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Induction of premature senescence in human fibroblasts through endoplasmic reticulum stress: protection by phenolic diterpenes

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Indução de senescência prematura em fibroblastos humanos através de stress no retículo endoplasmático: proteção por diterpenos fenólicos

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ABSTRACT

Induction of premature senescence in human fibroblasts through endoplasmic reticulum stress: protection by phenolic diterpenes

Population ageing is increasing worldwide due to the increase of lifespan and to sociological reasons. This is however associated with a higher incidence of age-related chronic disorders, such as neurodegenerative and cardiovascular diseases, diabetes and cancer. Therefore, new approaches to promote a healthy ageing are needed. The accumulation of damaged and misfolded proteins are present during the ageing process and known to be associated with endoplasmic reticulum (ER) stress and to the progression of many age-related diseases. Based on this, an *in vitro* method to induce premature senescence in normal human fibroblasts through induction of ER stress was developed in the present study in order to test potential anti-ageing compounds that acts by dealing with protein damage and associated ER stress.

Tunicamycin (TUN) was successfully used to induce ER stress in normal diploid human skin fibroblasts since it triggered the unfolded protein response (UPR) signaling, as shown by the up-regulation of BiP, ATF6-α, CHOP and spliced XBP1. Treatment of fibroblasts with TUN significantly inhibited cell growth without inducing cell death (even by apoptosis), and it affected cell morphology in a way that resembled a senescent phenotype. Induction of premature senescence was confirmed by the appearance of several markers of cellular senescence, such as induction of G1 arrest, presence of SA β-Gal positive cells, increased cells' green autofluorescence and expression of p16. Finally, this model was used to test the potential protective effect of carnosic acid - a natural phenolic diterpene. Although preliminary, the results suggested us that carnosic acid is able to prevent against the TUN-induced premature senescence corroborating the anti-ageing potential of this compound shown by previous results from the group.

This work contributed to the development of a new model of induction of premature senescence in human fibroblasts, which can then be used to test compounds applicable for nutritional ageing interventions.

RESUMO

Indução de senescência prematura em fibroblastos humanos através de stress no retículo endoplasmático: proteção por diterpenos fenólicos

O envelhecimento da população está a acontecer de uma forma crescente devido ao aumento da esperança média de vida e por razões sociais. Contudo, este fenómeno está associado ao aumento da incidência de doenças crónicas relacionadas com o envelhecimento, tais como doenças neurodegenerativas e cardiovasculares, diabetes e cancro. Portanto, novas abordagens são necessárias de modo a promover um envelhecimento saudável. A acumulação de proteínas danificadas está presente durante o processo de envelhecimento e é reconhecido a sua associação ao stress do retículo endoplasmático (ER) e com a progressão de muitas doenças relacionadas com o envelhecimento. Deste modo, no presente estudo foi desenvolvido um método *in vitro* de indução de senescência prematura em fibroblastos humanos normais através da indução de stress do ER, de modo a testar compostos com potencial anti-envelhecimento que atuam por modulação dos níveis de proteínas danificadas e o associado stress do ER.

A tunicamicina (TUN) foi usada com sucesso em fibroblastos diplóides normais isolados de pele humana para indução de stress do ER, uma vez que ativou a *unfolded protein response* (UPR) demonstrado pelo aumento observado da expressão de BiP, ATF6-α, CHOP e *spliced* XBP1. O tratamento dos fibroblastos com TUN inibiu significativamente o crescimento celular sem, porém, induzir morte celular (incluindo apoptose), mas afetou significativamente a morfologia das células de tal modo que mimetizava um fenótipo de senescência. A indução de senescência prematura foi confirmada através da presença de diversos marcadores de senescência celular, tais como a indução da paragem do ciclo celular em G1, presença de células marcadas com β-Gal, aumento da autofluorescência verde e da expressão de p16. Finalmente, este modelo foi usado para testar os potencias efeitos protetores do ácido carnósico - um diterpeno fenólico. Embora preliminares, os resultados sugerem que este composto previne contra a senescência prematura induzida pela TUN, corroborando assim o seu potencial efeito anti-envelhecimento anteriormente demonstrado pelo grupo.

Este trabalho contribuiu para o desenvolvimento de um novo modelo de indução de senescência prematura em fibroblastos humanos, o qual pode ser usado para testar compostos aplicáveis em intervenções nutricionais para o envelhecimento.

"Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less."

Marie Curie

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LIST OF ABBREVIATIONS

ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BiP	Immunoglobulin Binding Protein
b-Zip	basic leucine zipper
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
CHOP	transcription factor C/EBP homologous protein
eIF2α	Eukaryotic initiation factor 2
ER	Endoplasmic Reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
ERSE	ER stress response element
FETA	Free Radical Theory of Aging
IRE1	Inositol requiring enzyme-1
Nfr2	Nuclear factor (erythroid-derived 2)-like 2
PERK	Protein kinase RNA (PKR)-like ER kinase
PETA	Protein Error Theory of Aging
Rb	Retinoblastoma
ROS	Reactive oxygen species
SIPS	Stress-Induced Premature Senescence
TUN	Tunicamycin
UPR	Unfolded Protein Response
XBP1	X-box binding protein 1

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INTRODUCTION

1. INTRODUCTION

Tissue degeneration and loss of physiological function is a common problem that accompanies Human ageing (Levine *et al.*, 2009). That loss of function is caused by a progressive damage of tissues. It is documented that during skin ageing the replicative cycle leads to exhaustion of the capacity of cells for division and for their specialized functions (Campisi, 1998). The apoptotic death pathways also play an important role to maintain the tissue homeostasis and its failure leads to accumulation of errors that contribute to several problems like skin ageing (Haake *et al.*, 1998, Levine *et al.*, 2009). Therefore, regulation of cell death program is of the utmost importance. To mimic the ageing process *in vitro* cells isolated from various tissues can be used, such as human skin diploid fibroblasts. This type of cells show a limited proliferation potential *in vitro* (Hayflick limit) before they enter a state of permanent growth arrest named as senescence (Hayflick, 1992). In this state cells remain metabolically active but completely indifferent to mitogenic stimuli (Hayflick *et al.*, 1961). It is documented in literature that after entering the senescence state, human fibroblast arrest in G1 phase of cell cycle (Stein *et al.*, 1995).

A group of heterodimeric enzymes named cyclin-dependent kinases (CDK's) (Nigg, 1995) are responsible to control the evolution of cell cycle and their activity is controlled through cellular inhibitor protein, the cyclin-dependent kinase inhibitors (CDKI's), that block the catalytic activity of the CDK's (Peter, 1997). The expression of CDKI's plays an important role in senescence and they are used as bio-markers.

During the last 50 years, the experimental work related with ageing research led to the formulation of several mechanistic theories of biological aging. Two theories of these have been the most studied in biogerontology research, the Free-Radical and the Protein Error (Rattan, 2006). The first, Free Radical Theory of Ageing (FRTA), was proposed in 1956 and take in consideration that the ageing phenomenon are related to a variety of ROS that can lead to molecular damage and functional disorders, diseases and death (Rattan, 2006). The second theory is known as Protein Error Theory of Ageing (PETA). Since errors in protein synthesis are much more frequent than errors in the synthesis of nucleic acids, this theory became more relevant to ageing (Rattan, 2006, 2008a). Several evidences that support PETA come from experiments that show that the induction and increased errors in proteins can accelerate the ageing of human cells and bacteria (Holliday, 1996; Rattan, 1996, 2003; Nyström, 2002).

The endoplasmic reticulum (ER) is a multifunctional organelle responsible for lipid biosynthesis, calcium storage and release and most important, membrane protein folding. An alteration of ER homeostasis leads to the formation of misfolded or unfolded proteins, ER stress and consequently activation of some defense mechanisms like the unfolded protein response (UPR) (Naidoo, 2009). The ER stress can have a significant role in the ageing process. Its malfunction can lead to several diseases, such as diabetes, inflammation and neurodegenerative disorders (Park *et al.*, 2013) as will be discussed later.

Diet and the consumption of different bioactive natural compounds can have a huge importance in the modulation of the ageing process. It is known that a diet rich in fruits vegetables and aromatic herbs are associated with a lower incidence of ageing-related disorders, such as neurodegenerative and cardiovascular diseases and some types of cancer (Willett, 1995; Temple, 2000; Liu, 2003; Neuhouser, 2004; Ferrari, 2004, Everitt *et al.*, 2006). Many reports already attributed beneficial effects of several non-nutrient natural compounds to the human health, such as flavonoids and other polyphenols, sulfuraphanes, among others (Stanner *et al.*, 2004; Willcox *et al.*, 2004; Obrenovich *et al.*, 2011, Gerhäuser, 2012). In particular, phenolic diterpenes like carnosic acid (CA) are known for their direct antioxidant effects as well as by the ability to induce intracellular antioxidant defenses (Martin *et al.*, 2003; Satoh *et al.*, 2008, Kelsey *et al.*, 2010). These properties are very important to the prevention of age-related diseases and to modulate the ageing process itself.

1.1. The Ageing Process

Ageing is an arising phenomenon characteristic of protected environments due to the survival of the individuals beyond the natural lifespan in the wild (Rattan *et al.*, 2005). It is a concept that cannot be defined just considering the genetic of the individuals. In fact, there is no genetic program that determine the survival duration of an organism as no genes have been found whose function is to cause ageing (Rose, 1991; Kirkwood *et al.*, 2000, Gavrilov *et al.*, 2001, Rattan, 2005). Instead, ageing varies among different species, among organisms inside the same species, in each tissue and organ from the same organism, among different type of cells and their compartments as well as macromolecules (Holliday, 2000, Rattan, 2000). Ageing can be considered a condition that requires alterations not only in the organism but also in their organs, tissues, cells and macromolecules (Holliday, 1995; Rattan, 1996, Rattan *et al.*, 2005). This phenomenon is characterized by a gradual

accumulation of molecular damage in nucleic acids, proteins and lipids that lead to a loss of biological functions of cells. The progression and consequences of ageing are dependent of the combination of the individuals' genetic background and environment (Rattan *et al.*, 2005).

The homeostasis of an organism is totally dependent of the success of the cellular maintenance, repair and turnover machinery. Their inefficiency and failure leads to an accumulation of molecular damage and errors that are in the origin of ageing and age-related diseases (Holliday, 2000). In a society where the medical care increasingly extends the lifespan of human beings resulting in an increased aged-population, the development and success of research in the prevention of the onset of age-related diseases is of great importance in order to improve life quality at older age (Rattan *et al.*, 2005). Diseases such Alzheimer and Parkinson (Wick *et al.*, 2000, Tezil *et al.*, 2013), cancer and cardiovascular problems (Wick *et al.*, 2000; Khurana *et al.* and Tezil *et al.*, 2013) as well as osteoporosis (Wick *et al.*, 2000) are characteristic of the old ages. Almost all of these diseases have in common the cell deterioration caused by accumulation of damaged macromolecules and right before culminating in cell death, some of these cells experiment a state of senescence where they gradually loss the capacity to proliferate (Jeyapalan *et al.*, 2008).

1.1.1. Cellular Senescence: a marker of the ageing process

The loss of ability of cells to divide is a characteristic of cellular senescence. This process is characterized by a limited potential of cells for proliferation in culture called the Hayflick limit. Hayflick and Moorhead showed in 1961 that human fibroblasts initially undergo robust cell division in culture but gradually the proliferation decline with time culminating in the cessation of cell division – replicative senescence (Hayflick *et al.*, 1961). The cells that loss their capacity to divide, remain metabolic active (Campisi, 2013) and viable for many weeks but cannot grow even in the presence of nutrients (Campisi, 2007). It was also shown that senescence could serve as a tool for fighting cancer since it could be used to halt the uncontrolled proliferation of cancer cells. The cell senescence was also considered a barrier to cellular repair since it takes the cells to a state of growth arrest (Hayflick, 1992).

Mitotic cells are those that have the ability to proliferate but also have the ability to maintain a state of quiescence (G0 state). The cells in G0 state are able to go back to a state of proliferation in response to appropriate signals, including the need for tissue repair or regeneration (Campisi, 2007). These cells, like fibroblasts are susceptible to undergo cellular senescence when challenged by certain stimulus, which induces the loss of their capacity for cell division and perform their normal biological functions.

The senescent phenotype (Figure 1) is induced by different stimuli (such dysfunctional telomeres; chromatin perturbations; DNA damage and strong mitogen signals caused by activated oncogenes) and stresses (as oxidative stress). This phenotype is characterized by growth arrest, apoptosis resistance and altered gene expression (in pathways related with immune response and inflammation, cytoskeleton, stress response, and metabolism) (Campisi *et al.*, 2007). On the other hand, the post-mitotic cells are incapable of proliferation. They are considered differentiated cells, such neurons, and not senescence cells.

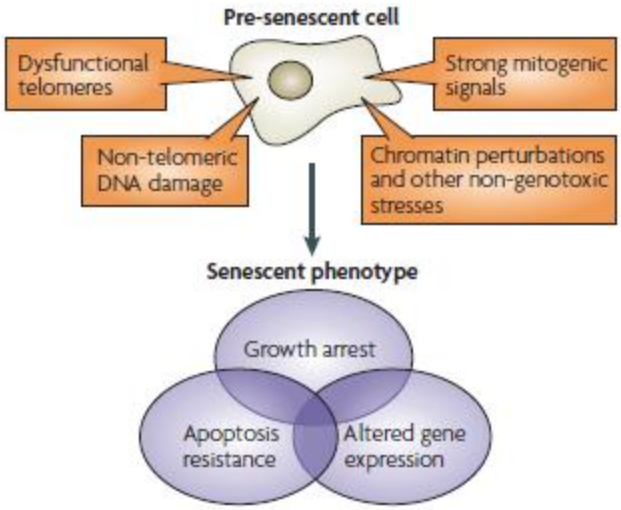

Figure 1: The senescent phenotype induced by multiple stimuli. (from Campisi *et al.*, 2007)

Special proteins are actively expressed in senescent cells that lead to irreversible growth arrest, such as the CDKI's, p21 (or CDKN1a, p21Cip1, Waf1 or SDI1) and p16 (or CDKN2a or p16INK4a) (Hara *et al.*, 1996, Campisi, 2007). These CDKI's are controlled by the p53 and Rb (retinoblastoma), two tumour-suppressor proteins involved in the cells' growth arrest. p21 is activated directly by p53 but the mechanism of activation of p16 is not very well understood (Gil *et al.*, 2006).

The senescent cells are known to be correlated with the secretion of proteins that can alter the microenvironment of cells that surround those (Alcorta *et al.*, 1996, Trougakos *et al.*, 2006). An example of this happens with senescent fibroblasts that have an altered gene expression and overexpress specific proteins, which induce inflammation and remodel the extracellular matrix (Campisi, 2007).

Cellular senescence is directly associated with ageing, since pathologies related with age accumulate senescent cells and their numbers increase with age (Campisi, 2007). There are diverse age-related diseases that present senescence-associated markers. Previous studies reported the presence of senescent endothelial cells in atherosclerotic plaques as other arterial lesions (Fenton *et al.*, 2001; Minamino *et al.*, 2002; Matthews *et al.*, 2006). Senescent cells were also detected in chrondrocyte clusters with mitotic activity nearly to osteoarthritic lesions (Price *et al.*, 2002) and in benign prostatic hyperplasia (disorder that affect aging male population) where the presence of senescent epithelial cells promote the continuum growth of the transition zone of the prostate (Choi *et al.*, 2000; Castro *et al.*, 2003). Other cases were the presence of senescent cells alter the microenvironment of tissues is aged skin. Here, the secretion of inflammatory cytokines, degradative enzymes and growth factors by dermal fibroblasts has been correlated with aged skin morphology (Jenkins, 2002; Hornebeck, 2003; Boukamp, 2005). It is also discussed that the presence of senescent cells contributes to the stimulation of growth and angiogenic activity in premalignant cells (Martens *et al.*, 2003; Bavik *et al.*, 2006; Coppe *et al.*, 2006) promoting the progression of these cells leading to carcinogenesis.

1.1.2. Premature Senescence: models in normal human fibroblasts

In order to better understand the senescence phenomenon *in vitro*, and to do not depend solely in the replicative senescence (which takes several months to be completed), methods of induction of premature senescence were developed. Several studies have shown that early passage normal human diploid fibroblasts can develop a phenotype similar to replicative senescence (such as the inability to replicate in response to diverse growth factors, reduced activity of certain cell cycle-related enzymes and enlarged cell size) in response to several stress factor such oxidants (Chen *et al.*, 1994; von Zglinicki *et al.*, 1995; de Magalhães *et al.*, 2004, Burova *et al.*, 2013), inhibitors of histone deacetylase (Ogryzko *et al.*, 1996), hyperactivation of RAS gene (Serrano *et al.*, 1997 and Lee *et al.*, 1999) and overexpression of E2F1 transcription factor (Dimri *et al.*, 2000). Stress-induced premature senescence (SIPS) is the designation of young cells that experience a state of premature senescence caused by diverse stimuli. Several stressors are in the origin of premature senescence and many models are used to induce that. It has been reported that mesenchymal stem cells *in vitro*, when subject to oxidative stress (Brandl *et al.*, 2011; Kim *et al.*, 2012, Ko *et al.*, 2012) or ionization radiation (Wang *et al.*, 2009; Prendergast *et al.*, 2011, Cmielova *et al.*, 2012) undergo stress-induced premature senescence. In the model of induction of premature senescence with oxidants, it was observed that after a 2 hours pulse

treatment with sub-cytotoxic concentrations of H_2O_2, cells lose the capacity to replicate and develop a senescent phenotype (Chen *et al.*, 1994, Chen, Q. *et al.*, 2000). This model of treatment causes a transient increase of p53 protein and inhibition of Rb hyperphosphorilation (Chen *et al.*, 1998).

SIPS exhibit common features with replicative senescence such as senescent morphology, senescence-associated β-galactosidase (SA β-gal) activity, growth arrest in G1 phase of the cell cycle, elevation of p21 and underphosphorylation of Rb (Chen *et al.*, 1998). SIPS differs from replicative senescence in that p53 is only transiently elevated (Chen *et al.*, 1998). These similarities turn the SIPS a viable model to study senescence *in vitro*.

1.2. Endoplasmic Reticulum: Response to Stress

The endoplasmic reticulum (ER) is a multifunctional organelle constituted by a network of membranes that extends throughout the nucleus to the cytoplasm (Naidoo, 2009). This complex organelle is responsible for multiple tasks like calcium storage, lipid biosynthesis and is the place where the synthesis, folding and maturation of secretory and membrane proteins occurs (Samali *et al.*, 2009). A balance between the protein load and the folding capacity is tightly regulated to maintain the homeostasis of cells (Oslowski *et al.*, 2011). Any change in this balance caused by physiological or pathological stimuli can easily lead to ER stress. The ER stress results from accumulation of misfolded and unfolded proteins that activate a complex signaling network known as unfolded protein response (UPR) (Oslowski *et al.*, 2011). But if for some reason the mechanism of UPR fails, ER stress can lead to cell death and disease (Kim *et al.*, 2008). ER stress is involved in various diseases with high incidence, such as diabetes, cancer and neurodegeneration (Ron *et al.*, 2007), which makes the ER stress pathways an important target for the study of new drugs against these diseases and ageing.

1.2.1. Role of Chaperones in Protein Folding

ER chaperones are a group of proteins important in the process of folding of proteins and in the maintenance of ER homeostasis. This process involves different chaperones such as the glucose regulated protein 78 (GRP78, also known as Immunoglobulin Binding Protein - BiP) and 94 (GRP94), lectins, calnexin, calreticulin, thiol-disulfide oxidoreductases, protein disulfide isomerase (PDI) and

ERp57. All these are responsible for the correct folding of proteins and many decreases with age (Naidoo, 2009).

BiP chaperone has a crucial role in the maintenance of cell homeostasis. In ER, it binds transiently to newly synthesized proteins and more permanently to misfolded, underglycosylated or unfolded proteins in order to correct them (Gething *et al.*, 1992, Gething, 1997) by interacting with the hydrophobic domains (Naidoo, 2009). BiP also functions to maintain the permeability barrier of the ER membrane by sealing the luminal end of the translocon pore before and early in translocation (Hamman *et al.*, 1998). When proteins are unable to fold properly they are retained in the ER and this accumulation of misfolded proteins results in the transcriptional up-regulation of many genes, including the one that encodes for BiP (McMillan *et al.*, 1994, McNees *et al.*, 1997, Lee, 2005). Such malfolded protein accumulation has been associated to neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, as well as prion protein diseases (Lee, 2005), and therefore, the correct function and activation of BiP is of high importance.

1.2.2. Unfolded Protein Response

When the homeostasis of ER is disrupted by physiological and pathological stimuli, misfolded and unfolded proteins accumulate in the lumen of ER, a condition known as ER stress (Liu *et al.*, 2003; Lai *et al.*, 2006, Bravo *et al.*, 2013). ER stress activates a set of signalling pathways via different transducer proteins, a process called UPR, that aims to reduce ER stress and restore homeostasis (Liu et al., 2003; Ron et al., 2007; Malhotra et al., 2007; Walter et al., 2011, Bravo et al., 2013). When the homeostasis of ER is disrupted, several ER sensors with transmembranar domains are triggered and a compensatory response is activated (Bravo *et al.*, 2013). Any increase of accumulation of misfolded or unfolded proteins in ER lumen, is noticed by the luminal sensor domains and then transduced to cytoplasm and nucleus by different pathways that act in parallel and/or in series (Schönthal, 2012; Park *et al.*, 2013). The process by which the UPR are trigered with accumulation of misfolded and unfolded proteins is not yet well understood. However, it is known that the BiP chaperone is involved (Gething, 1999, Naidoo, 2009). In unstressed cells, besides their chaperone function, much of the BiP is bonded to the UPR protein mediators keeping them inactive. With accumulation of misfolded and unfolded proteins, BiPs are released from UPR protein transducers to bind the hydrophobic region of unfolded proteins via a substrate binding domain to facilitate their proper folding or to otherwise send them to degradation by the ER-associated protein degradation (ERAD) machinery in the cytosol (Figure

2) (Ahner *et al.*, 2004, Jarosch *et al.*, 2003). The ERAD process can be divided on four steps: recognition, retrotranslocation, ubiquitination and degradation in the proteossome (Yoshida *et al.*, 2001).

Although questioned by more recent works, many authors consider BiP as a master regulator of the UPR (Kohno *et al.*, 1993), since upon ER stress, BiP dissociates from the UPR transmembranar protein sensors, resulting in their activation (Figure 2) (Oslowski *et al.*, 2011). The first one is PKR-like ER Kinase (PERK), a transmembranar kinase that after ER stress oligomerizes, autophosphorylates and directly phosphorylates eIF2α (Oslowski *et al.*, 2011). The phosphorylated eIF2α avoids the construction of ribossomal initiation complexes thereby reducing the mRNA translation (Harding *et al.*, 2000a). However, some mRNA is still translated in particular the ones involved in the UPR for ER stress restoration (Oslowski *et al.*, 2011). Phosphorilated PERK also have the function to activate the b-ZIP transcription factor ATF4 that regulates de several UPR genes such CHOP (Harding *et al.*, 2000b), and to phosphorylate the Nrf2 transcription factor that is involved in the up-regulation of several antioxidant and protective genes (Naidoo, 2009). The second UPR regulator is the Inositol Requiring Enzyme 1 (IRE1 protein,) a transmembrane serine/ threonine kinase that senses ER stress by its N-terminal luminal domain (Urano *et al.*, 2000a). IRE1 dimerizes and autophosphorylates to be active. Two isoforms of this protein are known: IRE1α that is expressed in all types of cells and is the most studied and the IRE1β (Oslowski *et al.*, 2011). IRE1 splices the X-box binding protein 1 (XPB-1) mRNA that encodes a basic leucine zipper (b-ZIP) transcription factor that upregulates UPR target genes such as chaperones for correct protein folding (Calfon *et al.*, 2002; Shen *et al.*, 2001, Yoshida *et al.*, 2001). The last UPR regulator is the Activating Transcription Factor 6 (ATF6) a transmembranar transcription factor that when activated under ER stress conditions transits to the Golgi where it is cleaved by S1P and S2P generating an activated b-ZIP transcription factor (Ye *et al.*, 2000). This form travels to the nucleus activating UPR genes responsible for protein folding, processing and degradation (Haze *et al.*, 1999, Yoshida *et al.*, 2000).

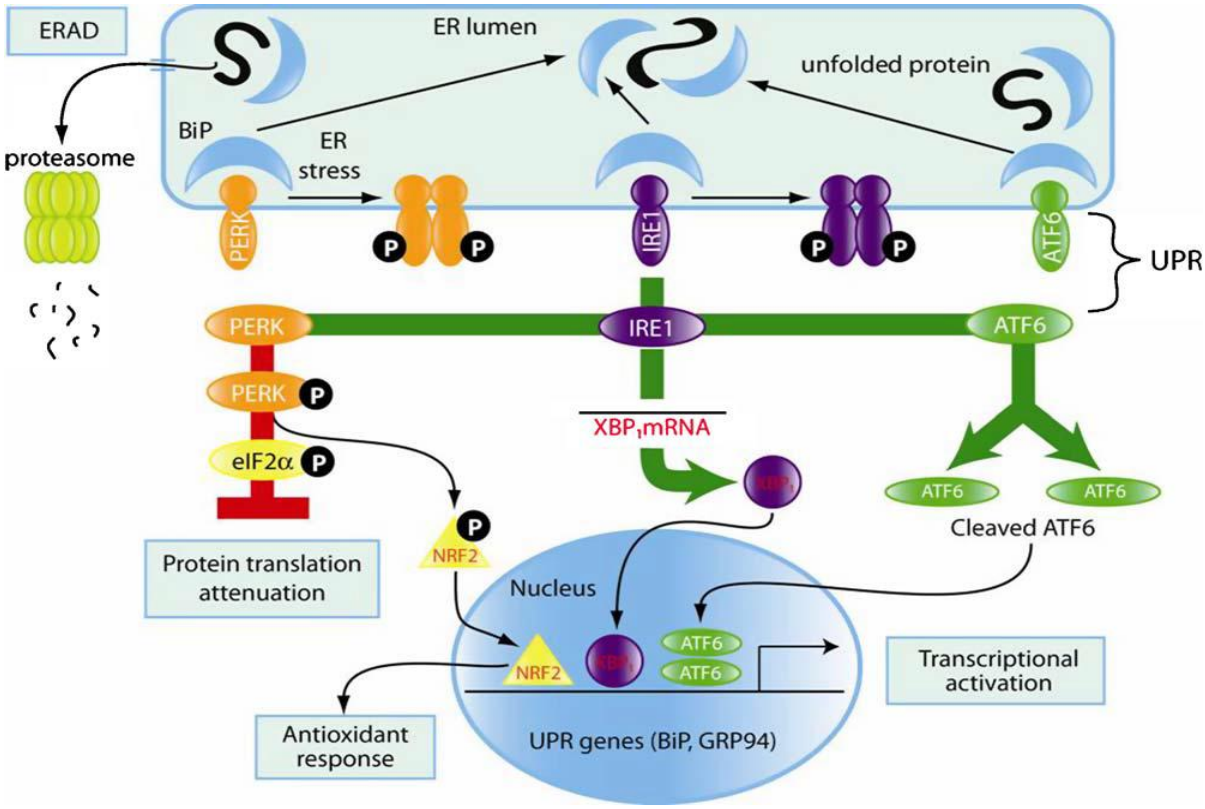

Figure 2: Scheme showing the activation of UPR and signaling of adaptative and protective responses to ER stress by the three UPR-protein transducers. Misfolded proteins are recognized by the BiP chaperone that if not properly folded are targeted for degradation by the ERAD machinery. Upon ER stress the transmembranar proteins PERK, IRE1 and ATF6 are activated regulating many transcriptions factors that upregulate genes with functions of adaptive response, feedback control, and cell fate regulation. (from Naidoo *et al.*, 2008)

Therefore, activated UPR aims an adaptive response to the ER stress by increasing the cellular protein-folding capacity and to reduce protein influx into the ER by inhibiting protein translation (Oslowski *et al.*, 2011, Bravo *et al.*, 2013). It also encompasses an increase in ERAD machinery and autophagy components to promote clearance of unwanted proteins, as well as a feedback control involving the negative regulation of UPR activation to prevent harmful hyperactivation when ER homeostasis is reestablished (Oslowski *et al.*, 2011, Bravo *et al.*, 2013). If these protective and adaptative measures to limit protein load and alleviate ER stress fails, UPR signal cell fate by activating pathways that leads usually to cell death by apoptosis, which plays an important role in the pathogenesis of ER stress-related disorders (Oslowski *et al.*, 2010).

1.2.3. ER Stress and Disease

When the ER stress persists for a long time and the UPR fails to restore protein homeostasis in the ER, to ultimately protect the organism against mal-functioning cells, a pathway of cell death by apoptosis is usually activated. UPR-associated cell death are known to contribute to the pathogenesis of a number human diseases, including diabetes mellitus, neurodegenerative disorders, chronic inflammation, and certain forms of conformational diseases that are characterized by a decreased ability of cells to respond to stress (Bartoszewska *et al.*, 2013). During ageing, it is also known that responsiveness of the UPR system to ER stress declines (Salminen *et al.*, 2010). Therefore, accumulation of misfolded proteins happens that quickly leads to the formation of protein aggregates. These small aggregates are very toxic for the cell and may impair the ubiquitin proteasome pathway that leads to even more accumulation of aggregates (Yoshida *et al.*, 2007). Thus, the failure of the cell machinery responsible for the degradation of proteins – the ERAD pathway, leads to several types of problems culminating in cell death and diseases (Park *et al.*, 2013).

Both type 1 and type 2 diabetes involve beta-cell dysfunction and/or apoptosis, which are associated with ER stress (Back *et al.*, 2012, Wang *et al.*, 2012). In an initial phase, type 2 diabetes (non insulin-dependent) is associated to a hyper-secretion of insulin that leads to insulin resistance in peripheral tissues (Shore *et al.*, 2011, Hetz *et al.*, 2013). This production of large amounts of insulin by beta-cells culminates in the induction of ER stress followed by beta-cell death. Therefore, in later phases of diabetes development, loss of beta-cells and lack of insulin production happens due to ER stress (Shore *et al.*, 2011). Neurodegenerative diseases like Alzheimer's or Parkinson's are correlated with unfolded proteins and protein aggregates that trigger the ER stress and cell death (Yoshida *et al.*, 2007, Sano *et al.*, 2013). In fact, neurons are thought to be sensitive to protein aggregates, and there are many reports showing that ER stress is involved in neurodegeneration (Forman *et al.*, 2003; Lindholm *et al.*, 2006, Yoshida *et al.*, 2007). Alzheimer's disease is the most common neurodegenerative disease, and is characterized by cerebral neuritic plaques of amyloid beta-peptide. Many reports suggest a strong causal relationship between ER stress and Alzheimer's disease, where is thought that accumulation of amyloid beta-peptide can trigger ER stress and that contributes to neurodegeneration (Yoshida *et al.*, 2007). Parkinson's disease is the second most common neurodegenerative disease and is characterized by a loss of dopaminergic neurons. Although it is still unclear whether the ER stress response is involved with Parkinson's disease, many findings strongly suggest a link between them. For example, Parkinson's disease is characterized by the presence of Lewy bodies, aggregates of alpha-

synuclein protein; and, one of the genes involved in the disease encodes the protein Parkin that is a ubiquitin-protein ligase (E3) involved in the ERAD machinery (Yoshida *et al.*, 2007). Among of other diseases, what is also related with ER stress is atherosclerosis (the accumulation of homocystein induces ER stress increasing the expression of BiP and CHOP); certain types of inflammation (for example in nervous system where interferon gamma induces ER stress); ischemia; heart, liver and kidney diseases (Yoshida *et al.*, 2007).

Considering the involvement of ER stress in several diseases and ageing, understanding its mechanisms of action and their modulation by natural food constituents can be helpful for the prevention and treatment of these diseases (Yoshida *et al.*, 2007; Park *et al.*, 2013).

1.2.4. Inducers of ER Stress

Several chemicals are capable of inducing ER stress *in vitro* like tunicamycin (TUN), thapsigargin, dithiothreitol (DTT), Brefeldin A and MG132 (Oslowski *et al.*, 2011). The inducers of ER stress are usually grouped in different classes depending of their mechanisms of action. In one of these classes are compounds that work as disruptors of Ca^{2+} homeostasis. The best known are the ionophore A23187 and the Ca^{2+} pump inhibitor thapsigargin. Since the Ca^{2+} concentration in ER is at high levels and ER chaperones like BiP need Ca^{2+} ions for their function, the use of these Ca^{2+} disruptors leads to ER stress (Doner *et al.*, 1990; Price *et al.*, 1992, Oslowski *et al.*, 2011).

Another class of ER stressors is composed by reducing agents. For the proper folding of proteins the formation of disulfide bonds is necessary between cysteine residues. This requires the presence of a very strong oxidative environment in the ER (Tu *et al.*, 2004, Xu *et al.*, 2005). The use of reducing agents will prevent the formation of these disulfide bonds and, consequently, ER stress occurs due to accumulation of misfolded proteins. The two most common reducing agents used are 2-β-mercaptoethanol and dithiothreitol (DTT) (Brostrom *et al.*, 1995; Fernandez *et al.*, 1996, Oslowski *et al.*, 2011).

In the case of brefeldin A, it induces ER stress by impeding the transport of proteins from ER to the Golgi and from Golgi apparatus to ER, leading therefore to accumulation of unfolded proteins (Oslowski *et al.*, 2011). On the other hand, MG132 is a chemical that inhibits the proteasome that belongs to the ERAD machinery, leading to an accumulation of misfolded and unfolded proteins in the ER (Oslowski *et al.*, 2011).

The last class of chemicals, and the most used, is of compounds that inhibit glycosylation of proteins. Most proteins are N-glycosylated, a post-translational modification very important for protein folding. A chemical that can inhibit protein glycosylation is TUN, an antibiotic produced by *Streptomyces lysosuperificus*. It acts by a mechanism that prevent UDP-N-acetylglucosamine-dolichol phosphate N-acetylglucosaminephospho transferase (GPT), in other words, TUN blocks the formation of correctly folded proteins by inhibition of glycoprotein biosynthesis in the ER (Oslowski *et al.*,2011).

1.3. Biological Effects of Carnosic Acid

Rosemary (*Rosmanirus officinalis* L.) is an aromatic plant with several medical properties. Both the herb and its oil are used in food processing for spicing and flavouring (Ho *et al.*, 1994). It is traditionally used as herb and has analgesic and anti-microbial properties (Newall, 1996, López-Jiménez *et al.*, 2013). Rosemary is known for it antioxidant effect. In fact, the European Union legislation allows the use of rosemary extracts by the food companies labelling it as "antioxidant: rosemary extract" (López-Jiménez *et al.*, 2013). The directives 2010/67/EU and 2010/69/EU refer the use of rosemary extracts as safe, effective and a natural alternative to synthetic antioxidants (López-Jiménez *et al.*, 2013). Carnosic acid (CA) is a natural occurring phenolic compound found in rosemary. It is a phenolic diterpene with special characteristics. CA is a compound with antioxidant properties that is being assigned to the prevention or even to the treatment of some diseases related with oxidative stress and inflammation (López-Jiménez *et al.*, 2013). In addition, CA possesses antimicrobial, antiobesity, antiplatelet and antitumor activities (Takahashi *et al.*, 2009; Kelsey *et al.*, 2010; Bernardes *et al.*, 2010; Yesil-Celiktas *et al.*, 2010, López-Jiménez *et al.*, 2013).

Besides being a direct antioxidant compound, CA induces also the expression of several intracellular antioxidant defences, such as phase II and antioxidant enzymes NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST), γ-glutamyl cysteine ligase (γ-GCL), ferritin and heme oxygenase-1 (HO-1) (Macé K *et al.*, 1998; Dinkova-Kostova, 2002; Iqbal *et al.*,2003, Itoh *et al.*, 2004). This antioxidant response is known to be mediated throught Nrf2 signalling. Nrf2 is a transcriptional factor that is also activated by the PERK pathway during ER stress (Cullinan et al.2003), and when it accumulates in the nucleus activates the transcription of genes that encode for phase II and antioxidant enzymes (Cullinan *et al.*, 2003, Itoh *et al.*, 2004).

Because CA possesses both antioxidant and promotes of defence pathways in cells, it can be viewed as a compound with potential to protect against ER stress, which will be tested in this project.

OBJECTIVES OF THE WORK

The research of our laboratory group has been focused in the study of the pharmacological properties of natural compounds. The age-related diseases have been the main target of the research goals and previous studies have been performed in order to test the anti-ageing effects of some polyphenols, such as quercetin, luteolin, rosmaniric acid, curcumin and phenolic diterpenes.

Related with the ageing process and its possible modulation by natural compounds, it is currently ongoing in the laboratory the funded FCT project NaturAge (PTDC/QUI-BIQ/101392/2008). In this project, the potential anti-ageing properties of natural compounds are being tested in human skin fibroblasts in models of SIPS and replicative senescence. In particular, phenolic diterpenes, such as CA, were shown to induce antioxidant defences in human skin fibroblasts through Nrf2 signaling and to prevent significantly H_2O_2-induced premature senescence (Carvalho *et al.*, 2013, unpublished data).

In the current master work, done under the NaturAge project, the objective of the study was to evaluate the ability of ER stress to induce premature senescence in normal human skin fibroblasts, and then to use this as model to study the anti-ageing properties of CA. Since the UPR that follows ER stress is known to activate Nrf2 signaling, because CA also activates Nrf2 in human fibroblasts we hypothesize that it would protect against ER stress-induced premature senescence. To follow the previous aims and hypothesis the following questions were addressed:

1) Is the inducer of ER stress TUN able to trigger cellular senescence in normal diploid skin human fibroblasts?

2) Is the phenolic diterpene CA able to protect against ER stress-induced premature senescence in fibroblasts?

MATERIAL AND METHODS

2. MATERIALS AND METHODS

2.1. Cell Culture and Treatment

Normal diploid adult human skin fibroblasts (ASF-2 cells) were kindly provided by Prof. Suresh Rattan (University of Aarhus, Denmark), which were isolated from a breast biopsy specimen from a consenting young healthy Danish woman (aged 28 years) (Lima C.F et al., 2011). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Biochrom KG; Berlin, Germany) and 1% antibiotic/antimycotic solution (100U/ml penicillin and 100 mg/ml streptomycin; Sigma-Aldrich) and grown under an atmosphere of 5% CO^2 in air at 37 °C. For subculturing, confluent cells were washed in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and treated with 0.25% Tripsyn-EDTA solution (Sigma-Aldrich) for cell detachment.

For experiments, cells were seeded at a density of 5×10^4 cell/ml onto 12-well culture plates for MTT, β-Gal and One Step Growth Curve assays (0.8 ml/well), 6-well culture plates for Western Blotting and Nuclear Condensation assays (2 ml/well), 60 mm petri dish for cytometry (5 ml/dish) and 100 mm petri dish for real-time PCR (10 ml/dish). To induce ER stress, cells were treated with TUN (AppliChem; Darmstadt, Germany) from stock solutions prepared in dimethyl sulfoxide (DMSO) and maintained at -20 °C in aliquots. Twenty-four hours after plating, the medium was discarded and replaced with fresh medium containing TUN with the desired concentration ensuring that the DMSO concentration did not exceed 0.5% (v/v). Controls received DMSO only. The time and concentration of TUN used for the different experiments is described below and/or in the Results section. Carnosic acid (CA; ChromaDex, Irvine, CA, USA) was used to test its ability to prevent TUN toxicity, and for that a stock solution was made in DMSO and used in cell culture as described for TUN.

2.2. Cell Viability: MTT Assay

The evaluation of cell viability was performed by a colorimetric assay using the dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as before (Lima C.F *et al.*,

2011). MTT assay relies on the capacity of cells to reduce the water soluble yellow-coloured MTT in a water-insoluble blue-coloured formazan that can be then measured spectrophotometrically after dissolution with appropriate solvent. Only live cells with active mitrochondria will reduce MTT, enabling this assay for cell viability and proliferation studies (Hatok J. *et al.*, 2009).

To evaluate the effect of different concentrations of TUN on cell viability, two incubation regimes were used: continuous incubation with TUN for 48 hours, and 4 hours-pulse incubation with TUN plus 44 hours with fresh medium without TUN. Two hours before the end of the incubation, 40 µl of 5 mg/ml MTT solution (Sigma-Aldrich) was added to each well. Then, medium was carefully removed and formazan crystals were dissolved with 800 µl of DMSO/etanol (1:1 v/v) solution. The absorbance of solution was read at 570 nm using a microplate reader spectrophotometer (SpectraMax Plus384, Molecular Devices; Sunnyvale, CA, USA) using 690 nm as reference. A well with solvent was used as blank. Cell viability was presented as percentage of the negative control (cells without TUN).

2.3. One Step Growth Curve

The effects of TUN 5 µM on cell proliferation was also estimated using a One Step Growth Curve, where the number of cells was estimated by cell counting in a Neubauer chamber. After incubating cells with TUN for 48 hours, medium was replaced by fresh medium without TUN and incubated for more 3 days (first 5 days-growth curve). Then, a second 5 days-growth curve in the absence of TUN was performed after subculturing cells and refreshing the medium 48 hours after cell seeding.

2.4. Cell Cycle Analysis by Flow Cytometry

For cell cycle analysis, TUN (1 and 5 µM) was incubated with ASF-2 cells using two incubation regimes: continuous incubation with TUN for 24 hours, and 4 hours-pulse incubation with TUN plus 20 hours with fresh medium without TUN. After incubation, cells were washed with PBS and collected for tubes after treatment with 0.25% Trypsin-EDTA solution. Cells were centrifuged and rinsed with PBS before fixation with ice-cold 70% (v/v) ethanol solution for 30 minutes. Then, cells were washed with PBS, resuspended in 500 µl of PBS, and incubated for 15 minutes at 37°C after addition of 50 µl of a

200 µg/ml RNase A solution. Later, 60 µl of 0.5 mg/ml propidium iodide (Sigma-Aldrich) stain solution was added and incubated at room temperature for 15 minutes before sample analysis. Cell cycle were analysed by flow cytometry using a Coulter Epics XL flow cytometer (Beckman Coulter; Inc., Miami, FL, USA) collecting data from at least 20,000 single cells per sample. The fit of cells to each cell phase was performed using the Flow Jo software (Tree Star, Ashland, OR, USA) as before (Xavier CP *et al.*, 2012).

2.5. Senescence-Associated β-Galactosidase (SA-β-Gal) Staining

Senescent cells were evaluated by the SA β-Gal staining assay as before (Dimri *et al.*, 1995).In this method, fixed cells are incubated with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), and when cleaved by β-galactosidase originates an insoluble blue compound observable by microscopy. β-Gal positive cells obtained at pH 6 are considered senescent (Bassaneze, V. *et al.* 2008, Debacq-Chainiaux *et al.*, 2009).

For this assay, cells were incubated with 5 µM TUN for 4, 24 and 48 hours followed by a recovery period in fresh medium without TUN up to 5 days. After that, cells were subcultured and seeded in new wells at a density of 5×10^4 cells/ml. One day after plating, cells were washed twice with PBS and fixed with 4% (w/v) paraformaldehyde for 5 minutes at room temperature. After washing cells with PBS, they were incubated with a fresh staining solution (1 mg/mL X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM, $MgCl^2$, 40 mM citric acid/phosphate buffer at pH 6.0) at 37 °C in a humidified chamber overnight. After that, β-Gal-positive (blue) cells were observed and counted using a phase-contrast microscope (Olympus IX71, Hamburg, Germany).

2.6. Cell Size and Lipofuscin-Related Green Autofluorescence Analysis by Flow Cytometry

Induction of senescence in human fibroblasts is associated with the increase of cell size and lipofuscin content (Bayreuther *et al.*, 1988). Lipofuscin are fine dense granular pigments that accumulate during ageing and result from non degradable material on lysosomes (Brunk *et al.*, 2002). Lipofuscin presents increase green autofluorescence and therefore it can be estimated in a flow cytometer (Brunk *et al.*,

2002, Sitte *et al.*, 2001). In this work, flow cytometry was used to detect both changes in cell size and lipofuscin-related green autofluorescence as done in a previous work (Martin-Ruiz *et al.*, 2004).

For this assay, cells were incubated with TUN (1 μM and 5 μM) using two incubation regimes: continuous incubation with TUN for 24 hours, and 4 hours-pulse incubation with TUN plus 20 hours with fresh medium without TUN. Then, cells were washed with PBS and collected for tubes after treatment with 0.25% Trypsin-EDTA solution. Cells were centrifuged for 5 minutes at 700 xg and at 4°C and resuspended in 500 μl of PBS. Cells were then analyzed on a flow cytometer (Beckman Coulter; Epics XL) counting at least 15,000 events in each sample measuring both cell size (FSC) and green autofluorescence. The data was treated using the FlowJo software.

2.7. Nuclear Condensation Assay

To estimate cell death by apoptosis, the presence of nuclear condensation in cells was performed as described previously (Dias *et al.*, 2013). Cells were incubated with TUN (1 μM and 5 μM) using two incubation regimes: continuous incubation with TUN for 48 hours, and 4 hours-pulse incubation with TUN plus 44 hours with fresh medium without TUN. After the incubation time, both floating and adherent (after treatment with 0.25% Trypsin-EDTA solution) cells were collected for tubes, washed with PBS, and fixed with 4% (w/v) paraformaldehyde in PBS for 15 minutes at room temperature. Cells were then washed with PBS and attached into a polylysine-treated slide using a Shandon Cytospin 4 (Thermo Scientific, Waltham, MA, USA) and nuclei stained with Hoechst. Apoptosis was identified by the presence of cells with condensated and fragment nuclei under a fluorescence microscope (Olympus IX71). The percentage of apoptotic cells were calculated from the ratio between cells presenting nuclear condensation and the total number of cells (nuclei stained with Hoechst) from a count higher than 500 cells per sample.

2.8. Western Blotting

The expression of different proteins after TUN treatment (1 μM and 5 μM) was monitored by western blotting. Two incubation regimes were used: continuous incubation with TUN for 24 hours, and 4 hours-

pulse incubation with TUN plus 20 hours with fresh medium without TUN. Protein extraction was then done after washing cells with PBS, where cell pellet was lysed at 4 °C with a strong lysis buffer (50 mM Tris-HCl pH 7.4, 190 mM NaCl, 9 mM EDTA, 0.1% (w/v) sodium deoxycholate. 0.1% (w/v) SDS, 1% (w/v) Triton-X 100) supplemented with 20 mM NaF, 20mM $Na_2V_3O_4$, 1mM phenylmethylsulfonyl fluoride, and 1x protease inhibitor cocktail (Roche, Mannheim, Germany). Cell lysate was centrifuged at 10,000 xg at 4 °C for 10 minutes and protein concentration quantified by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using bovine serum albumin (BSA) as protein standard.

For western blot analysis, 20 μg/ml of protein of each sample was separated by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel during approximately 1 hour at 150 V and electroblotted to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare, Bucks, UK) during 40 minutes at 15 V using a semi-dry electroblotting system (Trans-blot SD semi-dry transfer cell, Bio-Rad Laboratories). The membranes were then blocked in TPBS (PBS with 0.05% (v/v) Tween-20) containing 5% (w/v) non-fat dry milk during 2 hours, washed in TPBS and incubated with primary antibody at the appropriate dilution overnight at 4 °C. The primary antibodies used were: anti-ATF6-α (H-280), anti-p27, anti-phospho-JNK and anti-JNK from Santa Cruz Biotechnology, Inc (Dallas, Texas, USA) and anti-GRP78 (BiP) from Abcam Inc. (Cambridge, MA, USA). Afterwards the membranes were washed with TPBS and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. The secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were from Santa Cruz Biotechnology, Inc. The immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) and acquired by a chemiluminescence detection system (Chemi Doc XRS; Bio-Rad Laboratories, Inc.). To ascertain comparative expression and equal loading of the protein samples, expression of β-actin was performed using an antibody from Sigma-Aldrich.

2.9. Study of Gene Expression by Real-Time PCR

The effect of TUN on the expression of chosen genes related with UPR and senescence was evaluated by real-time PCR. Human fibroblasts were incubated with TUN (1 μM and 5 μM) during 6 hours and, after washing with PBS, cells were collected for tubes after treatment with 0.25% Trypsin-EDTA solution, centrifuged, homogeneized in the appropriate volume of RNA Lysis Buffer (from the kit bellow) and cell lysate kept at -80 °C until use.

2.9.1. Total RNA isolation

Total RNA of human fibroblasts was extracted using the SV Total RNA Isolation System kit (Promega, Madison, WI, USA) following manufacturer's instructions. In brief, lysate was thawed on ice, mixed with RNA Dilution Buffer and centrifuged. Then, the supernatant was transferred to a fresh tube and placed in a heating block at 70°C for 3 min. After adding 95% ethanol, the mixture was transferred to a spin column in a collection tube, centrifuged, washed with RNA Wash Solution (RWA) and then the DNase treatment was made. Finally, the tube was washed with RWA and the isolated RNA eluted into an elution tube.

Total RNA concentration and purity were determined in the NanoDrop 1000 spectrophotometer (Thermo Scientific). The ratio of absorbance at 260 nm and 280 nm (A260/A280) was used to assess the purity of RNA. An absorption ratio of approximately 2.0 was accepted as "pure" for RNA.

2.9.2. cDNA synthesis

cDNA synthesis was performed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following manufacturer's instructions. 1 µg of extracted total RNA was reverse transcribed using a thermal cycler (MyCycler, Bio-Rad Laboratories, Inc) using the following protocol 5 minutes at 25 °C; 60 minutes at 42 °C and 5 minutes at 85 °C. The obtained cDNA was stored at -20 °C.

2.9.3. Real-time PCR

After cDNA synthesis, three genes were chosen (Table 1) to study their expression by real-time PCR. The primers used were designed as a previous work (Oslowski *et al.* 2011, *Tani et al.*, 2010) and are detailed in Table 1. It was previously confirmed its specificity to the chosen human genes using an online tool (Primer Blast) thereby authenticating their alignment. The primers were synthesized by STAB Vida, Lda (FCT UNL, Caparica, Portugal). The lyophilized primers were dissolved in nuclease-free water to obtain a concentration of 100 µM. The primers specificity and efficiencies were verified in a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) by performing a melting curve. Real-time PCR efficiencies were calculated according to Pfaff (2001) from the data provided by software CFX Manager (Bio-Rad Laboratories, Inc). For the real-time PCR reaction a mastermix was prepared with the

following components: 10 µl of mix from SsoFast EvaGreen Supermix kit (Bio-Rad Laboratories, Inc.) following the manufacturer's instruction, 1 µl of forward primer and 1 µl of reverse primer, 7 µl of water. Then the mastermix (19 µl) was filled in a proper multiplate for PCR reactions and 1 µl of cDNA (50 ng) was added as PCR template. For amplification and detection a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) was used. The following real-time PCR protocol was used for all genes: denaturation program (3 minutes at 95 °C), amplification and quantification program repeated 40 times (10 seconds at 95 °C; 30 seconds at 60 °C with a single fluorescent measurement) followed by melting curve program (65 °C to 95 °C with a heating rate of 0.5 °C/s and continuous fluorescent measurements). GAPDH (primers available in the laboratory and design by us) was used as internal control. Results were obtained by the comparative Ct method (Pfaffl, 2001), and expressed as fold change with respect to the control.

Table 1: List of primers used to detect ER stress and senescence markers by real-time PCR ([1]Oslowski *et al.*, 2011 and [2]Tani *et al.*, 2010).

Gene (Human)	Primer Forward	Primer Reverse	Product Length (bp)
CHOP[1]	AGAACCAGGAAACGGAAACAGA	TCTCCTTCATGCGCTGCTTT	67
sXBP1[1]	CTGAGTCCGAATCAGGTGCAG	ATCCATGGGGAGATGTTCTGG	59
usXBP1[1]	CAGCACTCAGACTACGTGCA		76
p16[2]	GTGGACCTGGCTGAGGAG	CTTTCAATCGGGGATGTCTG	132
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	123

2.10. Statistical Analysis

As appropriate, Students' *t*-test or one-way ANOVA followed by the Student-Dunnetts' post-hoc test was used to perform statistical analysis for MTT, β-Gal assay, cell cycle, cell size and real-time PCR data. GraphPad Prism 5.0 software (San Diego, CA, USA) was used and P-values ≤ 0.05 were considered statistically significant.

RESULTS

3. RESULTS

3.1. Tunicamycin Inhibits the Growth of Human Fibroblasts

The first objective of this work was to test whether the induction of ER stress by TUN was able to induce premature senescence in normal human skin fibroblasts (ASF-2 cells). For that, first the effect of different concentrations of TUN on cell viability was studied and measured by the MTT reduction assay. Two incubation procedures were used: continuous incubation with TUN for 48 hours, and a 4 hours-pulse incubation with TUN followed by 44 hours with fresh medium without TUN (recovery period). This continuous and pulse incubation with TUN was generally followed along all the work.

As shown in Figure 3A (white bars), TUN decreased cell growth by about 30% in a concentration-dependent manner from 0.02 to 0.5 μM in the continuous incubation. From 1 to 20 μM the cell growth continued to be further inhibited but much more slightly and no significant cell death was observed, as shown by the absence of floating cells under microscopic observations (Figure 3B). With the 4 hours-pulse incubation (Figure 3A – grey bars) cell growth was only significantly decreased at the concentration of 5 μM of TUN, where about 30% inhibition was also reached. Similarly to the continuous incubation, no significant cell death was observed with the highest TUN concentration tested (Figure 3B).

To verify whether TUN induced cell death by apoptosis in ASF-2 cells the nuclear condensation assay was performed. We observed that TUN did not induced apoptosis after 48 hours of treatment until the concentration of 20 μM (data not shown). These results confirmed that TUN is not cytotoxic to human fibroblasts in the conditions used.

Besides the absence of floating cells and the visible decrease of cell number along with increasing concentrations of TUN, it is also interesting to observe in Figure 3B that cell morphology changed considerably with induction of ER stress. Control cells present normal morphology like being small, thin and elongated with parallel arrangement in culture; on the contrary, TUN-treated cells (specially with higher concentrations) present increased cell size with flattened and irregular morphology, loss of parallel arrangement and presence of dense cytoskeletal actin filaments and pigments (Figure 3B). These are morphological changes typical of normal fibroblasts undergoing senescence (Rattan, 2008b),

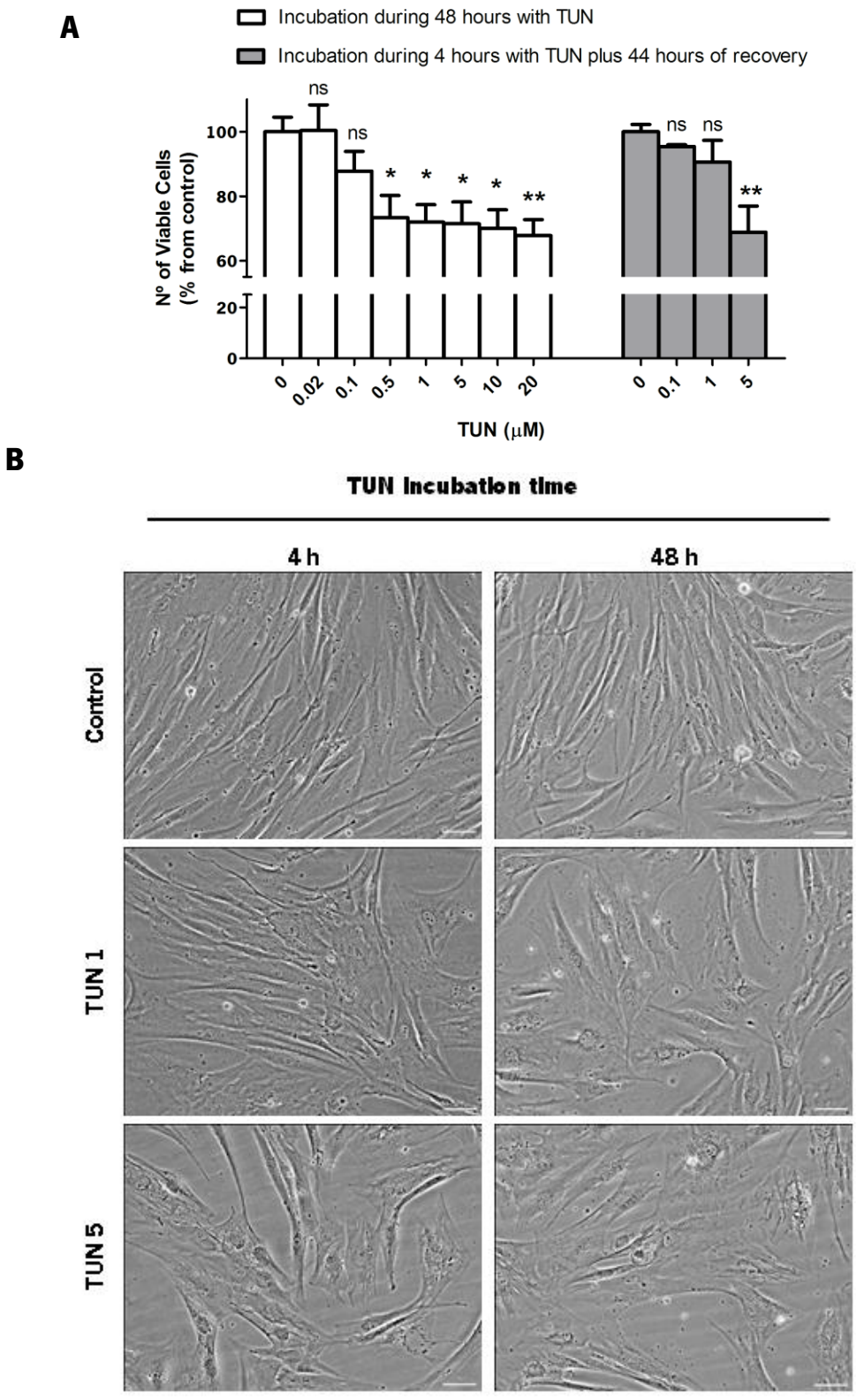

Figure 3: Effect of tunicamycin (TUN) in cell viability of human fibroblasts. (**A**) Effect of different concentrations of TUN on cell viability as measured by the MTT assay, after 48 hours incubation both in a continuous and a 4 hours-pulse TUN treatments. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value < 0.05,

(cont. Figure 3) ** P-value < 0.01, when compared with the respective control by the one-way ANOVA followed by the Student Dunnett's post-hoc test, NS, not significant when compared with control (P-value > 0.05) (**B**) Effect of TUN 1 μM (TUN 1) and 5 μM (TUN 5) in cell morphology after 48 hours of incubation with a continuous or a 4 hours-pulse treatment. Bar indicates 100 μm.

and therefore this is a good indication that TUN induced inhibition of cell growth associated with a senescent-like phenotype.

Based in these results, it was chosen the concentration of 1 μM and 5 μM for the following studies related with induction of UPR and senescence by TUN in normal human fibroblasts.

3.2. Tunicamycin Induced the Unfolded Protein Response (UPR)

Tunicamycin is known to induce ER stress followed by the UPR in human cells (Oslowski *et al.*, 2011). To confirm that in our cellular model, different UPR parameters were evaluated by western blot and real-time PCR. After 24 hours of incubation the protein levels of the ER chaperone BiP and the UPR mediator ATF6-α changed significantly after TUN treatment both in the continuous and pulse incubation (Figure 4A). With accumulation of unfolded and/or misfolded proteins due to ER stress, BiP dissociates from the UPR mediators activating them. Activation of UPR signaling pathways results then in the transcriptional activation of several genes including BiP in order to increase ER protein folding capacity (Naidoo, 2009). As shown in Figure 4A, BiP levels increased remarkably with TUN treatment, and in a concentration-dependent manner in the pulse assay, demonstrating the activation of the UPR.

In the case of ATF6-α, after its dissociation from BiP due to ER stress, it transits to the Golgi where the full-length protein (90 kDa) is proteolytically cleaved to an active transcription factor of 50 kDa that translocates to the nucleus to activate UPR genes (Oslowski *et al.*, 2011). Our results (Figure 4A) show that TUN induced a decrease or total disappearance of the inactive form of the protein (90 kDa band), most likely due to its cleavage due to the UPR activation. Unfortunately, with the antibody used, we were not able to observe the processed active form at 50 kDa (data not shown). An interesting observation is that with TUN treatment, mainly at higher concentrations and/or longer exposure time, a second band is present with a lower molecular weight (around 80 kDa) than that of the full-length and

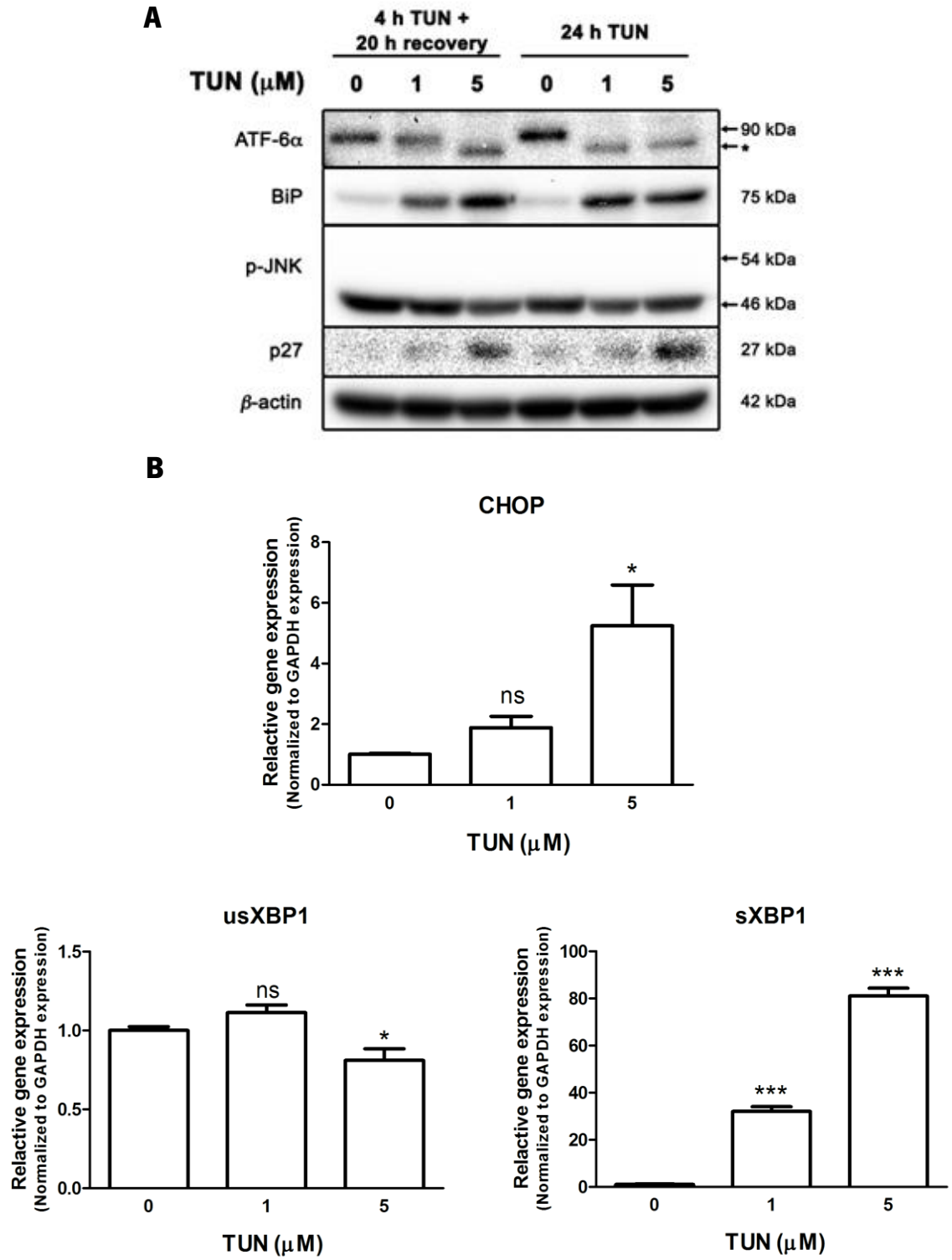

Figure 4: Effect of tunicamycin (TUN) on UPR signalling. (**A**) Effect of TUN (1 and 5 μM) in the levels of ATF6-α, BiP, p-JNK and p27 as measured by western blot, after 24 hours incubation both in a continuous and a 4 hours-pulse TUN treatments. β-Actin was used as loading control. The asterisk denotes the unglycosylated form of the full-length ATF6-α. Shown blots are representative of 3 independent experiments with similar results. (**B**) Effect of TUN (1 and 5 μM) for 6 hours in the levels of CHOP, unspliced XBP1 (usXBP1) and spliced XBP1 (sXBP1) mRNA as measured by real-time PCR. Values are mean ± S.E.M. of at least 3 independent

(cont. Figure 4) experiments. * P-value < 0.05, ** P-value < 0.01 and *** P-value < 0.001, when compared with control by the one-way ANOVA followed by the Student-Dunnett's post-hoc test, NS, not significant when compared with control (P-value > 0.05).

glycosylated one (Figure 4A). That band corresponds to the unglycosylated form of the full-length ATF6-α protein (Haze *et al.*, 1999), and, therefore, is in line with the direct effect of TUN, that is the inhibition of protein glycosylation. Overall, these western blot results indicate that TUN at 1 and 5 μM successfully induced the UPR response in a similar magnitude in the continuous incubation, where as in the pulse incubation there was a concentration-dependent effect of TUN, which is in agreement with the results of cell growth inhibition above.

The activation of other UPR signalling pathways, in particular the ones mediated by IRE1α and PERK regulators was evaluated by real-time PCR after 6 hours of treatment (Figure 4B). The activation of PERK, among other targets, leads to the up-regulation of CHOP that is associated with ER stress-mediated apoptosis (Oslowski *et al.*, 2011). We observed that the levels of CHOP mRNA increased in a concentration-dependent manner, attaining a significant 5x increase at the concentration of 5 μM of TUN (Figure 4B).

The activation of IRE1α signalling was evaluated measuring the levels of spliced XBP1. According to Oslowski *et al.*, 2011, IRE1α function as an endoribonuclease that cleaves a 26-nucleotide intron from the mRNA of the X-box binding protein (XBP) 1, originating a spliced product (sXBP1) (Figure 5). This spliced mRNA encodes for a protein that function as a potent factor of genes that restore the homeostasis of ER. As shown in Figure 4B, the expression levels of spliced XBP1 increased remarkably with TUN treatment in a concentration-dependent manner, whereas unspliced XBP1 (usXBP1) decreased significantly only at the highest concentration of TUN. Therefore, both the mRNA levels of CHOP and spliced XBP1 confirms that the UPR is triggered in human fibroblasts treated with TUN. Besides splicing of XBP1, the IRE1α mediator is also involved in the activation of JNK pathway through ASK1, which is involved in the activation of apoptosis (Nishitoh *et al.*, 2002, 2008 and Oslowski *et al.*, 2011). Although the expression of the UPR-related gene CHOP associated with apoptosis increased after TUN treatment (Figure 4B), JNK signaling decreased as shown by the phosphorylation levels of JNK measured by western blot (Figure 4A). This observation is in agreement with the absence of apoptosis shown by the lack of nuclear condensation.

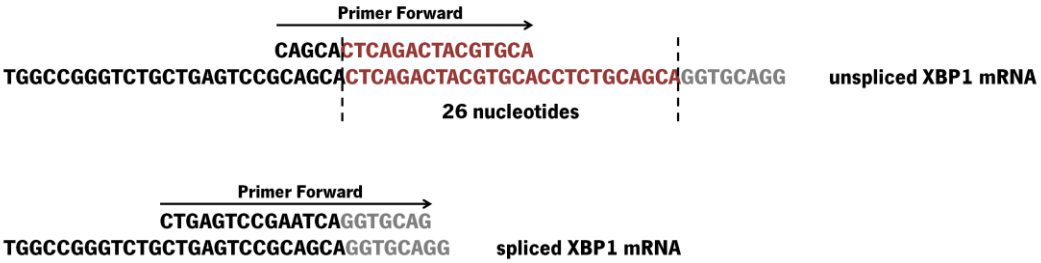

Figure 5: Representation of spliced product obtained from cleavage of 26-nucleotide intron of XBP1 mRNA and the localization of primer forward primers on unspliced and spliced XBP1 mRNA.

3.3. Tunicamycin Induces a Senescence-Like Phenotype in Normal Human Skin Fibroblasts

Considering that TUN inhibited cell growth and triggered the UPR, we then asked whether this ER stress would culminate in induction of premature senescence. First, to confirm TUN ability to inhibit cell growth, the cell cycle progression was analyzed by flow cytometry. As shown in Figure 6, TUN induced a clear increase in the number of cells at G0/G1 phase in both incubation regimes. This was accompanied by a significant decrease in the number of cells synthesizing DNA (S phase) and a noticeable decrease of cells at G2/M phase. This G1 arrest induced by TUN was concentration-dependent in both incubation regimes (Figure 6). Regarding the comparison between the two incubation regimes used, no appreciable difference was noticed, although the effect was stronger in the continuous incubation. Interestingly, a G1 arrest is what is observed in normal fibroblasts undergoing replicative senescence (Cristofalo *et al.*, 1989) or premature senescence using other inducer models (Zubova *et al.*, 2005. With the cell cycle analysis we also never observed a sub-G1 phase typical of DNA fragmentation due to apoptosis (Figure 6B), corroborating once more that TUN did not trigger apoptosis in human fibroblasts in the conditions used here.

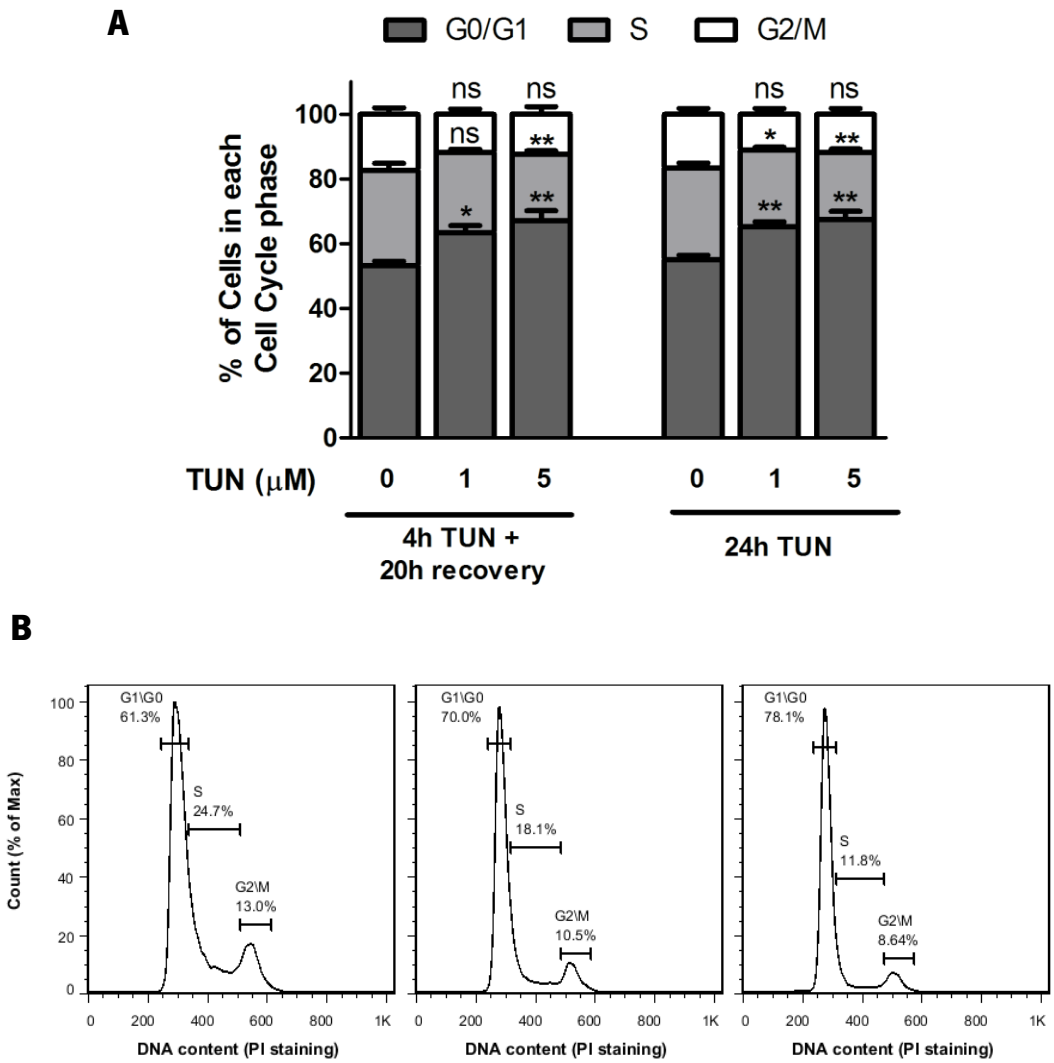

Figure 6: Effect of tunicamycin (TUN) in cell cycle progression. (**A**) Effect of TUN (1 and 5 μM) in the cell cycle phases as measured by flow cytometry, after 24 hours incubation both in a continuous and 4 hours-pulse TUN treatments. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value < 0.05 and ** P-value < 0.01, when compared with the respective phase control by the one-way ANOVA followed by the Student-Dunnett's post-hoc test, NS, not significant when compared with control (P-value > 0.05). (**B**) Representative results of the effect of the 4 hours-pulse of TUN experiment on the distribution of single cells through the phases of cell cycle after 24 hours of incubation.

During senescence of human skin fibroblasts cells increase in cell size and accumulate dense bodies containing UV-fluorescent pigments such as lipofuscin (Rattan, 2008b, Brunk *et al.*, 2002). Therefore, measuring both the increase of cell size and cells' green autofluorescence by flow cytometry can be helpful to study senescence and to distinguish senescent cells (Martin-Ruiz *et al.*, 2004). As can be

observed in Figure 7A, in the control condition it was represented a group of 25% of total cells presenting higher cell size and/or green autofluorescence, which were considered cells with a senescent-like phenotype.

This box was then maintained fixed during the TUN treatments and, as shown in Figure 7A&B, an increase from 25 % (control) to 33 % and 47 % of cells with a senescent phenotype was obtained with TUN 1 and 5 μM, respectively. Only measuring mean green autofluorescence a significant increase in this parameter was also observed dependent on TUN concentration (Figure. 7B). The mRNA levels of p16, a well-known ageing marker associated with inhibition of cell cycle (Campsi, 2007), was also measured by real-time PCR. As shown in Figure 7C, TUN induced significantly the expression of p16, and therefore this result agrees with the ability of TUN to induce senescence. The protein levels of another cell cycle inhibitor, p27, were also increased with TUN treatment (Figure 4A) corroborating thus that the cell cycle is stopped after ER stress.

Additionally, a well-known biochemical biomarker for senescence - the SA β-Gal staining - was performed 5 days after TUN treatment by different periods of time. As shown in Figure 8, TUN 5 μM increased in a time-dependent manner the number of cells presenting SA β-Gal staining. Overall, these results support that TUN induces premature senescence in normal human skin fibroblasts and seems to be irreversible, since after 5 days in culture cells still did not increase in cell number as followed by microscopic observations.

To confirm whether the stoppage of cell cycle was or not irreversible, a growth curve in two steps of 5 days each was performed. In the first one step growth curve of 5 days, cells were incubated with TUN 5 μM during 48 hours followed by 3 days of recovery in fresh medium (Figure 9B, left panel).

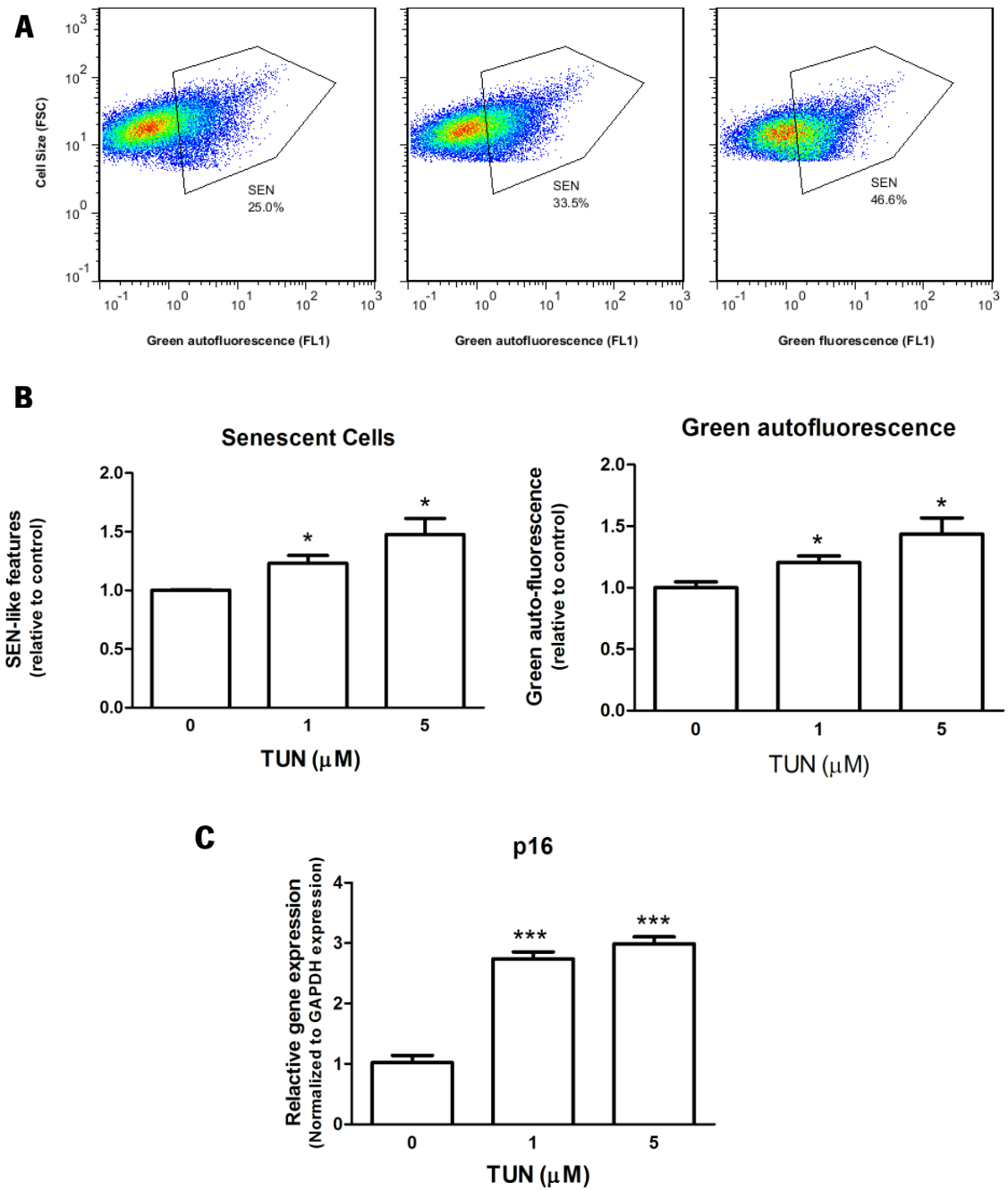

Figure 7: Effect of tunicamycin (TUN) in cellular senescence parameters. (**A**) Representative experiment of the effect of TUN (1 and 5 μM) in cell size and cells' green autofluorescence as measured by flow cytometry, after 24 hours incubation with an initial 4 hours-pulse TUN treatment. The percentage of cells inside the box were considered senescent (SEN) based in the last quartile of cells in the control condition with higher cell size and/or green autofluorescence. (**B**) Effect of TUN in the number of cells considered senescent (left panel) and cells' mean green autofluorescence (right panel) as measured by flow cytometry. Cells were treated with TUN (1 and 5 μM) during 4 hours followed by a period of recovery of 20 hours in fresh medium. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value < 0.05, when compared with control by the one-

(cont. Figure 7) way ANOVA followed by the Student-Dunnett's post-hoc test. (**C**) Effect of TUN (1 and 5 μM) for 6 hours in the levels of p16 mRNA as measured by real-time PCR. Values are mean ± S.E.M. of at least 3 independent experiments. *** P-value < 0.001, when compared with control by the one-way ANOVA followed by the Student-Dunnett's post-hoc test.

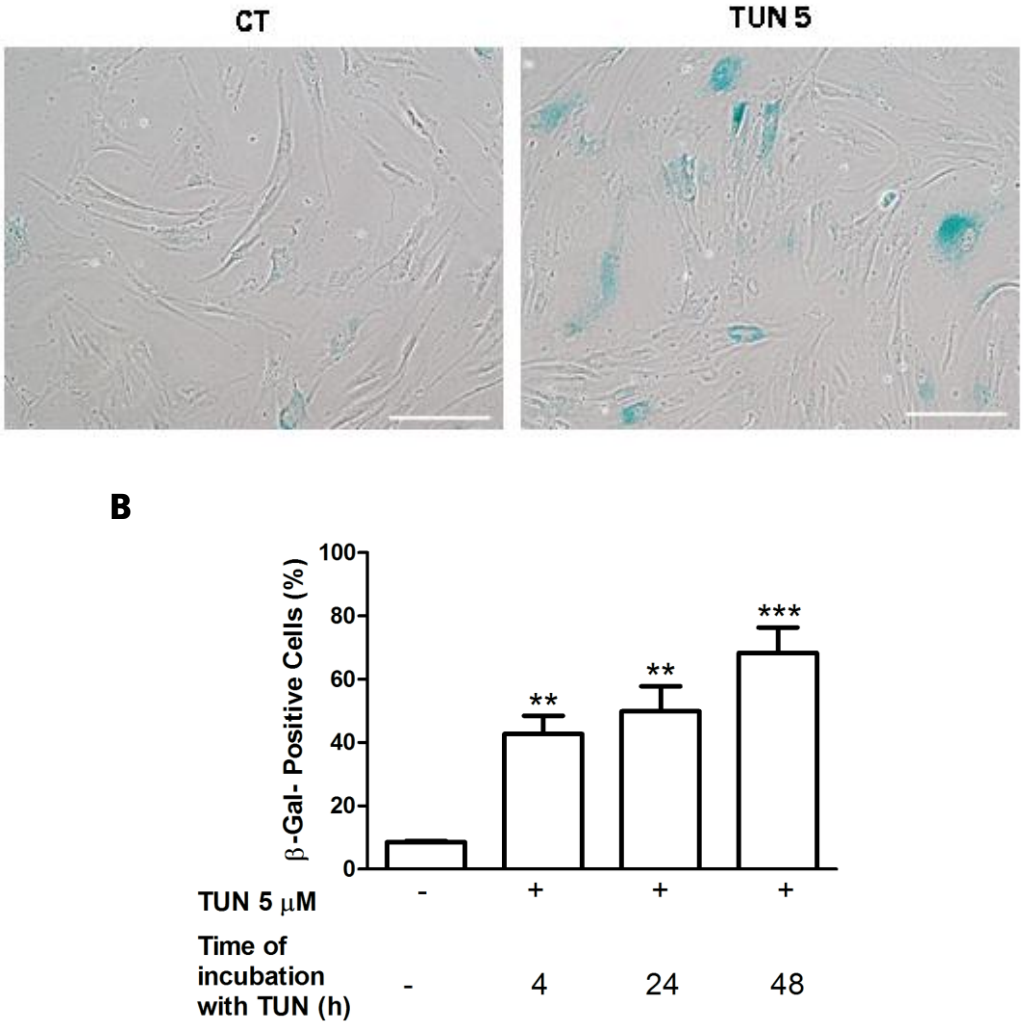

Figure 8: Effect of tunicamycin (TUN) in the senescence-associated (SA) β-Gal staining of human fibroblasts. (**A**) Representative images from the effect of TUN 5 μM (TUN 5) in the SA β-Gal positive cells, 5 days after 4 hours-pulse treatment with TUN. Bar indicates 200 μm. (**B**) Quantitative analysis of the effects of TUN in the SA β-Gal-positive cells. The fibroblasts were treated with TUN 5 μM during 4, 24 or 48 hours followed by a recovery period up to 5 days in fresh medium. Values are mean ± S.E.M. of at least 3 independent experiments. ** P-value < 0.01, *** P-value < 0.001, when compared with control by the one-way ANOVA followed by the Student-Dunnett's post-hoc test.

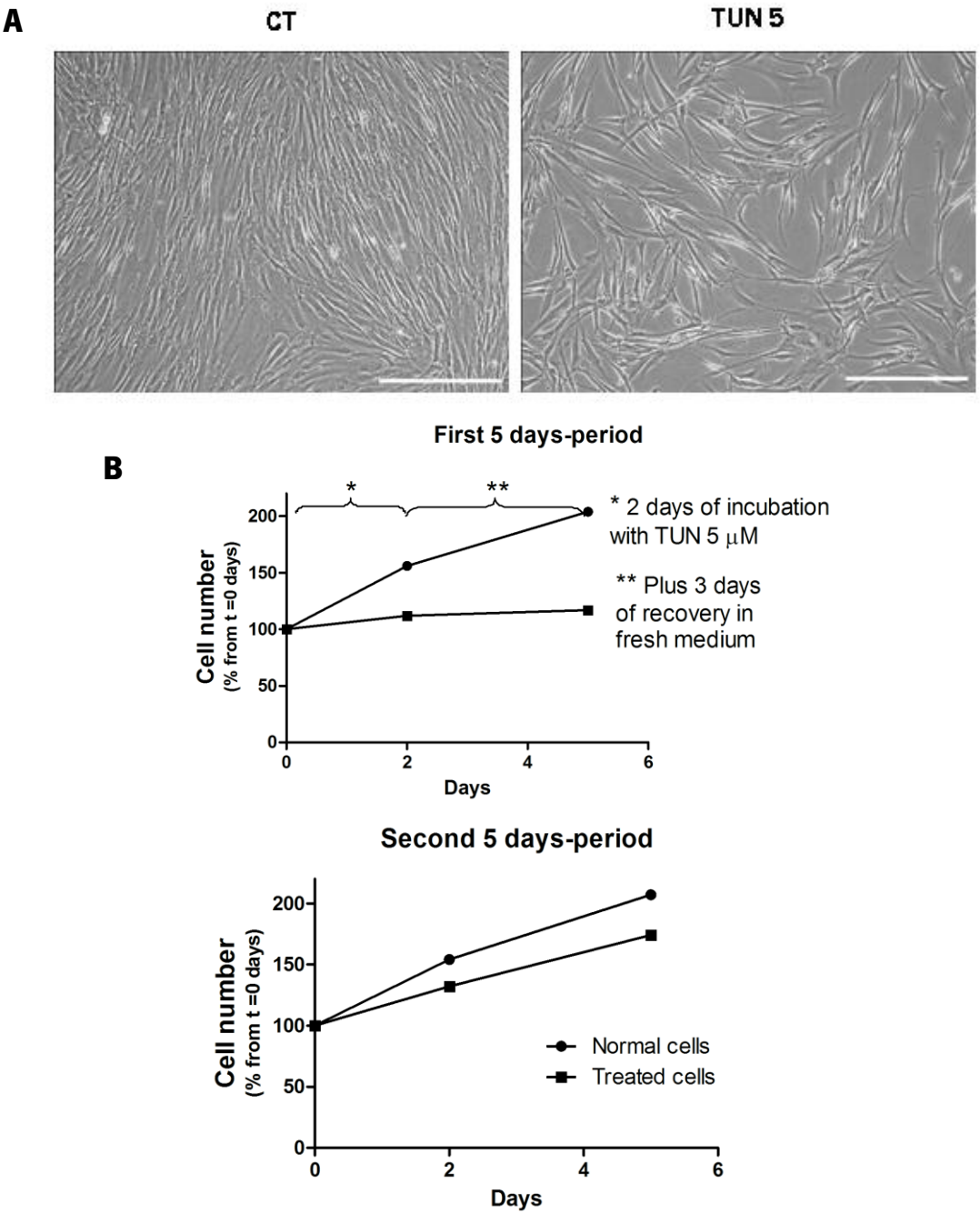

Figure 9: Reversibility of senescence induced by tunicamycin (TUN) in human fibroblasts. (**A**) Representative images of fibroblasts after 5 days in culture with or without an initial TUN 5 µM (TUN 5) treatment for 48 hours. Bar indicates 500 µm. (**B**) Effect of TUN 5 µM treatment for 48 hours in cell growth during two steps of 5 days as measured by cell counting. In the first one step growth curve of 5 days, cells were incubated with TUN 5 µM during 2 days followed by 3 days of recovery in fresh medium (left panel). Control and treated cells were then counted and subcultured to new wells at the same cell density, and a second one step growth curve of 5 days was followed without TUN treatment (right panel). Shown data are representative of two independent experiments with similar results.

As expected, control cells grew considerably as opposed to TUN-treated cells, where they maintained basically the same cell number after cell seeding – a typical response of cells undergoing senescence. After this 5 days time period, the difference in cell number between control and TUN-treated cells is well visible in Figure 7A. Then, control and TUN-treated cells were subcultured to the same cell density, and a second one step growth curve of 5 days was performed without TUN treatment. As shown in Figure 7B (right panel), control cells grew as usual, and cells that were previously treated with TUN also increased in cell number along the time but at lower rate. However, of notice in this experiment is that the growth arrest induced by TUN in human fibroblasts typical of senescence was not irreversible. Although several markers of cellular senescence were observed after TUN treatment, such as induction of G1 arrest, presence of SA β-Gal positive cells, increased cells' green autofluorescence and expression of p16, probably a percentage of cells not irreversibly arrested (non senescent) stopped cell cycle for recovery from ER stress-induced damage through a successful UPR, and then began to proliferate. This hypothesis needs, however, to be confirmed in further experiments. In fact, in a preliminary experiment (data not shown), a first treatment of cells with TUN protected them to a second TUN insult done after 5 days of a recovery period, probably due to the induction of the UPR due to the first treatment dose.

3.4. Carnosic Acid Prevents Against TUN-Induced Premature Senescence in Human Fibroblasts

After characterizing the effects of TUN in normal human fibroblasts in culture where it induced a senescence-like phenotype in thecell population, the second objective of the work was to test whether the phenolic diterpene CA, known to induce stress-associated cellular antioxidant defences (Carvalho *et al.*, 2012 and Satoh *et al.*, 2008), will be able to protect against the premature senescence induced by TUN. For that, cells were pre-incubated with the natural compound CA at 10 μM for 16 hours followed by incubation with fresh medium containing 5 μM of TUN during 4 hours. Then, cells were left in fresh medium for recovery during 5 days (with a mid-time medium refreshment) and cell growth was estimated by the MTT assay.

The used concentration of CA was previously shown to be toxic non, to not affect cell growth and to able to induce cellular antioxidant defences (Carvalho *et al.*, 2012). Here, the ability of CA to induce antioxidant defences, such as HO-1 and NQO1 enzymes, was also confirmed by western blot (data not shown).

As observed in Figure 10, a 4 hours-pulse incubation with TUN 5 μM inhibited significantly cell growth due to cell cycle arrest and induction of a senescent-like phenotype described as above. When the cells were pre-treated with CA there was a slight but significant protection against the inhibition of cell growth induced by TUN (Figure 10B). By microscopic observations, CA pre-treated condition presented also a higher number of cells with the typical parallel arrangement and less number of cells with morphological features of senescent cells, such as increased cell size, flattened and irregular morphology, and presence of dense cytoskeletal actin filaments and pigments (Figure 10A). Thus, CARN was able to prevent in some magnitude normal human fibroblasts from TUN-induced premature senescence. This needs, however, to be confirmed in further experiments measuring, for example, SA β-Gal staining and cells' green autofluorescence.

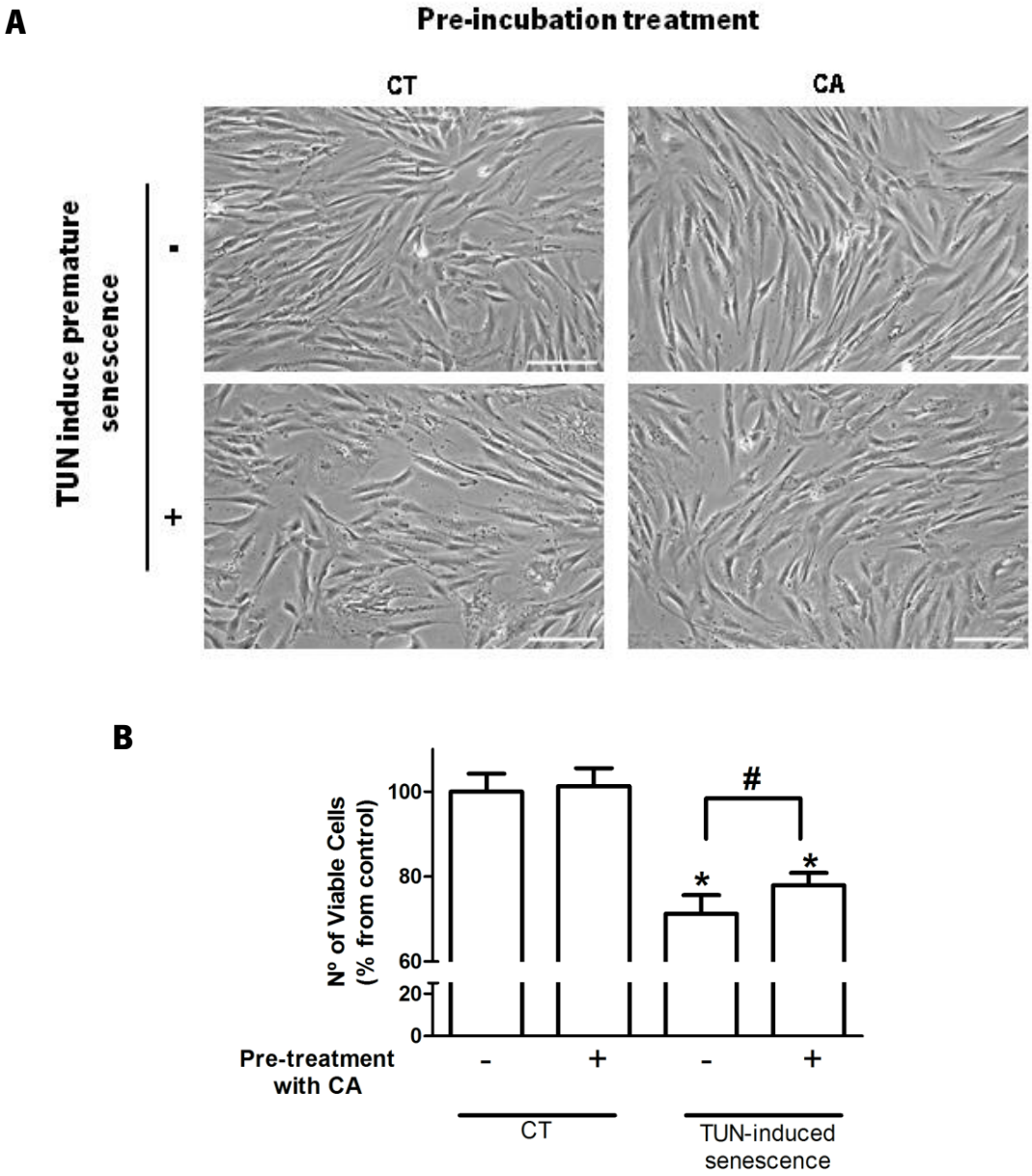

Figure 10: Ability of carnosic acid (CA) to protect against premature senescence induced by tunicamycin (TUN). Normal human fibroblasts were pre-treated with CA during 16 hours in order to activate the cellular protection pathways. After that, cells were treated with TUN 5 μM during 4 hours followed by a recovery period in fresh medium for 5 days (with a mid-time medium refreshment). In (**A**) are representative images of cells in the end of the experiment. Bar indicates 200 μm. In (**B**) is the estimation of cell growth by the MTT assay. Values are mean ± S.E.M. of 3 independent experiments. * P-value < 0.05, when compared with the respective control non-treated with TUN; # P-value < 0.05, when compared with each other (compares the effect of pre-treatment with CA). Statistical analysis was done using the Students' *t*test.

Discussion

4. DISCUSSION

Human lifespan is increasing over the years due to the improvements in the leaving conditions, diet and the health care system (Vaupel, 2010). The higher percentage of elderly people (over 65 years old) is associated, however, with a higher incidence of age-related chronic disorders such as diabetes, cancer, neurodegenerative and cardiovascular diseases, among others (Rahman, 2007). These diseases affect human health at old age inflicting suffering to the people and their families as well as imposing a high morbidity and mortality rates. Therefore, finding new strategies to stimulate a healthy ageing would be an important approach to prevent age-related diseases. Considering that a balanced diet with a high consumption of fruits vegetables and aromatic herbs are generally associated with a decreased risk of some age-related diseases (Stanner *et al.*, 2004), interventions through nutritional factors can be a good approach to stimulate a healthy ageing. In the present work we aimed to develop an *in vitro* method to induce premature senescence in normal human fibroblasts in order to be used to test compounds efficacious against protein damage and associated ER stress, known as an important factor in the development of certain age-related diseases as well as the ageing process itself.

Cellular ageing is characterized by a progressive loss of physiological fitness, leading to increased cell damage, and failure of important biological functions (Campsi, 2013). It is well known that human cells in culture do not proliferate indefinitely but instead are "mortal", reaching a limit of divisions called Hayflick limit (Hayflick *et al.*, 1961). The cells approach this limit by slowing down their division until entering in senescence, a state of total dormancy of the cells (Campisi *et al.*, 2007). After the discovery of the senescent phenotype in cultured cells, the replicative senescence of human cells were applied in the research associated to several pathological diseases, such as in cancer therapies (Kuilman et al., 2010), treatment of atherosclerotic plaques (Fenton *et al.*, 2001; Minamino *et al.*, 2002; Matthews *et al.*, 2006) and lesions (Price *et al.*, 2002), and ageing interventions resulting in lifespan extension (Haines *et al.*, 2013).

During the ageing process, an important role is attributed to damage to proteins, which is associated with the accumulation of inactive or partial active enzymes (Rothstein, 1979, 1989; Rattan, 1996; Gershon, 1979 and Rosenberg, 1991). In senescent cells, it is also reported the accumulation of abnormal enzymes and proteins due to protein misfolding and/or unfolding (Gafni, 1990; Rattan, 2008a). To overcome the time needed to follow the replicative senescence, several protocols of induction of premature cellular senescence in early passage cells have been developed. Premature

senescence can be triggered by exposure of normal diploid human fibroblasts to subcytotoxic concentrations of different stressors, such as hydrogen peroxide, ethanol, UV light and γ-irradiation (Ryazanov *et al.*, 2002). These stressors induce senescence by induction of reactive oxygen species (ROS) and/or direct damage against DNA. However, it is also known that accumulation of damaged proteins also happens, and when that accumulation turns prolonged, stress to the ER is triggered (Lai *et al.*, 2007 and Oslowski *et al.*, 2011). Since induction and increased damaged to proteins can accelerate the ageing of human cells (Rattan, 1996, 2003), in this project we attempted to develop a method of premature senescence in normal human fibroblasts using an inducer of ER stress. In our experimental model, TUN was used, which is a drug that inhibits the glycolisation of proteins, leading to their misfolding and accumulation in the ER causing ER stress and activation of the UPR pathway (Zamarbide *et al.*, 2013).

Incubation of normal diploid human skin fibroblasts with TUN resulted in cell growth inhibition in a concentration-dependent manner, but not in cell death. As expected by previous results (Samali *et al.*, 2010; Bull *et al.*, 2012), TUN induced the UPR signaling as a mechanism of defense against the ER stress, in view of the increase of BiP protein levels and cleavage of ATF6-α*,* as well as increased expression of CHOP and spliced XBP1 mRNAs. Contrary to previous results using different cell types (Ming-Zhi *et al.*, 2010 and Jung *et al.*, 2012), TUN did not induce apoptosis in human fibroblasts, as shown by the absence of nuclear condensation, lack of a fraction of cells at the sub-G1 phase by flow cytometry, and decreased levels of phospho-JNK. However, CHOP expression increased, and this protein in generally associated with ER stress-mediated apoptosis.

Interestingly, after TUN treatment cells presented s morphology typical of senescent cells, such as increased cell size with flattened and irregular morphology, loss of parallel arrangement and presence of dense cytoskeletal actin filaments and pigments. In addition, cell cycle stopped at G0/G1 phase, which is also what is observed after replicative senescence (Pignolo *et al.*, 1997, Mao *et al.*, 2012). To understand whether TUN was inducing premature senescence to the human fibroblasts or simply the cell cycle was being arrested to solve the ER stress through the UPR activation, markers of cellular senescence were evaluated. TUN treatment induced an increase in cell size and green cells' autofluorescence (a measure of ageing-associated lipofuscin content), as well as an increase in the expression of p16 and in the number of SA β-Gal positive cells. An increase in all of these parameters is characteristic of senescent cells, and is considered hallmarks of ageing (Brunk *et al.*, 2002, Rattan, 2008b). To clarify whether this senescent-like phenotype would be clearly associated with induction of

premature senescence, we then tested whether the growth arrest was irreversible. One week after TUN treatment, cells began to growth almost as good as the control cells. That indicates that at least a fraction of cells were not irreversibly growth arrested and, therefore, were not senescent. Other approaches that induces premature senescence, such as treatment of human fibroblasts with hydrogen peroxide (H_2O_2) that makes use of induction of oxidative stress to onset cellular senescence (Kiyoshima *et al.*, 2012 and Wang *et al.*, 2013), two pulse treatments with the stressor is needed to suitably induce premature senescence (Chen *et al.,* 2004). This was also attempted here with a time period of one week between the two treatments, but a protective response was obtained instead of a more pronounced senescent phenotype (preliminary experiment). This happened with TUN probably due to the protective response elicited by it – the UPR. In future experiments it can be attempted a short time between the pulse incubations with TUN to try to avoid the protective UPR response.

Nevertheless, because in the short time (one week), TUN treatment resembled well a senescent phenotype, the protective role of a known antioxidant phenolic diterpene was studied. We have chosen the natural compound CA, which is well known for its antioxidant effects (Chipault *et al.*, 1952) and its ability to induce intracellular antioxidant defenses (al-Sereiti *et al.*, 1999). Previous results in our laboratory showed the ability of CA to increase cellular antioxidant defenses through Nrf2 activation in human fibroblasts, which afforded further protection against an oxidative insult and against H_2O_2-induced premature senescence (Carvalho *et al.*, 2013, unpublished results). Also Satoh *et al.*, 2008 documented the ability of CA to induce an antioxidant protection in cerebrocortical neurons against oxidative stress, excitotoxicity, and ischemic injury via Nrf2-mediated induction of phase 2 antioxidant enzymes. That showed the capacity of this diterpene to function as a prospective therapeutic compound. A previous report by Itoh *et al.*, 2004 also showed that when cells were treated with CA, cellular antioxidant defenses increased via transcriptional activation of the Nrf2, such as the phase 2 and antioxidant enzymes, HO-1, NQO1, γ-GCL, ferritin, and GST. Since the activation of Nrf2 transcriptional pathway is triggered by the UPR-mediated PERK phosphorylation,which is crucial for cell homeostasis during ER stress (Cullinan *et al.*, 2003), we hypothesize that the pre-treatment of human fibroblasts with a compound able to induce antioxidant defenses through Nrf2 would protect against the TUN-induced premature senescence. In fact, in previous studies, cells knockout for Nrf2 revealed an increase of apoptosis and a decrease of cell survival in response to TUN treatment (Cullinan *et al.*, 2003), showing how important is Nrf2 signalling for cell survival after TUN treatment. Interestingly, in our work we observed a slight but significant protection of CA against the inhibition of cell growth induced by TUN. In addition, less number of cells with a morphology resembling senescent cells was

visible in the CA-treatment condition. It seems, therefore, that CA was also able to prevent against the TUN-induced premature senescence, although more studies is needed to confirm this and the involvement of Nrf2 induction by the phenolic diterpene in its protective response.

All these results indicate that ER stress can trigger a senescent phenotype in human fibroblasts and corroborate the importance of damage to proteins in the ageing process. This model of premature senescence developed here with TUN can be useful to find new compounds that may modulate the ageing process and ER stress in diseases associated with it, such as diabetes and neurodegenerative disorders. Finally, CA presented an interesting protective potential against the TUN-induced premature senescence in normal human fibroblasts and may be viewed in the near future as a nutritional approach for ageing interventions.

Final Remarks and Future Prespectives

5. FINAL REMARKS AND FUTURE PRESPECTIVES

5.1. Final Remarks and Conclusions

Accumulation of damaged proteins is known to be an important factor contributing to the ageing process. Unfolded and misfolded proteins induce ER stress and, if unsolved by the UPR, it can contribute to the progression of age-associated diseases, such as neurodegenerative disorders and diabetes. In this work, a protocol for induction of premature senescence triggered by ER stress was developed in normal diploid human skin fibroblasts using TUN as a stressor agent. TUN induced growth inhibition of human fibroblasts caused by cell cycle arrest at G1/G0 phase without inducing significant cell death. The UPR was triggered after TUN treatment confirming, therefore, the induction of ER stress due to inhibition of protein glycosylation.

After TUN treatment fibroblasts presented a morphology typical of a senescent state, in particular cells were bigger, flattened and with irregular edges, they loss their typical parallel arrangement and presented dense filaments and pigments. In addition, cells were growth arrested for about one week. Induction of premature senescence by TUN was confirmed by the presence of a high percentage of cells positive for SA β-Gal staining and containing the lipofuscin-associated green autofluorescence, as well as a high expression of p16 mRNA. However, one week after TUN treatment cells began to proliferate, probably because not all cells (non-senescent ones) were irreversibly growth arrested and were only dealing with the ER stress through a successful UPR.

Using this TUN-induced premature senescence model in normal human fibroblasts, the protective potential of the phenolic diterpene CA was assessed. Although preliminary, our results showed the capacity of CA to significantly prevent senescence-associated features induced by TUN in human fibroblasts.

With this work we contributed to the development of a new approach to study compounds with potential anti-ageing properties through ameliorating the ER stress due to accumulation of misfolded and damaged proteins. The potential of CA to possess positive effects during the ageing process and to prevent age-related diseases, such as Alzheimer and diabetes, was also corroborated with this work. In addition, these results agree with the assumption that consumption of fruits, vegetable and food spices

promotes a healthy ageing due to the presence of bioactive phytochemicals, which can be viewed as interesting nutritional modulators in ageing interventions.

5.2. Future Prespectives

Despite the global advance performed in this work, further experiments need to be done to answer and clarify remaining questions.

As previous mentioned, the induction of premature senescence by the ER stress inducer TUN was not attained for all cells, and after one week cells began to proliferate. A hypothesis raised was that a fraction of cells were not irreversibly growth arrested and, after recover from the ER stress, these cells began to proliferate overriding senescent ones. This needs to be clarified in following experiments, for example by sorting cells in a cell sorter after TUN treatment based in the intensity of green autofluorescence and cell size, as done in a previous work (Martin-Ruiz *et al.*, 2004). The method of induction of premature senescence through of ER stress can also be significantly improved. A possible strategy to increase the percentage of senescent cells can be by imposing to cells two pulse incubations with TUN separated by a determined time period. Another strategy would be to use two different ER stress inducers that act by different mechanisms, incubating cells with one followed by the other.

As mentioned before, the protective potential of CA against TUN-induced premature senescence needs to be confirmed by measuring more senescent-associated parameters, such as SA β-Gal positive cells and p16 expression. The role of Nrf2 signalling in the protection afforded by CA can be studied by siRNA against this transcription factor. Would also be interesting to study other natural compounds in this model of premature senescence by using compounds known to have anti-ageing effects, such as carnosine (Boldyrev *et al.*, 2013).

5.3. Funding Acknowledgements

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