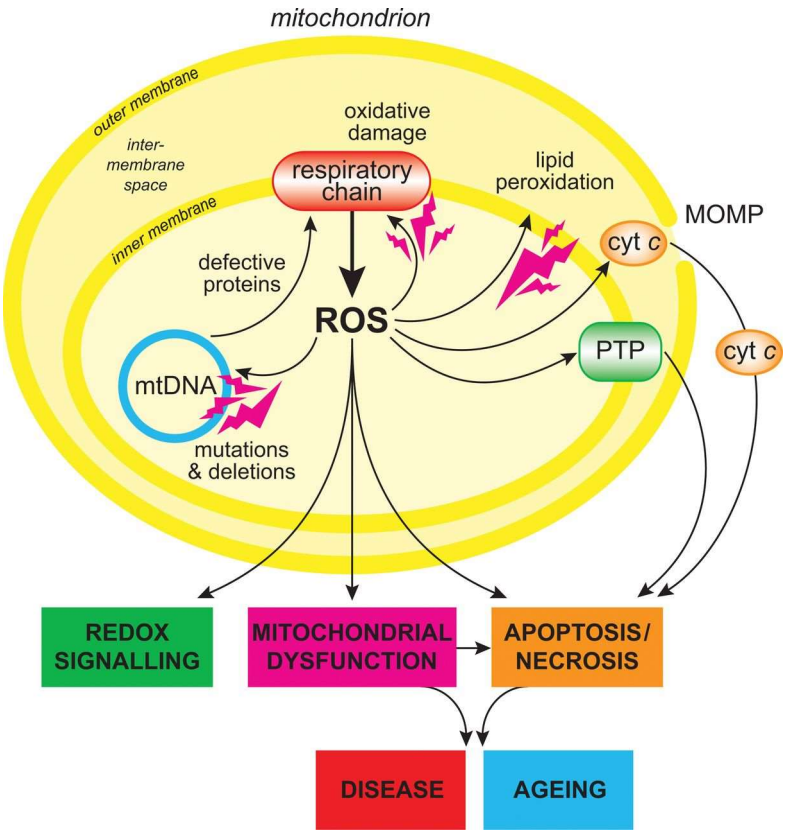


Figure 2. Mitochondrial dysfunction. ROS production by mitochondria can lead to oxidative damage of mitochondrial proteins, membranes, and DNA, impairing the ability of mitochondria fulfil metabolic functions. Mitochondrial oxidative damage can also increase the tendency of mitochondria to release intermembrane space proteins such as cytochrome c (cyt c) to the cytosol by mitochondrial outer membrane permeabilization (MOMP) and thereby activate the cell's apoptotic machinery. In addition, mitochondrial ROS production leads to induction of the mitochondrial permeability transition pore (PTP), which cause the inner membrane permeable to small molecules in situations such as ischaemia/reperfusion injury. Consequently, it is

unsurprising that mitochondrial oxidative damage contributes to a wide range of pathologies. In addition, mitochondrial ROS may act as a modulable redox signal, reversibly affecting the activity of a range of functions in the mitochondria, cytosol, and nucleus (Murphy, 2009). Image used under permission [418] (see annex).

A long-term imbalance between RS generation and antioxidant endogenous defence mechanisms result in global oxidative stress state, which all may cause irreversible damage to DNA, proteins, and lipids (Martindale *et al*., 2002). In the last decades, evidence indicates that chronic and acute excess generation of RS under pathophysiologic conditions is a pivotal stimulus parameter for the disease's development and the appearance of a wide range of pathologies, including malignant diseases, type II diabetes, atherosclerosis, chronic inflammatory processes, ischemia/reperfusion injury, and several neurodegenerative diseases (Orrenius *et al*., 2007; Raha and Robinson, 2000).

6 Free radical theory of aging

Aging can be defined as a progressive accumulation of diverse harmful changes in cells and tissues that potentiate the risk of disease and death. Genetic and environmental factors can cause these changes. The free radical theory of aging proposes that the aging process is initiated by free radical reactions, that could be responsible for progressive deterioration of biological systems over time, caused by the free radicals' capacity of produce random changes (Harman, 2003). The progressive mitochondrial dysfunction leads to rise the production of ROS which in turn cause more mitochondrial deterioration over the time increasing cellular damaged. As a chronological age advance, increases the accumulation of cellular stress and damage, as consequence of an increased ROS production (López-Otín *et al*., 2013).

Aging also is a consequence of environmental damage, caused by oxidant atmosphere, UV-irradiation, chemical and physical pollutants, as well as, drugs and dietary contaminants (Fisher *et al*., 2002; Bickers and Athar, 2006).

The skin is the organ that provides the interface between the organism and the environment being the first protect barrier against external injuries, preventing invasion of pathogens and fending off chemical and physical assaults, as well as avoid the loss of water and heat (Proksch, *et al*., 2008). The skin is constantly exposed to stress increasing the levels of free radicals leading to cellular damage (Rittié and Fisher, 2002). Biological skin damage can also occur. Leucocytes are a source of ROS in dermal skin, since when activated, they produce ROS by expressing high amounts of nicotinamide

adenine dinucleotide phosphate (NADPH) oxidase. This membranal enzyme releases $^{\cdot-}O_2$ in extracellular space, which is converted in H_2O_2, in turn is converted in $^{\cdot}OH$, and $^1O_2$ (Pillai *et al*., 2005), to respond to microorganism invasion or to degrade damage tissues structures

The major functional manifestations of skin ageing occur as a consequence of structural and compositional remodelling of normally long-lived dermal extracellular matrix proteins (Naylor *et al*., 2011). Aged skin dermal fibroblasts decrease the collagen synthesis of extracellular matrix and a consequent reduced of elasticity and the emergence of fine wrinkles (Varani *et al*., 2006).

7 Mitochondrial antioxidant defence system

Cellular redox environment plays a pivotal role in redox homeostasis, and the imbalance between pro-oxidant and antioxidants favour oxidative stress condition and result in disease. To limit ROS at physiologic levels and to prevent oxidative-induced damage of structural macromolecules, cells are provided with an antioxidant defence mechanism. This system consists in the interaction of enzymatic antioxidants such as glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) together with non-enzymatic antioxidants, such as, α-tocopherol, ubiquinone, β-carotene, ascorbate, and glutathione (GSH) (Briganti and Picardo, 2003). For instance, lipid soluble free radical scavenging α-tocopherol present in mitochondrial membranes reduce lipid peroxides, and then is regenerated by lipid soluble $CoQH_2$ and water soluble ascorbic acid and GSH in water/membrane interface (Lass *et al*., 1999; Niki *et al*., 1982; Andreyev *et al*., 2005); GSH (L-γ-glutamyl-L-cysteinyl glycine) is the principal non-enzymatic thiol involved in antioxidant defence, which can be found in its free form or bound to proteins. Reduced (GSH), in response to oxidative stress, can be converted in oxidised glutathione (GSSG), in which the thiol group is oxidised, and form disulphide bound (–S–S–) with another oxidised glutathione. GSSG can be reduced back to GSH by glutathione reductase (GR) enzyme. The regenerated GSH by GR, present in mitochondrial matrix, utilizes NAD(P)H as source of reducing equivalents (Andreyev *et al*., 2005). Alternatively, the pool of GSH can be replenished by uptake from cytosol / extracellular environment and/or by *de novo* synthesis of GSH (Andreyev *et al*., 2005). The selenium-dependent phospholipid hydroperoxide glutathione peroxidase (GPx) is an enzyme that catalyses the reaction of hydroperoxides with GSH to form GSSG and reduced hydroperoxide products (Chance *et al*., 1979; Andreyev *et al*., 2005). GPx is specific to its electron donnor (GSH) but nonspecific for hydroperoxides, as it reduces

phospholipid hydroperoxides, H_2O_2, cholesterol peroxide, and thymine hydroperoxide yielding non-radicalar end-products and GSSG (Thomas *et al*., 1990; Maiorino *et al*., 1991; Bao *et al*., 1997; Andreyev *et al*., 2005); Glutathione-S-transferase (GST) catalyse the addition of GSH to toxic molecules protecting mitochondria from oxidative damage.

Superoxide dismutase (SOD) is one of the detoxifying enzymes responsible for converting $^{\bullet-}O_2$ to H_2O_2 at very high rates, avoiding its attack to iron-sulphur clusters. The SOD family includes copper-zinc-SOD (CuZnSOD or SOD1) in the cytosolic and mitochondrial intermembrane space, and manganese-SOD (MnSOD or SOD2) in the mitochondrial matrix. Consequently, CAT is an enzyme that converts H_2O_2, when produced in excess from $^{\bullet-}O_2$ dismutation, in H_2O and O_2 (Andreyev *et al*., 2005); Thioredoxin (TRx) are small globular proteins that participate in many cellular functions, including antioxidant defence. Their active reduced form is maintained by thioredoxin reductase (TRxR), which use NADPH as source of reducing equivalents. In mitochondria, TRx play an important role in furnish electrons to thioredoxin-dependent peroxidase in order to reduce H_2O_2 and hydroperoxides (Miranda-Vizuete *et al*., 2000; Powis and Montfort, 2001).

Under pathologic conditions, some constituents of antioxidant defence system may be compromised leading to an unbalance between ROS production and removed to keep physiologic levels.

8 Dietary antioxidants

Under physiological concentrations, ROS are required for normal cell function including intracellular signalling and redox regulation. In fact, several cytokines, growth factors, hormones, and neurotransmitters use ROS as secondary messenger in intracellular transduction (Nordberg and Arner, 2001). Although cells have their own antioxidant mechanism defence to prevent the excessive ROS production (Rijken and Bruijnzeel, 2009), the antioxidant mechanism defence can be overwhelmed resulting in an oxidative stress status (Rijken and Bruijnzeel, 2009). In this scenario, dietary antioxidants obtained through human diet can reinforce the endogenous antioxidant defence system. Human diet contains a wide range of phytochemical polyphenols, synthetized by plants as secondary metabolites (Crozier *et al*., 2009), which despite the limited number of molecular scaffolds have the capacity to polymerase originating more than 4000 different derivatives.

Polyphenols can be characterized by having at least one aromatic ring with one or more hydroxyl groups and, based on their structural properties, can possess different

biological properties, including antioxidant, anticancer, anti-inflammatory activities. Their beneficial effects have been demonstrated by preventing and/or age-related diseases such as cardiovascular disease, osteoporosis, neurodegenerative disease, cancer and diabetes mellitus (Crozier *et al*., 2009; Quideau *et al*., 2011; Abbas *et al*., 2017). Although it was initially thought that their antioxidant activity was mainly due to their scavenger ROS capacity, several mechanisms of action have now been preferred to account for polyphenols antioxidant activity, such as metal transition chelation, inhibition of enzymes involved in ROS overproduction, and modulation of ROS-eliminating enzymes activity (Benfeito *et al*., 2013).

Epidemiological evidence suggests that polyphenolic-enriched diet showed beneficial effects on the prevention of oxidative stress-related conditions (Diplock *et al*., 1998). Consequently, polyphenols have gained attention due to their remarkable antioxidant effects, with their beneficial effects having been studied in several disease models (Diplock *et al*., 1998). Resveratrol, found in red fruits, grapes, and red wine, is one of the most studied polyphenols, and is suggested to regulate $^{\cdot-}O_2$ and $^{\cdot}OH$ formation in mitochondria, while inhibiting lipid peroxidation induced by Fenton reaction products (Zini *et al*., 1999). Curcumin, obtained from Curcuma longa rhizome, is described to exert an antioxidant effect due to the inhibition of lipid peroxidation, H_2O_2 scavenging activity and transition metals chelation (Ak and Gülçin, 2008; Chainani-Wu, 2003). Rosmarinic acid, which can be extracted from rosemary plant, has also antioxidant and anti-inflammatory properties by inhibiting macrophages and astrocytes-mediated ROS generation (Frankel *et al*., 1996; Qiao *et al*., 2005). Quercetin, found in onions, apples, broccolis, and berries present also free radicals' scavenger and iron chelation properties (Murota and Terao, 2003; Wach *et al*., 2007). Epigallocathechin-3-gallate, found in green and black tea, and red wine, is another well-described dietary antioxidant (Khan, and Mukhtar, 2007).

Phenolic acids are a family of polyphenolic compounds with remarkable antioxidant activity which have been extensively studied due to their structural simplicity. Moreover, phenolic acids represent of about 1/3 of the total antioxidant content ingested through human diet (Rice-Evans *et al*., 1996; Scalbert and Williamson, 2000). Phenolic acids can be sub-divided into hydroxybenzoic (HBA) and hydroxycinnamic (HCA) acids (Manach *et al*., 2004). HBAs comprise seven carbon atoms (C6-C1), whereas HCAs containing nine carbon atoms (C6-C3) with a double bound in the side chain (Manach *et al*., 2004; Teixeira *et al*., 2013b; Teixeira *et al*., 2017b).

HBA can be found in some fruits such as berries, wine, or red fruits, being tea and wine an important source of HBA derivatives (Tomás-Barberán and Clifford, 2000). HCA are substantially more abundant in nature that HBAs, being highly present in fruits, such

as apples, pears, berries (El-Seedi *et al*., 2012), being coffee, fruits, and their juices the main dietary sources of HCAs (Radtke *et al*., 1998). The radical scavenger properties of HBAs and HCAs can break the redox chain reactions, due to their hydrogen atom donating ability and stabilization of the resulting phenoxyl radical (Teixeira *et al*., 2013a). HCAs have a larger ability to H-donate than HBAs thought due to the –CH=CH-COOH side chain promoting radical stabilization by resonance (Rice-Evans *et al*., 1996). Catecholic and gallolyl acids present metal chelation properties due to aromatic substitution pattern, which improve their antioxidant activity (Perron and Brumaghim, 2009). The potencial redox active properties are due its benzene ring-bound hydroxyl groups, which can donate the hydrogen atom or one electron the ROS, stabilizing free radicals, that to stabilise the phenoxyl radical generated, react with another radical forming a stable quinone structure (Sandoval-Acuna *et al*., 2014).

9 Xenohormesis

Plant toxins or phytoalexins are product of self-defence, once the plant cannot move away from the stressors like environmental extreme variation of temperature, water or nutrient low availability, or predation (Hooper *et al*., 2010). Since animals normally depend upon plants for their food supply, they have adapted their metabolism and defence mechanisms to sense toxins/bioactive substances produced by stressed plants. The evolution promotes the ability of animals' sense stress signalling molecules produced by plants in their environment. So, an organism senses chemical cues from other species about the status of environment or food supply and responds to them in a way that is beneficial (Surh, 2011). Polyphenols are one of the secondary plant metabolites produced by most of these plants in response to stress (Howitz and Sinclair, 2008). The interaction of chemicals from different organisms (plants vs. animals) can also induce a hormetic response, the so-called xenohormesis. These xenohormetic phytochemicals, which alert animals to adversity, can stimulate their stress response and eventually fortify cellular defence capacity (Hooper *et al*., 2010). These phytochemicals that cause toxicity to microorganisms, insects and pests eating plants, at the subtoxic doses ingested by humans as part of diet, are considered to induce mild cellular stress responses. Several type of chemicals, including 1) redox-active bisphenols, quinones and phenylenediamines; 2) Michael acceptors; 3) isothiocyanates; 4) dimercaptans; 5) hydroperoxides; 6) metals; and 7) some polyphenols (Stefanson *et al*., 2014).

Most diet-derived polyphenols are Michael acceptors or can be metabolized as such (Stefanson *et al*., 2014). Consequently, polyphenols pro-oxidant activity can induce mild

oxidative stress, a process that can result in an up-regulation of cytoprotective genes expression.

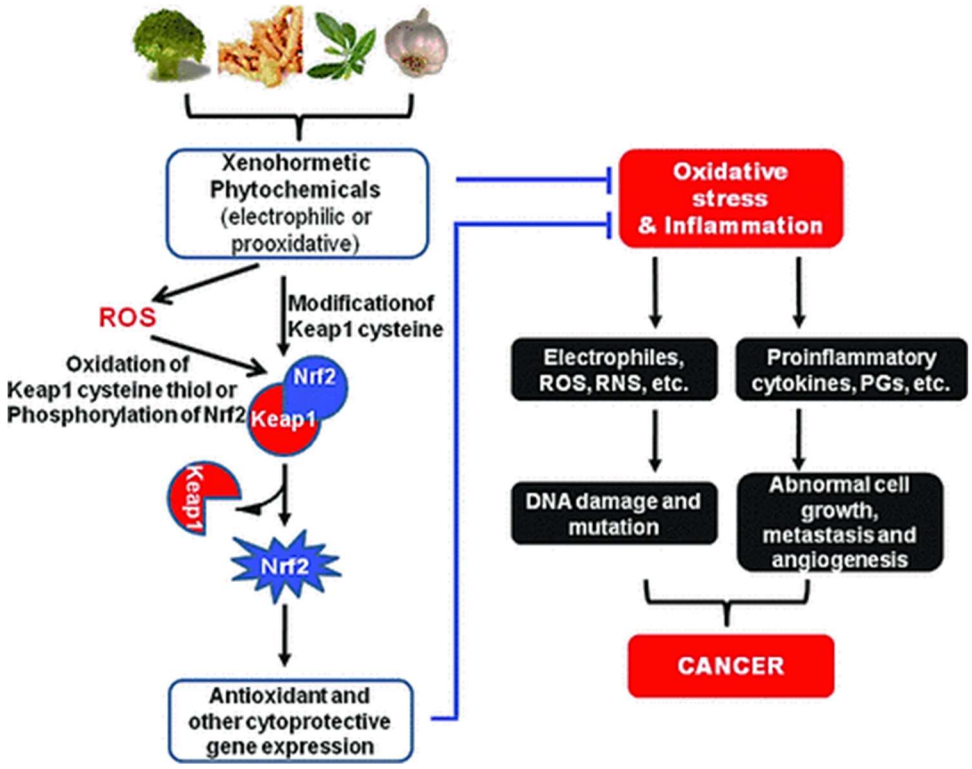


Figure 3. Xenohormetic mechanism of action. Phytochemicals induce protective response through a slightly and transient increase of ROS levels that trigger the genes expression of antioxidant defence system(Surh, 2011). Image used under permission [4] (see annex).

10 (In)efficacy of dietary antioxidants: the reality

Epidemiological evidences consider a diet rich in fruits and vegetables healthier and capable to decrease the incidence of several pathological conditions (Diplock, 1991). Nevertheless, the scientific evidence is lacking to understand the biochemical mechanism of these beneficial effects and antioxidant therapy has enjoyed relative success in human clinical trials, as controversial data and little benefits in humans have been attained (Benfeito *et al*., 2013; Hasnain and Mooradian, 2004; Steinhubl, 2008).

Polyphenols do not satisfy all chemical specifications to be considered a good bioavailable drug. Beyond their low lipophilicity, other features such as solubility, stability due to gastric and colonic pH, metabolism by gut microflora, abortion across intestinal wall, active efflux mechanism, and first-pass metabolic effects reduce their bioavailability and represents a limitation to polyphenols efficacy (Upadhyay and Dixit, 2015). Polyphenols are treated as xenobiotics in human body, and metabolised and eliminated as efficiently as possible (Crozier *et al*., 2009). Antioxidants are modified by xenobiotic

metabolising enzymes, such as the cytochrome-P450 and conjugating enzymes of phase II detoxification, which play a major role in the metabolism of polyphenols. This metabolic detoxifying process may considerably decrease a compound efficacy and result in the formation of derivatives that are more suitable for excretion than the parent molecules. For instance, in hepatic metabolism, polyphenols are conjugated to glucuronic acid and/or sulphate. Such conjugation reactions block hydroxyl groups and might impair antioxidant function (Stahl *et al*., 2002). The native phenols may suffer oxidation either directly or mediated by oxidative enzymes, as well as, undergo a series of condensation reactions to produce high molecular weight complex molecules more easily eliminated by human body. The transport of nutrients and drugs, such as polyphenols, in human body is through the blood. However polyphenolic metabolites circulate in its free form in plasma and blood, a significant amount is bound to plasma proteins, such as albumin, which significantly reduce their bioavailability (Manach *et al*., 2004).

Antioxidants must not only act per se, but they also must take part of the antioxidant defense network system, which combine the action of small molecules with protective and repair enzymatic systems. In that way, the decrease of the endogenous antioxidant status may be compensated by an up-regulation of other endogenous defenses. However, the reinforcement of the endogenous antioxidant system with exogenous antioxidants acquired from diet or administered, with a net increase in antioxidant defenses is not always straightforward (Murphy, 2014).

While the exogenous antioxidants regularly are dispersed throughout the body, the ROS generation, and the consequent damage generally are localised in particular cell types or organelles, such as mitochondria. Consequently, antioxidant amount available in ROS-generation place (mitochondria) may not be sufficient to exert their beneficial effects (Murphy, 2014).

Although many antioxidants reveal promising results in *in vitro* trials their use into preclinical/clinical trials have not revealed such benefits (Firuzi *et al*., 2011; Schmidt *et al*., 2015). Bioavailability still is one of the main limitations, and so great efforts have been done to develop drug delivery systems such as liposomes, phospholipid complexes, and smart antioxidants carriers to improve their bioavailability.

11 Mitochondrial pharmacology

Mitochondrial pharmacology is a feasible strategy because therapies that impact on a few common cellular damaging pathways can treat patients with a wide range of

primary and secondary mitochondrial disorders, which make mitochondria an appealing pharmacological target. Most of the strategies to improve mitochondrial function aim to ameliorate the consequences of primary defects rather than address the cause of dysfunction. In mitochondrial pharmacology field three types of strategies were developed to modulate mitochondria dysfunction: 1) create molecules that selectively accumulate within mitochondria; 2) use molecules to bind targets within mitochondria so that their function is dependent of the specific location; 3) regulate the cellular functions outside mitochondria to fine tune mitochondrial function (Smith *et al*., 2012).

Targeting biomolecules to mitochondria is a strategy that allows directly delivery of bioactive molecules to mitochondria, avoiding side effects in extra-mitochondrial space and reducing the biomolecules concentration required to exert the effect. Covalent attachment of bioactive molecules to lipophilic cation, such as triphenylphosphonium (TPP^+), due to its lipophilicity and positive ionic charge, allows it to cross plasma and mitochondrial membranes. This feature effectively lowers the activation energy to membrane passage enabled by $m\Delta\Psi$ (Smith *et al*., 2011).

Mitochondrial penetrating peptides are a class of peptides that can be used to target mitochondrial function, possessing the necessary balance of charge and lipophilicity that provide electrostatic driving force to cross energized barriers like cellular and mitochondrial membranes (Horton *et al*., 2008; Yousif *et al*., 2009). Szeto-Schiller (SS) peptides are compounds used to target bioactive molecule to the MIM. These peptides can penetrate a variety of cell types without requirements for specific transporters or receptors, and its mitochondrial uptake is not dependent of $m\Delta\Psi$. Mitochondria may also be used as bioreactor, with mitochondria-targeted molecules reacting inside the organelle to yield a new compound, or to generate a bioactive molecule inside mitochondria that diffuse to extramitochondrial space and react with its target (Smith *et al*., 2011).

The second strategy is the modulation of intramitochondrial specific targets or process with drugs. This approach can be used in cancer therapy by utilizing molecules that disrupt the sequestration of pro-apoptotic proteins by antiapoptotic proteins BCL-2, which allows the permeabilization of MOM, release of apoptotic factors such as cytochrome *c* from IMS to cytosol, leading to apoptosis (Fulda *et al*., 2010).

Moreover, manipulating processes such as fission, fusion, and autophagy allows cells to degrade damage mitochondrial. Upregulation of mitophagy eliminate damage mitochondria, while inhibition of mitochondrial fission process through inhibition of dynamin related protein-1 (DRP-1) avoids MOM permeabilization and apoptotic cell death (Smith *et al*., 2011).

In ischemia/reperfusion (I/R) injury, oxidative stress, ATP depletion and calcium dyshomeostasis lead to a cyclophilin D-dependent permeability transition pore (PTP) formation causing cell death. Cyclosporin A is a classic drug that has been proposed to inhibit the formation of PTP (Smith *et al*., 2011).

Finally, the pharmacological manipulation of mitochondrial function, through small biomolecules that interfere in endogenous pathways that control mitochondrial gene expression and enable mitochondrial function in response to damage is also a promising strategy (Smith *et al*., 2011). Mitochondrial biogenesis and respiration can be stimulated by peroxisome proliferated-activated receptor gamma co-activator (PGC-1α), through the upregulation of nuclear respiratory factor (NRF) and NRF-2 gene expression. Consequently, it modulates the expression of the transcription factor A mitochondrial (TFAM), which is important to mtDNA replication and transcription (Ventura-Clapier *et al*., 2008).

12 Mitochondria-targeted molecules using lipophilic cations

During the past decade considerable progress in developing mitochondria-targeted antioxidants has been made. Since then, the covalent attachment of the lipophilic cations, such as TPP^+ has been established as a general and robust method to target small bioactive molecules, enzymes, and probes to mitochondria in vivo (Smith *et al*., 2011). These compounds are known to pass through all biological membranes and accumulate within mitochondria more easily than their non-targeted parent molecules. TPP^+ act as carrier to mitochondria, that due to its lipophilic characteristics and positive charge delocalised over a large and hydrophobic surface area, cross easily through lipid bilayers.

Lipophilic cationic derivatives are rapidly and extensively taken up *in vivo* by mitochondria driven by the large $m\Delta\Psi$, which is negative inside (Smith *et al*., 2011). The Nernst equation adequately describes the membrane potential dependent uptake of lipophilic cations, increasing 10-fold for every ~60 mV of $m\Delta\Psi$. In this way, it is estimated that a hundred-fold uptake of the antioxidant within mitochondria *in vivo* occurs (Porteous *et al*., 2010). The extent of uptake of a TPP^+ derivative depends on the plasma $\Delta\Psi$ and $m\Delta\Psi$, the cell volume, the external media, and the number of mitochondria within a given cell. Consequently, the amount of compound found within mitochondria can substantially differ according different cell types (Reily *et al*., 2013). Furthermore, the extend of TPP^+

derivatives anchoring to the MIM is dependent upon their hydrophobicity, the length of the linker unit and the functionalization of the bioactive molecule (Anders, 2013).

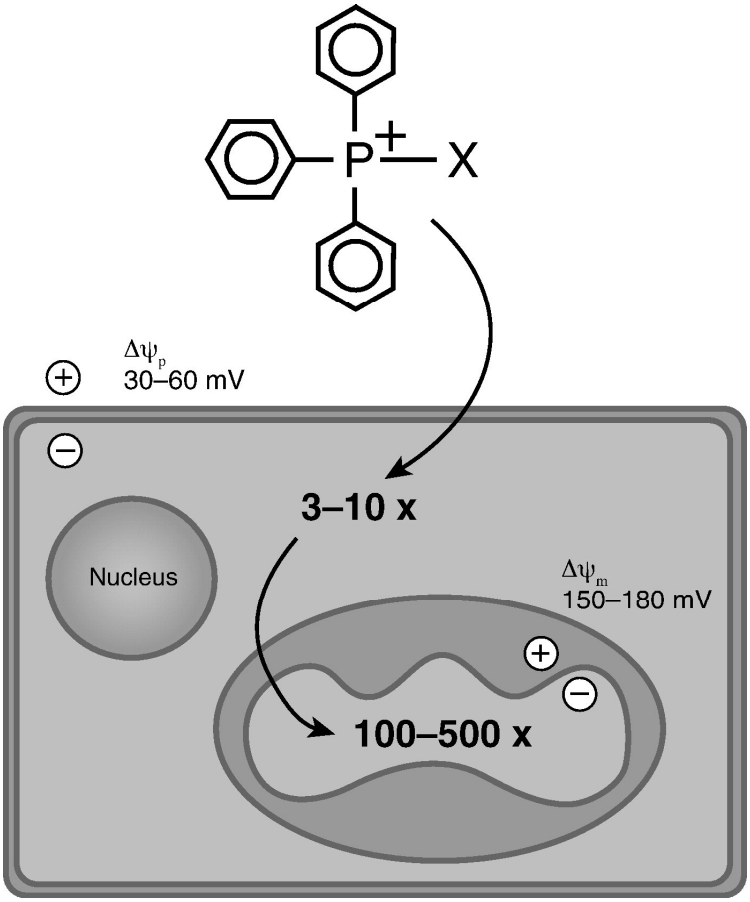


Figure 4. Uptake of triphenylphosphonium cations by mitochondria within cells. The drug (X) attached to lipophilic cation get in cytoplasm driven by the plasma membrane potential ($\Delta\psi_p$) and goes to mitochondrial matrix driven by mitochondrial inner membrane potential ($\Delta\psi_m$). The moiety, X, could be an antioxidant or a probe of mitochondrial function (Murphy, 2008). Image used under permission [1029] (see annex).

The selective targeting of pharmacophores or bioactive molecules to concentrate within mitochondria, significantly decrease the external dose required, while the specific sequestration in mitochondria avoid toxic side effects and minimise the metabolism (Smith *et al*., 2011). Once inside mitochondria, TPP^+ derivatives are primarily positioned on the mitochondrial matrix upon surpassing the phospholipid bilayer, maintaining the linker and bioactive molecule positioned within the MIM (Apostolova *et al*., 2015). Within mitochondria, these derivatives can elicit beneficial effects by diverse mechanisms, namely by scavenging ROS and preventing membrane lipid peroxidation and/or control mitochondrial redox signalling (Smith *et al*., 2012).

13 Development of novel mitochondria-targeted polyphenols

Polyphenols of plant origin have lately received increased attention, namely the ones which are taken exogenously from the diet (as nutritional supplements). Phenolic acids (e.g., HBAs and HCAs) have already been proved to be suitable scaffolds for the rational design and development of new antioxidants, which are of utmost importance in pharmaceutical, chemical and food industries. The results obtained so far confirmed the importance of exploring natural phenolic systems as safer templates to build new antioxidants that can lead to drug candidates, through rational design approaches.

Bearing in mind the importance of dietary polyphenols, a drug discovery program was established to develop mitochondriotropic antioxidants (Teixeira *et al*., 2018a) based on HBA (Teixeira *et al*., 2017b) and HCA cores (Teixeira *et al*., 2017a). Consequently, HBA and HCA were attached to TPP^+, and their antioxidant protective properties within mitochondria were screened. It was demonstrated that these novel constructs effectively accumulate within mitochondria, can scavenge free radicals, protect biomembranes against lipid peroxidation and chelation activity (Teixeira *et al*., 2017a; Teixeira *et al*., 2017b). Along the project, three compounds emerged due to their noteworthy properties: $MitoBEN_2$, (Figure 5A), $MitoCIN_4$ (Figure 5B) and $MitoCIN_6$ (Figure 5C).

$MitoBEN_2$ (Figure 5A) is a mitochondrial-targeted antioxidant based on gallic acid conjugated with TPP^+ showing remarkable antioxidant and iron-chelation properties and capable to prevent mitochondrial lipid peroxidation due to $m\Delta\Psi$-driven mitochondrial accumulation (Teixeira *et al*., 2017b). $MitoBEN_2$ has a large safety margin toward rat cardiomyoblasts (H9c2), normal human dermal fibroblasts (NHDF), and human hepatocyte (HepG2) cells, protecting them against oxidative stressors (Teixeira *et al*., 2017b).

$MitoCIN_4$ (Figure 5B) is a mitochondrial-targeted antioxidant based on natural dietary caffeic acid connected to lipophilic TPP^+ through an alkyl spacer (Teixeira *et al*., 2017a; Teixeira *et al*., 2012). $MitoCIN_4$ expectedly accumulated within mitochondria driven by the $m\Delta\Psi$ without affecting mitochondrial morphology and polarization. Moreover, $MitoCIN_4$ showed remarkable antioxidant and iron-chelation properties, and can inhibit oxidative damage either in isolated liver mitochondria or hepatic cells. Additionally, it was found that $MitoCIN_4$ can play a role in the maintenance of intracellular GSH homeostasis by increasing its supply (Teixeira *et al*., 2017a).

MitoCIN$_6$ (Figure 5C) is a mitochondrial-targeted antioxidant based on natural dietary caffeic acid conjugated with TPP$^+$ through an alkyl spacer longer than MitoCIN$_4$, and less one hydroxyl group at phenyl ring than MitoCIN$_4$ (Teixeira *et al*., 2017a; Teixeira *et al*., 2012). MitoCIN$_6$ also is accumulated within mitochondria driven by mΔΨ without affecting mitochondrial morphology and polarization. Antioxidant capacity and iron-chelation also are properties found in MitoCIN$_6$ as well as the inhibition of oxidative damage in isolated liver mitochondria and hepatic cells. Besides, MitoCIN$_6$ was able to induce the maintenance of intracellular GSH homeostasis by increasing its supply (Teixeira *et al*., 2017a).

14 Objectives of the present work

Human skin is the first protect barrier against external injuries, preventing invasion of pathogens and defending of chemical and physical assaults, as well as avoid the loss of water and heat (Proksch *et al*., 2008). Moreover, skin provides the interface between the organism and environment and is constantly exposed to chemical and physical pollutants. Consequently, dietary contaminants and drugs can also manifest their beneficial or toxic effects in the skin cells (Bickers and Athar, 2006).

Phytochemicals such as polyphenols have been described as electrophilic and antioxidant molecules, which can exert health beneficial effects at low doses. The use of diet-derived polyphenols can modulate the cells metabolic processes leading to an up-regulation of the cytoprotective antioxidant defences. The demand for restoring the cells redox state highlight agents that can induce the up-regulation or activate antioxidant response as first-line drugs to be used pharmacologically as bioactive molecule to decrease oxidative stress-induced conditions (Maulik *et al*., 2013).

Despite the polyphenols low bioavailability constrains, they can be important tools for drug discovery processes. Bearing this in mind, new mitochondriotropic antioxidants based on HBA (MitoBEN$_2$) and HCA (MitoCIN$_4$ and MitoCIN$_6$) cores were developed by covalently attachment to TPP$^+$. As part of the long-term project, we used human dermal fibroblasts to understand the long-term mechanism of action of the developed mitochondria-targeted antioxidants. Moreover, we intended to investigate if mitochondria-targeted antioxidants are able to stimulate a hormetic response.

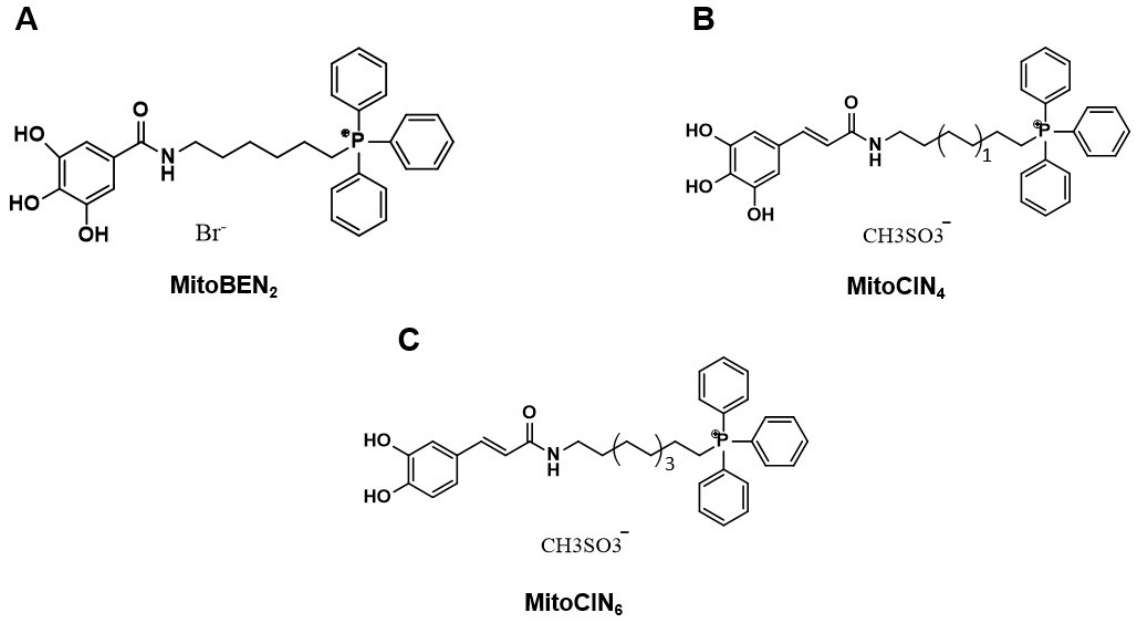


Figure 5. Molecular structure of mitochondria-targeted antioxidants studied in this project. MitoBEN$_2$, a hydroxybenzoic derivative molecule and MitoClN$_4$ and MitoClN$_6$, a hydroxycinnamic derivative molecule (Teixeira *et a*l., 2017a; Teixeira *et al*., 2017b).

Material and methods

2.1. Common reagents

The common reagents used in this work include: Dulbecco's modified Eagle's medium (D5030), L-glutamine, glucose, galactose, sodium bicarbonate, sodium pyruvate, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), bovine serum albumin (BSA) (catalog #A1595), dimethyl sulfoxide (DMSO), DL-Dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), sulforhodamine B sodium salt (SRB), trypan-blue solution, sodium chloride (NaCl), resazurin sodium salt, trichostatin A, bafilomycin A, *tert*-butyl hydroperoxide (*t*-BHP), Bradford reagent, phenylmethanesulfonyl fluoride (PMSF), tween 20, 2-mercaptoethanol (Sigma-Aldrich Chemical Co., Saint Louis, Missouri, USA); fetal bovine serum (FBS), penicillin-streptomycin (10.000 U/mL), 0.05% Trypsin–EDTA (Gibco-Invitrogen, Grand Island, New York, USA); acetic acid, methanol, magnesium chloride ($MgCl_2$), potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), sodium phosphate monobasic (NaH_2PO_4), sodium hydrogencarbonate ($NaHCO_3$), sodium hydroxide (NaOH) (Merck, Whitehouse Station, New Jersey, USA); CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation, Madison, Wisconsin, USA); fluorescent probes Hoechst 33342 (from Molecular Probes, Life Technologies, New York, NY, USA); Lysotracker Green DND-26, TMRM (Tetramethylrhodamine, Methyl Ester, Perchlorate) (ThermoFisher Scientific, Waltham, Massachusetts, USA); glycine, tris base (nzytech, Lisbon, Portugal); cell lysis (Cell Signaling Technology, Danvers, Massachusetts, USA); Laemmli buffer (Bio-Rad, Hercules, California, USA).

2.2. Solutions preparation

2.2.1. Low glucose cell culture medium

Cell culture medium was composed of Dulbecco's Modified Eagle Medium (DMEM-D5030), supplemented with 5 mM glucose, sodium bicarbonate (3.7 g/L), HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) (1.19 g/L), L-glutamine (0.876 g/L), sodium pyruvate (0.11 g/L), 10 % fetal bovine serum (FBS), 1 % penicillin-streptomycin.

2.2.2. Glucose-free cell culture medium (OXPHOS medium)

Glucose-free cell culture medium (OXPHOS medium) was composed of Dulbecco's Modified Eagle Medium (DMEM-D5030), supplemented with galactose (1.80 g/L), sodium bicarbonate (3.7 g/L), HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) (1.19 g/L), L-glutamine (0.876 g/L), sodium pyruvate (0.11 g/L), 10 % fetal bovine serum (FBS), 1 % penicillin-streptomycin.

2.2.3. Phosphate-buffered saline (PBS)

The PBS was prepared with 15.44 mM KH_2PO_4, 1.55 M NaCl, 27 mM KCl and 27.09 mM Na_2HPO_4, pH 7.2. PBS was prepared as a 10X solution.

2.2.4. Acetic acid solution in methanol, 1% (v/v)

The acetic acid solution 1 % (v/v) was prepared with 5 mL of acetic acid added to 495 mL methanol. The homogenized solution was stored at −20°C.

2.2.5. Acetic acid solution in MilliQ-purified water, 1% (v/v)

For acetic acid solution 1% (v/v), 10 mL of acetic acid was added to 990 mL MilliQ-purified water. This solution was stored at room temperature.

2.2.8. Tris-NaOH

For Tris-NaOH, 10 mM, pH 10 0.64 g of Tris base was dissolved in 400 mL of MilliQ-purified water. pH was adjusted to 10.5 with 1 M NaOH and the solution was brought to a final volume of 500 mL with MilliQ-purified water.

2.2.9. Microscopy medium

The microscopy medium was composed by 12 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM HEPES, 5 mM $NaHCO_3$, 1.2 mM Na_2SO_4 and 5 mM glucose prepared in MiliQ water and pH 7.4. This medium solution was filtered with a 0.2 porosity filter in sterile condition. The medium was used for fluorescent microscopy analyses.

2.2.10. Resazurin solution

The resazurin solution (1 mg/mL) was prepared in PBS (1X) solution. 10 mg of resazurin sodium salt was added to 10 mL of PBS and the solution was filtered with 0.2 µm pore filter and stored at −20 ºC.

2.2.11. Sulforhodamine B (SRB) solution

The SRB solution was prepared with 0.25 g of sulforhodamine B dissolved in 500 mL of 1 % (v/v) acetic acid in MilliQ-purified water. The solution was maintained protected from light, at 4 °C.

2.2.12. Running buffer

The running buffer was prepared with 150 g of glycine, 30 g of Tris base and 10 g of SDS, dissolved in MilliQ-water until make up 1 L. The running buffer was prepared as a 10X solution and was stored at room temperature.

2.2.13. Transfer buffer

The transfer buffer was prepared with 145 g of glycine and 30.35 g of Tris base, dissolved in MilliQ-water until make up 1L. The running buffer was prepared as a 10X solution and stored at room temperature. Before to be used the transfer, buffer was diluted to 1X with 100 mL of 10X transfer buffer, 100 mL of methanol, 500 µL of (10%) SDS, and MilliQ-water until make up 1L, that solution was kept at 4 °C.

2.2.14. Washing buffer or Tris-buffered saline, 0.1% tween 20 (TBS-T)

The washing buffer was prepared with 90 g of NaCl, 500 mL of 1 M Tris pH 8.0, 10 mL of tween 20 and MilliQ-water until make up 1L. The washing buffer was prepared as a 10X solution and was stored at room temperature.

2.2.15. Cell lysis buffer

The cell lysis buffer 1X was prepared with 100 µL of 10X cell lysis, 1.5 µL of 200 mM PMSF and MilliQ-water until make up 1 mL.

Biological Assays

2.3. Cell line

Normal human dermal fibroblasts (NHDF) were furnished from Lonza Group AG (Basel, Switzerland). They were acquired from dermis of adult skin and were cryopreserved at the end of primary culture. Lonza's human dermal fibroblasts are guaranteed through 15 population doublings when using FGM™-2 Growth Media. All cells test negative for mycoplasma, bacteria, yeast, and fungi. HIV-1, hepatitis B and hepatitis C are not detected for all donors and/or cell lots. Dermal fibroblasts are characterized by morphological observation throughout serial passage. Fibroblasts originate locally from mesenchymal cells and are permanent residents of connective

tissue, they are responsible for producing and maintain the extracellular matrix components, synthetize, and release collagen, elastin, glycosaminoglycans, proteoglycans and multiadhesive glycoproteins. Morphologically fibroblasts have more abundant and irregularly branched cytoplasm, its nucleus is large, ovoid, euchromatic, and has a prominent nucleolus (Mescher, 2013).

2.4. Cell culture and treatment regime

Normal human dermal fibroblasts (NHDF), were cultured in Dulbecco Modified Eagle's Medium (DMEM, D5030) supplemented with 10 % fetal bovine serum (FBS), 1 % antibiotic penicillin-streptomycin, 1.1915 g/L 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid buffer (HEPES), 3.7 g/L sodium bicarbonate, 0.876 g/L L-glutamine, 0.11 g/L sodium pyruvate and 0.90 g/L glucose. All cells were cultured in monolayer in adherent tissue culture dishes at 37 °C in a humidified atmosphere of 5 % CO_2, cells were passaged by trypsinization using standard methods when reaching 80-90 % confluence, and NHDF cells were only used between passage 11 to 17 in cultures in log-phase growth. NHDF cells were seeded (15,000 cells/cm^2) and allowed to proliferate for 24 h during which they reached 40–60 % confluence. Next, cells were incubated for 72 h in the presence of vehicle (0.1 % DMSO; CT) and the mitochondria-targeted antioxidants MitoBEN$_2$ (12.5 μM), MitoCIN$_4$ (12.5 μM) and MitoCIN$_6$ (0.39 μM) in "regular" DMEM or "OXPHOS" culture medium.

2.5. Nuclei number

Nuclei number was measured by staining cells with Hoechst 33342. Thirty minutes before the time point, all culture medium was removed and replaced for low glucose cell culture medium without FBS at 37 °C for 30 minutes containing Hoechst 33342 (1μg/mL). After incubation time, the solution was replaced for 100 μL of fresh microscopy medium. All images were collated at 40X magnification using the InCell Analyzer 2200 high-throughput epifluorescence imaging microscope.

2.5. Sulforhodamine B assay

Sulforhodamine B (SRB) assay was used for cell mass determination based on the measurement of cellular protein content (Vichai and Kirtikara, 2006). Briefly, after treatment, the cell culture medium was removed, and wells were washed with PBS (1X). Cells were then fixed by adding 1 % acetic acid in 100 % methanol overnight at −20 °C. The fixation solution was discarded, and the plates were dried in an oven at 37 °C, and then 70 μL of 0.05 % SRB in 1 % acetic acid solution was added and incubated at 37 °C for 1 hour. The wells were then washed with 1 % acetic acid in water and dried. Next, 125 μL of Tris-NaOH (pH 10) was added to resuspend the SRB dye, and optical density

was measured at 510 nm and the background measurement at 620 nm in Biotek Cytation 3 reader (Biotek Instruments, Winooski, VT, USA).

2.6. Oxygen consumption and Extracellular acidification rate

Cells were seeded in 96-well plate in the same conditions described above at a density of 5000 cells/100µL/well. After incubation time, oxygen consumption was measured at 37 °C using a Seahorse XF^{e}96 Extracellular Flux Analyzer (Seahorse Bioscience, Germany). In addition, an XF^{e}96 sensor cartridge for each cell plate was placed in a 96-well calibration plate containing 200 µL/well calibration buffer and left to hydrate overnight at 37 °C. The cell culture medium was replaced one hour before the time point and was incubated at 37 °C in low buffered free-serum minimal DMEM (102353, Bioscience) medium supplemented with 1mM pyruvate, 6 mM glutamine and 5 mM glucose, and the pH was adjusted to 7.4 to allow the temperature and pH of the medium to reach equilibrium before the first-rate measurement. Oligomycin, FCCP, rotenone and antimycin A were prepared in DMSO.

For oxygen consumption rate (OCR) measurements, 2 µM oligomycin injected into reagent delivery port A. 1 µM FCCP injected into port B, which followed the injection of oligomycin was diluted in low-buffered serum-free DMEM medium. One µM rotenone and 1 µM antimycin A injected into reagent delivery port C was diluted in low-buffered serum-free DMEM medium and the pH adjusted to 7.4 with 1 M NaOH. 25 µL of compounds was then pre-loaded into the ports of each well in the XF^{e}96 sensor cartridge. The sensor cartridge and the calibration plate were loaded into the XF^{e}96 Extracellular Flux Analyzer for calibration. When the calibration was complete, the calibration plate was replaced with the study plate. Three baseline rate measurements of OCR of the NHDF cells were made using a 3 min mix, 5 min measure cycle. The compounds were then pneumatically injected by the XF^{e}96 Analyzer into each well, mixed and OCR measurements made using a 3 min mix, 5 min measure cycle.

For extracellular acidification rate (ECAR), three baseline rate measurements of ECAR of the NHDF cells were made using a 3 min mix, 5 min measure cycle. The compounds were then pneumatically injected by the XF^{e}96 Analyzer into each well, mixed and ECAR measurements made using a 3 min mix, 5 min measure cycle. Results were analysed by using the Software Version Wave Desktop 2.6.

2.7. Mitochondrial copy number

Mitochondrial DNA copy number measurement was performed using quantitative polymerase chain reaction (qPCR). RNase-treated total DNA was first isolated using the

Qiagen DNeasy kit according to the manufactor's recommended protocol. DNA abundance and purity were assessed in a NanoDrop 2000 spectrophotometer. 1µg DNA was used as template for qPCR based on amplification of cytochrome B (encoded on the mitochondrial genome; variable number in each cell) and beta-2-microglobulin (encoded on the nuclear genome; fixed number in each cell) using a Roche LightCycler and Roche FastStart DNA Master SYBR Green protocols. Each reaction was performed in duplicate. Each qPCR experiment contained parallel reactions in which standards with serial dilutions of purified amplicon used as template; reactions with no template served as negative control. The specificity of each reaction for a single product was verified by melting analysis. The cycle number of linear amplifications for each sample was compared with the five-point standard curve to determine the number of template copies present at the start of each reaction. To estimate the mitochondrial copy number relative to nuclear genomes, the number of copies of cytochrome B template was divided by the number of copies of beta-2-microglobulin template.

2.8. Western blotting

To obtain total cellular extracts, all cells were harvested with PBS-EDTA and washed once with PBS 1X. In order to collect total cells, two centrifugation steps were performed for 5 min at 1000xg (4 °C). Cellular pellet was resuspended in cell lysis buffer 1X (Bio-Rad, 9803) supplemented with 100 µM phenylmethylsulfonyl fluoride (PMSF). Protein contents were determined by the Bradford method using bovine serum albumin (BSA) as a standard. After denaturation at 100 °C for 5 min in Laemmli buffer (from Bio-Rad) and sonicated 3 three times (cycles of 15 seconds), an equivalent amount of proteins (20–50 µg) was separated by electrophoresis on 12 % SDS–polyacrylamide gels (SDS–PAGE) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5 % milk in TBS-T (50 mM Tris–HCl, pH 8; 154 mM NaCl and 0.1 % tween 20) for 2 h at room temperature, membranes were incubated overnight at 4 °C with the antibodies directed against the denatured form of OXPHOS complexes cocktail (1:1000; ab110411, Abcam, Cambridge, UK), Sirtuin 1 (1:1000; ab110304, Abcam, Cambridge, UK), Sirtuin 3 (1:1000; 1675490S, Cell Signalling Technology, Danvers, Massachusetts, USA) and Actin (1:5000; MAB1501, Chemicon international-Fisher Scientific, Hampton, New Hampshire, USA). Membranes were further incubated with goat anti-mouse IgG (1:2500) and goat anti-rabbit IgG (1:2500) secondary antibodies, for 1 h at room temperature. Membranes were then incubated with the ECF detection system (from GE Healthcare, Chicago, Illinois, USA) and imaged with the Biospectrum—Multispectral imaging system (UVP; LLC Upland, CA; Cambridge, UK). The densities of each band were calculated with Quantity One Software (Bio- Rad). The

membranes were later stained with Ponceau S solution (Sigma-Aldrich, Saint Louis, Missouri, USA) to confirm equivalent protein loading in each lane, an accurate method to confirm equal protein load.

2.9. Intracellular pH

Cytosolic pH (pHc) was measured using the pH-sensitive reporter molecule BCECF-AM (2,7-Bis-(2-Carboxyethyl)-5-(and-6)- Carboxyfluorescein acetoxymethyl ester). The fluorescence of H^+-bound form, excited at 440 nm and H^+-unbound form excited at 490 emitted at 530 nm was recorded and the ratio between the emission signal obtained at 490 nm at 440 nm excitation was used as a measure of intracellular pH. Briefly, cells were loaded with BCECF-AM by incubating them in microscopy medium for 15 min at 37 °C and 5 % CO_2 in the dark. Then, cells were washed 3 times with microscopy medium without BCECF and fluorescence signals were quantified using a microplate reader (Cytation 3; BioTek US, Winooski, VT, USA). The protonated and deprotonated forms of BCECF were excited at 440 nm and 490 nm, respectively, and BCECF fluorescence was quantified at 530 nm. The ratio between the emission signal obtained following 490 and 440 excitation was used as a measure of cytosolic pH (pHc).

2.10. Cell metabolic activity

Cell metabolic activity was assessed through the resazurin reduction assay. After the incubation time, the culture medium was removed, and cells were incubated for 1 hour with 80 µL of culture medium supplemented with 10 µg/mL resazurin. The amount of resazurin reduced to resorufin, indicative of metabolic activity, was measured fluorimetrically with 540 nm excitation and 590 nm emission in Biotek Cytation 3 reader (Biotek Instruments, Winooski, VT, USA).

2.11. Intracellular ATP levels

Cells were seeded in 150 µL of culture medium, in a white opaque-bottom, 96-well plate and then subjected to the different treatments. After incubation time, intracellular ATP levels were measured by using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, Wisconsin, USA) following manufacturer's instructions. Briefly, 100 µL of culture medium was removed from the wells and 50 µL of medium containing CellTiter-Glo® Reagent (CellTiter-Glo® Buffer + CellTiter-Glo® Substrate) was added to the cells. Contents were mixed for 2 minutes on an orbital shaker to induce cell lysis and, after 10 minutes of incubation at 22 °C, the luminescence signal was monitored in a Cytation 3 reader (BioTek Instruments Inc., USA). ATP standard curve was also generated following manufacturer's instructions. Luminescence signal is proportional to the amount of ATP present in solution.

2.12. Vital epifluorescence microscopy

Vital epifluorescence microscopy was used to detect the presence of acidic bodies (lysosomes) after treatment of NHDF cells with mitochondria-targeted antioxidants. Cells were seeded in 6 well plates with a glass coverslip in each well, at a density of 30,000 cells/mL with a final volume of 3 mL per well. Bafilomicin (0.5 µM, 4h) and trichostatine (2 µM, 24 h) were used as negative control and positive control, respectively, which bafilomicin inhibits the autophagosome formation and trichostatine enhances autophagy. Thirty minutes before the end of the incubation, cells were incubated with TMRM (100 nM) and Lysotracker Green (75 nM) in microscopy medium at 37 °C and 5 % CO_2 in the dark. Images were acquired using a Nikon Eclipse TE2000U microscope (Nikon Instruments, Amsterdam, The Netherlands) equipped with a x40 Plan Fluor 0.6 NA objective (Nikon) and analysed with ImageJ Fiji program (version Win64).

2.13. Cell proliferation rate

Cell proliferation rate was assessed through measurement of cell mass with the sulforhodamine B assay. Cells were cultured in low glucose and OXPHOS medium in the presence of mitochondria-targeted antioxidants and the SRB assay was performed at respective time points (0 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h) as previous described.

2.14. Oxidative stress protection

The antioxidant efficiency of mitochondria-targeted antioxidants in the presence of oxidative stressors was evaluated in both cells culture medium (low glucose and OXPHOS medium. Cells were seeded in 96-well plate and incubated with MitoBEN_2 (12.5 µM), MitoCIN_4 (12.5 µM), MitoCIN_6 (0.39 µM) for 72 h. Then, the oxidative stress-induced agent *t*-BHP (250 µM) was added to culture medium of NHDF cells for 3 hours more. After incubation time, cellular metabolic activity was determined as previously described.

2.15. Statistics

Data were analyzed in GraphPad Prism 5.0 software (GraphPad Software, Inc.), with all results being expressed as means ± SEM for the number of experiments indicated. The student's t-test for comparison of two means, and one-way and two-way ANOVA with Dunnet multiple comparison post-test was used to compare more than two groups with one and two independent variable respectively were used in data analysis. Significance was accepted with *P<0.05, **P<0.01, ***P<0.0005, ****P<0.0001.

Results and discussion

In the realm of hormetic responses, a low dose of a toxin can activate an adaptive compensatory process following an initial disruption in cell homeostasis. Therefore, a toxin which in high doses is toxic to cells or organisms, when administrated in low doses can trigger an adaptative mechanism defence, in a biphasic or non-linear response to potentially harmful substances (Mattson, 2008). The transient increase in stressor agents, such as ROS, can induce a hormetic mechanism response. Thus, oxidant agents may act as potential regulators of several intracellular pathways, activating the gene expression of oxidative stress defence system, allowing the cell to avoid the injuries caused by these kind of stressors agents (Finkel, 2012). In this scenario, mitochondria play an important role. Mitochondria can adapt to external factors and can respond to energetic demand by producing effectors that activate multiple pathways, the majority related to OS in a process called mitochondrial hormesis or mitohormesis (Yun *et al*., 2014; Willems *et al*., 2015).

To dissect the long-term mechanism of action of novel mitochondria-targeted antioxidants MitoBEN$_2$ (gallic acid derivative) and MitoCIN$_4$ and MitoCIN$_6$ (caffeic acid derivatives), we used human dermal fibroblast. Skin is constantly subjected to external and internal stresses and dermal fibroblasts play not only an important role in the synthesis of extracellular matrix components, essential for the skin well-being, as they also are very vulnerable to stress agents (Menon, 2002; Proksch *et al*., 2008; Naylor *et al*., 2011).

3.1. Mitochondria-targeted antioxidants cytotoxicity in normal human dermal fibroblasts (NHDF)

In a previous work using human skin fibroblasts (HSF), we demonstrated that mitochondria-targeted antioxidants (MitoBEN$_2$, MitoCIN$_4$ and MitoCIN$_6$) long-term treatment (72h) dose-dependently decreased cell viability only at concentration above those required for their antioxidant activity, 12.5 μM, 12.5 μM, and 0.39 μM, respectively (Teixeira *et al*., unpublished work). Herein, we used NHDF to test if and whether mitochondria-targeted antioxidants long-term treatment may trigger a mitochondrial hormetic response. Initially mitochondria-targeted antioxidants cytotoxicity was assessed in this cell line at concentrations that previously showed no cytotoxic and

remarkable antioxidant activity. The cytotoxicity was assessed using two different methods: nuclei staining with Hoechst 33342 for quantification of viable cell number; and sulforhodamine B (SRB) assay for determination of cell/protein mass. Despite the eventual metabolic and phenotypic differences between cell lines (HSF vs. NHDF), MitoBEN$_2$, MitoCIN$_4$ and MitoCIN$_6$ do not reveal cytotoxic effects at the tested concentrations, measured as both nuclei number (Figure 6 A) and cell mass (Figure 6 B). In fact, MitoCIN$_4$ and MitoCIN$_6$ promoted a significant increase in cell mass (Figure 6). MitoBEN$_2$, MitoCIN$_4$ and MitoCIN$_6$ are chemical and structurally different, derived from HBA and HCA, respectively and with different lipophilic profiles, which may explain their different cytotoxicity profile toward NHDF. Importantly, the mitochondria-targeted antioxidants did not cause cytotoxicity in NHDF cells at the same concentrations performed in HSF.

The TPP delocalized cations are hydrophobic and can freely pass through the phospholipid bilayers of the plasma membrane and other organelles, without the requirement for a specific uptake mechanism (Reily *et al*., 2013), and despite the TPP^+-uptake is not uniform across different tissues and different organs, that depends of the mitochondrial quantity by cell and its mΔΨ (Smith *et al*., 2011; Smith *et al*., 2012). In this case, the difference in concentrations of mitochondria-targeted antioxidants used is mainly due to differences in the lipophilicity profile of antioxidants.

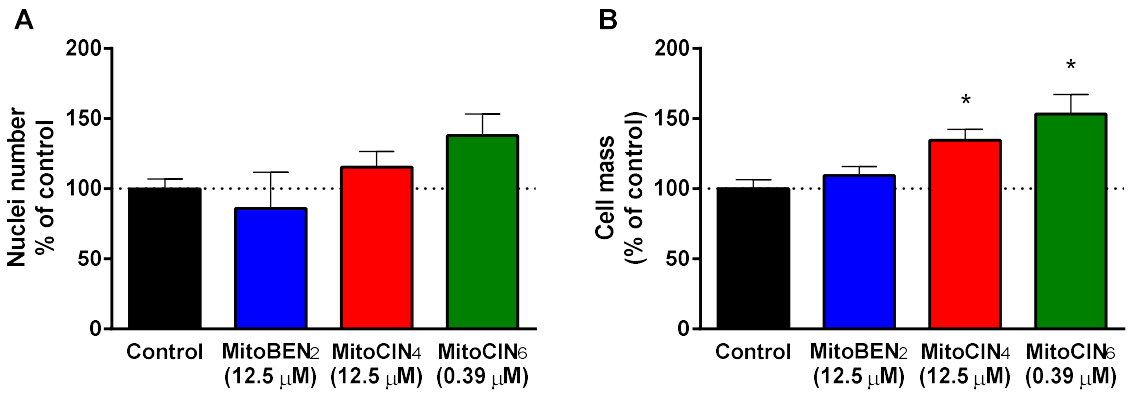
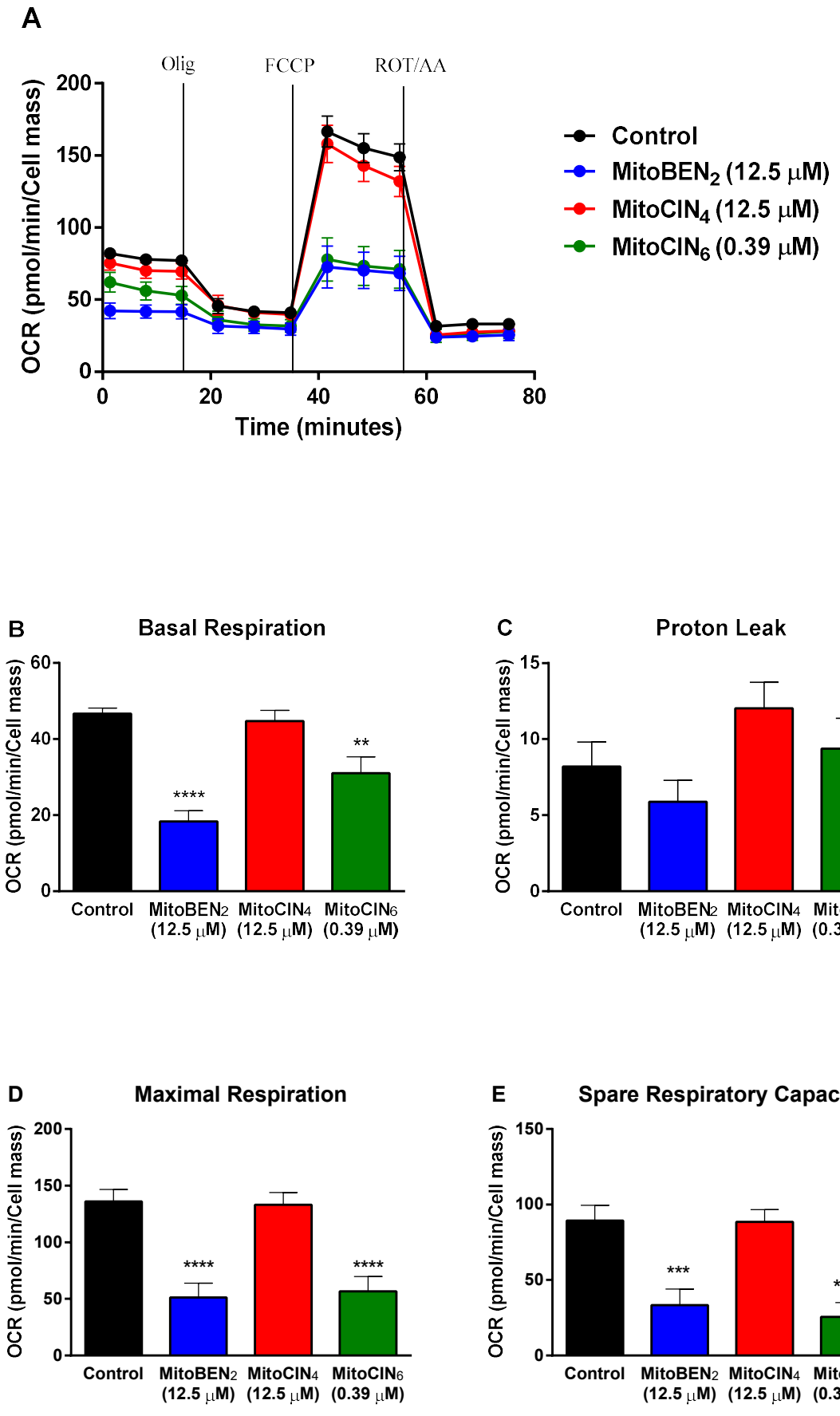


Figure 6. Cytotoxicity of mitochondria-targeted antioxidants on NHDF cells. NHDF cells were seeded and incubated during 72 hours with mitochondria-targeted antioxidants: MitoBEN$_2$ (12.5 μM), MitoCIN$_4$ (12.5 μM) and MitoCIN$_6$ (0.39 μM) cytotoxicity was evaluated through changes in A) nuclei number and B) cell mass of NHDF cells. Cell number was determined by measuring the number of nucleus in living cells stained with Hoechst 33342, while cell mass was measured through the SRB assay. Data are means ± SEM of three and seven respectively independent experiments and the results are expressed as percentage of control (control = 100 %), which represents the cells without any treatment in the respective time point. Statistically significant compared with control group using one-way ANOVA. Significance was accepted with *P<0.05 vs. control.

3.2. Mitochondrial oxygen consumption

In cells that have sufficiently active glycolysis to support metabolism while mitochondrial function is manipulated, all the major aspects of mitochondrial coupling and respiratory control can be measured in a single experiment (Brand and Nicholls, 2011). The analysis of the effects of the potential therapeutic mitochondria-targeted antioxidants on both oxygen consumption rate (OCR) - mitochondrial respiration - and extracellular acidification rate (ECAR), which indirectly an provide information on glycolytic rates, of whole cells was performed using the "mitostress test". The "mitostress test" uses selected mitochondrial inhibitors which allow determination of six main parameters that describe key aspects of mitochondrial function in a cellular context: basal OCR, ATP-linked OCR, proton leak OCR, maximal OCR, spare respiratory capacity, and non-mitochondrial OCR.

Next, the mitochondrial function of cells treated with mitochondria-targeted antioxidants was assessed at concentrations that are pharmacologically active and at which cytotoxic effects were not visible, through measurements of OCR using the Seahorse XF^e96 Extracellular Flux Analyser. The oxygen consumption was measured in cells pre-treated with mitochondria-targeted antioxidants for 72 hours, and several parameters related with oxygen consumption were measured following the acute administration of specific metabolic inhibitors, such as oligomycin, FCCP, rotenone and antimycin A (Figure 7 A). After oligomycin injection, F_1F_o-ATP-synthase was inhibited to consequently halt ATP production, and the oxygen consumed related with energy respiration. An injection of FCCP followed, a mitochondrial membrane uncoupler which dissipate the proton gradient through MIM forcing the maximal oxygen consumption to replace the protons gradient. Finally, the injection of rotenone and antimycin A inhibiting the ETC, stopped the mitochondrial oxygen consumption by mitochondria (Dranka *et al*., 2011).


A
Olig
FCCP
ROT/AA
OCR (pmol/min/Cell mass)
Time (minutes)
Control
MitoBEN2 (12.5 μM)
MitoCIN4 (12.5 μM)
MitoCIN6 (0.39 μM)
B
Basal Respiration
C
Proton Leak
D
Maximal Respiration
E
Spare Respiratory Capacity
Control
MitoBEN2 (12.5 μM)
MitoCIN4 (12.5 μM)
MitoCIN6 (0.39 μM)

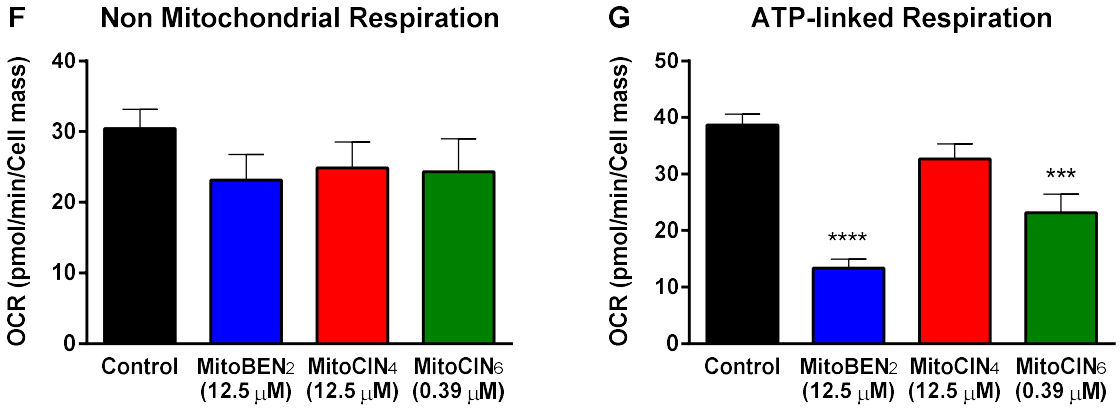


Figure 7. Effect of mitochondria-targeted antioxidants on mitochondrial oxygen consumption of NHDF cells. NHDF cells were seeded and incubated during 72 hours with non-toxic concentrations of MitoBEN$_2$ (12.5 μM), MitoCIN$_4$ (12.5 μM) and MitoCIN$_6$ (0.39 μM) and (A) the Seahorse XF^{e}96 Extracellular Flux Analyzer measured cellular oxygen consumption rate (OCR). Several respiratory parameters were evaluated: (B) cell basal respiration; (C) proton leak (minimal OCR after oligomycin injection minus non-mitochondrial derived OCR); (D) maximal respiration (maximal measurement after FCCP addition minus non-mitochondria derived OCR); (E) spare respiratory capacity (maximal respiration minus basal respiration); (F) non mitochondrial respiration (minimal OCR measurement after rotenone and antimycin A addition); and (G) oxygen consumption associated with ATP production (basal respiration minus proton leak). Data are means ± SEM of eleven independent experiments and the results are expressed as pmol O_2 / min / cell mass for OCR. Statistically significant compared with control group using one-way ANOVA. Significance was accepted with **P<0.01, ***P<0.0005, ****P<0.0001 vs. control.

In general, treating cells with MitoCIN$_4$ (12.5 μM) did not affect mitochondrial function, while treating cells with MitoBEN$_2$ (12.5 μM) or MitoCIN$_6$ (0.39 μM) decreased mitochondrial OCR, at concentrations without cytotoxic effect.

MitoBEN$_2$ decreased mitochondrial function as measured by the significant decrease in basal respiration (Figure 7 B), maximal respiration (Figure 7 D), spare respiratory capacity (Figure 7 E) and consumed oxygen for ATP production (Figure 7 G). Following the same trend, MitoCIN$_6$ also decreased mitochondrial function as measured by the significant decreased in basal respiration (Figure 7 B), maximal respiration (Figure 7 D), spare respiratory capacity (Figure 7 E), and consumed oxygen for ATP production (Figure 7 G). In MitoCIN$_4$-treated cells, although a slight increase in proton leak (Figure 7 C) paralleled by a slight decrease in oxygen consumed for ATP production (Figure 7 G) was observed, MitoCIN$_4$ (12.5 μM) did not affect mitochondrial function.

Validation of mitochondria-targeted antioxidants as potential therapeutic agents is necessary to understand how cells respond to changes in its physical properties, employing different cationic moieties and varying the carbon chain linkers to attach functional groups (HBA or HCA antioxidants). At concentrations levels which are found

to be pharmacologically active, mitochondrial respiration is frequently reduced in cells treated with triphenylphosphonium (TPP^+) cation-derived agents bearing molecules with antioxidant properties, such as ubiquinol, piperidine nitroxide TEMPOL or α-tocopherol (Reily *et al*., 2013). Comparing the mitochondrial respiration of $MitoCIN_4$ and $MitoCIN_6$ (both HCA derivatives), the results suggest the negative dependence of mitochondrial respiration of the length of alkyl-TPP^+ chain.

Gallic acid and caffeic acid exhibit different effects on mitochondrial respiration, with gallic acid inhibiting mitochondrial respiration by decreasing OCR on basal and maximal respiration, while caffeic acid keep mitochondrial respiration parameters in normal levels even in short anoxia-reoxygenation events (Feng *et al*., 2008; Gu *et al*., 2012). Comparing $MitoBEN_2$ with $MitoCIN_4$ having with the same alkyl-TPP^+ chain length, a decrease of OCR on basal and maximal respiration in $MitoBEN_2$ treated cells was observed, probably due to the presence of a gallic acid moiety in its structure.

The observed effects of $MitoBEN_2$ and $MitoCIN_6$ on mitochondrial function, in the absence of cytotoxic effects, may correspond to disruption in cell homeostasis, which might be beneficial rather than detrimental. In fact, a mild inhibition of mitochondrial respiratory chain could be a protective response associated to a moderate elevation of mtROS protection (Zelenka *et al*., 2015). Despite the well-known described toxicological effects of TPP^+, the data showed that $MitoCIN_4$ did not affect mitochondrial function of NHDF cells.

3.3. Evaluating mitochondrial DNA copy number

Evidence suggests that mtDNA copy number variation might be related with altered mitochondrial function. For instance, mtDNA copy number is significantly increased in apoptotic tumour cells and may serve to protect tumour cells against apoptosis, while reduced mtDNA copy number significantly increased the ROS levels in tumour cells increasing the sensitivity of tumour cells to chemotherapeutic drugs (Mei *et al*., 2015). Moreover, depletion in mtDNA copy number is associated with decreased mitochondrial function (Yu *et al*., 2009). In that trend, we assessed the effect of mitochondria-targeted antioxidants on mtDNA copy number using qPCR. The ratio of a mtDNA gene (cytochrome B) by nuclear gene (β-2-microglobulin) allows determining the number of mtDNA molecules per cell.

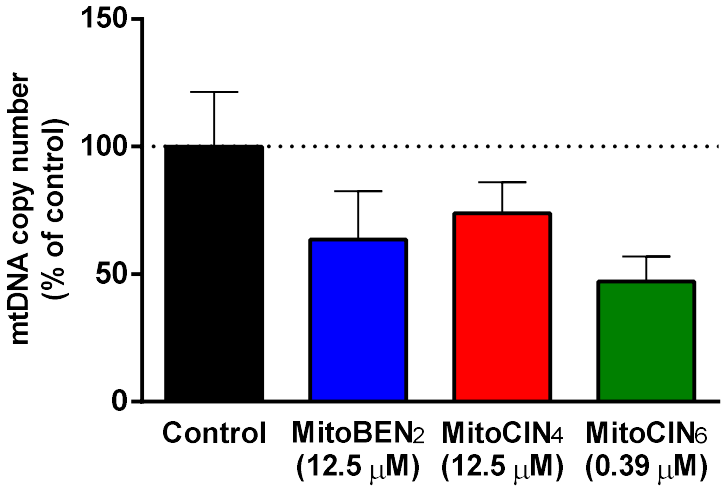


Figure 8. Effect of mitochondria-targeted antioxidants on mtDNA copy number of NHDF cells. NHDF cells were seeded and incubated during 72 hours with non-toxic concentrations: $MitoBEN_2$ (12.5μM), $MitoCIN_4$ (12.5μM) and $MitoCIN_6$ (0.39μM) and the mtDNA copy number was evaluated by qPCR based on amplification of cytochrome B (encoded on the mitochondrial genome; variable number in each cell) and β-2-microglobulin (encoded on the nuclear genome; fixed number in each cell) using a Roche LightCycler and Roche FastStart DNA Master SYBR Green protocols. Data are means ± SEM of six independent experiments and the results are expressed as percentage of control (control = 100 %), which represents the cells without any treatment in the respective time point. Statistically significant compared with control group using one-way ANOVA.

In general, mitochondria-targeted antioxidants decreased mtDNA copy number, although the results were not significant. This effect is more evident in $MitoBEN_2$ and $MitoCIN_6$, a fact that could be explained by the decrease in mitochondrial oxygen consumption. In $MitoCIN_4$-treated cells, the decrease in mitochondrial DNA copy was not so evident (Figure 8).

Although not statistically significant, the data obtained seemed to be consistent with the observed decreased in oxygen consumption by mitochondria. The observed decrease in mtDNA copy number in cells treated with mitochondria-targeted antioxidants, more noticeable in $MitoBEN_2$ and $MitoCIN_6$, may be explained by the increase in ROS production by mitochondria and/or the occurrence of mitochondrial fusion/fission events. These events are described as a protection mechanism of mitochondria towards mild oxidative stress (Youle and Van Der Bliek, 2012) to maintain mtDNA levels, fidelity, and toleration to high levels of mtDNA mutations (Chen *et al*., 2010). Other antioxidants such as resveratrol seems to induce the mitochondrial biogenesis by increasing the mtDNA copy number (Lagouge *et al*., 2006), while the mitochondria-targeted antioxidant MitoQ had no effect on mtDNA copy number (Rodriguez-Cuenca *et al*., 2010).

3.4 OXPHOS complexes

The decrease of basal oxygen consumption rate suggests a decrease of mitochondrial function and/or a decrease of mitochondrial mass in a general way, supported by the slight decrease of mtDNA copy number. Mitochondrial respiration and function are linked to mitochondrial electron transport chain, which is coupled with ATP synthase forming the mitochondrial oxidative phosphorylation (OXPHOS) system.

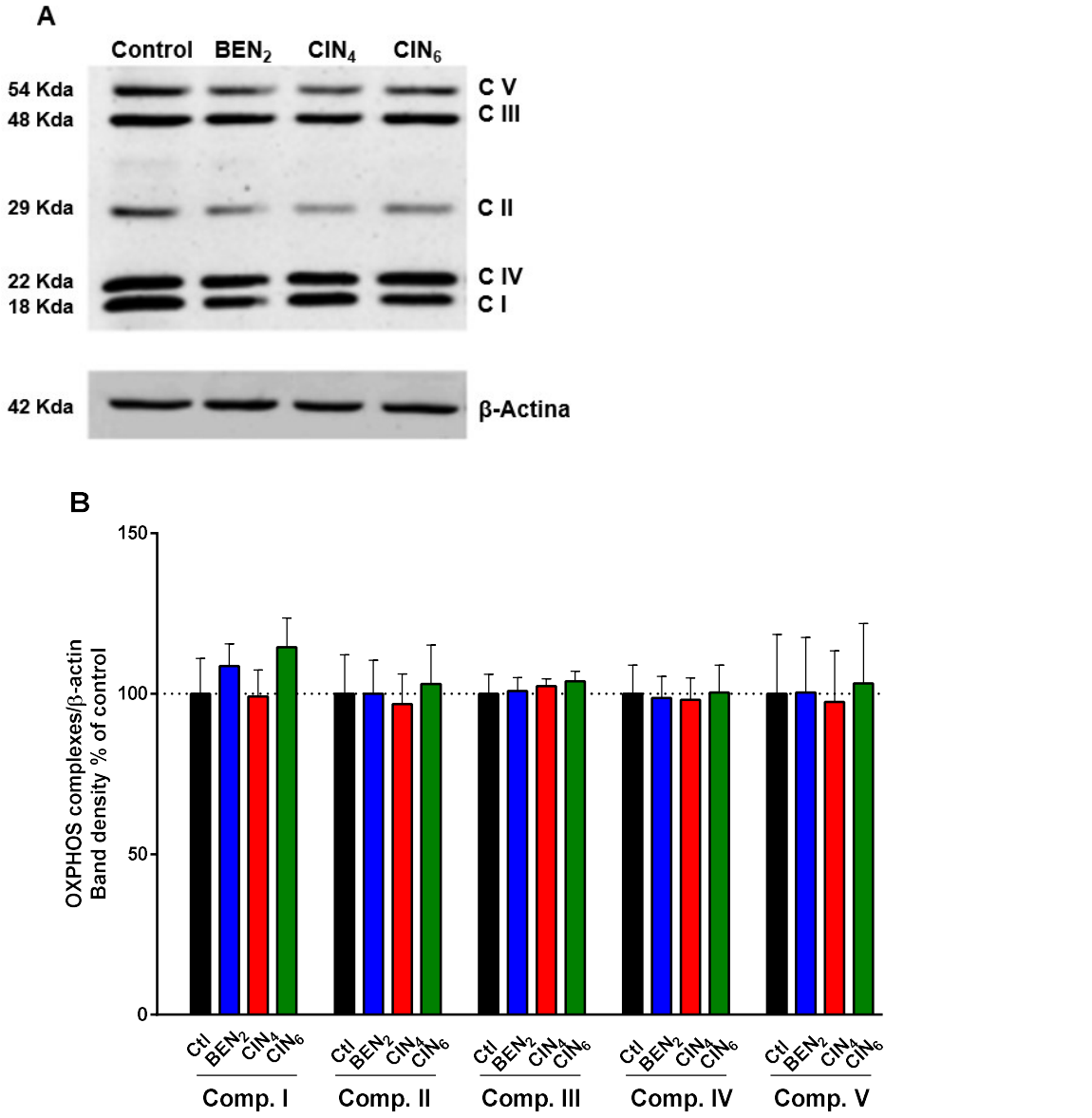


Figure 9. Effect of mitochondria-targeted antioxidants on mitochondrial OXPHOS complexes protein content of NHDF cells. NHDF cells were seeded and incubated during 72 hours with non-toxic concentrations: MitoBEN$_2$ (12.5μM), MitoCIN$_4$ (12.5μM) and MitoCIN$_6$ (0.39μM). (A) Western blot signal in cells treated with MitoBEN$_2$, MitoCIN$_4$ and MitoCIN$_6$. (B) Western blot analysis of mitochondrial OXPHOS complexes protein content. Each data point of different OXPHOS subunit levels was normalized to β-actin protein levels. Data are means ± SEM of six independent experiments and the results are expressed as percentage of control (control = 100 %), which represents the cells without any treatment in the respective time point.

Consequently, we next investigated the effect of mitochondria-targeted antioxidants on mitochondrial OXPHOS complexes protein content.

The western blot analysis revealed that neither MitoBEN$_2$, MitoCIN$_4$ nor MitoCIN$_6$ treatment altered the OXPHOS complexes protein content, including nuclear-encoded complex I NDUFB8 subunit (Emahazion and Brookes, 1998), complex II SDHB subunit coded by nuclear DNA (Baysal *et al*., 2000), nuclear genome complex III subunit core 2 (Jang *et al*., 2013), mtDNA-encoded complex IV MTCO1 subunit (Vissing *et al*., 2013) and nuclear-encoded complex V subunit alpha (Papa *et al*., 2017).

Despite the non-significant decrease of mtDNA copy number and the decrease of mitochondrial respiration in MitoBEN$_2$- and MitoCIN$_6$-treated cells, there was no impact on mitochondrial OXPHOS content, supporting the idea of a possibility mitochondrial fusion to maintain OXPHOS integrity. Another phenolic compounds such as epigallocatechin-3-gallate seems to restore mitochondrial biogenesis in Down Syndrome patients' cells (Valenti *et al*., 2013), while the mitochondria-targeted antioxidant MitoQ increased of mitochondrial respiration and increased of OXPHOS enzymes content (Plecitá-Hlavatá *et al*., 2009).

These results suggest that, particularly in MitoBEN$_2$- and MitoCIN$_6$-treated cells, mitochondria still have the total amount of OXPHOS machinery for proper function, such as energy production, although probably working at lower metabolic rates.

3.5. Mitochondria-targeted antioxidants did not change ATP levels or overall metabolic activity

Under certain stress situations, such as mitochondria lower function, cells can find alternative pathways to adapt their metabolism and function in order to supply their requirements and, ultimately, for surviving. The reduced basal oxygen consumption found particularly in MitoBEN$_2$ and MitoCIN$_6$ -treated cells suggest that cells appeal to another energy source rather than oxidative phosphorylation.

With this is mind, we next focused our interest in understanding how and whether mitochondria-targeted antioxidants affect cellular energetic competence. To do so, metabolic activity and intracellular ATP levels were measured, using the resazurin reduction assay and the commercially available CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega), respectively. Resazurin dye, which can be reduced by cellular dehydrogenases of viable cells to resorufin, allows the estimation of cellular metabolic activity. The intracellular ATP levels were quantified throughout the mono-oxygenation

of luciferin catalysed by luciferase in the presence of Mg^{2+}, ATP and molecular oxygen. Luciferase emits a luminescent signal that is converted into nmols ATP (Hannah *et al*., 2001).

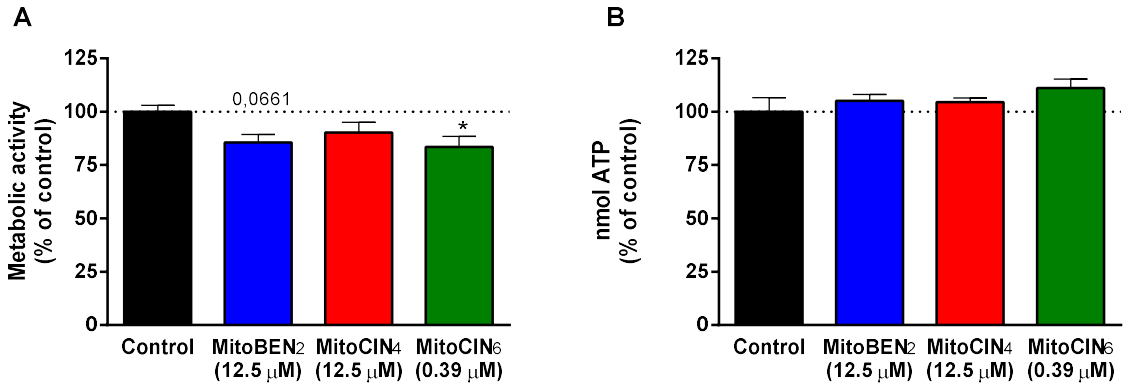


Figure 10. Effect of mitochondria-targeted antioxidants on metabolic competence of NHDF cells. NHDF cells were seeded and incubated during 72 hours with mitochondria-targeted antioxidants in non-toxic concentrations: $MitoBEN_2$ (12.5μM) and $MitoCIN_4$ (12.5μM) and $MitoCIN_6$ (0.39μM), and the metabolic competence was evaluated through measurements of A) cellular metabolic activity and B) intracellular ATP levels. Cell metabolic activity was measured through the resazurin reduction assay and ATP levels were measured through the commercially CellTiter-Glo Luminescent Cell Viability Assay Kit. Data are means ± SEM of six and three respectively independent experiments and the results are expressed as percentage of the control (control = 100 %), which represents the cells without any treatment in the respective time point Statistically significant compared with control group using one-way ANOVA. Significance was accepted with *P<0.05 vs. control.

Under these conditions, $MitoBEN_2$ and $MitoCIN_6$ significantly decreased cellular metabolic activity (Figure 10 A), while metabolic activity of $MitoCIN_4$-tretaed cells was not altered (Figure 10). Surprisingly, treating NHDF cells with mitochondria-targeted antioxidants did not alter intracellular ATP levels (Figure 10B). These results suggest that NHDF cells treated with mitochondria-targeted antioxidants, even with decreased mitochondrial O_2 consumption and reduced dehydrogenases activity, have the ability to generate ATP from other sources, probably from glycolysis.

Despite the mitochondrial function is compromised in $MitoBEN_2$- and $MitoCIN_6$-treated cells, the intracellular ATP levels remain unchanged, probably due a metabolic shift on energy supply from OXPHOS to glycolysis. That is a feature that was already observed by treating cells with some polyphenols, which are able to regulate the key pathways of the carbohydrate's metabolism, including glycolysis upregulation (Bahadoran *et al*., 2013). In fact, cells treated with gallic acid showed increased capacity for glucose uptake (Prasad *et al*., 2010), Fink *et al*. also found a glycolysis upregulation in cells treated with MitoQ that also decreased mitochondrial function (Fink *et al*., 2012). The capacity of cells to maintain the energy supply reveal that mitochondriotropic

antioxidants may trigger a compensatory hormetic response, which first induce a stressor transient condition that will initiate an adaptive response by cells, the decrease of mitochondrial function is compensated by another source of energy in an adaptative response protecting the cell against stressor events such as energy deprivation (Calabrese *et al*., 2012).

3.6. Effect of mitochondria-targeted antioxidants on physiological pH

In most mammalian cells, cellular energy in the form of ATP is generated by the integrated action of the glycolysis pathway in the cytosol, and the TCA cycle and OXPHOS system in the mitochondrion. Alterations in cellular energy metabolism often induce extracellular acidification, the rate and mechanism of which depend on the cell type and used energy substrate (Teixeira *et al*., 2018b). The mild decrease on cell metabolic activity in MitoBEN$_2$- and MitoCIN$_6$-treated cells probably due to poor contribution of mitochondrial diaphorase (Borra *et al*., 2009), which may decrease their capacity to produce equivalent reducers essential to supply mitochondrial ETC, not paralleled by cells' reduced capacity to generate ATP, raise the question whether cells fulfil their energetic requirements.

Next, we evaluated the effect of mitochondria-targeted antioxidants on extracellular acidification, as an indirect indicator of the glycolytic pathway, through measurement of extracellular acidification rate (ECAR) using the Seahorse XF^{e}96 Extracellular Flux Analyser. The ECAR was assessed in basal conditions in cells treated with mitochondria-targeted antioxidants for 72h. In addition, intracellular acidification was also measured using the pH-sensitive reporter molecule BCECF-AM. The fluorescence of H^+-bound form, excited at 440 nm and H^+-unbound form excited at 490 emitted at 530 nm was recorded and the ratio between the emission signal obtained at 490 nm at 440 nm excitation was used as a measure of intracellular pH.

Under these conditions, MitoBEN$_2$- and MitoCIN$_6$- significantly increased ECAR (Figure 11 A), while MitoCIN$_4$ had no effect on this parameter (Figure 11 A), when compared with control cells. The observed increase may probably due to an increased glycolysis flux and may explain why MitoBEN$_2$- and MitoCIN$_6$-treated cells, at concentrations with minimal effects on metabolic activity, display similar intracellular ATP levels and cell mass when compared with control cells. Despite the increased ECAR

observed in MitoBEN$_2$- and MitoCIN$_6$-treated cells, no alterations were observed in intracellular acidification (Figure 11 B).

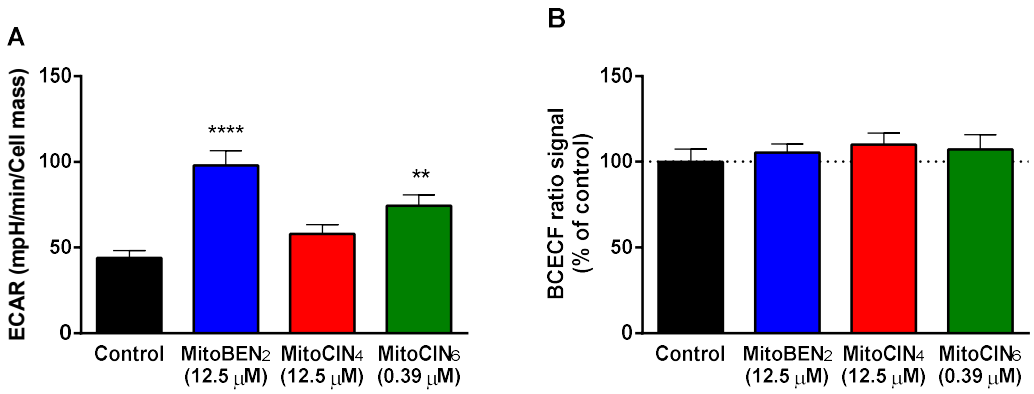


Figure 11. Effect of mitochondria-targeted antioxidants on physiological pH of NHDF cells. NHDF cells were seeded and incubated during 72 hours with mitochondria-targeted antioxidants at non-toxic concentrations: MitoBEN$_2$ (12.5μM) and MitoCIN$_4$ (12.5μM) and MitoCIN$_6$ (0.39μM), and their effects A) on extracellular acidification rate (ECAR) and B) intracellular pH was evaluated. Basal ECAR was measured using the Seahorse XF^{e}96 Extracellular Flux Analyzer. Data are means ± SEM of eleven independent experiments and the results are expressed as mpH / min / cell mass, while intracellular pH was measured using the pH-sensitive reporter molecule BCECF-AM. The fluorescence of H^+-bound form, excited at 440 nm and H^+-unbound form excited at 490 emitted at 530 nm was recorded. Data are means ± SEM of three independent experiments and the results express as % of control (control = 100 %) of the H^+-unbound/H^+-bound ratio. Statistically significant compared with control group using one-way ANOVA. Significance was accepted with **P<0.01, ****P<0.0005 vs. control.

Recent work described that fibroblasts can respond to mitochondrial dysfunction by up regulating the glycolytic pathway (Zelenka *et al*., 2015). Lactate as a bio-product of glycolytic pathway increase the extracellular acidification, a common feature also observed in MitoBEN$_2$- and MitoCIN$_6$-treated cells. Intracellular pH (pHi) has an important role in the maintenance of normal cell function, and hence this parameter has to be tightly controlled within a narrow range (Madshus, 1988). Lactate is easily deprotonated at physiologic pH increasing the intracellular [H^+], that is kept in a tight range of concentrations by the extrusion of protons by antiporter Na^+/H^+ channels or the lactate export machinery by monocarboxylate transporter (Ferrick *et al*., 2008; Tian and Bae 2012).

The data suggest that the metabolic shift from OXPHOS to glycolysis in MitoBEN$_2$- and MitoCIN$_6$-treated cells can supply the cell´s energy demand without affecting the maintenance of normal cell´s function, while the intracellular pH homeostasis is maintained, probably by protons extrusion or lactate export.

3.7. Effect of mitochondria-targeted antioxidants on cellular metabolic profile

To highlight the potential shift on metabolic profile of cells treated with mitochondria-targeted antioxidants, we next plotted the data from oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). These data facilitate the visual interpretation about the preference of cells' energy source obtained by both control and cells treated with mitochondria-targeted antioxidants.

In basal conditions, MitoBEN$_2$- and MitoCIN$_6$-treated cells showed increased ECAR, while the OCR decreased, suggesting that cells shifted their metabolic profile from an aerobic to a more glycolytic status (Figure 12). In MitoCIN$_4$-treated cells, this trend was not observed, as these cells presented normal OCR and ECAR when compared to control cells (Figure 12).

Several molecules may induce a shift on cells' energy metabolism from mitochondrial respiration to glycolysis without detrimental effects (Gohil *et al*., 2010). In fact, walnut phenolic extract upregulates the glycolytic pathway, although promoted the transcription of genes associated with mitochondrial function (Choi *et al*., 2018).

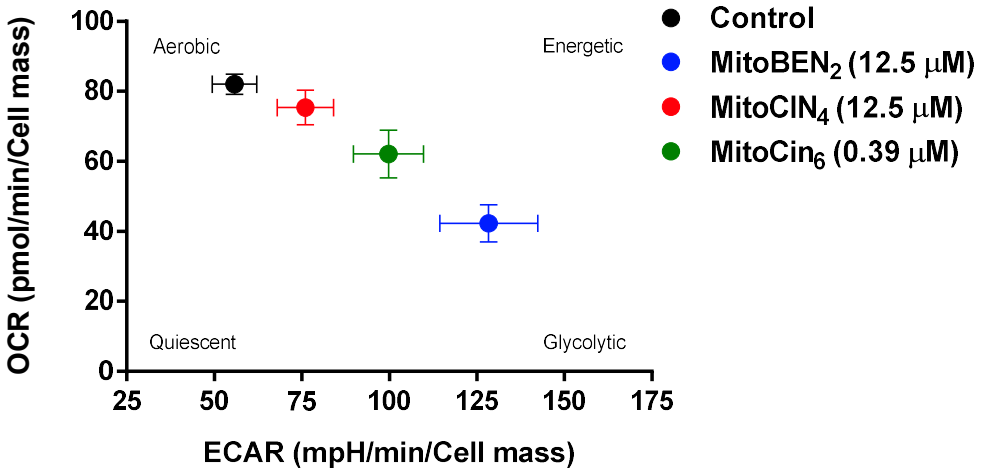


Figure 12. Energy map of NHDF cells treated with mitochondria-targeted antioxidants. Cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse XF^{e}96 Extracellular Flux Analyzer. Data are means ± SEM of eleven independent experiments and the results are expressed as pmol O_2 / min / cell mass or mpH / min / cell mass for OCR or ECAR, respectively.

As described above, another mitochondria-targeted antioxidants may also decrease OXPHOS pathway and increase glycolysis (Fink *et al*., 2012), suggesting a metabolic

shift from an aerobic pathway to a glycolytic pathway. The pro-oxidant property of such mitochondria-targeted antioxidants induces a mild and transient increase of ROS triggering protecting pathways, which may activate hypoxia response through Hif-1α transcription factor activation, that induce glycolytic pathways a way to decrease the ROS formation (Kim *et al*., 2006), promoting a hermetic-like response that a transient potential harmful feature triggers a protector mechanism defence (Pan, 2011).

3.8. Mitochondria-targeted antioxidants do not change sirtuin 1 and 3 protein content

In order to test if and whether mitochondria-targeted antioxidants effects on cells' metabolism were modulated by Sirt1 and/or Sirt3, we measured the protein content by Western Blotting after cell treatment.

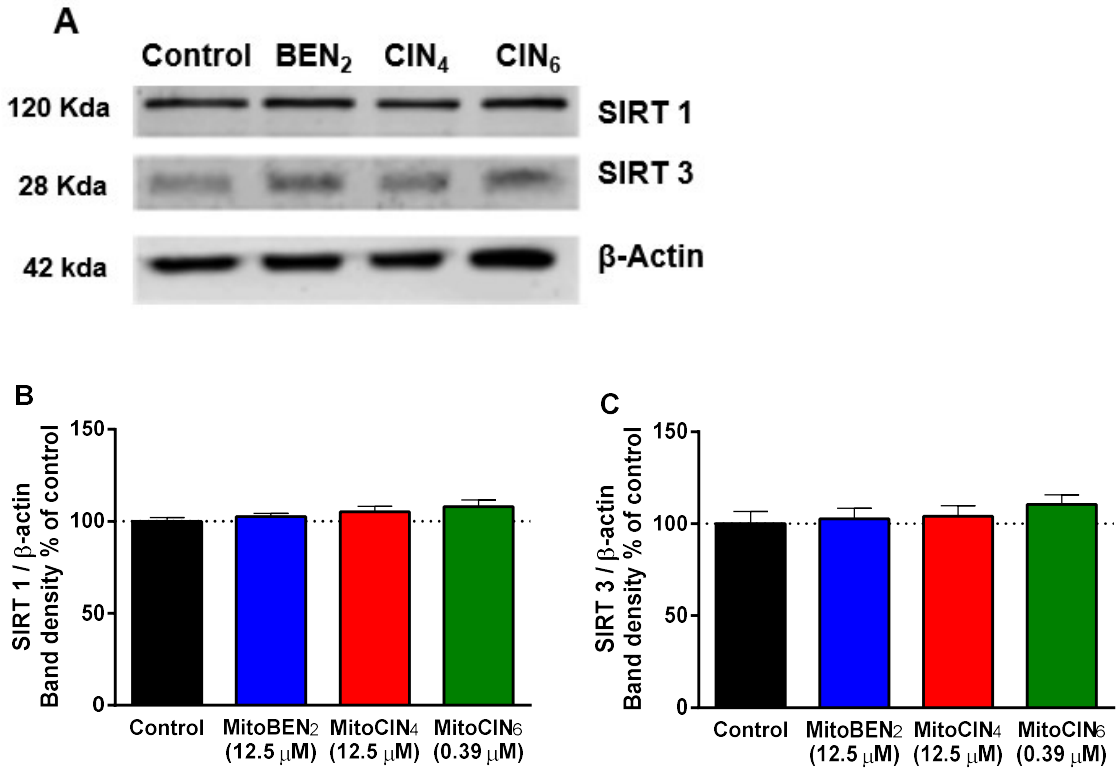


Figure 13. Effect of mitochondria-targeted antioxidants on metabolic regulators SIRT1 and SIRT3 protein content in NHDF cells. NHDF cells were seeded and incubated during 72 hours with non-toxic concentrations: MitoBEN$_2$ (12.5μM), MitoCIN$_4$ (12.5μM) and MitoCIN$_6$ (0.39μM). (A) Western blot signal in cells treated with MitoBEN$_2$, MitoCIN$_4$ and MitoCIN$_6$. Western blot analysis of (B) cytoplasmic SIRT1 and (C) mitochondrial SIRT3 protein levels. Each data point of different sirtuins levels was normalized to β-actin protein levels. Data are means ± SEM of six independent experiments and the results are expressed as percentage of the control (control = 100 %), which represents the cells without any treatment in the respective time point.

Western blot analysis revealed that, despite the impact of mitochondria-targeted antioxidants on cell's metabolism, in particular $MitoBEN_2$ and $MitoCIN_6$, these mitochondria-targeted antioxidants did not alter Sirt1 or Sirt3 protein content.

Some polyphenols such as resveratrol can induce Sirt1 activation, which allow the activation of cellular pathways (Scapagnini *et al*., 2014) including enhanced expression of SOD2 and activation of PGC-1α pathway (Ristow, 2014). At the end, resveratrol may confer protective effect to cells and increase life expectancy (Wood *et al*., 2004). Sirt3 downregulation induces the upregulation of Hif-1α that activate the glycolytic pathway (Finley *et al*., 2011).

Despite the observed effect of mitochondria-targeted antioxidants on cell metabolism, changes in Sirt1 and Sirt3 protein content was not observed, which suggest that these metabolic modulators were not involved in the $MitoBEN_2$ and $MitoCIN_6$ biological effects.

3.9. Effect of mitochondria-targeted antioxidants on mitophagy

To evaluate if mitophagy quality control mechanisms were affected in the presence of mitochondria-targeted antioxidants, cells were incubated with two fluorescence dyes: lysotracker green and TMRM. Lysotracker green is a fluorescent acidotropic dye for labelling and tracking acidic organelles in living cells, able to stain autophagosomes, while TMRM is a cell-permeant dye that accumulates in active mitochondria with intact $\Delta\Psi m$. In order to regulate mitophagy quality control mechanisms, Bafilomycin A (0.5 μM for 4 h), which inhibits the fusion between autophagosomes and lysosomes (Yamamoto *et al*., 1998), and Trichostatin A (2 μM for 24 h), a histone deacetylase inhibitor that enhances autophagy (Shao *et al*., 2016) were used as controls.

The images were analysed in FIJI software, in which red pixels represents the TMRM dye and correspond to energized mitochondria, while green pixels represent the lysotracker green dye staining acidic lysosomal bodies. The yellow pixels suggest co-localization of both probes, which is in turn, is indicative of the occurrence of mitophagy quality control mechanisms.

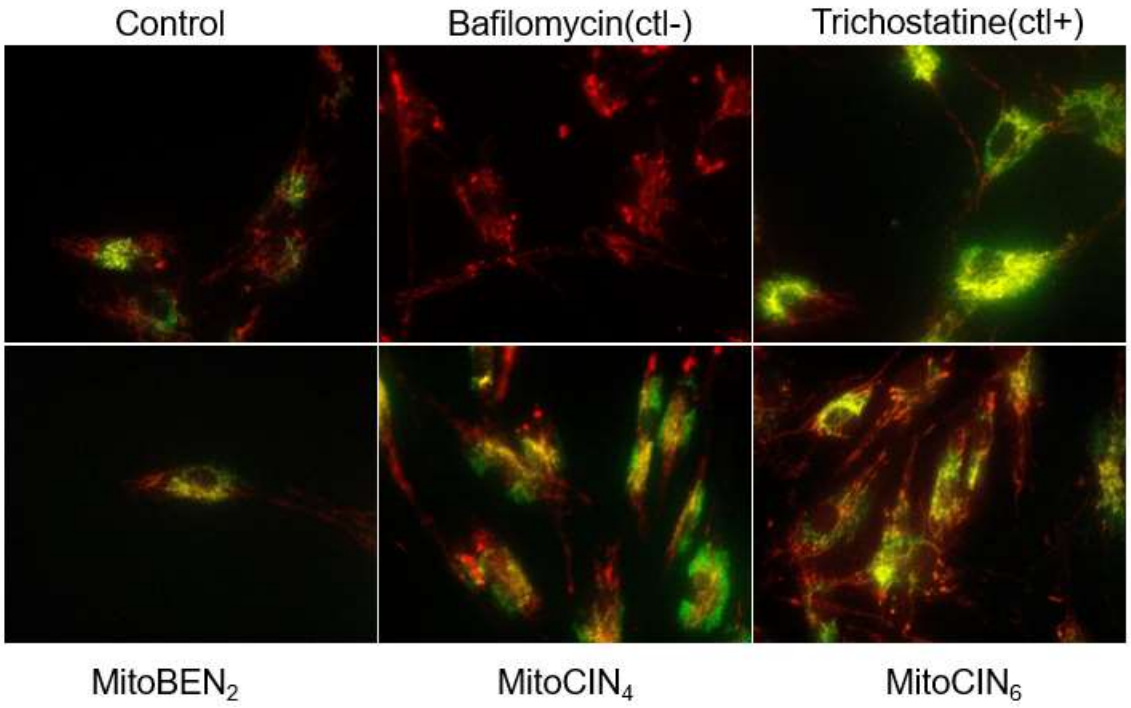


Figure 14. Effect of mitochondria-targeted antioxidants on mitophagy quality control mechanism of NHDF cells. Typical fluorescence images of NHDF cells stained with TMRM and lysotracker green after 72 h treatment with vehicle (CT) and mitochondria-targeted antioxidants at non-toxic concentrations: $MitoBEN_2$ (12.5µM) and $MitoCIN_4$ (12.5µM) and $MitoCIN_6$ (0.39µM). Bafilomycin (0.5 µM), an autophagy inhibitor and trichostaine (2 µM), an autophagy inducer were used as controls. The images are representative of three independent experiments and were acquired using a Nikon Eclipse TE2000U microscope (Nikon Instruments, Amsterdam, The Netherlands) equipped with a x40 Plan Fluor 0.6 NA objective (Nikon). Total magnification was 400x.

Mitophagy quality control mechanism is a process that occurs naturally allowing cells to repair and remove damaged mitochondria or part of mitochondrial network. After treatment with mitochondria-targeted antioxidants, lysotracker green-labelled acidic bodies (mostly mysosomes) were still co-localized with polarized mitochondria, which may suggest the occurrence of initial steps of mitophagy in which polarized mitochondria are translocated to acidic bodies (Figure 14). Although $MitoBEN_2$ and $MitoCIN_6$ had no visible effect on mitophagy, $MitoCIN_4$ appeared to increase the number of merged signals, when compared to control cells. The results suggest the appearance of multiple large lysosomes that co-localized with the mitochondrial network (Figure 14).

Maintenance of mitochondrial homeostasis requires the removal of damaged and generation of fresh and functional mitochondria (Palikaras *et al*., 2015). Impaired mitochondria generate more ROS that induce impaired mitochondrial degradation by mitophagy process. On the other hand, low ROS levels can induce mitophagy, keeping the right balance between mitochondrial biogenesis and mitochondrial degradation, which avoids the accumulation of dysfunctional mitochondria or damage

macromolecules (Sena *et al*., 2012). Mitophagy promotes an healthy cell phenotype upon mild attenuation of mitochondrial function. Moreover, mitophagy confers resistance to various stressors, including starvation, genotoxicity, and mitochondrial oxidative stress. In fact, deleterious mutations in mtDNA can be selectively eliminated through mitophagy (Suen *et al*., 2010). In mitochondria-targeted antioxidants-treated cells seems that the self-removal of damage mitochondria by mitophagy is upregulated. In fact, some polyphenols such as resveratrol and quercetin have been shown to alter mitophagy transcriptome mediated by FOXO3, a signalling to potentiate Parkin-PINK1 mitophagy in cardiac and hepatic cells. For instance, attenuating myocardial infarction in rats subjected to ischemia/reperfusion injury, and protecting mitochondrial damage in ethanol-induced liver injury through mitophagy (Tan and Wong, 2017). Mitochondria-targeted antioxidant MitoQ restore the mitochondrial network in diabetic kidney disease mediated mitophagy, which the damaged mitochondrial fragments were degraded, whereas the mitochondrial fission was inhibited (Xiao *et al*., 2017). The mitophagy also may be induced by hypoxia mediated Hif-1α activation, to protect cells from damaged mitochondria and from the overproduction of ROS, upregulating the glycolytic pathway coffering an alternative energy supply (Zhang *et al*., 2008).

3.10. MitoBEN2 and MitoCIN6 activity depends on mitochondrial function

To understand if and whether mitochondrial function is essential for the observed effects of mitochondria-targeted antioxidants on NHDF cells, we next performed a series of experiments in a cell culture medium without glucose, the so called OXPHOS medium (in which glucose is replaced by galactose, and pyruvate and glutamine are supplemented). This strategy force cells to generate most, if not all, of their ATP using mitochondrial OXPHOS at the expense of pyruvate and glutamine. In cells, galactose can be converted into glucose, a process that require energy having the conversion of galactose into glucose a zero net energy yield (Cohn and Segal, 1973). Here, this strategy was used in NHDF cells cultured in OXPHOS medium to evaluate the effects of mitochondria-targeted antioxidants on nuclei number, cell metabolic activity and mass.

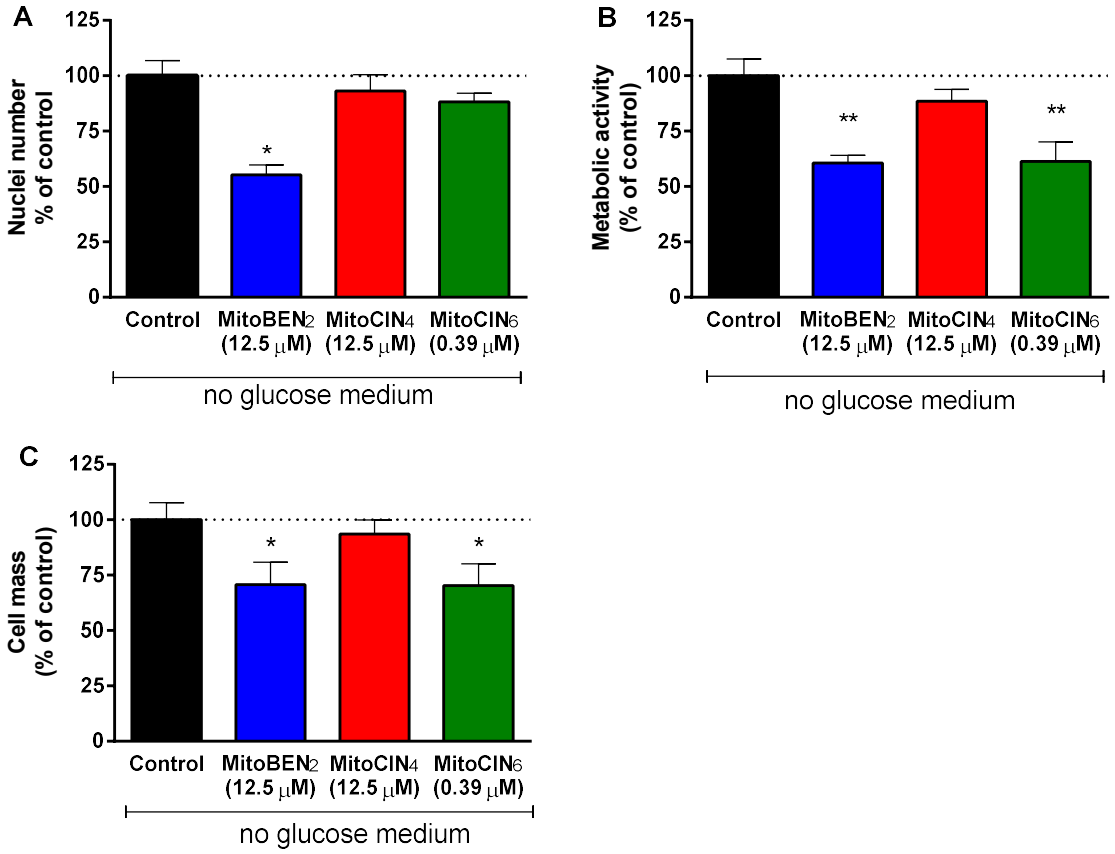


Figure 15. Cytotoxicity of mitochondria-targeted antioxidants on NHDF cells cultured in OXPHOS medium. NHDF cells were seeded and incubated during 72 hours in OXPHOS medium with non-toxic concentrations of mitochondria-targeted antioxidants: MitoBEN$_2$ (12.5μM), MitoCIN$_4$ (12.5μM) and MitoCIN$_6$ (0.39μM) and their cytotoxic profile was estimated through the evaluation of A) nuclei number, B) cell metabolic activity and C) cell mass. Cell number was determined by measuring the number of nucleus in living cells stained with Hoechst 33342, while cell metabolic activity was measured through the resazurin reduction assay and cell mass was measured through the SRB assay. Data are means ± SEM of three independent experiments and the results are expressed as percentage of control (control = 100 %), which represents the cells without any treatment in the respective time point. Statistically significant compared with control group using one-way ANOVA. Significance was accepted with *P<0.05, **P<0.01 vs. control.

We previous demonstrated that mitochondria-targeted antioxidants (MitoBEN$_2$, MitoCIN$_4$ and MitoCIN$_6$) long-term treatment (72h) did not reveal cytotoxic effects at the tested concentrations, measure as both nuclei number (Figure 6 A) and cell mass (Figure 6 B), while MitoBEN$_2$ and MitoCIN$_6$ decreased cell metabolic activity in cells cultured in glucose medium (Figure 10). In cells cultured in OXPHOS medium, the effects of mitochondria-targeted antioxidants were more noticeable. In fact, MitoBEN$_2$ significantly decreased nuclei number (Figure 15 A), as well as cell mass (Figure 15 B) and metabolic activity (Figure 10 C). Similarly, MitoCIN$_6$ cytotoxic effects were more noticeable in cells cultured in OXPHOS medium (Figure 15), while MitoCIN$_4$ did not reveal cytotoxic effects also in cells cultured in OXPHOS medium (Figure 15).

Literature describes OXPHOS medium as capable to increase mitochondrial capacity and force cells to use exclusively OXPHOS for their ATP energetics demand (Marroquin *et al*., 2007). Since the ATP production from galactose oxidation to pyruvate via glycolysis is zero, galactose-grown cells are forced to use mitochondria for their ATP supply and are, hence, more susceptible than glucose-grown cells to compounds that are uncouplers or OXPHOS inhibitors (Nadanaciva and Will, 2011). Moreover, this strategy is also using to unmask some mitochondrial defects, which are normally hidden when cells can completely able to use glycolysis to produce ATP.

It was reported that some polyphenols such as resveratrol and gallic acid esters decrease mitochondrial function, through the reduction of F_1F_o-ATP-synthase activity (Zheng, and Ramirez, 2000). These data pointed out the importance of mitochondrial function to sustain the cells' well function. When cells depend exclusively on mitochondria to supply energy demands and, in MitoBEN$_2$- and MitoCIN$_6$-treated cells where mitochondrial function is compromised, the cytotoxic effects of both mitochondria-targeted antioxidants were more noticeable.

3.11. Proliferation rate depends of metabolic pathway

Culturing cells in OXPHOS medium forces cells to use exclusively mitochondria for fulfil energy requirement, which increase the chances to reveal possible mitochondrial defects. We next evaluated the effects of mitochondria-targeted antioxidants in cell proliferation rate of cells cultured in both glucose and OXPHOS medium.

In cells cultured in glucose medium, cells treated with mitochondria-targeted antioxidants (MitoBEN$_2$, MitoCIN$_4$ and MitoCIN$_6$) for 72h growth equally, when compared to control cells, as no effects were observed on cell proliferation rate (Figure 16 A). In fact, it seems that treating cells with MitoCIN$_4$ and MitoCIN$_6$ slightly increased cell proliferation rate. As we showed before, even in situation where mitochondrial function was decreased, cell well function was not compromised. This suggests a kind of adaptative response for survival and to supply energy requirements.

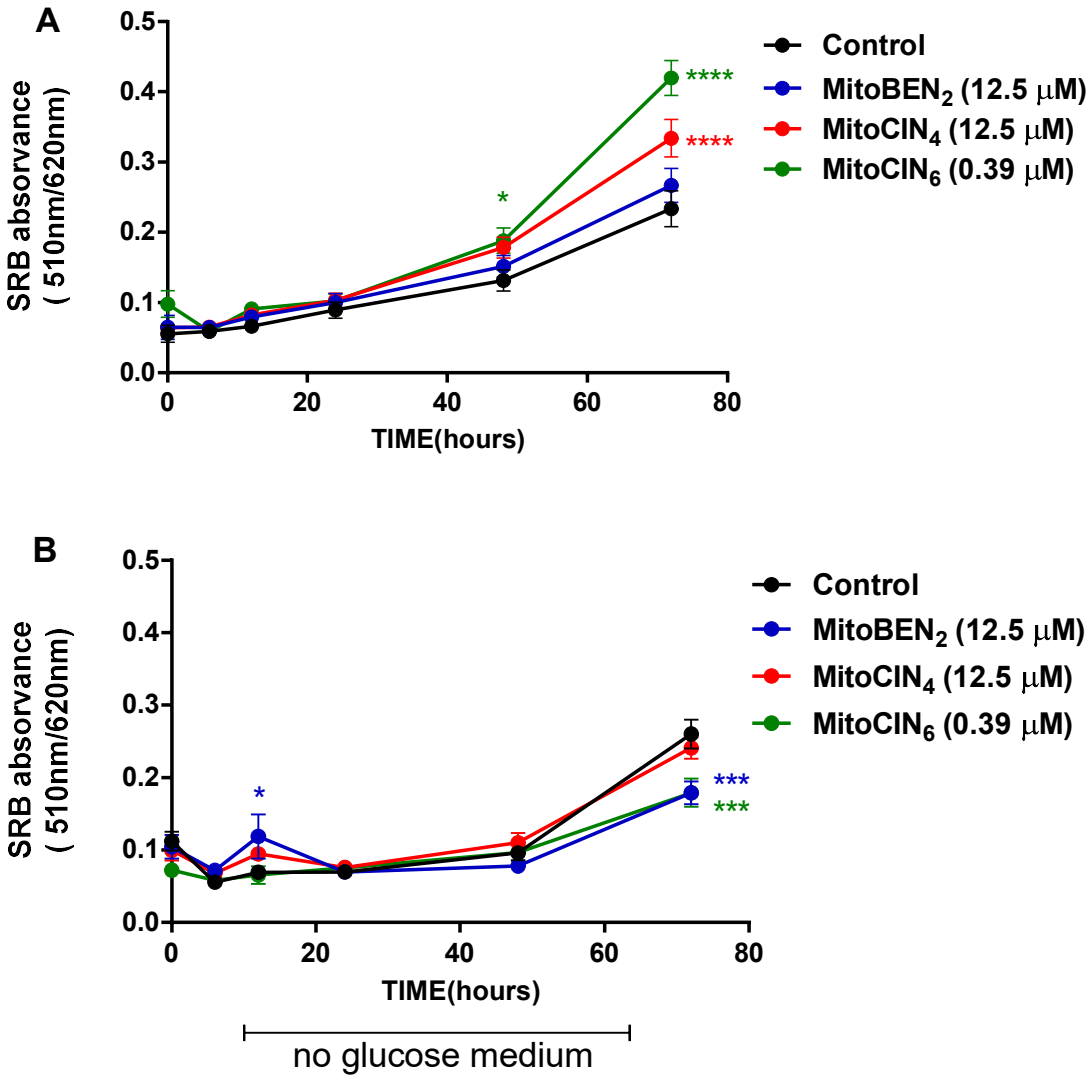


Figure 16. Effect of mitochondria-targeted antioxidants on cell proliferation rate. NHDF cells were seeded and incubated during 72 hours in A) glucose and B) OXPHOS medium with non-toxic concentrations of mitochondria-targeted antioxidants: MitoBEN$_2$ (12.5μM), MitoCIN$_4$ (12.5μM) and MitoCIN$_6$ (0.39μM). Cell proliferation rate was estimated by measuring cell mass through the SRB assay at different time points. Data are means ± SEM of four independent experiments and the results are expressed as the absorbance of SRB at 510 nm. Statistically significant compared with control group using two-way ANOVA. Significance was accepted with *P<0.05, ***P<0.001, ****P<0.0001 vs. control.

On the other hand, in cells cultured in OXPHOS medium, the effects of mitochondria-targeted antioxidants were more noticeable, in particular with MitoBEN$_2$ and MitoCIN$_6$ (Figure 16 B). As expected, MitoBEN$_2$- and MitoCIN$_6$-treated cells, when cells are forced to use exclusively OXPHOS for energy supply, they showed decreased capacity to sustain proliferation. These results are in agreement with the idea that mitochondrial function is decreased in MitoBEN$_2$- and MitoCIN$_6$-treated cells, at least at the concentrations tested here. Moreover, the data reinforced the idea that whatever the effect of mitochondria-targeted antioxidants in NHDF cells, they are dependent of the

mitochondrial well function. In MitoCIN$_4$-treated cells, as the mitochondrial function is not affected, cells can properly proliferate in both glucose and OXPHOS medium. These results evidence the metabolic shift in MitoBEN$_2$- and MitoCIN$_6$-treadted cells. The proliferating cells depend of the glycolytic pathway to keep growing, where glucose and other nutrients cannot be scarce and the ATP may never be limiting. Moreover, the acetyl-CoA and NADPH provided from pyruvate oxidation and the pentose phosphate pathway, respectively, are required for macromolecular synthesis (Vander Heiden *et al*., 2009). When the glucose was replaced by galactose the substrates for macromolecules synthesis were furnished essentially from pyruvate and glutamine metabolization, which may not be sufficient to maintain the cell´s proliferative capacity.

3.12. Antioxidant activity of mitochondrial-targeted molecules

The central focus on finding mitochondria-targeted drugs is related with the discovery and development of antioxidants able to block mitochondrial oxidative damage (Smith *et al*., 2012). Antioxidants that can specifically reduce mitochondrial ROS generation in pathological processes can be also considered putative drugs to improve age-associated events and to prolong life span. At the end, mitochondria-targeted phenolic acid antioxidants main goal is to protect, directly or indirectly, cells against oxidative stress-induced cell damage.

Next, we evaluated antioxidant activity of mitochondria-targeted antioxidants against *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative damage. *t*-BHP is common chemical agent used as a model for evaluating cellular mechanisms of alterations resulting from oxidative stress damage in cells and tissues. *t*-BHP leads to the production of peroxyl and alkoxyl radicals, that induce peroxidation of membrane phospholipids, leading to a loss of membrane fluidity and permeability. Conversion of *t*-BHP to *t*-butanol requires the enzymatic reaction of GPx, which consume reduced glutathione, and consequently decrease the cellular antioxidant capacity (Kučera *et al*., 2014).

NHDF cells were first treated with mitochondria-targeted antioxidants at non-toxic concentrations for 72 hours, and then oxidative stress was induced by adding *t*-BHP for an incubation period of three hours. In both glucose and OXPHOS medium, treating cells with *t*-BHP promote a significant reduction in cell metabolic activity of about 25 %.

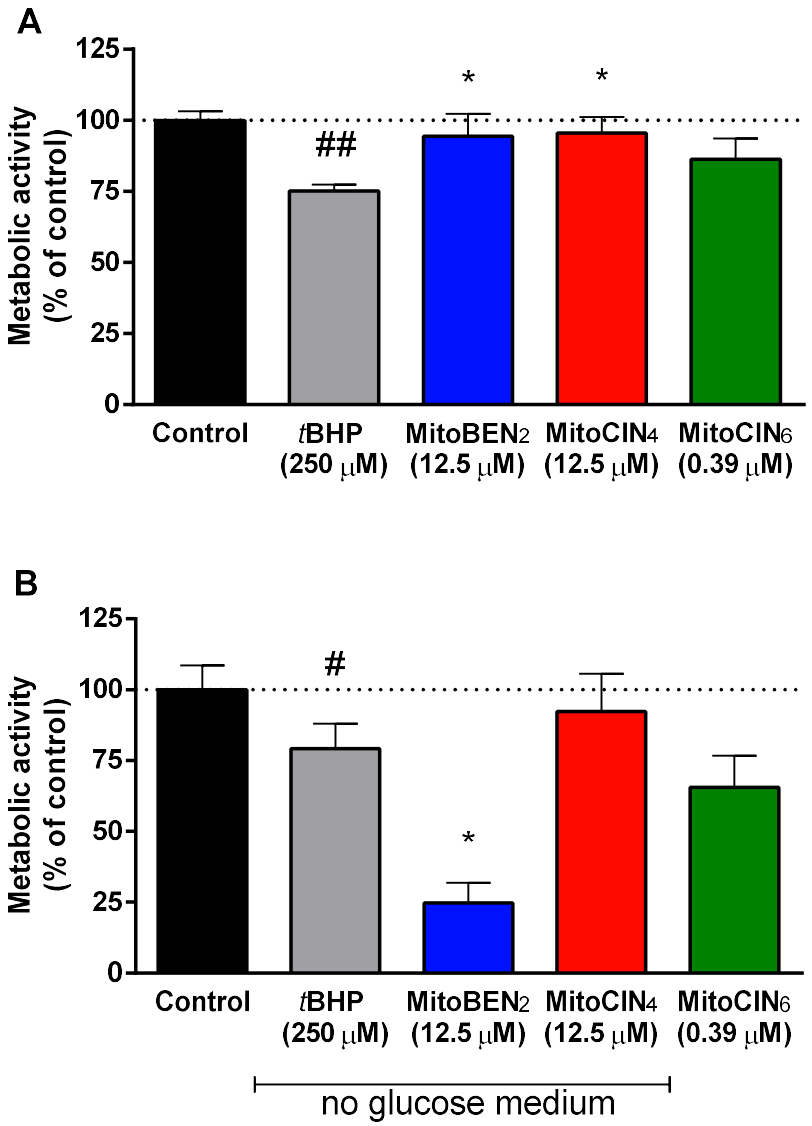


Figure 17. Antioxidant effect of mitochondria-targeted antioxidants on NHDF cells. NHDF cells were seeded and incubating for 72 hours in A) glucose or B) OXPHOS medium with non-toxic concentrations of mitochondria-targeted antioxidants: MitoBEN$_2$ (12.5μM), MitoCIN$_4$ (12.5μM) and MitoCIN$_6$ (0.39μM). Then, oxidative stress was induced by adding *t*-BHP 250 μM for three hours more. Antioxidant activity was determined through changes in metabolic activity using resazurin reduction assay. Data are means ± SEM of four independent experiments and the results are expressed as percentage of control (control = 100 %), which represents the cells without any treatment in the respective time point. Statistically significant compared between control group and *t*-BHP treatment and between *t*-BHP treatment and mitochondria-targeted antioxidants using one-way ANOVA. Significance was accepted with #P<0.05, ##P<0.01, *P<0.05 respectively.

In cells cultured in glucose, mitochondria-targeted antioxidants (MitoBEN$_2$, MitoCIN$_4$ and MitoCIN$_6$) long-term treatment (72h) significantly prevented *t*-BHP-induced cell damage, although this effect was not clearly observed in MitoCIN$_6$-treated cells (Figure 17 A). Interestingly, in cells cultured in OXPHOS medium, the antioxidant activity of mitochondria-targeted antioxidants, namely MitoBEN$_2$ and MitoCIN$_6$, was not observed

(Figure 17 B). In fact, MitoBEN$_2$- and MitoCIN$_6$-treated cells not only lost their antioxidant activity as they aggravated the oxidative damage induced by *t*-BHP, clearly in OXPHOS medium MitoBEN$_{\mathbf{2}}$- and MitoCIN$_{\mathbf{6}}$-treated cells decreased their dehydrogenase activity, essential for furnish NADPH required to reduce glutathione that is oxidised by Gpx in the reduction of *t*-BHP in *t*-butanol (Kramer *et al*., 1988), while in cells cultured in glucose those equivalent reducers are provided by cytosolic dehydrogenases as bioproduct of glycolytic pathway.

MitoCIN$_4$, even in cells cultured in OXPHOS medium maintained its remarkable antioxidant properties (Figure 17 B). In summary, MitoBEN$_2$ and MitoCIN$_6$ capacity to protect cells against the acute induced oxidative damage, was lost when cells were forced to use exclusively mitochondria for energy supply. MitoCIN$_4$-treated cells maintained its remarkable antioxidant effects in both glucose and OXPHOS medium.

The data indicates that the antioxidant activity of mitochondria-targeted antioxidants is somehow dependent on mitochondrial well-function.

Conclusions

Polyphenols such as HBA and HCA display heterogeneous effects at the subcellular level. Being antioxidants, HBA and HCA were expected to exert their activity by directly scavenge free radicals. Still it appears that the mechanism is mainly based on an (in)direct effect, through modulation of ROS-eliminating enzymes and redox homeostasis may be observed. This suggests that polyphenols might be useful for mitigating oxidative stress and/or modulate redox signalling.

The data clearly points out that mitochondria-targeted antioxidants, in particular, $MitoBEN_2$ and $MitoCIN_6$, promoted a decrease in mitochondrial function leading to a shift on cells' metabolic profile. Cells treated with mitochondria-targeted antioxidants can up-regulate the glycolytic pathway to supply cells' energy requirements in order to protect cells from the oxidative stress. In $MitoBEN_2$- and $MitoCIN_6$-treated cells, where a shift on cells metabolic profile was observed, mitochondrial well-function seems to be crucial. In fact, in cells cultured in OXPHOS medium, which force the exclusive use of mitochondria as source of energy production, $MitoBEN_2$ and $MitoCIN_6$ cytotoxic effects were more evident. Moreover, in cells cultured in OXPHOS medium, $MitoBEN_2$ and $MitoCIN_6$ lost their antioxidant capacity. On the other hand, $MitoCIN_4$ maintained its remarkable antioxidant properties in both aerobic (OXPHOS medium) or more glycolytic (glucose medium) conditions, probably through modulation of mitophagy to eliminate damaged mitochondria allowing cells to regulate mitochondrial function and increase their resistance to oxidative stress.

Finally, our results suggest that the tested polyphenol variants potentially display hormetic effects, which might be part of their mode-of-action. In this sense, these molecules could be of future use as therapeutic agents for oxidative stress-related diseases or as active ingredients to prevent skin aging.

Future perspectives

Facing the inconclusive results obtained in order to understand the long-term effects of mitochondria-targeted phenolic acid antioxidants, the future work will attempt to test new several hypotheses:

1) Evaluate the effect of this mitochondria-targeted molecules in isolated mitochondria to assess their effects on ETC complexes activity.

2) Deep understanding the mechanism behind metabolic shift, in MitoBEN$_2$- and MitoCIN$_6$-treated cells, measuring directly lactate and pyruvate production through nuclear magnetic resonance (NMR).

3) Evaluate the role of proteins, such as pyruvate dehydrogenase or hexokinase, on metabolic adaptation from OXPHOS for glycolytic pathway, by western blotting.

4) Understand if and whether AMPK, a metabolic pathway energetic sensor, is capable of regulate the bioenergetics pathways mediated by mitochondria-targeted antioxidants.

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