Escola Superior de Saúde
Instituto Politécnico do Porto

Ana Rita Soares Barbosa da Costa

Oxidative stress in neurodegenerative diseases using yeast as a model

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Sempre chegamos ao sítio aonde nos esperam.

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Abstract

Nowadays, the ageing of population is conducting to a rise in the number of individuals with age-related illnesses, in which neurodegenerative diseases (ND) are included. Regardless of many variations in etiology and diverse mechanisms of cell injury, most of ND share high levels of oxidative stress, which have been highly referred not only as an underlying factor, but also as a feature. Thus, an actual challenge is to evaluate the role of antioxidants on oxidative stress states and evaluate the magnitude of possible therapeutic effects of these agents.

In the present study, the toxic effect of hydrogen peroxide (H_2O_2) and the antioxidative function of vitamin C were investigated using *Saccharomyces cerevisiae* as a model. The results demonstrated that H_2O_2 exposure decreased yeast cells viability in a dose-dependent manner and that, at an optimal concentration, vitamin C was able to revert its effects.

Also, a High Performance Liquid Chromatography method for determining 3-nytrotirosine, a biomarker of oxidative stress, and octopamine, a neurotransmitter similar to norepinephrine, was performed. However, results shown to be inconclusive.

Taken together, the present study demonstrated that H_2O_2 induced yeast cell death and that these adverse effects were partially rescued by combined exposure with vitamin C. These results improved the understanding of the reversal effect of antioxidant treatment and, therefore, may be helpful on providing insights on a natural antioxidant-based therapy for ND.

Key words: neurodegenerative diseases, oxidative stress, *Saccharomyces cerevisiae*, vitamin C.

Resumo

Atualmente, o envelhecimento da população tem vindo a conduzir a um aumento do número de indivíduos com patologias relacionadas com a idade, nas quais se incluem as doenças neurodegenerativas (ND). Independentemente das várias etiologias e dos diversos mecanismos de lesão celular, a maioria das ND apresenta altos níveis de *stress* oxidativo, fator referido não apenas como uma causa subjacente, mas também como característica. Assim, torna-se imperativo avaliar o papel dos antioxidantes nos estados de *stress* oxidativo e estimar a magnitude dos possíveis efeitos terapêuticos desses agentes.

No presente trabalho, o efeito tóxico do peróxido de hidrogénio (H_2O_2) e a função antioxidante da vitamina C foram estudados, usando como modelo a levedura *Saccharomyces cerevisiae*. Os resultados demonstraram que a exposição a H_2O_2 diminuiu a viabilidade celular de forma dependente da concentração e que, com uma concentração ótima, a vitamina C pôde reverter esses efeitos.

Além disso, foi utilizado um método de Cromatografia Líquida de Alta Eficiência para quantificar 3-nitrotirosina, um biomarcador de *stress* oxidativo, e octopamina, um neurotransmissor similar à norepinefrina. No entanto, os resultados demonstraram não ser conclusivos.

Em suma, o presente estudo permitiu demonstrar que o H_2O_2 é capaz de induzir a morte celular e que esses efeitos adversos foram parcialmente revertidos pela exposição combinada com vitamina C. Estes resultados possibilitaram ainda a compreensão do efeito do tratamento antioxidante e, por esse motivo, podem ser úteis para estudar a possibilidade de uma terapia natural baseada em antioxidantes para as ND.

Palavras-chave: doenças neurodegenerativas, *stress* oxidativo, *Saccharomyces cerevisiae*, vitamina C.

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$^{\bullet}NO_2$	Nitrogen dioxide
$^1O_2$	Singlet oxygen
3-NT	3-nitrotyrosine
8-OHdG	8-hydroxy-2′-deoxyguanosine
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
ATCC	American Type Culture Collection
Aβ	Amyloid-β
CAG	Cytosine-adenine-guanine
CAT	Catalase
CFU	Colony forming units
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAD	Diode-array detector
DHA	Dehydroascorbate
ECD	Electrochemical detector
fALS	Familial Amyotrophic lateral sclerosis
Fe	Iron
FUS	Fused in sarcoma
GABA	Gamma-aminobutyric acid
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GSSG	Glutathione disulfide
H_2O	Water
H_2O_2	Hydrogen peroxide
HD	Huntington's disease
HPLC	High Performance Liquid Chromatography
HTT	Huntingtin
IsoP	Isoprostane
LC	Locus coeruleus
MAP	Microtubule-associated protein
MDA	Malondialdehyde

Mn	Manganese
MT	Microtubule
NaCl	Sodium chloride
ND	Neurodegenerative diseases
NE	Norepinephrine
NFT	Neurofibrillary tangles
NO	Nitric oxide
NOS	Nitric oxide synthases
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide anion
OD_{600}	Optical density at 600 nm
$ONOO^-$	Peroxynitrite
Ox-LDL	Oxidized low-density lipoprotein
PD	Parkinson's disease
PolyQ	Polyglutamine
PRX	Peroxiredoxin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
sALS	Sporadic Amyotrophic lateral sclerosis
SD	Standard deviation
SOD	Superoxide dismutase
SOD1	Copper-zinc superoxide dismutase 1
SOD2	Manganese-superoxide dismutase
SOD3	Extracellular-superoxide dismutase
TAAR	Trace amine-associated receptors
TARDBP	Transactive response DNA-binding protein
TFA	Trifluoroacetic acid
Tyr	Tyrosine
UV	Ultraviolet
VCP	Valosin containing protein
WHO	World Health Organization
WT	Wild-type
YEPD	Yeast Extract Peptone Dextrose
α-syn	α-synuclein

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Chapter I – State of the art

1. Neurodegenerative diseases

With the increase in life expectancy, neurodegenerative diseases (ND) are becoming highly prevalent in our society. They represent a heterogeneous group of hereditary and/or sporadic conditions characterized by chronic and progressive loss of neurons, which leads to nervous system dysfunction (Uttara, Singh, Zamboni, & Mahajan, 2009; Pereira, Bessa, Soares, Leão, & Saraiva, 2012; Kim, Kim, Rhie, & Yoon, 2015). This dysfunction can cause either ataxia or dementia, depending on the type of alterations caused: functional or sensorial (Uttara et al., 2009).

Neuronal loss is associated with gliosis and with some proteins' misfolding into an abnormal three-dimensional conformation, which makes these proteins more propitious to aggregate and form extracellular and/or intracellular deposits of amyloid fibrils and inclusions containing protein fibrils. These findings represent the hallmarks of many ND and the composition of the aggregates is shown to be specific of each disease (Figure I). For example, intracellular α-synuclein (α-syn) aggregates characterize Parkinson's disease (PD); huntingtin (HTT) aggregates characterize Huntington's disease (HD); and aggregates of tau protein and amyloid-β (Aβ) peptide characterize Alzheimer's disease (AD). Besides that, all ND have in common an impaired mitochondrial function, defects in ubiquitin-proteasome system, changes in iron metabolism, excitotoxicity and inflammation events, and increased oxidative damage (Tabner, El-Agnaf, German, Fullwood, & Allsop, 2005; Halliwell, 2006; Tenreiro & Outeiro, 2010; Chen, Chunyan, & Kong, 2012; Ramanan & Saykin, 2013). Some of the most frequent ND are briefly described next.

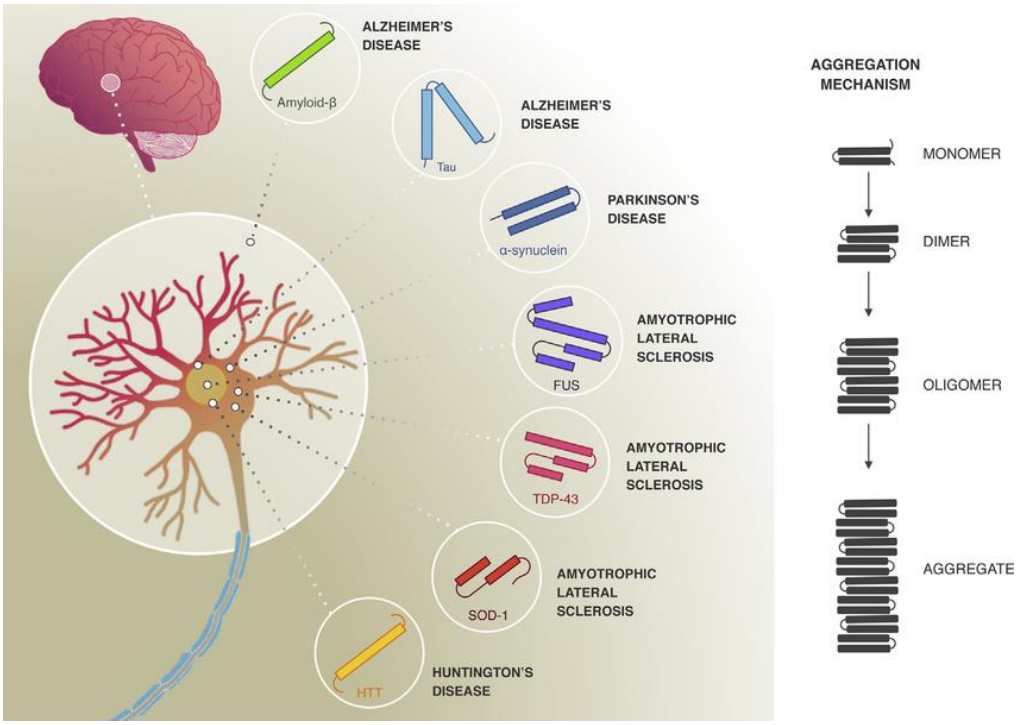


Figure I | Protein aggregation in ND.

Natively unfolded protein monomers form cross β-sheet assemblies, which evolve into oligomers, and finally form highly ordered fibrillar aggregates. This process is associated with neurodegeneration and produces insoluble protein deposits, which compose the hallmarks of different ND.

(Eftekharzadeh, Hyman & Wegmann, 2016)

i. Alzheimer's disease

AD is the most common cause (60-70%) of dementia affecting the elderly population. It is an age-related disorder causing the progressive loss of the higher cerebral functions, such as memory, language and cognitive thinking. Therefore, patients with AD tend to change their behavior and mood at the same time they lose self-sufficiency (Mega, Cummings, Fiorello, & Gornbein, 1996; Luca, Luca, & Calandra, 2015; Ahmadinejad, Møller, Hashemzadeh-Chaleshtori, Bidkhori, & Jami, 2017).

Pathologically, AD is characterized by the presence of extracellular amyloid plaques (also known as senile plaques) composed by Aβ peptide. Aβ peptides (length ranging from 38 to 43 amino acids) are proteolytic products of amyloid precursor protein (APP) and are cleaved by β- and γ- secretases (Prasad & Bondy, 2014; Chen & Petranovic, 2015). γ- secretases produce the C-terminal at the end of the Aβ peptide and they usually produce the Aβ40 peptide, the most frequent and soluble form in biological fluids; more rarely, they can

generate the Aβ42 peptide, the longer form of Aβ peptides and the most predominant in cerebral plaques. Since Aβ42 peptide is more hydrophobic and, thus, more prone to aggregate, it becomes more cytotoxic and may initiate the progressive pathophysiology of AD (Figure II) (Zou, Gong, Yanagisawa, & Michikawa, 2002; Pereira et al., 2012; Chen & Petranovic, 2015).

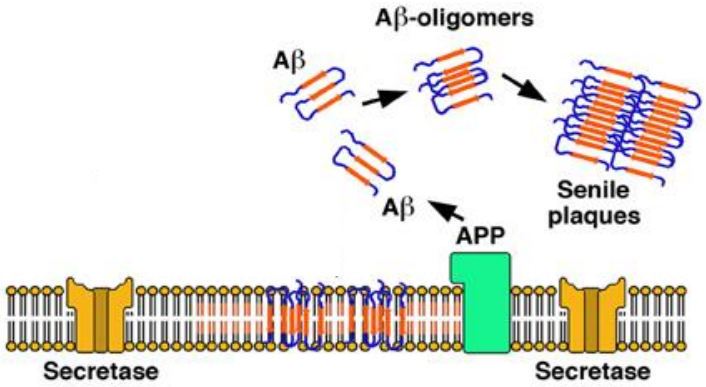


Figure II | Formation of senile plaques in AD.

Aβ peptide is generated by proteolytic cleavage of APP by secretases. Aβ peptide undergoes aggregation, with the formation of oligomers, which will deposit as senile plaques in brain.

Adapted from: Pizzimenti et al., 2013

Another hallmark of AD is the intracellular accumulation of neurofibrillary tangles (NFT) in the brain, composed by aggregates of hyperphosphorylated tau protein. Under normal circumstances, tau is a highly soluble microtubule-associated protein (MAP), predominantly found in neurons, that is involved in microtubule (MT) stabilization. MTs serve as essential 'tracks' for normal trafficking of cellular cargo along the lengthy axonal projections of neurons (Braun, 2012; Nussbaum, Seward, & Bloom, 2013; Luca et al., 2015). However, under pathological conditions, the balance of tau binding to the MTs is disturbed – it is though that the path from normal tau bound to the MTs to large aggregate structures as NFTs begins with the detachment of tau from the MTs (which can be caused by an increased rate of phosphorylation or a decreased rate of dephosphorylation) (Figure III), resulting in an abnormal increase in the levels of the unbound tau fraction. Thus, the resultant higher cytosolic concentration of tau increases the probability of pathogenic conformational changes, which may lead to its aggregation. If tau forms aggregates, its normal function of stabilizing MTs is lost and leads to a disturbance in the normal structural and regulatory functions of the cytoskeleton, which compromises axonal transport and consequently

contributes to neurodegeneration, as observed in AD (Ballatore, Lee, & Trojanowski, 2007; Noble, Hanger, Miller, & Lovestone, 2013).

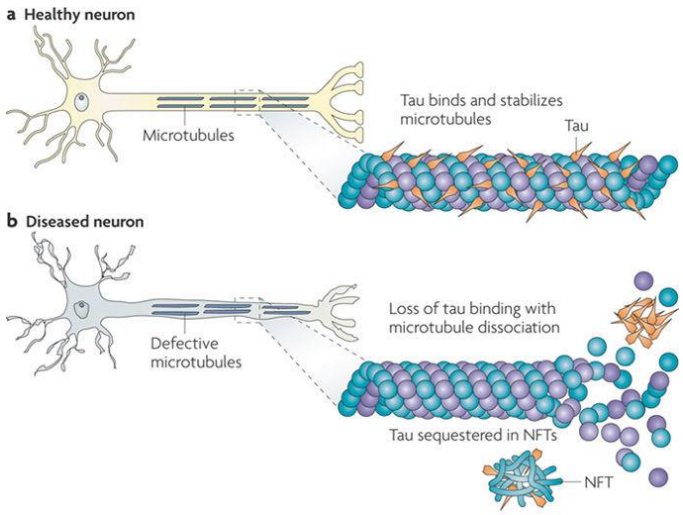


Figure III | Tau in healthy neurons and in pathological conditions.

a| Tau facilitates MT stabilization. **b|** Tau function is impaired in AD, probably as a result of both tau hyperphosphorylation, which reduces the binding of tau to microtubules, and the aggregation of hyperphosphorylated tau into NFTs, which reduces the amount of tau that is available to bind microtubules. Loss of tau function leads to MT instability and contributes to neurodegeneration.

Brunden, Trojanowski, & Lee, 2009

ii. Parkinson's disease

PD is the second most common ND and the most prevalent movement disorder, presenting clinical features as resting tremor, bradykinesia, rigidity and postural instability (Pereira et al., 2012; Franssens et al., 2013). Although there are strong evidences that PD is associated with environmental risk factors (Campdelacreu, 2014), there is a large spectrum of genetic factors underlying PD pathophysiology.

Some genes were found to be related with PD genetic etiology (including *PINK1*, *PARK2*, *DJ-1* and *LRRK2*), but the α-syn gene (*SNCA*) was the first to be identified as to play a major role (Lin & Beal, 2006; Saiki, Sato, & Hattori, 2011), with three-point mutations (A53T, A30P and E46K), as well as locus duplication and triplication being highly associated with familial forms of the disease (Saiki et al., 2011; Chesi, Kilaru, Fang, Cooper, & Gitler, 2012; Pereira et al., 2012). Besides that, α-syn is also related to sporadic PD, being the major constituent of Lewy bodies, the proteinaceous neuronal inclusions that are the hallmark of

neurodegeneration found in PD (Eriksen, Przedborski, & Petrucelli, 2005; Johri & Beal, 2012).

The common feature of mutations of wild-type (WT) α-syn and duplications/triplications of its gene is the elevated levels of protein generated, causing α-syn to be toxic and leading to an increased rate of neuronal cell death, especially dopaminergic neurons (Figure IV) (Perez & Hastings, 2004; Olanow & Brundin, 2013). On the contrary, some studies claim that the aggregation of α-syn in Lewy bodies may allow soluble α-syn levels to fall below a critical limit needed for optimal dopaminergic neuronal function, since α-syn regulates dopamine homeostasis (Perez & Hastings, 2004; Saiki et al., 2011).

As the impairment of the dopaminergic function causes the symptoms abovementioned, and it is closely related to α-syn, its function should be further elucidated in order to allow the comprehension of the exact mechanisms behind PD.

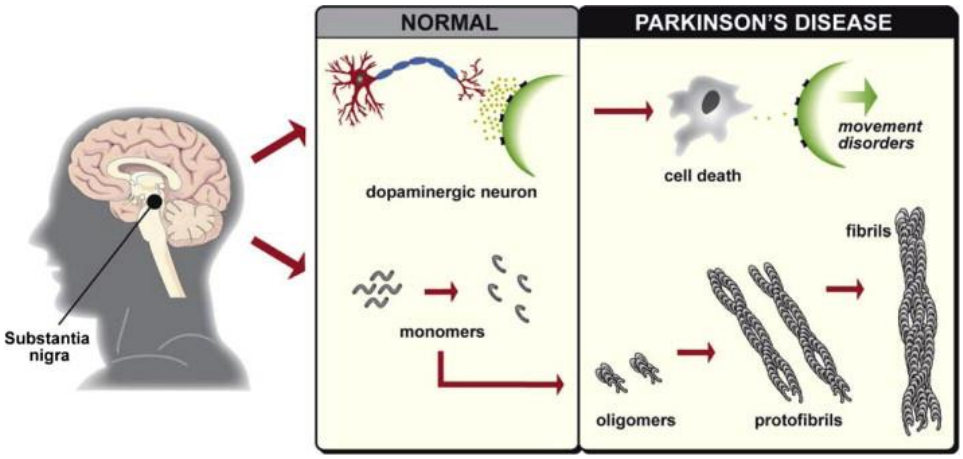


Figure IV | Pathogenesis of PD.

Formation of α-syn fibrils and the loss of dopaminergic neurons in the substantia nigra are observed in patients with PD. α-syn is normally a monomeric unstructured protein which undergoes conformational changes and forms fibrils. These fibrils lead to dopaminergic neurons death and, consequently, movement disorders.

Ruipérez, Darios, & Davletov, 2010

iii. Huntington's disease

HD is an autosomal dominant inherited ND characterized by progressive decline of motor, behavioral and cognitive functions, with patients demonstrating loss of self and spatial consciousness, depression, dementia and anxiety. Usually, these symptoms begin in young

adult life and occur over 10-20 years, ending invariably in death (Labbadia & Morimoto, 2013; Dayalu & Albin, 2015).

At the molecular level, HD is caused by a mutation in the *HTT* gene, located on chromosome 4, that encodes HTT, a protein ubiquitously expressed by neurons throughout the central nervous system (CNS). HTT contains a polyglutamine (polyQ) tract encoded by uninterrupted cytosine-adenine-guanine (CAG) trinucleotide repeats in the first exon of *HTT*; however, this mutation causes an expansion of the CAG trinucleotide repeat, resulting in an excessively elongated polyQ tract, which is associated with protein aggregation and gain-of-function toxicity due to the expansion (Figure V). To note, while normal individuals have up to 35 CAG repeats, HD patients have expansions of 36 or more repeats, with ≥42 causing HD with 100% certainty. Individuals with a repeat length of 36-41 can present signs or symptoms of the disease within a normal life span (Pereira et al., 2012; Labbadia & Morimoto, 2013; Dayalu & Albin, 2015; Glajch & Sadri-Vakili, 2015; Jimenez-Sanchez, Licitra, Underwood, & Rubinsztein, 2017).

Mutant HTT is highly prone to aggregation and, thus, the formation of cytoplasmic aggregates and nuclear inclusions all over the brain is one of the hallmarks of HD. Initially, the disease is characterized by the degeneration of the striatum, due to specific loss of medium spiny neurons; yet, with the disease progression, other regions are affected, including neocortex, cerebellum and hippocampus. All these findings have a direct correlation with the typical symptoms of HD (Roos, 2010; Johri & Beal, 2012; Ayala-Peña, 2013; Labbadia & Morimoto, 2013).

Despite its monogenic nature, HD pathogenesis is so complex that, nowadays, treatment is limited to suppressing chorea (the abrupt, irregular and unpredictable movements of the arms and legs) and treating psychiatric symptoms, since there are no known disease-modifying medications currently available (Jimenez-Sanchez et al., 2017).

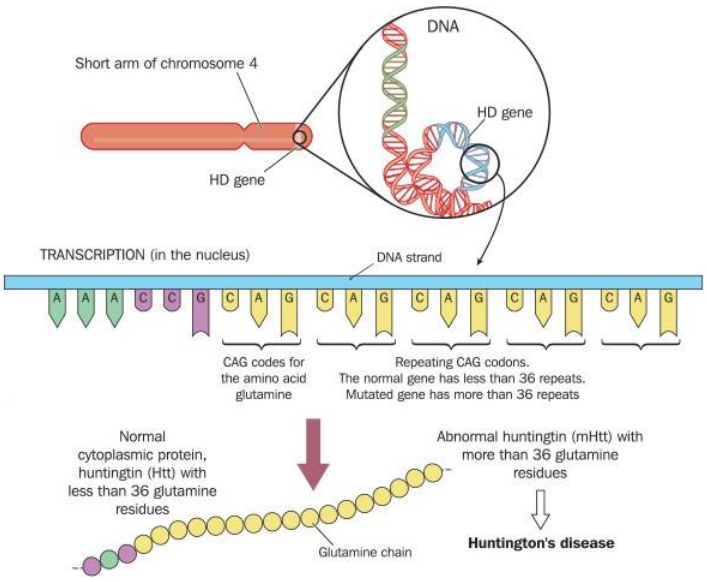


Figure V | Pathogenesis of HD.

Expansion of CAG codons encoding polyQ leads to an abnormal HTT protein with more than 36 glutamine residues, which is the underlying cause of HD.

Adapted from: Huntington's Disease Society of America. Retrieved from http://hdsa.org/what-is-hd/.

iv. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive ND that targets selectively the upper and lower motor neurons in cortex and spinal cord (Johri & Beal, 2012; Al-Chalabi et al., 2014). The degeneration reflects in primary lateral sclerosis, as a result of the impairment of upper motor neurons, and muscular atrophy and spasticity, as well as weakness, as a result of the loss of lower motor neurons (Figure VI) (Gordon, 2013).

About 90% of cases are sporadic (sALS) and it is thought that they have both genetic and environmental causes, such as advanced age and smoking; despite that, the major cause remains unclear. On the other side, 10% of the ALS reported cases are familial (fALS) and inherited in an autosomal dominant, autosomal recessive or X-linked mode (Gordon, 2013; Cirulli et al., 2015).

Among the genes implicated in fALS, there are several studies reporting a pathogenic role for the copper-zinc superoxide dismutase 1 (*SOD1*), transactive response DNA-binding protein of 43 kD (*TARDBP*), fused in sarcoma (*FUS*), angiogenine and valosin containing protein (*VCP*) genes. Besides these, there are approximately 15 more genes involved in the pathogenesis of ALS; however, only mutations in the referred ones are associated with the

disease, while the remainder are associated with unusual phenotypes or have been reported in a few number of cases (Hardiman, Berg, & Kiernan, 2011; Gordon, 2013; Cirulli et al., 2015).

The first discovered mutation was in the *SOD1* gene, in chromosome 21, and is responsible for about 20% of fALS (Bowser, Cudkowicz, & Kaddurah-Daouk, 2006; Johri & Beal, 2012). SOD1 is a metal-binding and homodimeric ubiquitous protein of 153 residues, which function is to catalyze the reduction of the superoxide anion ($O_2^{\bullet-}$) to oxygen (O_2) and hydrogen peroxide (H_2O_2). More than 170 different mutations in *SOD1* are involved in fALS, but most of these mutations show little changes in enzymatic function, suggesting that toxicity derives from a gain of toxic function, instead of a loss of native function. Additionally, it is known that protein aggregates are also a common feature of ALS and appear as a result of *SOD1* mutation (as well as on other involved genes), in spite of the causal relationship between aggregates and neuronal cell death is not conclusively reported. Thus, similarly to what happens in the other ND, protein aggregates are associated with neurodegeneration (Andersen & Al-Chalabi, 2011; Gordon, 2013; Cirulli et al., 2015; Sangwan et al., 2017).

It is important to note that several ALS genes show pleiotropy, which means that the same gene mutation can result in different phenotypes; this fact turns ALS in an unpredictable disease that may progress at variable rates and, therefore, makes it difficult to find a molecular path to its stop or delay its progression (Bowser et al., 2006; Al-Chalabi et al., 2014).

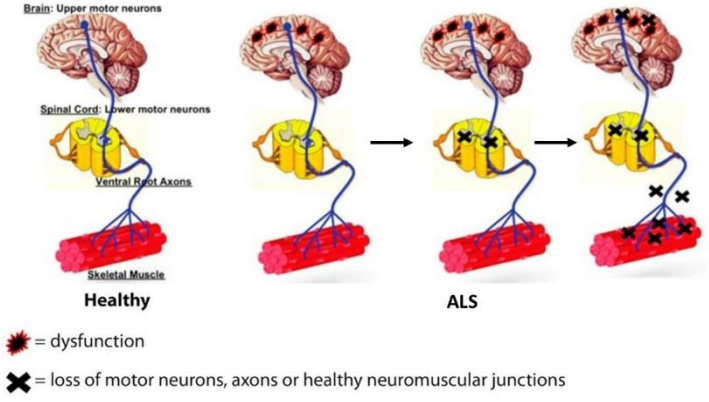


Figure VI | Model of ALS pathogenesis and progression.

Dysfunction (red/black symbols), but not cell death (black x symbols), of corticospinal motor neurons occurs at early and presymptomatic stages, leading to a presymptomatic loss of spinal motor neurons. This loss, which affects the neuromuscular junctions, results in the appearance of the characteristic symptoms of the disease.

Adapted from: Thomsen et al., 2014

2. Oxidative stress in neurodegenerative diseases

The well-known impact and association between oxidative stress and many human diseases led to a high number of experiments in this research area. Diseases such as AD, PD, ALS, among others, are truly associated to oxidative stress (Ahmadinejad et al., 2017). Despite the knowledge of some hallmarks of these ND, their etiology is not fully clarified; although, it is known that oxidative stress has a central role in their development (Li, O, Li, Jiang, & Ghanbari, 2013; Kim et al., 2015; Liu, Zhou, Ziegler, Dimitrion, & Zuo, 2017).

Oxidative stress is a condition characterized by an imbalance between the production of free radicals, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS), and the antioxidants levels, resulting in damage to cells (Tabner et al., 2005; Gandhi & Abramov, 2012; Pokusa & Trančíková, 2017).

The main ROS molecules are presented in Figure VIII. ROS are produced from molecular O_2 in consequence of normal cellular metabolism. These molecules are characterized by containing one or more unpaired electrons, working as electron donors, and can be divided into 2 groups: free radicals and nonradicals. Molecules containing one or more unpaired electrons, and thus giving reactivity to the molecule, are called free radicals. When free radicals share their unpaired electrons, nonradical forms are generated. Although these forms are not reactive, they can easily lead to free radical reactions in organism; thus, the presence of unpaired electrons makes molecules highly reactive, which leads to oxidation. Consequently, damage of several macromolecules occurs (e.g. lipids, proteins, polysaccharides and nucleic acids) (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Poprac et al., 2017; Shefa et al., 2017).

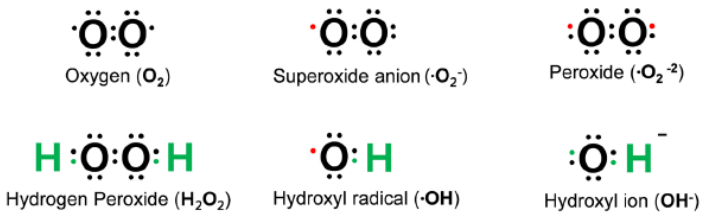


Figure VII | Main ROS molecules.

The consecutive reduction of oxygen through adding electrons cause the formation of a variety of ROS.

Kim et al., 2015

RNS are a result of excessive production of nitric oxide (NO), which is a molecule that contains one unpaired electron and is, therefore, a free radical. NO is generated in biological

systems by nitric oxide synthases (NOS) and, since ROS are also a product of normal metabolism, NO, in the presence of $O_2^{\bullet-}$, is converted in peroxynitrite ($ONOO^-$), which is the most toxic RNS. Along with $ONOO^-$, other derivative oxidants from NO, represented in Table I, also have the capacity of acting as oxidative agents, causing DNA fragmentation and lipid oxidation (Valko et al., 2007; Griendling et al., 2016).

Table I | Major biological RNS.

Formula	Name	Reaction of formation
NO	Nitric oxide	L-arginine $\xrightarrow{NOS}$ L-citrulline + NO
$ONOO^-$	Peroxynitrite	$NO + O_2^{\bullet-} \longrightarrow ONOO^-$
ONOOH	Peroxynitrous acid	$ONOO^- + H^+ \longrightarrow$ ONOOH
$^{\bullet}NO_2$	Nitrogen dioxide	ONOOH $\longrightarrow$ $^{\bullet}NO_2 + OH$
N_2O_3	Dinitrogen trioxide	$NO + {}^{\bullet}NO_2 \longrightarrow N_2O_3$

Several exogenous and endogenous causes are associated with oxidative stress (Figure VIII). The exogenous and environmental causes comprise ionizing radiation of X-, γ- or cosmic rays, α particles from radon decay, oxidizing chemicals and ultraviolet (UV) A radiation from solar light. The endogenous causes have an intracellular source, mostly resulting from signaling pathways and metabolic and/or inflammation processes (Kryston, Georgiev, Pissis, & Georgakilas, 2011; Birben et al., 2012; Ahmadinejad et al., 2017).

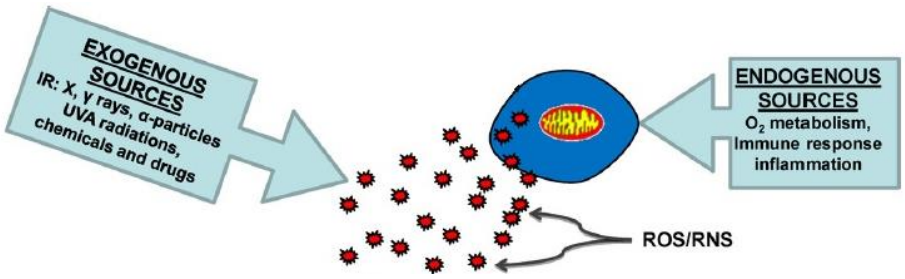


Figure VIII | Exogenous and endogenous sources of oxidative stress.

IR: Ionizing Radiation; ROS/RNS: Reactive Oxygen/Nitrogen Species.

Adapted from: Kryston et al., 2011

Nonetheless, since ROS and RNS are molecules which occur naturally in the body during metabolic processes, at controlled levels (low to moderate concentrations), they both play

important and beneficial roles in numerous physiological processes. For instance, ROS are involved in growth, apoptosis, cell signaling and immune response, whereas RNS contribute to the regulation of apoptotic cell death, leukocyte adhesion, blood pressure regulation and angiogenesis. Nevertheless, when ROS/RNS levels exceeds a certain threshold and the system's ability to neutralize and eliminate them, their deleterious effects become apparent and may conduct to lipid peroxidation, protein misfolding and aggregation, and DNA mutations (Pokusa & Trančíková, 2017; Poprac et al., 2017).

Several studies indicate that neuronal cells are very susceptible to oxidative injury due to (i) their high polyunsaturated fatty acid content in membranes, that are prone to oxidation; (ii) their rich content in metal ions – which accumulate in brain as a result of ageing and can be a trigger for ROS formation; (iii) exposure to high O_2 concentration due to ventilation, and (iv) weak antioxidant defense (Chen et al., 2012; Kim et al., 2015; Liu et al., 2017). The major factor for the high susceptibility of the brain to oxidative stress is its great O_2 consumption, since brain corresponds to only 2% of body mass, but requires about 20% of the total O_2 production. Together, these conditions can lead to free radicals' generation in a concentration that can exceed the scavenging ability of endogenous antioxidants, resulting in an oxidant state of the brain (Abramov, Berezhnov, Fedotova, Zinchenko, & Dolgacheva, 2017; Pokusa & Trančíková, 2017).

As abovementioned, the pathogenesis of several ND is a result of misfolded proteins' accumulation. According to Liu et al. (2017), the aggregation of these modified proteins is a trigger for inflammatory response in the brain, which induces ROS release and consequent oxidative stress. On the other hand, Ganguly, Chakrabarti, Chatterjee, and Saso (2017) suggest that proteinopathy is not the only promoting factor of disease, but acts together with oxidative stress and mitochondrial dysfunction to cause neurodegeneration. Still, Chen et al. (2012) propose that ROS toxicity contributes to protein misfolding. Thereafter, the pathophysiology of ND is certainly associated with oxidative stress (Bisht & Dada, 2017), despite it is still not known whether it is a cause or a consequence.

i. Biomarkers of oxidative stress

According to World Health Organization (WHO), a biomarker is any substance, structure or process that can be measured in the body or its products and can influence or predict the

incidence of outcome or disease. Thus, biomarkers can be markers of exposure, effect or susceptibility (WHO, 2001).

Biomarkers of oxidative stress are molecules modified by interactions with ROS and RNS in the microenvironment and other molecules that change in response to increased redox stress. DNA, lipids (including phospholipids), carbohydrates and proteins are examples of molecules that can be modified by excessive ROS/RNS, leading to deleterious damages to these molecules (Ho, Galougahi, Liu, Bhindi, & Figtree, 2013; Marrocco, Altieri, & Peluso, 2017).

Since free radicals are highly reactive and have a short half-life, they are difficult to measure. Therefore, accurate assessment of oxidative stress associated molecules, such as H_2O_2 and NO, in human tissues or biological fluids is challenging. Thereby, the oxidative stress state has to be assessed by indirect methods, involving the measurement of biomarkers, since they are stable end products that usually accumulate at detectable concentrations and reflect specific oxidation pathways (Shah, Mahajan, Sah, Nath, & Paudyal, 2014; Griendling et al., 2016).

The interaction between ROS/RNS and specific molecules produces different end products. Hence, lipid oxidation biomarkers include malondialdehyde (MDA), isoprostane (IsoP) and oxidized low-density lipoprotein (Ox-LDL); DNA oxidation biomarker is mainly the 8-hydroxy-2′-deoxyguanosine (8-OHdG) product; and protein oxidation is markedly given by S-glutathionylation of cysteine residues and nitration of tyrosine (Tyr) residues (Figure IX). Among all these referred biomarkers, lately, the protein nitration has been highly considered as a very promising approach for the assessment of oxidative stress (Ho et al., 2013; Shah et al., 2014; Teixeira, Fernandes, Prudêncio, & Vieira, 2016).

Tyr is a non-essential amino acid present in most proteins found in nature. It is moderately hydrophilic, which makes it frequently surface-exposed in proteins, allowing subsequent modifications, namely nitration. Nitration is mediated by RNS like $ONOO^-$ and nitrogen dioxide ($^{\bullet}NO_2$), and generally occurs in a two-step process involving the formation of a Tyr radical generated by different oxidative steps, followed by a reaction with $^{\bullet}NO_2$. This process results in the formation of 3-nitrotyrosine (3-NT), a very stable molecule that is considered a good biomarker of protein oxidation (Frijhoff et al., 2015; Marrocco et al., 2017).

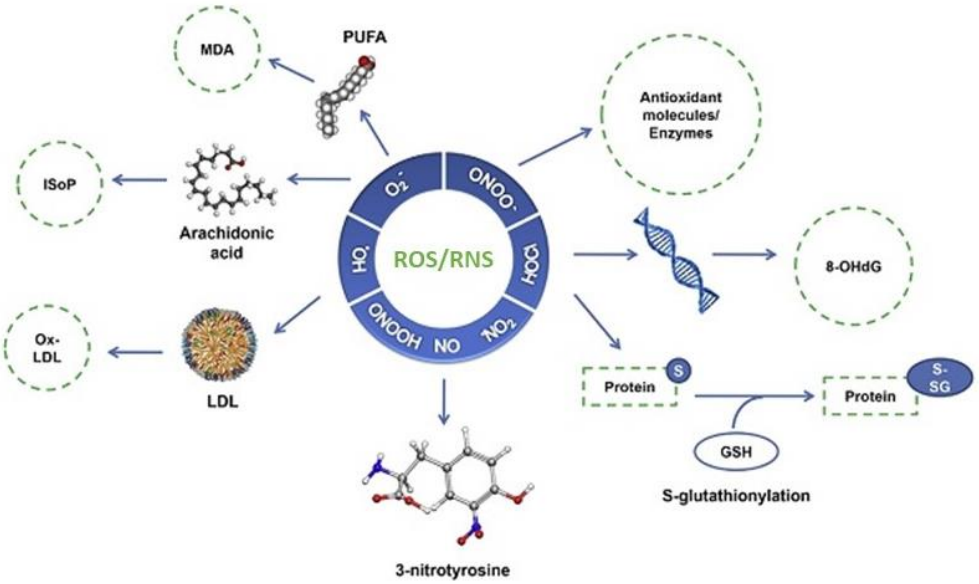


Figure IX | Formation pathways of the most common biomarkers of oxidative stress.

Adapted from: Teixeira et al., 2016

Moreover, 3-NT has been implicated in age-related diseases, thus being also an excellent biomarker for oxidative damage that is known to occur in ND (Marrocco et al., 2017).

ii. Methods for quantification of 3-NT

Since 3-NT has raised great interest due to its potential as a biomarker of oxidative stress and as a parameter for therapeutic monitoring of several diseases involving oxidative stress, some efforts have been made to develop more sensitive and specific methods for its quantification (Guvenç, Aksoy, Das, Atmaca, & Yavuz, 2014; Teixeira, Prudêncio, & Vieira, 2017).

The first approaches for 3-NT analysis in biological samples included immunological methods, performed using antibody-based methods as Western blot or immunohistochemistry. Because many of these assays have not a defined and validated accuracy, precision and limit of quantification, chromatography-based methods began to be developed. In fact, among the other techniques used for 3-NT detection, chromatographic methods reveal a better performance, since the concentrations, even in pathological conditions, are in the lower nM-range and this type of analysis has a great sensibility. This set of methods was already used to detect 3-NT in several biological samples, including plasma, serum, urine and cerebrospinal fluid (CSF) (Ryberg & Caidahl, 2007; Radabaugh, Nemirovskiy, Misko, Aggarwal, & Mathews, 2008; Teixeira et al., 2017).

Among the chromatographic methods used for detection and quantification of 3-NT, High Performance Liquid Chromatography (HPLC) is one of the most extensively used in combination with different detectors, such as UV, electrochemical detector (ECD) or diode-array detector (DAD) (Yang, Zhang, & Pöschl, 2010; Meyer, 2010).

HPLC is a useful method to separate, identify, quantify and purify a certain compound of a mixture. It is basically a highly improved form of column liquid chromatography, in which a sample is injected, at high pressure, into a column that holds packing material (the stationary phase), with further pumping of the mobile phase through the column. The detection is then accomplished through the retention times exhibited by the molecules. The retention time is based on differences in the migration rate through the column, since different molecules have different affinities for the mobile phase and the stationary phase used in the separation (Meyer, 2010; Teixeira et al., 2016).

The separation of the molecules itself is not visible unless a detection method is used. Regarding the existing options, HPLC-DAD (Figure X) is the most commonly used method for *in vitro* 3-NT analysis, taking into account that it allows the simultaneous detection at diverse wavelenghts, enabling a more complete analysis (Skoog, Holler, & Crouch, 2017; Teixeira et al., 2017).

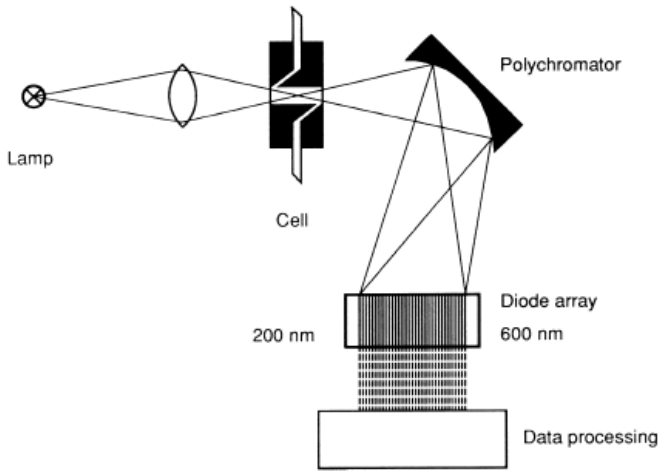


Figure X | DAD system.

Meyer, 2010

Briefly, DAD consists of a light source from a deuterium or tungsten lamp that passes directly through a detector cell, being subsequently divided spectrally in a polychromator. The spectral light then reaches the diode array (a chip with large number of light sensitive

diodes) and each diode obtains a fraction of the information, which is posteriorly read by the electronics for data processing and translating (Meyer, 2010; Skoog et al., 2017).

3. Antioxidant defenses

Although cells are constantly exposed to the effects of ROS deriving from either external sources or endogenous metabolism, they have developed effective antioxidant defenses to cope with the toxic effects of oxidants (Guaiquil, Vera, & Golde, 2001). Antioxidants are molecules that inhibit or quench free radical reactions and delay or protect against oxidative damage to a target cell (Pamplona & Costantini, 2011). Antioxidants exists both in the intra and extracellular environment and can be divided into two categories, based on their activity: enzymatic and non-enzymatic (Table II).

Table II | Main enzymatic and non-enzymatic antioxidants.

Adapted from: Birben et al., 2012

Enzymatic antioxidant defenses		Non-enzymatic antioxidant defenses	
Name	**Acronym**	**Chemical name**	**Name**
Superoxide dismutase	SOD	β carotene	-
Catalase	CAT	Ascorbic acid	Vitamin C
Glutathione peroxidase	GSH-Px	α-tocopherol	Vitamin E
Peroxiredoxin	PRX	Glutathione	GSH

i. Enzymatic antioxidants

Enzymatic antioxidants are the first and primary defense system against ROS and work by breaking down and removing free radicals. The antioxidant enzymes convert dangerous oxidative products to H_2O_2 and then to water (H_2O), in a multi-step process (Birben et al., 2012).

Since $O_2^{\bullet-}$ is the main ROS produced, its dismutation by superoxide dismutase (SOD) has a crucial importance for cells. There are three identified different forms of this enzyme: cytosolic SOD1; mitochondrial manganese (Mn)-SOD (SOD2); and extracellular-SOD (SOD3) (Kim et al., 2015).

SOD eliminates $O_2^{\bullet-}$ by converting it to H_2O_2 trough the reaction 1:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

Despite H_2O_2 is less reactive than $O_2^{\bullet-}$, it stills being toxic and must be removed. In order to do that, other enzymes, such as catalase (CAT) and glutathione peroxidase (GSH-Px), work coordinately to eliminate H_2O_2 (Kim et al., 2015; Nimse & Pal, 2015).

CAT is mainly located in peroxisomes, but it is also found in the cytoplasm and mitochondria. This enzyme is responsible for the conversion of H_2O_2 to H_2O and O_2 (reaction 2) using either iron (Fe) or Mn as a cofactor for its activity. CAT has the highest turnover rates of all the enzymes, being able to convert approximately 6 million molecules of H_2O_2 each minute. Thus, it is the most effective protection against high levels of oxidative stress (Rahman, 2007; Nimse & Pal, 2015).

$$2\ H_2O_2 \rightarrow O_2 + H_2O \quad (2)$$

On the other hand, GSH-Px, located in cytosol and mitochondria, catalyzes the reduction of H_2O_2 to H_2O using glutathione (GSH) as an electron donor (reaction 3). It competes with CAT for H_2O_2 as a substrate and, contrary to it, GSH-Px is the major source of protection for low levels of oxidants (Rahman, 2007; Kim et al., 2015; Nimse & Pal, 2015).

$$H_2O_2 + GSH \rightarrow GSSG + H_2O \quad (3)$$

Despite their differences, there is a clearly synergistic effect on the scavenging of $O_2^{\bullet-}$ by these three enzymes, as shown on Figure XI.

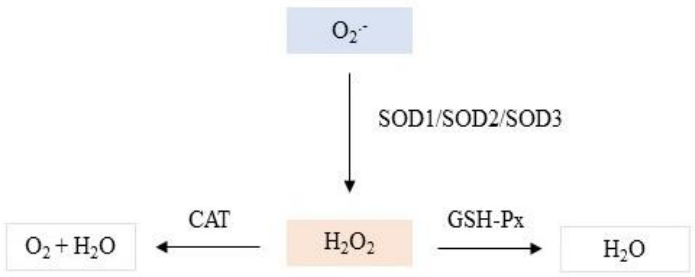


Figure XI | Combined activity of SOD, CAT and GSH-Px on eliminating $O_2^{\bullet-}$.

Furthermore, peroxiredoxin (PRX) is also an important antioxidant enzyme which, together with its partner proteins – thioredoxins –, has a strong catalytic power and promotes a very fast reaction on reducing H_2O_2 to H_2O, as well as catalyzing the reduction of other organic hydroperoxides and $ONOO^-$ (Kim et al., 2015). There are six PRX isoforms with different locations (Netto & Antunes, 2016; Fisher, 2017), as shown on Table III.

Table III | PRX isoforms and respective locations.

PRX isoform	Location
1	Cytoplasm, nuclei, mitochondria and peroxisomes
2	Cytoplasm and nuclei
3	Mitochondria
4	Cytoplasm, nuclei and lysosomes
5	Cytoplasm, nuclei, mitochondria and peroxisomes
6	Cytoplasm

As presented, PRX are abundant in eukaryotic cells, being more than 1% of total cellular proteins; this fact makes it responsible for the reduction of up to 90% of mitochondrial H_2O_2 and nearly 100% of cytoplasmic H_2O_2 (Netto & Antunes, 2016; Fisher, 2017).

ii. Non-enzymatic antioxidants

The non-enzymatic antioxidants are considered the second defense system against free radicals (Peng et al., 2014). Despite there are two types, natural and synthetic non-enzymatic antioxidants (Lü, Lin, Yao, & Chen, 2010), only the first ones will be briefly revised.

Non-enzymatic antioxidants include a diversity of low molecular weight compounds, such as vitamins, β-carotene and GSH, which provide protection against oxidative damage caused by ROS, and also enhance the endogenous enzymatic antioxidants function, by synergistically scavenging the reactive free radicals. Their low molecular weight also represents an advantage to eliminate ROS at sites not accessible for larger enzymes (Pamplona & Costantini, 2011; Peng et al., 2014).

GSH is a tripeptide synthesized from glutamate, cysteine and glycine and is highly abundant in all cell compartments. GSH comprises a system that maintains the intracellular reducing environment and acts as a defense against excessive generation of injurious ROS (Kim et al., 2015; Nimse & Pal, 2015). Glutathione is present in several redox forms, among which the most important are its reduced form, the GSH itself, and oxidized form, glutathione disulfide (GSSG). In normal conditions, the predominant form is the reduced form, in a ratio of 100:1, with GSH constituting up to 98% of the total glutathione pool. Thence, GSH/GSSG ratio is a major determinant of oxidative stress (Birben et al., 2012; Mironczuk-Chodakowska, Witkowska, & Zujko, 2018).

GSH shows its antioxidant effects by two ways:

1) It detoxifies H_2O_2 and lipid peroxides via action of GSH-Px. GSH donates its electron to H_2O_2 to reduce it into H_2O and generates its oxidized form:

$$H_2O_2 + 2\ GSH \xrightarrow{GSH\text{-}Px} 2\ H_2O + GSSG \quad (4)$$

GSSG is then reduced again into GSH by GSH reductase, using NADPH as an electron donor:

$$GSSG + NADPH + H^+ \xrightarrow{GSH\ reductase} 2\ GSH + NADP \quad (5)$$

It is also important to refer that GSH-Px is involved in the protection of cell membrane from lipid peroxidation, mediating the transference of protons from GSH to membrane lipids, protecting them against oxidant injuries (Birben et al., 2012; Kim et al., 2015; Mironczuk-Chodakowska et al., 2018).

2) It regenerates vitamins C and E back to their active forms, after their reactions with free radicals (Rahman, 2007; Pamplona & Costantini, 2011).

In fact, despite having a different mechanism, GSH work together with some enzymatic antioxidants to create a network to scavenge ROS and removing H_2O_2, avoiding oxidative damage, as shown on Figure XII (NG, Schafer, Buettner, & Rodgers, 2007).

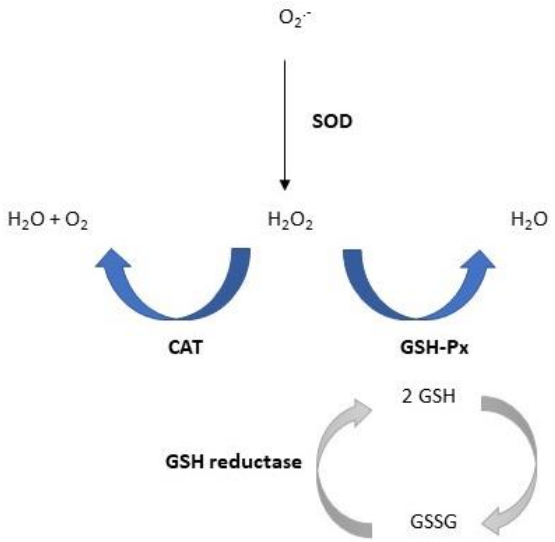


Figure XII | Network of antioxidants for H_2O_2 removal.

β-carotene is a member of the carotenoids group, which are considered provitamins due to their particularity of being converted into active vitamin A. Carotenoids contain conjugated double bounds and their antioxidant activity comes from their ability to delocalize unpaired electrons. This fact gives carotenoids an important role on the protection of cell membranes against ROS, due to their chemical reactivity with free radicals. Furthermore, carotenoids can scavenge peroxyl radicals, disrupting the reaction sequence, and, consequently, preventing the damage of cellular lipids. β-carotene, particularly, acts as a quencher of singlet oxygen ($^1O_2$), preventing oxidative damage (Pham-Huy, He, & Pham-Huy, 2008; Nimse & Pal, 2015).

Vitamin E is the collective term given to a group of fat-soluble compounds with antioxidant activity. Naturally occurring vitamin E includes eight forms of lipophilic molecules, namely the alpha, beta, gamma and delta classes of tocopherol and tocotrienol. α-tocopherol is the most active form in humans, and given its fat-soluble property, it is the major powerful membrane bound antioxidant, safeguarding cell membranes from damage by free radicals (Rahman, 2007; Pham-Huy et al., 2008; Wu, Cheng, & Wanga, 2017).

The main function of vitamin E is to protect against lipid peroxidation by transferring a hydrogen atom to a lipid free radical, such as peroxyl and alkoxyl, resulting in the corresponding non-radical product of the lipid and an α-tocopheroxyl radical (Figure XIII). Thus, it functions to terminate the lipid peroxidation chain reaction and, since the resultant α-tocopheroxyl radical is relatively stable and insufficient to initiate lipid peroxidation itself, it can be reduced to its original form by vitamin C (Pamplona & Costantini, 2011; Nimse & Pal, 2015).

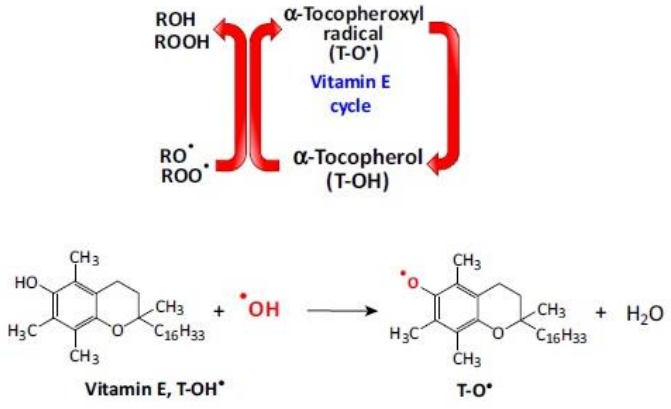


Figure XIII | Antioxidant mechanism of vitamin E.

Lipid peroxyl radicals (ROO•) are scavenged by vitamin E (α-tocopherol, T-OH), resulting in the formation of lipid hydroperoxide and a radical of vitamin E (α-tocopheroxyl radical, T-O•).

Adapted from: Poprac et al., 2017

Vitamin C, also referred as ascorbate or ascorbic acid, is a water-soluble free radical scavenger, which is synthesized from glucose in the liver of some mammalian species, allowing the maintenance of needed physiological levels. However, humans lack the functional enzyme for the final step of its synthesis, being strictly dependent on exogenous sources (Covarrubias-Pinto, Acuña, Beltrán, Torres-Díaz, & Castro, 2015).

Vitamin C is a water-soluble antioxidant that counteracts the damaging effects of oxidative stress through its ability to quench $^1O_2$ and other reactive species. In addition, vitamin C is able to neutralize ROS before they can attack lipids. It acts as an antioxidant by donating 2 of its electrons, preventing other compounds from being oxidized. By donating an electron to a lipid radical to terminate the lipid peroxidation chain reaction, an ascorbyl radical is formed. Despite being a radical, it is stable when compared to other free radicals and is considered a good free-radical scavenger. The loss of a second electron generates dehydroascorbate (DHA), which does not have any antioxidant capacity, but can be converted back in ascorbate (Figure XIV). Along with this mechanism, which enables a reaction with nearly all relevant reactive species, vitamin C also acts as a coantioxidant, by regenerating α-tocopheroxyl back to its reduced state (Heo, Hyon-Lee, & Lee, 2013; Prior, 2014; Nimse & Pal, 2015; Ashor, Siervo, & Mathers, 2016; Poprac et al., 2017).

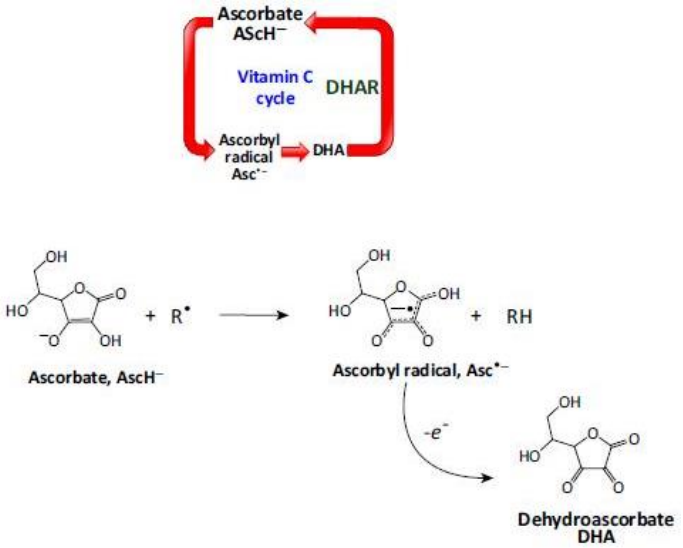


Figure XIV | Antioxidant mechanism of vitamin C.

The reaction of vitamin C (ascorbate, $AscH^-$) with a free radical ($R^\bullet$) leaves behind a vitamin C radical (ascorbyl radical, $Asc^{\bullet-}$). $Asc^{\bullet-}$ is transformed to a more stable DHA, which is reduced back to ascorbate by the action of dehydroascorbate reductase (DHAR).

Adapted from: Poprac et al., 2017

4. Vitamin C in neurodegenerative diseases

Vitamin C is an antioxidant with multiple cellular functions. It plays a role as an enzymatic cofactor, participates in detoxification processes and, among other functions, modulates synaptic activity and neuronal metabolism (Covarrubias-Pinto et al., 2015).

Vitamin C homeostasis is highly regulated, with the greatest concentration found in the brain. Since the brain is responsible for 25% of total body glucose utilization, such elevated activity leads to a high oxidative metabolism, causing the brain to be dependent on antioxidants for protection against pathological situations (Heo et al., 2013; Covarrubias-Pinto et al., 2015).

Vitamin C enters the CNS via sodium-dependent vitamin C transporter type 2, from the plasma to the CSF across the choroid plexus epithelium. On the other hand, if large amounts of DHA are present in the blood, it can rapidly enter the CNS via glucose transporters present in the blood-brain barrier endothelium. Once in the CSF, either vitamin C or DHA enters the neurons through the same respective transporters, and then, DHA can be reduced to vitamin C. This uptake results in a higher than 10-fold gradient between the concentration of vitamin C in brain (160 μmol/L) and plasma (40 to 60 μmol/L) (Heo et al., 2013; Pisoschi & Pop, 2015; Moretti, Fraga, & Rodrigues, 2017).

The main role of vitamin C in the CNS is to provide antioxidant protection against oxidative damage. Thus, a vitamin C deficiency has a negative consequence on brain function, particularly during development. A high growth rate in a developing brain is responsible for an increased cellular metabolism; if there is not a sufficient antioxidant system, this state promotes a redox imbalance. For this reason, levels of vitamin C in brain must be increased during early life. Along with the antioxidant function, vitamin C is also considered a neuroprotective agent, since it protects neurons from glutamate excitotoxicity, which is associated with ND as well (Covarrubias-Pinto et al., 2015; Kocot, Luchowska-Kocot, Kiełczykowska, Musik, & Kurzepa, 2017).

As already abovementioned, redox imbalance and oxidative stress are observed during aging and ND. The origin of oxidative stress in ND could be related with a redox imbalance promoted by (i) a deficiency of antioxidant defense enzymes, (ii) vitamin C supply and (iii) an altered vitamin C homeostasis in the CNS. In fact, failure to supply or maintain vitamin C concentrations in the brain can increase the oxidative status of the cells (Covarrubias-Pinto et al., 2015; Moretti et al., 2017).

Kocot et al. (2017) refers that a decreased concentration of vitamin C in nervous tissue is accompanied with neurological disorders; at the same time, Acuña et al. (2013) suggests that a failure in neuronal vitamin C uptake is related with neurodegenerative events. Moreover, plasma vitamin C levels decline with age and ND are often associated with aging. Thus, these postulates constitute further evidence pointing to the role of vitamin C in neurodegeneration (Kocot et al., 2017; Moretti et al., 2017).

Given its importance in brain, in the past few years there have been a growing interest in study vitamin C based treatments for ND and demonstrating its beneficial effects. For example, Murakami et al. (2011) demonstrated that a 6-month treatment with vitamin C restored behavioral deficits and reduced the formation of Aβ oligomers in a mouse model of AD. They also presented that a decrease in Aβ oligomerization was accompanied by a decreased oxidative damage in brain. Sil et al. (2016) used a colchicine-induced rat model of AD to demonstrate that vitamin C administration resulted in recovery of memory along with prevention of neurodegeneration in the hippocampus. Referring to HD, and knowing that it is linked to a deficit of vitamin C release into striatal extracellular fluid (Rebec, Barton, & Ennis, 2002), Rebec, Conroy, and Barton (2006) proved that a 3-day injection of sodium ascorbate returned the level of striatal extracellular vitamin C in a mouse model of HD. Finally, regarding multiple sclerosis, which is associated with neurodegeneration, increased oxidative stress and demyelination, vitamin C seems to be useful for its prevention or treatment, once, besides its property of scavenging free radicals, it contributes for the collagen synthesis, which is associated with myelin formation (Moretti et al., 2017).

The current knowledge on the effects of vitamin C in neurodegenerative processes is summarized in Figure XV.

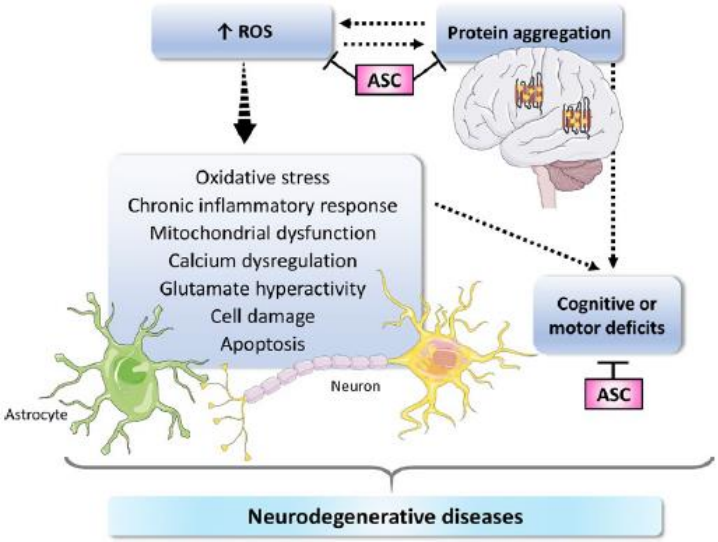


Figure XV | Effects of vitamin C.

ND are associated with increased ROS in the brain. The large amount of ROS and impaired antioxidant defenses observed in these diseases, lead to neuronal oxidative stress, chronic inflammatory response, mitochondrial dysfunction, calcium dysregulation, glutamate hyperactivity, cell damage and apoptosis. Most importantly, excessive ROS play a role in events associated with protein aggregation and cognitive dysfunction by causing damage to proteins, lipids and DNA. Clinical and nonclinical studies have indicated that vitamin C (ASC) has neuroprotective effects by reducing oxidative stress and formation of protein aggregates, which may contribute to the reduction of cognitive and motor decline observed in these pathologies.

Moretti et al., 2017

Despite there are many studies concluding that vitamin C may result as a treatment for some ND, through its capacity of diminishing oxidative stress and the protein aggregation, other studies did not show the same results, being inconclusive as to the relationship between vitamin C and disease (Moretti et al., 2017). Therefore, more studies are needed to support the current results and clarify the knowledge in this field.

5. Neurotransmitters in neurodegenerative diseases

The two most important neurotransmitters in the brain are glutamate and gamma-aminobutyric acid (GABA), which control excitatory and inhibitory neurotransmission, respectively. Thus, in the CNS, maintaining an appropriated balance between glutamate and GABA is critical for a normal neuronal function (Rowley, Madsen, Schousboe, & White, 2012; Li et al., 2016).

GABA is synthesized from glutamate by the enzyme glutamic acid decarboxylase. Therefore, it follows that glutamate should be present at right concentrations and that cells should have an accurate sensitivity to it; also, it is essential to maintain adequate extracellular levels of glutamate, as it is unceasingly released from the cells and, thus, should be continually removed at appropriated rates from the extracellular fluid in order to keep the balance. Both excess and lack of glutamate are harmful, with the excess causing a process referred as excitotoxicity, which happens when glutamate excite nerve cells to their death (Campos-Peña & Meraz-Ríos, 2014; Zhou & Danbolt, 2014; Li et al., 2016).

An altered balance in neurotransmission was found to be related to ND. In fact, neurotransmitters are implicated not only in the development of symptoms (such as seizures, a typical hallmark of PD) (Rowley et al., 2012), but also in the process of neurodegeneration (Li et al., 2016). For instance, in PD, there is an imbalance of GABA and glutamate in the extrapyramidal system, a part of the brain's motor system involved in the coordination of movements. In this disease, a typical GABA deficiency and a glutamate excitotoxicity are present (Werner & Coveñas, 2014). Also, in patients with early PD that have not the motor symptoms yet, other features such as decreased sense of smell and depression (shown to precede the classical motor alterations), have been described as undoubtedly linked to the deficit of GABA (Błaszczyk, 2016). In case of AD, the same pattern of imbalance is related, with an extracellular elevation of glutamate causing excitotoxicity, which correlates with the loss of memory and learning ability in these patients, resulting from the cell death (Campos-Peña & Meraz-Ríos, 2014). Concerning to ALS, Bosch, Damme, Bogaert, and Robberecht (2006) refers that excitotoxicity is not only a part of the pathogenic mechanism responsible for the disease, but could also be a cause for the vulnerability of motor neurons during the course of the disease. In HD, the role of the imbalance between glutamate and GABA is similar to other ND, being a cause of great impact on the appearance of symptoms and disease progression (André, Cepeda, & Levine, 2010).

Along with the GABAergic/glutaminergic system, also the adrenergic system seems to play an important role in neurodegeneration. This system includes the neurotransmitters epinephrine and norepinephrine (NE), which content is greatly decreased throughout the brain due to the prominent pathology found in the locus coeruleus (LC) (LeWitt, 2012). In fact, LC is the major noradrenergic nucleus of the brain and projects to areas prominently involved in cognitive function. Thus, there is a clear relation between the loss of this sole

source of NE – and consequent reduced levels of NE – and neurodegeneration (Vazey & Aston-Jones, 2012).

The loss of NE-neurons is a common feature of ND as AD and PD. In both diseases, a reduction of NE levels accelerates the disease progression and pathology as well as worsening the clinical symptoms. Hence, an increase in the content of NE may be beneficial in reducing the damage in the brain and have been studied as a therapeutic option (LeWitt, 2012; Vazey & Aston-Jones, 2012).

Similar to NE, octopamine is a monoamine synthesized from the aminoacid tyrosine (Figure XVI) and is responsible for adrenergic signaling in invertebrates. Along with its function of regulating heart beat and aggressive behaviours, octopamine is known to play a critical role in basic neural functioning (Bauknecht & Jékely, 2017; Woude & Smid, 2017).

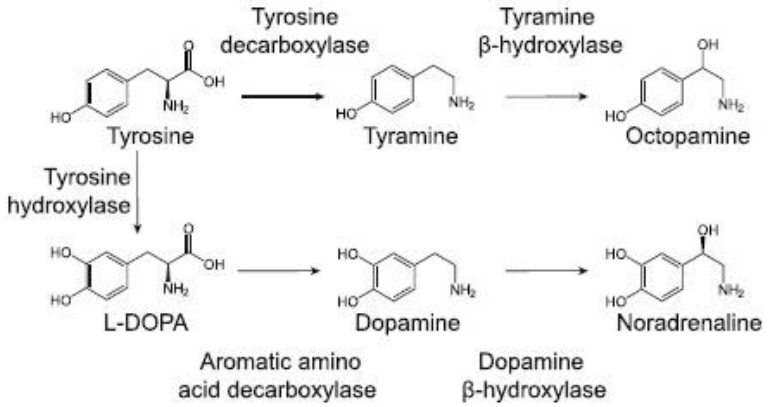


Figure XVI | Biosynthesis of octopamine and norepinephrine from tyrosine.

Synthesis of both octopamine and norepinephrine starts from the aminoacids tyrosine. By decarboxylation, tyrosine is converted to tyramine by the enzyme tyrosine decarboxylase and the hydroxylation to octopamine occurs through the action of tyramine β-hydroxylase.

Adapted from: Bauknecht & Jékely, 2017

Vertebrates, including humans, are absent of octopamine receptors, which suggests that this neurotransmitter has not a role in human physiology. Though, low circulating levels are found in plasma, leading octopamine to be classified as one of the primary trace amines. Sideways with this knowledge, in recent years, a group of receptors named trace amine-associated receptors (TAAR) have been identified, at the same time as the discovered that octopamine can bind a TAAR subtype that is responsible for the modulation of the release of monoamines in the brain. This fact confirms the ancient presence of octopamine in mammalian brain (Bauknecht & Jékely, 2017; Beaumont, Cordery, James, & Watson, 2017).

In 2010, D'Andrea and colleagues assessed the plasma levels of octopamine in *de novo* PD patients and found a decreased concentration of their levels when compared to controls. Unlike these findings, in the same patients, there was no changes in plasmatic NE, giving arise to the hypothesis that PD is characterized by abnormalities of tyrosine decarboxylase (the enzyme responsible for the formation of octopamine) rather than tyrosine hydroxylase. Given the importance of this enzyme in producing trace amines, this study suggests that octopamine circulating levels may be a promise as a biomarker of early PD (D'Andrea et al., 2010).

Given the growing interest in the potential of this monoamine in ND, some methods for its detection have been developed. Most of them include techniques as the abovementioned HPLC, since it is a very sensitive technique able to detect octopamine even in concentrations at the range of ng/mL (D'Andrea et al., 2010; Beaumont et al., 2017; Cook, Brent, & Breed, 2017; Woude & Smid, 2017).

6. *Saccharomyces cerevisiae* as a model of neurodegenerative diseases

In ageing populations, ND are becoming high prevalent and will become a major cause of death among society. For these reasons, multiple complementary experimental approaches have been studied in the last years in order to be helpful to elucidate the mechanisms underlying these complex diseases and allow the development of possible treatments and therapeutics (Miller-Fleming, Giorgini, & Outeiro, 2008; Pereira et al., 2012; Franssens et al., 2013; Gitler, Dhillon, & Shorter, 2017).

In this context, the yeast *Saccharomyces cerevisiae*, the best-studied eukaryotic unicellular organism, has become widely employed as a model system to study the mechanisms underlying human aging processes as well as ND, such as AD, PD, ALS and HD (Miller-Fleming et al., 2008; Ocampo & Barrientos, 2008). At first sight, it might not seem clear to use such a simple and unicellular organism to study complex brain disorders, since the complexity of the yeast cells is very different from human neurons. Although, basic processes involved in neurodegeneration are well conserved from yeast to human (Figure XVII), enabling the use of yeast disease models to represent key features and molecular events of cellular pathology of neurodegeneration (Franssens et al., 2013; Bonanomi, Visentin, Invernizzi, Tortora, & Regonesi, 2015). Also, the high degree of conservation is evidenced by the considerable proportion (47%) of yeast essential genes that can be

successfully replaced by orthologous human genes. Consistently, near 30% of the genes involved in human diseases may have a *S. cerevisiae* orthologue (Winderickx et al., 2008; França, Lima, & Eleutherio, 2017).

When compared to higher organisms, yeasts are shown to possess many strong advantages. Among these, are included the rapid growth – with a doubling time of approximately 90 min – short generation time, ease of manipulation, non-pathogenicity, ease of DNA transformation (with an efficient process of introduction of genes and modifications in the genome) and, most important, it is amenable to analysis. Besides these useful characteristics, *S. cerevisiae* has its limitations, as it cannot recapitulate the cellular interactions occurring in the human brain in case of a ND. Similarly, proteins and pathways required for the development of multicellular organisms are not present in yeast (Miller-Fleming et al., 2008; Pereira et al., 2012; Franssens et al., 2013).

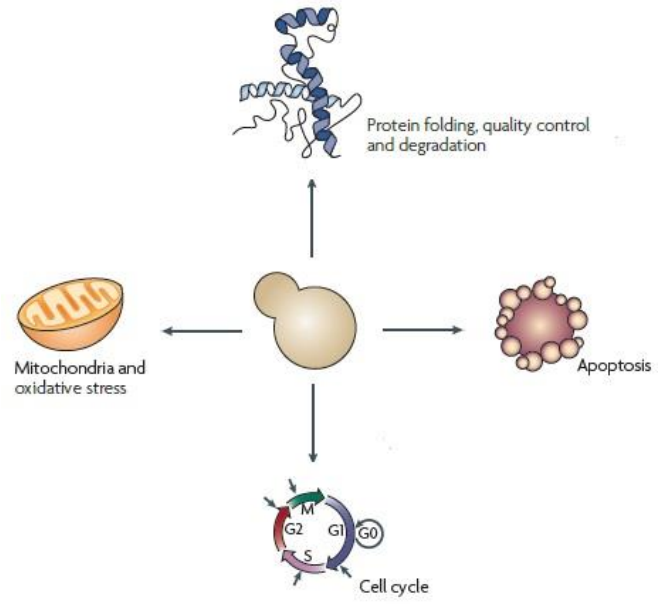


Figure XVII | Yeast conserved cellular mechanisms underlying ND.

Several cellular pathways of high relevance to neurodegeneration are conserved in yeast, such as mitochondrial dysfunction, oxidative stress, protein folding, quality control and degradation machineries, apoptosis and cell cycle.

Adapted from: Khurana & Lindquist, 2010

Few aspects of yeast cellular biology deserve a specific reference in the context of ND. As already mentioned, ND are associated with protein misfolding and aggregation; in yeast, these processes are easily studied because there is a high conservation of the protein quality system. Mitochondrial dysfunction and oxidative stress are, as well, referred as involved in

the pathogenesis of ND; in yeast, as in mammalian cells, the central organelle responsible for ROS production is mitochondria, thus allowing the study of defects that would be injurious for mammalian cells. At least, and concerning to apoptosis, an event that is also been implicated in neurodegeneration, it is considered that the mechanisms of cell death occurring in yeast are likely to be relevant to neuronal loss. Combining all these features, *S. cerevisiae* may actually be a powerful first-line system for the study of ND (Ocampo & Barrientos, 2008; Tenreiro & Outeiro, 2010; Franssens et al., 2013).

For this particular aim, a yeast model must manifest the course of human disease and capture the critical events preceding cell death. Two distinct strategies can be used when modeling a human disease in yeast: if a homologue of the gene implicated in the disease is present in yeast genome, its function can be studied directly, either through its deletion or overexpression; otherwise, if the gene underlying the disease is absent in yeast, it can be modeled via heterologous expression (Tenreiro & Outeiro, 2010; Barrera et al., 2016). To date, the elements of ND modeled in yeast include α-syn (PD), Aβ peptide and tau protein (AD), HTT (HD) and frataxin (Friedreich's ataxia) (Pereira et al., 2012; Breitenbach et al., 2013). In some of these diseases, the human disease genes have yeast homologues (as *PARK7* involved in PD and *SOD1* in ALS) and so its functional analysis can be performed directly (Pereira et al., 2012; Menezes, Tenreiro, Macedo, Santos, & Outeiro, 2015); notwithstanding, in diseases such as PD, AD and HD, the most important genes involved are restricted to vertebrates and so the yeast models are constructed by heterologous expression of gene (Sharma et al., 2006; Ocampo & Barrientos, 2008). This process is highly efficient in yeast due to the ease of DNA transformation and the availability of selectable markers that allow the introduction of multiple self-replicating plasmids (Sharma et al., 2006; Breitenbach et al., 2013; Franssens et al., 2013).

Given the described characteristics, recognizing the usefulness of the so-called humanized yeast as a model to understand the features of ND will help and may lead to the development of mechanism-based therapeutics for currently incurable diseases (Miller-Fleming et al., 2008; Gitler et al., 2017).

7. Objectives

As previously stated, despite the production of ROS being a common process under physiological conditions, an imbalance in its levels leads to oxidative stress, a condition highly associated to ND. On the other hand, vitamin C is referred as a powerful antioxidant which major concentration is found precisely in the brain. Thus, and combining this knowledge with the fact that *S. cerevisiae* has been widely and successfully used as model for ND, the objectives of this work were study the induction of an oxidative stress state in yeast using H_2O_2, and study the antioxidant effect of vitamin C, assessing whether and at which concentrations it can revert the oxidative stress condition.

Another aim of the present study was to assess how oxidative stress state varies with the abovementioned different treatments induced in *S. cerevisiae* with the analysis of 3-NT by HPLC. Knowing that 3-NT is a biomarker of oxidative stress, its quantification is helpful for a better understanding of how powerful the effects of H_2O_2 and vitamin C are.

Lastly, using the fact that some neurotransmitters are present at decreased levels in case of a ND, the main goal of this project was to assess octopamine levels by HPLC. Despite, in the current literature, there are no evidences of its production by *S. cerevisiae*, the objective was to evaluate its presence or absence and its levels, possibly relating it to the oxidative stress state. With this data, the objective was to describe a possible relationship between oxidative stress and the reduction of neurotransmitters levels.

Chapter II – Materials and methods

1. Yeast strain and growth conditions

All experiments were performed with *Saccharomyces cerevisiae* (ATCC® 9763™) obtained from American Type Culture Collection (ATCC) (Manassas, USA). The lyophilized *S. cerevisiae* was rehydrated in sterile distilled water at room temperature for 2 h, accordingly ATCC instructions. To initiate a culture, several drops of suspension were plated on solid Yeast Extract Peptone Dextrose (YEPD) medium (1% yeast extract, 2% bacteriological peptone, 2% glucose, 1,5% agar) (Sigma-Aldrich, Missouri, USA) and plates were incubated at 30°C for 48 h. For the following experiments, new cultures were established from the first one.

For all subsequent experiments, after an initial culture on solid medium on the abovementioned conditions, cells were harvested for 10 mL of liquid YEPD medium (1% yeast extract, 2% peptone, 2% glucose) (VWR, Pennsylvania, USA) and grown also at 30°C for 48 h. Then, yeast cells were harvested for liquid YEPD medium, with the ratio of flask volume:medium of 5:1, until the culture reached an optical density at 600 nm (OD_{600}) between 0,080 and 0,100 (0.5 McFarland standard).

2. Growth curve analysis

Initial cultures were incubated at 30°C under 120 rpm continuous shaking to provide sufficient aeration. *S. cerevisiae* growth was measured at 1 h intervals over a period of 10 h by measuring the absorbance at an OD_{600} using a UV-Vis spectrophotometer (GENESYS™ 20 Visible Spectrophotometer, Thermo Fisher Scientific, Wilmington, EUA). The experiment was stopped after the stationary phase had been achieved. The yeast growth was also determined based on the viable cell counts [colony forming units (CFU)]. Thence, 100 µL of culture at each time point were tenfold serially diluted in a 0,85% sodium chloride (NaCl) (Carlo Erba Reagents, Val de Reuil, France) solution. Then, drop dilution assay was performed by plating 10 µL of the tenfold dilutions on YEPD agar. After an incubation period of 48 h at 30°C, the cell counts (CFU/mL) were determined and a growth curve of $\log_{10}$ CFU/mL versus incubation time was plotted. Similarly, a growth curve of absorbance versus incubation time was also designed.

3. Effect of H_2O_2 and vitamin C on yeast cells viability

i. Effect of H_2O_2 on yeast cells viability

For oxidative stress experiments, exponential cultures (OD_{600} between 1,502 and 1,882) were equally divided into four flasks: one acted as a control and was not exposed, and the others were treated individually with 1, 3 and 5 mM H_2O_2 (hydrogen peroxide 30%, Merck, New Jersey, USA) for 3 h with 120 rpm shaking at 30°C. Each 45 min after exposure (until reaching 3 h), 100 µL of each culture were tenfold serially diluted and drop dilution assay was performed as abovementioned.

ii. Effect of vitamin C on yeast cells viability

To assess the antioxidant potential of vitamin C, exponential cultures (OD_{600} between 1,502 and 1,882) were equally divided into four flasks: one acted as a control and was not exposed, and the others were exposed individually to 1, 2 and 4 mg/dL vitamin C (BDH Prolabo®, Pennsylvania, USA) for 3 h with 120 rpm shaking at 30°C. Each 45 min after exposure (until reaching 3 h), 100 µL of each culture were tenfold serially diluted and drop dilution assay was performed as abovementioned.

iii. Combined effect of H_2O_2 and vitamin C on yeast cells viability

To assess whether vitamin C is protective against the H_2O_2 effects, three different experiments were carried out with different combinations of the oxidant and the antioxidant agents. Therefore, at different experiments, exponential cultures (OD_{600} between 1,502 and 1,882) were divided into four flasks and exposed to: 5 mM H_2O_2 + 4 mg/dL vitamin C; 5 mM H_2O_2 + 2 mg/dL vitamin C; 5 mM H_2O_2 + 1 mg/dL vitamin C; 3 mM H_2O_2 + 4 mg/dL vitamin C; 3 mM H_2O_2 + 2 mg/dL vitamin C; 3 mM H_2O_2 + 1 mg/dL vitamin C; 1 mM H_2O_2 + 4 mg/dL vitamin C; 1 mM H_2O_2 + 2 mg/dL vitamin C; 1 mM H_2O_2 + 1 mg/dL vitamin C. The exposure lasted for 3 h with 120 rpm shaking at 30°C. Each 45 min after exposure (until reaching 3 h), 100 µL of each culture were tenfold serially diluted and drop dilution assay was performed as abovementioned.

iv. Viability assessment after exposure

For all the experiments abovementioned (3i to 3iii), cell viability after exposure was assessed by CFU counting after 48 h incubation at 30°C on YEPD agar plates. CFU/mL of cell growth was calculated, data were recorded and a graph of $\log_{10}$ CFU/mL versus incubation time was

plotted. To ensure reproducibility, the protocol was carried out in triplicate and repeated three times.

4. 3-NT and octopamine measurement by HPLC

For 3-NT measurement, HPLC method was performed according Teixeira et al. (2017); for octopamine assessment, the method is being developed.

i. Equipment and software

All experiments were performed on a Hitachi LaChrom Elite®HPLC system (Hitachi High − Technologies Corporation, Tokyo, Japan) composed by HTA L-2130 LaChrom Elite quaternary pumps, L-2200 LaChrom Elite autosampler, L-2300 LaChrom Elite column heater and L-2455 LaChrom Elite photo DAD. EZChrom Elite Compact Software Version 3.3.2. (Agilent Technologies, Inc., Santa Clara, CA, United States) was used for data collection and analysis.

ii. Mobile phase

The mobile phase was prepared according to the following proportions: 0.5% CH_3COOH:MeOH:H_2O (15:15:70, v/v) [acetic acid (Merck S.A., Algés, Portugal); methanol (Carlo Erba Reagents, Val de Reuil, France); ultrapure water obtained from the Water Purification System TKA Barnstead™ GenPure™ capsule 0.2 µm (Thermo Fisher Scientific, Wilmington, EUA)] and was filtered through a 0.45 µm filter membrane.

iii. Calibration standards

0,5 mg/mL 3-NT (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and 1 mg/mL octopamine (Sigma-Aldrich, Missouri, USA) stock solutions were prepared using the mobile phases as solvents and filtered through a filter membrane device. Standard solutions of 0,2; 0,1; 0,05; 0,02; 0,01 and 0,005 mg/mL were prepared by diluting the stock solution with the desired mobile phase and were used for calibration purposes.

iv. HPLC conditions

Chromatographic conditions used in all experiments were: flow rate of 1 mL/min; detection in the range 190–400 nm; volume of injection of 25 µL and oven temperature of 25°C.

v. Sample preparation

5 mL of *S. cerevisiae* culture were collected from the initial time and each 45 min after exposure to H_2O_2, vitamin C and H_2O_2 + vitamin C in all tested concentrations, while previous described experiences were being carried out. In order to be prepared for HPLC analysis, samples were initially submitted twice to a cycle of freezing (10 min) and thawing (10 min) at -80°C and room temperature, respectively, aiming the disruption of cell wall. Subsequently, also at room temperature, cells were sonicated for 15 min (Silent Crusher S at 47–63 Hz) and then centrifuged for 10 min at 14500 rpm. The obtained supernatant was filtered through a filter membrane device of 0.2 µm (Whatman™, Maidstone, UK) and spiked with different concentration levels of both 3-NT and octopamine. Non-spiked supernatants were tested in simultaneous.

vi. Viability assessment

After the previous protocol to disrupt cell wall, and before the filtering step, 10 µL of yeast cells samples were mixed with 10 µL of methylene blue 1% (PanReac AppliChem, Chicago, USA) to perform a microscopic analysis (Primo Star, ZEISS, VWR, Primo Star iLED; VisiCam 5.0: 630-1032) and assess yeast viability and cell wall integrity.

5. Statistical analysis

Concerning to results based on viable counts, data are presented as the mean of three independent measurements in triplicate ± standard deviation (SD) and were analyzed using IBM SPSS Statistics Version 23.0 (IBM Corp. in Armonk, NY). Given the reduced sample size (n=9), differences between the groups were determined using a nonparametric test (Kruskal-Wallis H), followed by post-hoc testing (Mann-Whitney U). The level for statistical significance was set at $p<0.05$.

Statistical comparisons for 3-NT and octopamine measurements were performed using Pearson Correlation test and $p<0.05$ was considered to indicate statistically significant differences.

Chapter III – Results

1. Growth curve of *S. cerevisiae* ATCC® 9763™

The normal growth curve of *S. cerevisiae* cultured under 30°C and 120 rpm continuous shaking is shown in Figure XVIII. Under these growth conditions, the lag phase of *S. cerevisiae* growth was extended for 2 h before the yeast cells entered the log phase, where a sharpest slope was observed for, approximately, 6 h. The stationary phase started after an incubation period of nearly 8 h.

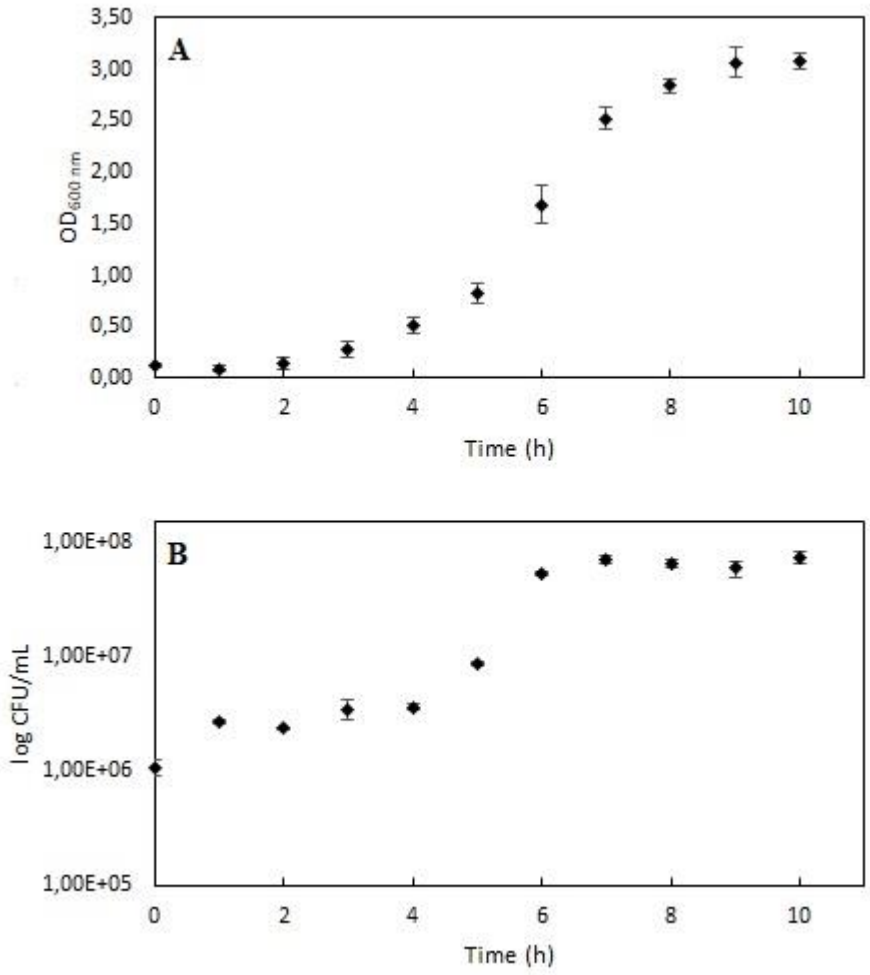


Figure XVIII | Growth curve of *S. cerevisiae*.

Cells were grown at 30°C and 120 rpm in YEPD medium. Culture density was measured by UV-Vis spectrophotometry (A) and corresponding CFU/mL were counted on YEPD agar (B). Data represent the mean ± SD of an experiment performed in triplicate (n=3).

The time point desired for the subsequent experiments was established at mid-log phase, where the OD_{600} reached a value between 1,502 and 1,882. At this point, yeast cells were in the necessary condition to be exposed either to H_2O_2 or vitamin C or both.

i. Specific growth rate

The logistic equation for the cell growth is described by the following equation (6) (Maier, 2015):

$$\ln X = \mu t + \ln X_0 \quad (6)$$

where X is the number of cells or the OD at a time, μ is the specific growth rate constant, t is the difference between log phase final and initial time, and X_0 is the initial number of cells or the initial OD value.

The times used to determine the specific growth rate were chosen by visual examination of a $\log_{10}$ plot of the data (see Figure XVIII). Examination of the graph shows that the exponential phase is from approximately 2 to 8 h. Thus, considering the data presented on Table IV, it was possible to calculate the specific growth rate of *S. cerevisiae* based upon viable counts (CFU/mL) and absorbance data. The result is always expressed in h^{-1} and presented in Table V.

Table IV | Data for specific growth rate calculation.

	Initial time (h)	Final time (h)	Δ t (h)	X_0	X
CFU/mL	2	8	6	$2{,}35 \times 10^6$	$6{,}60 \times 10^7$
Absorbance				0.141	2.840

Table V | Specific growth rate of *S. cerevisiae*.

Data	μ (h^{-1})
CFU/mL	0.55
Absorbance	0.50

2. Effect of H_2O_2 on cell viability

To assess if H_2O_2 has an injurious effect on *S. cerevisiae* cells and to determine which concentration causes a significant decrease in yeast cells viability, exponential cultures were exposed to different concentrations of H_2O_2 for 3 h, performing a dose-response and a time course experiment. The results shown on Figure XIX represent the time course of the experiments and the $\log_{10}$ viable counts, making noticeable that, at the tested concentrations, H_2O_2 decreases cell viability. In fact, almost 90% of yeast cells died after a 3 h incubation

with 5 mM H_2O_2, with CFU/mL constantly decreasing each 45 min. Approximately 80% died after the same incubation period with 3 mM H_2O_2, also with a continuous decreasing of CFU/mL, even though in a less extension than the higher concentration. At 1 mM H_2O_2 exposure, the toxic effects of H_2O_2 were stable and only about 15% of yeast cells died after 3 h.

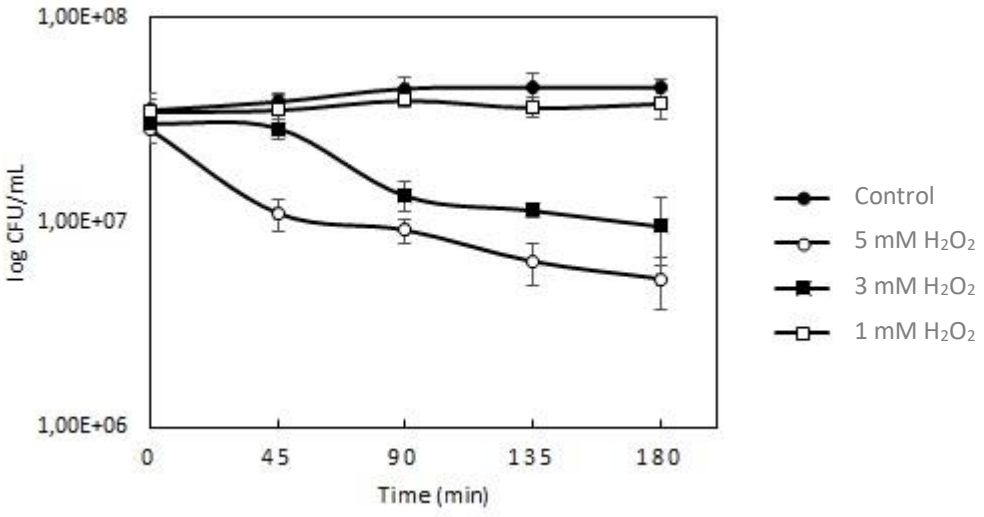


Figure XIX | Effect of H_2O_2 on cell viability.

For 3 h, cells were exposed at 5 mM (○), 3 mM (■) and 1 mM (□) H_2O_2 and the results were compared with the Control (●). Data represent the mean ± SD of three experiments performed in triplicate (n=9).

Respecting to statistical analysis, results (see Appendix I) demonstrated no statistically significant differences between control and the exposed groups at the initial time of exposure (t = 0 min) ($p>0.05$). However, the results proved that there was a significant decrease in cell viability since the first 45 min until the 180 min post- H_2O_2 5 mM and 3 mM exposure when compared to the control ($p<0.05$). Also, when comparing the effects of 5 mM H_2O_2 with the effects caused by the two other concentrations, results have shown a statistically significant difference, with $p<0.05$, confirming that the higher concentration causes a more pronounced decrease in cell viability. When considering the effects of the lower concentration of H_2O_2, there was no statistically significant differences when compared to the control ($p>0.05$). Although, when compared to 3 mM and 5 mM H_2O_2, significance appeared ($p<0.05$) and, once again, corroborated the fact that higher concentrations of H_2O_2 are toxic to cells, causing its unviability.

ii. Specific death rate

The death phase can be described by the following equation (7) (Maier, 2015):

$$k_d = \frac{\ln X - \ln X_0}{t_1 - t_0} \quad (7)$$

where k_d is the specific death rate, X is the number of cells at a time, X_0 is the initial number of cells, and t_1 and t_0 are, respectively, the final and initial time.

Since only 5 mM and 3 mM H_2O_2 caused a significant cell death, only the effects of these two concentrations were calculated in terms of death rate. By observation of Figure XIX, the times used to determine the specific death rate caused by 5 mM H_2O_2 were the initial time of exposure (t = 0 min) and the final time (t = 180 min = 3 h), given the cell death occurs from the first moment. Relatively to 3 mM H_2O_2, the chosen initial and final times were, respectively, t = 45 min (= 0,75 h) and t = 180 min (= 3 h), since the cell death starts right after the first 45 min of exposure and lasts until the end of the experience. Considering the data presented on Table VI, it was possible to calculate the specific death rate of *S. cerevisiae* caused by 5 mM and 3 mM H_2O_2, based upon viable counts (CFU/mL) (Table VII).

Table VI | Data for specific death rate calculation.

$[H_2O_2]$	t_0 (h)	X_0 (CFU/mL)	t_1 (h)	X (CFU/mL)
5 mM	0	$2,85x10^7$	3	$5,28x10^6$
3 mM	0,75	$2,88x10^7$	3	$9,67x10^6$

Table VII | Specific death rate of *S. cerevisiae* caused by 5 mM and 3 mM H_2O_2.

$[H_2O_2]$	k_d (h^{-1})
5 mM	- 0.56
3 mM	- 0.48

3. Effect of vitamin C on cell viability

To examine the effect of vitamin C on *S. cerevisiae* cell viability and at which concentration it has the major effect, exponential cultures were exposed to different concentrations of vitamin C for 3 h, performing a dose-response and a time course experiment. As shown in Figure XX, the viability counts of all vitamin C tested concentrations were similar to untreated control and almost overlap its CFU/mL value during all time course.

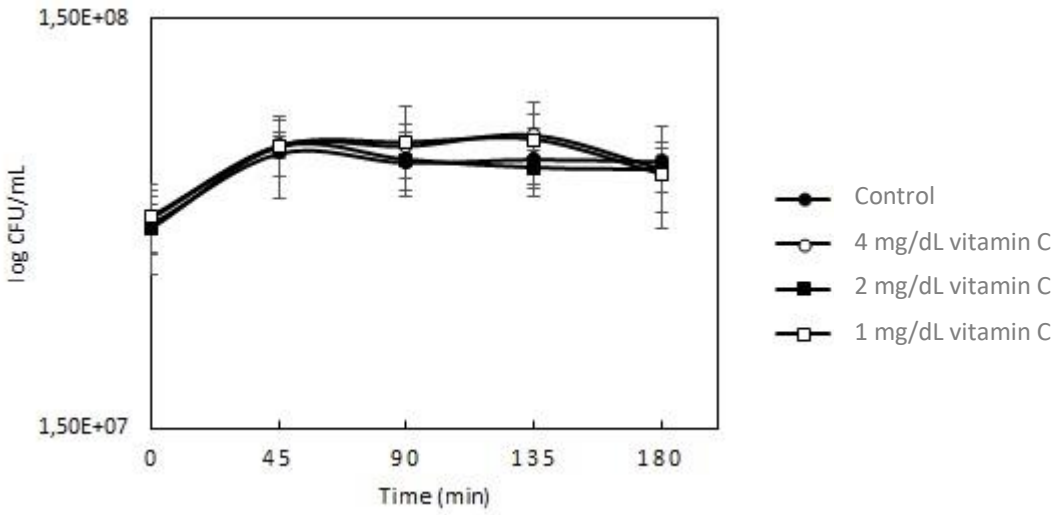


Figure XX | Effect of vitamin C on cell viability.

For 3 h, cells were exposed at 4 mg/dL (○), 2 mg/dL (■) and 1 mg/dL (□) vitamin C and the results were compared with the Control (●). Data represent the mean ± SD of three experiments performed in triplicate (n=9).

Statistically, results (see Appendix II) showed no differences between control and the exposed groups ($p>0.05$).

4. Effect of vitamin C on H_2O_2 induced oxidative stress

To assess whether vitamin C is protective against oxidative stress induced by H_2O_2, yeast exponential cultures were exposed to both vitamin C and H_2O_2 for 3 h at different concentration combinations. In order to compare the effects caused by H_2O_2, vitamin C and both agents together at corresponding concentrations, results were compared with the control in terms of percentage. Therefore, considering the control as 100% of viability during all time exposure, results were expressed in % control. Figures XXI, XXII and XXIII show, respectively, the results relating to exposure at 5, 3 and 1 mM H_2O_2 and different concentrations of vitamin C.

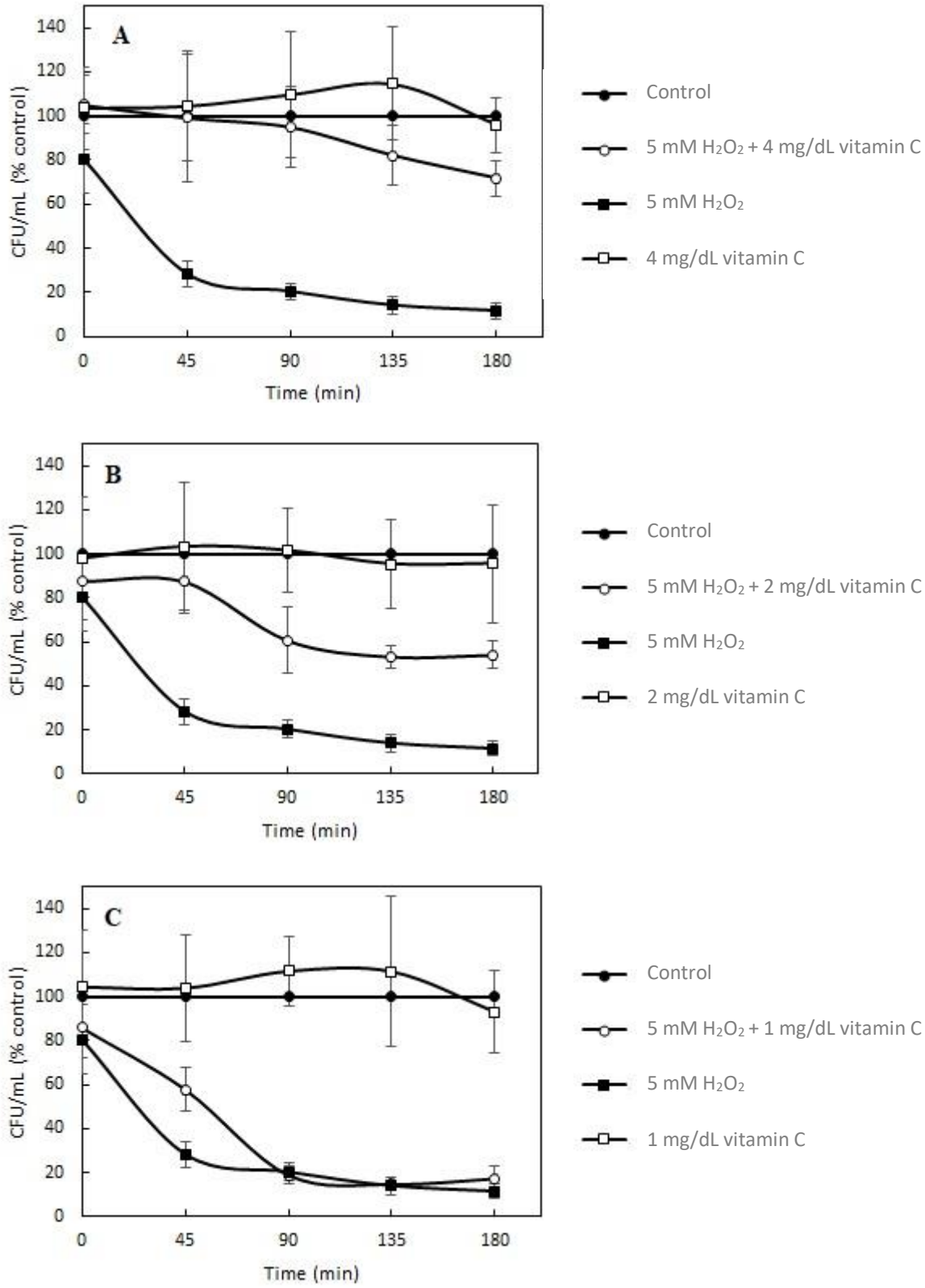


Figure XXI | Effect of different concentrations of vitamin C on 5 mM H_2O_2 induced cell death.

For 3 h, cells were exposed at 5 mM H_2O_2 in combination with **(A)** 4 mg/dL vitamin C; **(B)** 2 mg/dL vitamin C; **(C)** 1 mg/dL vitamin C. Results of isolated exposure to 5 mM H_2O_2 and 4, 2 and 1 mg/dL vitamin C are also represented. Results were compared with the Control (●) and are expressed in % control. Data represent the mean ± SD of three experiments performed in triplicate (n=9).

When comparing the 5 mM H_2O_2-exposure effect on cell viability with the effect of 5 mM H_2O_2 + 4 mg/dL vitamin C, it showed a statistically significant difference from the first 45 min of exposure ($p<0.001$), demonstrating that 4 mg/dL vitamin C could reverse the toxic effects of 5 mM H_2O_2. In effect, comparing the almost 90% of death caused by 5 mM H_2O_2 itself, 4 mg/dL vitamin C reverted H_2O_2 effect in more than 30%, with the percentage of cell death being 28% at the end of 3 h. However, differences ($p<0.05$) were found between control and the 5 mM H_2O_2 + 4 mg/dL vitamin C exposed group, showing that the reversal of toxic effects by vitamin C was not complete, since it was not similar to control [Appendix III (A)].

Considering the results obtained with the combination of 5 mM H_2O_2 + 2 mg/dL vitamin C and comparing to the extent of cell death caused by 5 mM H_2O_2 itself, differences were also found ($p<0.01$) from the first 45 min until the end of the experience, showing that 2 mg/dL vitamin C was also able to counteract the toxic effects of 5 mM H_2O_2. In fact, in combination with 2 mg/dL vitamin C, the percentage of death caused by 5 mM H_2O_2 was reduced to 46% after a 3 h exposure. Still, differences were found between control and the combination-exposed group ($p<0.001$), demonstrating that, once again, the reversal was not complete [Appendix III (B)].

Concerning the results obtained with 5 mM H_2O_2 + 1 mg/dL vitamin C, when compared with 5 mM H_2O_2 itself, no differences were found at any time ($p>0.05$), indicating that 1 mg/dL vitamin C was not sufficient to reverse the effects of this H_2O_2 concentration. Indeed, in combination with 1 mg/dL vitamin C, the percentage of cell death caused by 5 mM H_2O_2 was reduced to 83% after a 3 h exposure, which means a difference of only 7%. Furthermore, it was found a significant difference between control and the 5 mM H_2O_2 + 1 mg/dL vitamin C exposed group ($p<0.001$), confirming the fact that 1 mg/dL vitamin C could not counteract the effects of H_2O_2 at the tested concentration [Appendix III (C)]. Also, through observation of graphic C represented on Figure XXI, it was possible to notice that its effect almost overhead the effect caused by 5 mM H_2O_2.

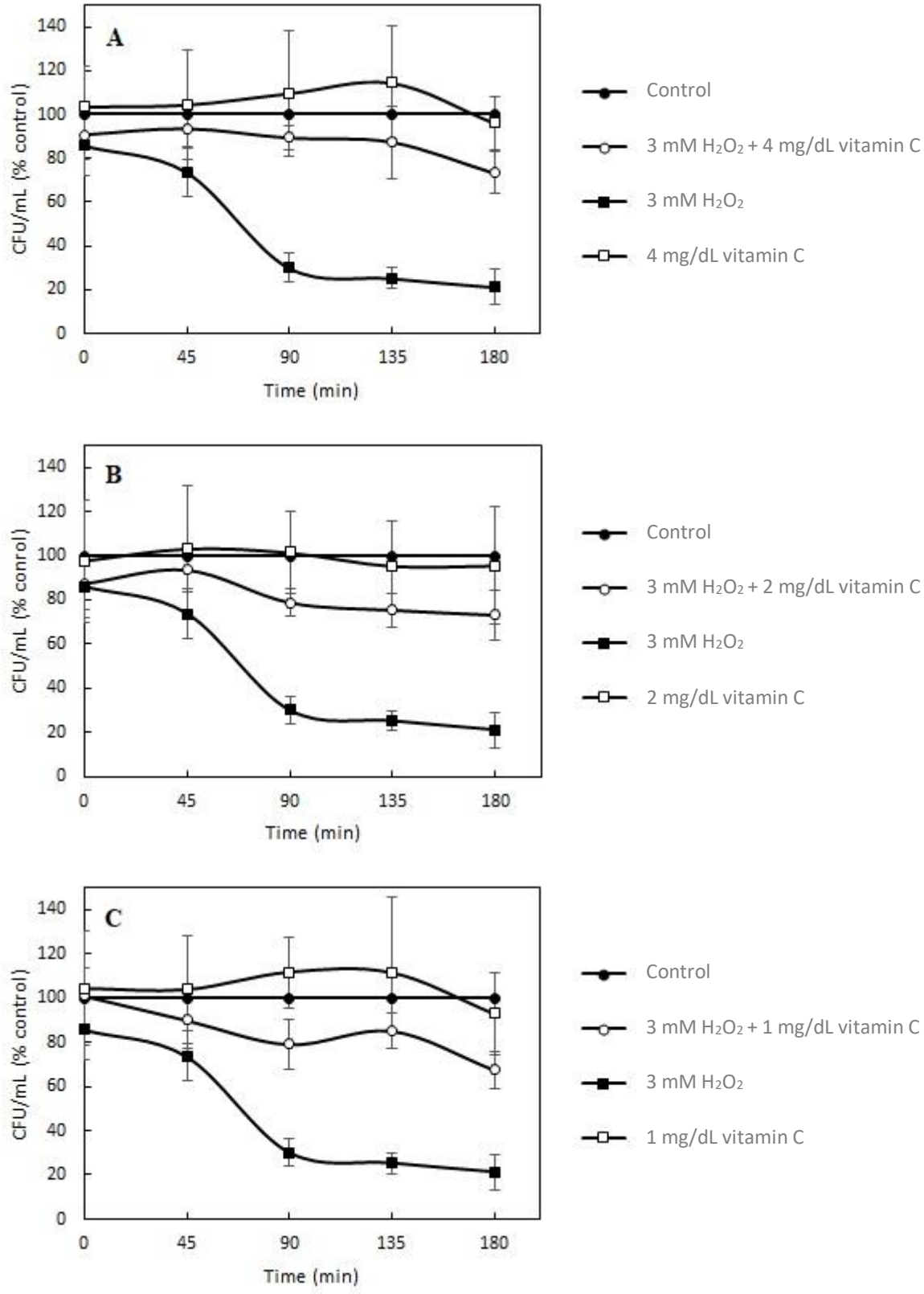
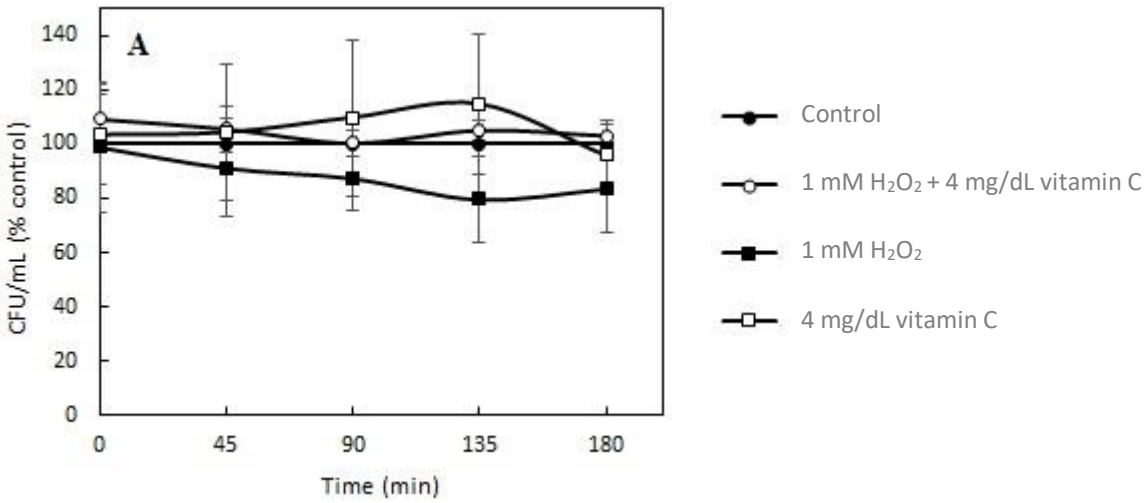


Figure XXII | Effect of different concentrations of vitamin C on 3 mM H_2O_2 induced cell death.

For 3 h, cells were exposed at 3 mM H_2O_2 in combination with **(A)** 4 mg/dL vitamin C; **(B)** 2 mg/dL vitamin C; **(C)** 1 mg/dL vitamin C. Results of isolated exposure to 3 mM H_2O_2 and 4, 2 and 1 mg/dL vitamin C are also represented. Results were compared with the Control (●) and are expressed in % control. Data represent the mean ± SD of three experiments performed in triplicate (n=9).

Comparing the 3 mM H_2O_2-exposure effect on cell viability with the effect of 3 mM H_2O_2 + 4 mg/dL vitamin C and with the effect of 3 mM H_2O_2 + 2 mg/dL, a statistically significant difference was found at the 90, 135 and 180 min of exposure ($p<0.05$); at 45 min, the difference between the effects of H_2O_2 and the effects of both combinations was not significant ($p>0.05$). This result showed that only from the 90 min forward, 4 mg/dL vitamin C and 2 mg/dL vitamin C were able to reverse significantly the toxic effects of 3 mM H_2O_2. In fact, regarding the percentage of death caused by 3 mM H_2O_2, which was around 80%, 4 and 2 mg/dL vitamin C reduced this value to 27% after 3 h, showing similar results. Then, comparing control with 3 mM H_2O_2 + 4 mg/dL vitamin C and with 3 mM H_2O_2 + 2 mg/dL vitamin C exposed groups, differences were found at 45, 135 and 180 min after exposure ($p<0.05$), which means that reversal is only complete at 90 min for both [Appendix IV (A) and (B)].

Relatively to the results obtained with the combination of 3 mM H_2O_2 + 1 mg/dL vitamin C and comparing to the effects caused by 3 mM H_2O_2 itself, differences were found ($p<0.05$) at 90 and 180 min after exposure. Also, at the final time, the percentage of cell death was 33%, using the combination 3 mM H_2O_2 + 1 mg/dL vitamin C, instead of the 80% caused by 3 mM H_2O_2. When compared to the control, differences were found since the first 45 min, showing that reversal of toxic effects of 3 mM H_2O_2, even when existent, was not complete [Appendix IV (C)].



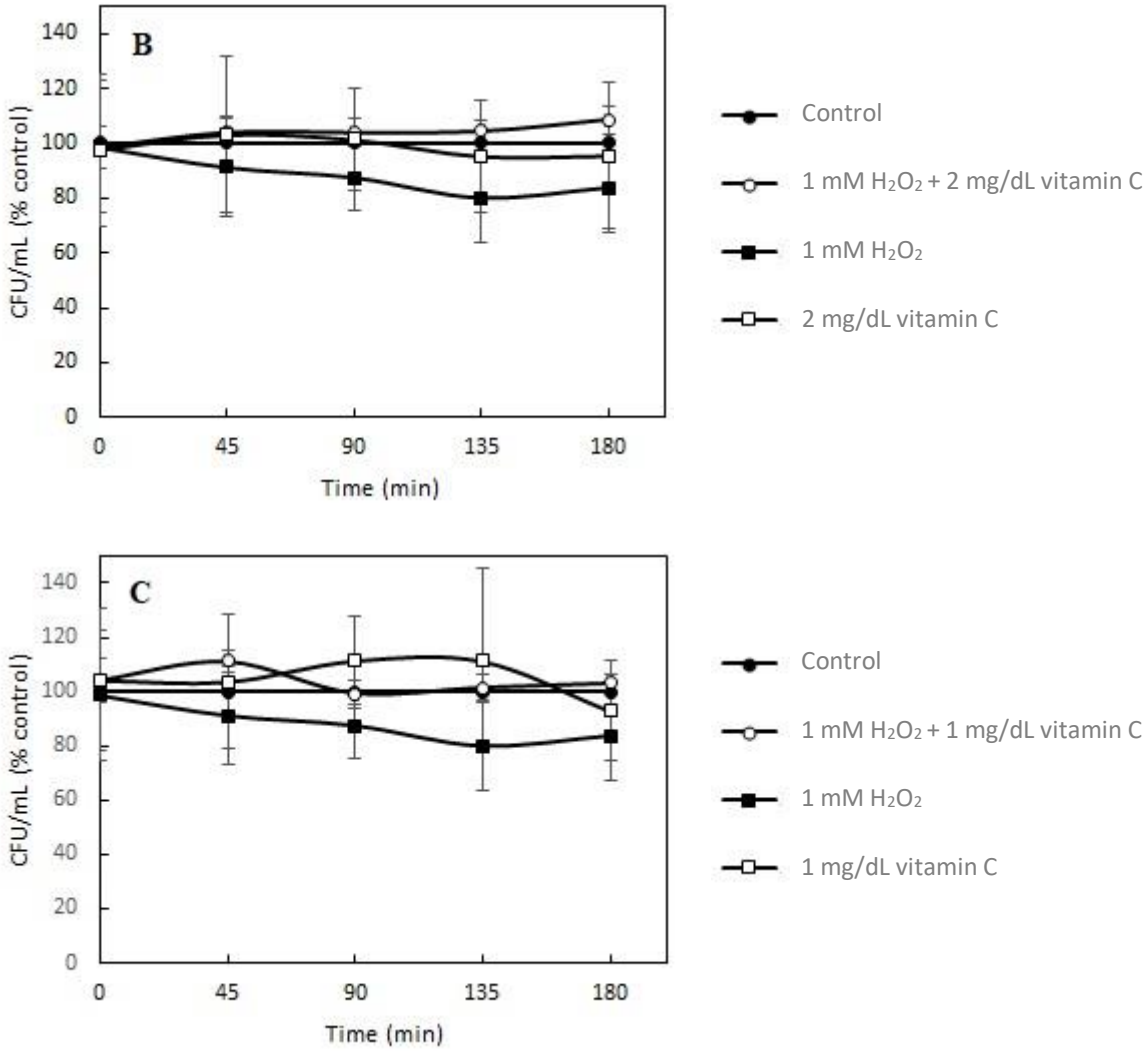


Figure XXIII | Effect of different concentrations of vitamin C on 1 mM H_2O_2 induced cell death.

For 3 h, cells were exposed at 1 mM H_2O_2 in combination with **(A)** 4 mg/dL vitamin C; **(B)** 2 mg/dL vitamin C; **(C)** 1 mg/dL vitamin C. Results of isolated exposure to 1 mM H_2O_2 and 4, 2 and 1 mg/dL vitamin C are also represented. Results were compared with the Control (●) and are expressed in % control. Data represent the mean ± SD of three experiments performed in triplicate (n=9).

Results of 1 mM H_2O_2 + 4 mg/dL vitamin C on cell viability were different ($p<0.05$) from those obtained with 1 mM H_2O_2 only at 180 min after exposure, which means, by observation of graphic A on Figure XXIII, that the effect of H_2O_2 is reversed. At the same time, no differences ($p>0.05$) were found between control and the group exposed to the combination, fact that confirms that the reversal was complete [Appendix V (A)].

The same pattern of results was obtained for 1 mM H_2O_2 in combination with 2 mg/dL vitamin C and with 1 mg/dL vitamin C, with both being different from 1 mM H_2O_2 itself and not different from control at 180 min [Appendix V (B) and (C)].

By observation of Figure XXIII, vitamin C, at any tested concentrations, was able to totally revert the H_2O_2 effect 3 h after the exposure to both agents, turning the 15% of cell death caused by 1 mM H_2O_2 in a value of % CFU/mL superior to the control.

5. 3-NT and octopamine assessment

i. Yeast cells viability

Light microscopy was used before the protocol for yeast cells wall disruption and after the freezing/thawing cycles. The results are shown on Figure XXIV.

Yeast cells subjected to the referred cycles were compared to the non-treated yeast (used as a control for a normal structure of *S. cerevisiae*) to assess if the temperature changes were enough to disrupt cell wall. Both samples were stained with methylene blue 1%.

By observation of Figure XXIV, in a non-treated condition, yeasts presented a cell wall normal integrity, as expected; however, even after the freezing/thawing cycles, the cell wall seemed not to be disrupted, since the coloration was well defined and *S. cerevisiae* showed its typical structure.

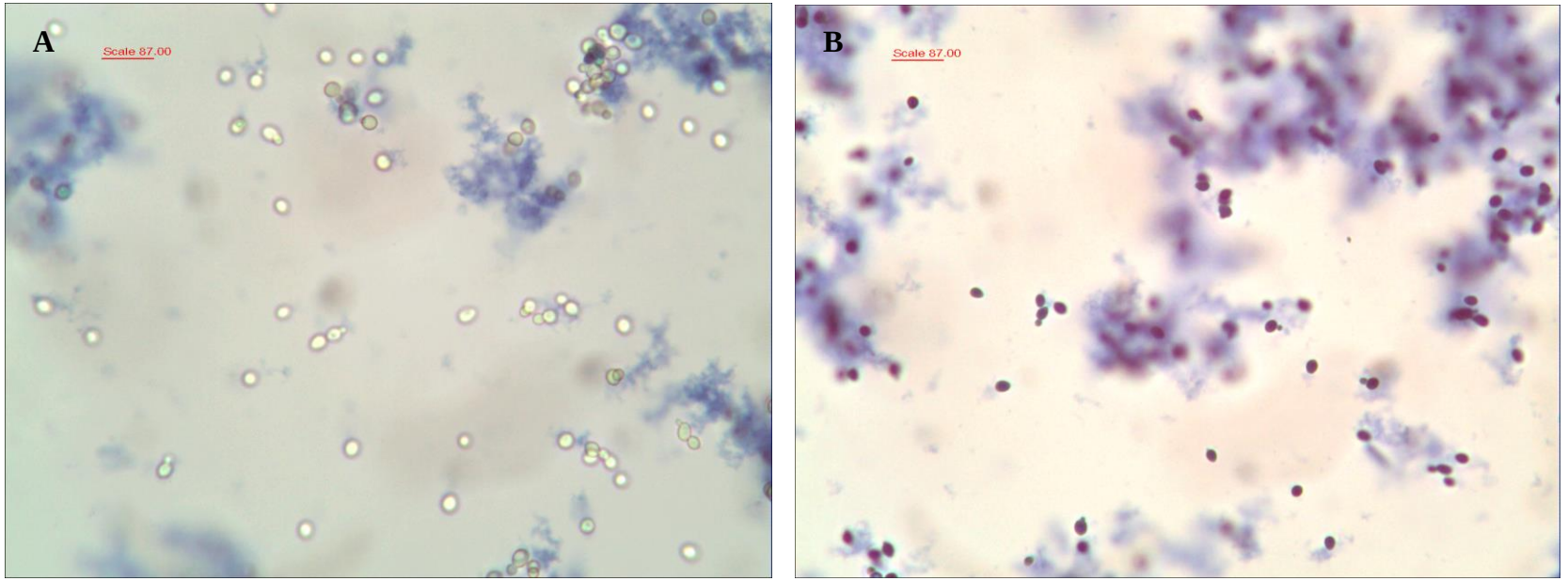


Figure XXIV | Light microscopy images of *S. cerevisiae.*

(A) control; **(B)** after freezing/thawing cycles. Methylene blue staining. (Primo Star iLED, ZEISS, EUA; 40X magnification)

ii. 3-NT and octopamine detection by HPLC

After the protocol for yeast cell wall disruption, control samples (non-exposed), as well as yeast cells samples exposed to 5, 3 and 1 mM H_2O_2 at 0, 45, 90, 135 and 180 min of exposure were subjected to a HPLC analysis.

Samples were spiked with 3-NT and octopamine and detection at 356 nm and 276 nm, respectively, was evaluated. The results are shown on Figures XXV and XXVI.

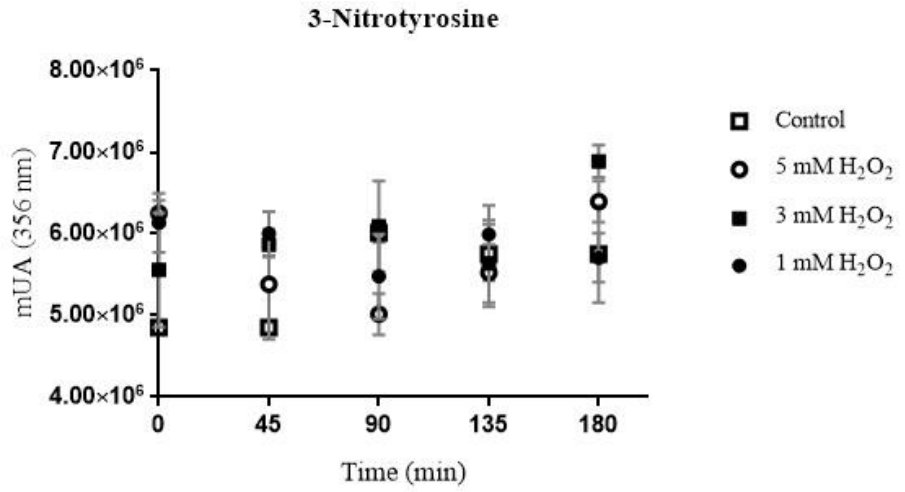


Figure XXV | Comparison of 3-NT levels at different oxidative stress exposure conditions.

Data represent the mean ± SD of an experiment performed in triplicate (n=3).

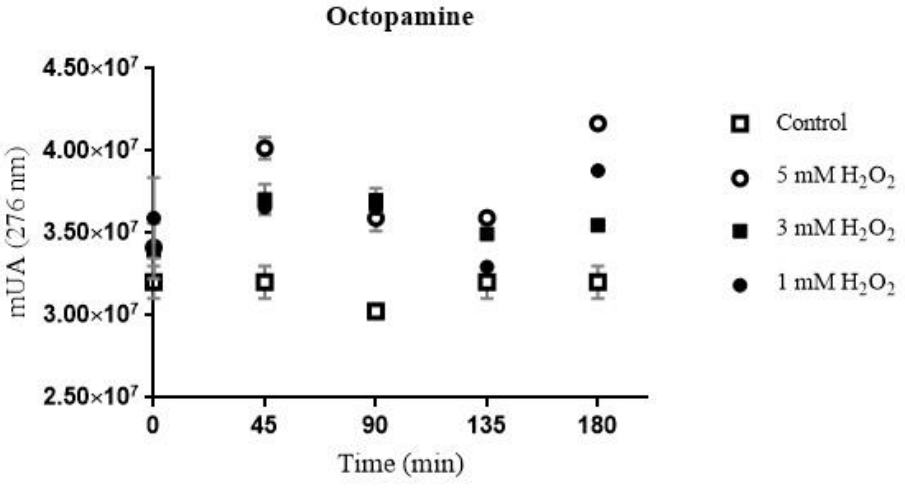


Figure XXVI | Comparison of octopamine levels at different oxidative stress exposure conditions.

Data represent the mean ± SD of an experiment performed in triplicate (n=3).

Despite there seems to be a slight alteration, with an apparent increase in both 3-NT and octopamine levels compared to control, no statistically significant results were found ($p>0.05$) between yeast samples exposed to H_2O_2 and control.

Non-spiked samples were also submitted to HPLC analysis; however, data is not shown since there was no detection.

Chapter IV – Discussion

Oxidative stress plays a central role in ND, either due to excess of ROS or antioxidant deficiency. A significant association between an abnormal oxidative stress status and ageing diseases have been demonstrated over the past years, with oxidative stress being pointed as a major cause of their development (Kim et al., 2015; Liu et al., 2017). Therefore, detailed investigation of the effects of oxidative stress on cells and the evaluation of the antioxidant capacity of natural compounds, as vitamins, may provide crucial insights into pathological and pharmaceutical approaches for these diseases.

In the present study, *S. cerevisiae* was used as a model since it is the best-studied eukaryotic unicellular organism, which has a high degree of conservation of genes and biological processes from yeast to human. Besides that, its ease of manipulation and rapid growth, among other favorable characteristics previously described (Miller-Fleming et al., 2008; Pereira et al., 2012; Franssens et al., 2013), made it a very useful model for this work, allowing the fulfillment of the objectives.

The establishment of a growth curve of *S. cerevisiae* in its optimal conditions (30°C and 120 rpm continuous shaking (Alloue-Boraud et al., 2015)) was imperative for subsequent studies, in order to evaluate the corresponding time and OD at which yeast was in mid-log phase, the time point desirable for exposure experiences, as described by Sharma et al. (2006) and Morano, Grant, and Moye-Rowley (2012). According to the achieved results, it was possible to describe three periods: 0-2 h corresponded to lag phase, where no growth occurs and yeast cells start acclimatizing to the medium; 2-8 h was the log phase, where cells rapidly grow and divide due to the high availability of nutrients; and 8-10 h was the stationary phase, where no growth occurs as a result of complete substrate consumption and high waste concentration.

Respecting to exponential phase, it was possible to calculate specific growth rate of *S. cerevisiae* based on absorbance reading and CFU counting and values of 0.50 h^{-1} and 0.55 h^{-1} were, respectively, obtained. This difference between the specific growth rate based on absorbance and viable counts has already been the subject of previous studies (Dalgaard & Koutsoumanis, 2001) and the general conclusion appears to be that growth parameters estimated based on viable counts data are more reliable, particularly at low cell densities. However, absorbance-based method is also valid (Dalgaard & Koutsoumanis, 2001).

It should be noted that specific growth rate is a constant that reflects the intrinsic properties of the microorganism, the limiting substrate and the temperature of growth. Thus, for a

temperature of growth of 30°C and glucose as a substrate, the reference interval for μ is 0.5-0.6 h^{-1} (Maier, 2015). Therefore, the specific growth rate calculated based on the established growth curve agrees with the reference values in both calculation methods. It is also important to mention that specific growth rate is valid only under the same experimental conditions used, since if the experiment was performed at different temperatures or in a different culture medium, it could be expected a different specific growth rate.

Since a significant association between abnormal oxidative status and ND has been extensively studied (Kim et al., 2015; Ahmadinejad et al., 2017; Liu et al., 2017), in the present project the role of H_2O_2 was initially examined. Concentrations of 1, 3 and 5 mM were chosen based on some previous studies using *S. cerevisiae* and H_2O_2 as an inductor of oxidative stress (Salmon, Evert, Song, & Doetsch, 2004; Fomenko et al., 2011; Pereira, Martins, & Saraiva, 2014). Despite higher concentrations can be used, as in the case described by Salmon et al. (2004), the range from 1 to 5 mM seems to be the most applied.

According with the results obtained in this study, it was found that the toxic effects of H_2O_2 induced a decrease in cell viability in a dose-dependent manner, independently of time exposure, since different concentrations produced different results over the same exposure time. While 5 mM and 3 mM H_2O_2 caused a significant decrease in viability, the lower concentration of H_2O_2 did not show significant deleterious effects on cell survival or proliferative capacity. Furthermore, the specific death rate caused by 5 mM and 3 mM H_2O_2 was calculated, since these concentrations caused a significant cell death ($p<0.05$). Values of -0.56 h^{-1} and -0.48 h^{-1}, respectively, were obtained, supporting the fact that 5 mM H_2O_2 caused a more noticeable cell death.

Despite *S. cerevisiae*, like all organisms, has effective antioxidant defense mechanisms to detoxify ROS and maintain the redox environment, when ROS overwhelm these defenses, a physiological dysfunction occurs and leads to cell death (Morano et al., 2012). In fact, 5 mM and 3 mM H_2O_2 seemed to overcome these mechanisms in yeast, while 1 mM was not sufficient to cause a deleterious effect probably because *S. cerevisiae* was able to counteract its action.

To assess the antioxidant properties of vitamin C, and knowing that the reference range values in plasma are 0.4 - 2 mg/dL (MAYO CLINIC: Mayo Medical Laboratories, s.d.), the upper limit concentration was chosen to test, aiming to study its antioxidant capacity and simulate a physiological condition. Additionally, 1 mg/dL and 4 mg/dL were tested, as these

concentrations represent, respectively, the half and the double of the initial. The objective was to assess if a lower concentration of vitamin C would maintain the antioxidant capacity and if a higher concentration would be more effective.

According to the obtained results, the tested concentrations of vitamin C itself had no effect on cell viability, maintaining it over the time. It was possible to demonstrate that the exposure of yeast cells to 1, 2 and 4 mg/dL vitamin C had similar results when compared with control.

Despite that, when a combined exposure to vitamin C and H_2O_2 was performed, it was shown that vitamin C protected cells from killing induced by H_2O_2. In fact, yeast cells exposed only to H_2O_2 were substantially more sensitive to cell death than those exposed to a combination of both agents, which means that an effective protection was achieved, except for the combination 5 mM H_2O_2 + 1 mg/dL vitamin C.

Despite a concentration of 1 mg/dL vitamin C is considered normal (comparing with the reference range), it was not sufficient to revert the deleterious effects of 5 mM H_2O_2; however, 2 mg/dL and 4 mg/dL were able to counteract H_2O_2 effects, which means that a higher vitamin C concentration is more effective against H_2O_2 induced oxidative stress.

Although it was found that 4 mg/dL reverted the effects of 5 and 3 mM H_2O_2, the reversal was not complete, since the results continued to be different from the control; only the reversal of 1 mM H_2O_2 effects was total, but, since 1 mM H_2O_2 effects were not significantly different from control ($p>0.05$), these results seems to have light relevance. The same happened when 1 mM H_2O_2 + 2 mg/dL vitamin C was tested.

Comparatively to 2 mg/dL and 1 mg/dL, 4 mg/dL vitamin C was found to be the optimal concentration for counteracting H_2O_2 effects, given it reduced the percentage of cell death caused by all tested concentrations of H_2O_2 in a higher extension. Thus, vitamin C was found to protect yeast cells from oxidative stress in a dose-dependent manner, and increasing the vitamin C dose, cells turn to be more resistant to oxidative damage.

Concerning to the HPLC results, it was expected that, the greater the concentration of H_2O_2 and, consequently, the larger cell death (as demonstrated on Chapter III), the greater would be the levels of 3-NT, since it is a biomarker of oxidative stress; however, results did not show significance.

Relatively to octopamine, it was expected to assess its levels and relate it with oxidative stress state induced by H_2O_2, aiming to evaluate whether a higher or lower concentration of H_2O_2 could influence octopamine levels. However, the obtained results were not significant.

This may be due to the fact that yeast cells wall had not been disrupted after the freezing/thawing cycles. Since the common approach for 3-NT quantification in biological samples is the previous cleavage of peptide bonds to release the free aminoacids from proteins in tissues or fluids (Delatour et al., 2007), Teixeira et al. (2017) referred the use of trifluoroacetic acid (TFA) to promote acid hydrolysis in *S. cerevisiae*. Nevertheless, TFA has a strong UV-absorption band, which can lead to HPLC baseline fluctuations that disturb high-sensitivity measurements (Choikhet, Glatz, & Rozing, 2003). Therefore, the use of TFA was not an option during the current work.

Additionally, Teixeira et al. (2017) also performed the HPLC method for 3-NT quantification in *S. cerevisiae* which suspension was made in ultrapure water, allowing, consequently, cell wall disruption and making it possible to detect 3-NT. During this work, and for 3-NT HPLC assessment, *S. cerevisiae* was suspended in its growing medium (liquid YEPD), which provides the optimal conditions for cells integrity.

Concerning to octopamine detection, the same pattern of results was obtained, supporting the fact that there was no cell wall disruption. Despite there are no current studies on octopamine assessment in yeast, this amine had been quantified using other matrices (Di Lorenzo et al., 2014; Guo, Wang, Wang, & Zhang, 2014). To obtain octopamine, extraction protocols were applied, either by chemicals (Latorre-Moratalla et al., 2009) or ultrasonification (Gatti, Lotti, Morigi, & Andreani, 2012). The last technique is also referred as an efficient process for *S. cerevisiae* cell disruption (Yeo et al., 2002; Bystryak, Santockyte, & Peshkovsky, 2015), and despite had been applied in this study, the frequency was lower than that stated in some studies (Yeo et al., 2002; Bystryak et al., 2015).

Since the results obtained with yeast samples exposed to H_2O_2 were not significant, we opted for not testing vitamin C exposed samples until the protocol for yeast cells lysis is improved and shown to be effective.

Chapter V – Conclusion

Considering the critical role of oxidative stress in ND, the manipulation of ROS levels may represent a treatment option to slow down neurodegeneration and reduce associated symptoms.

In this context, our results reinforce the already described deleterious effects of H_2O_2 as an oxidative stress inducer and the antioxidant properties of vitamin C. With the combination of both agents, it was found that 4 mg/dL vitamin C was the optimal concentration, between the tested ones, for reversal of oxidative stress. Despite it was not complete, in case of ND, even an incomplete reversal could possibly be helpful.

Also concerning to vitamin C, 1 mg/dL was shown not to be effective against 5 mM H_2O_2, despite being a value contained in the reference range. Extrapolating this result to the context of ND, where high levels of oxidative stress are present, it seems to be important to maintain an optimal concentration of vitamin C in plasma and near to the higher limit.

The extent of yeast cell protection depended on the concentration of vitamin C and the degree of oxidative challenge. Reliably, these results showed that the effects of vitamin C against oxidative stress occurred in a dose-dependent manner.

Concerning to the determination of 3-NT and octopamine by HPLC, a clear improvement of general method is needed in order to assess its levels and to establish a correlation between them and an oxidative stress state, evaluating also the importance of vitamin C.

In short, we considered that the results obtained with this study may be helpful on understanding the role of natural antioxidants on oxidative stress and may provide crucial insights into therapeutic options for treatment of ND.

Chapter VI – Future Perspectives

In a short-term time, our aim is to improve the protocol for *S. cerevisiae* cells lysis to allow the measurement of 3-NT and octopamine and draw a conclusion about their levels and relate them to the effect of H_2O_2 and vitamin C.

Also, it is pretended to evaluate the proteins, by Western blot, in yeast cells exposed to H_2O_2 to assess whether the exposure to oxidative stress is able to cause protein aggregation, a common feature of ND.

In the future, we aim to test the same conditions of oxidative stress and antioxidant defenses using a ND yeast model to study and compare the cellular response between a normal situation and a pathological one.

Thus, using cell and molecular biology methods, it is our aim to construct *S. cerevisiae* mutants for Familial Amyloidotic Polyneuropathy, since it is an inherited ND for which the role of oxidative stress is poorly studied. We also intend to study the significance of using vitamin C as an antioxidant and possible therapeutic option, as well as assess its influence on neurotransmitters levels. To that, it is our goal to improve the HPLC method for quantification of octopamine and develop other methods for detection of GABA and glutamate, since they are the most important neurotransmitters in CNS. With GABA and glutamate quantification and the well-known variation patterns in ND, we could establish a ratio that could be indicative of an oxidative stress condition.

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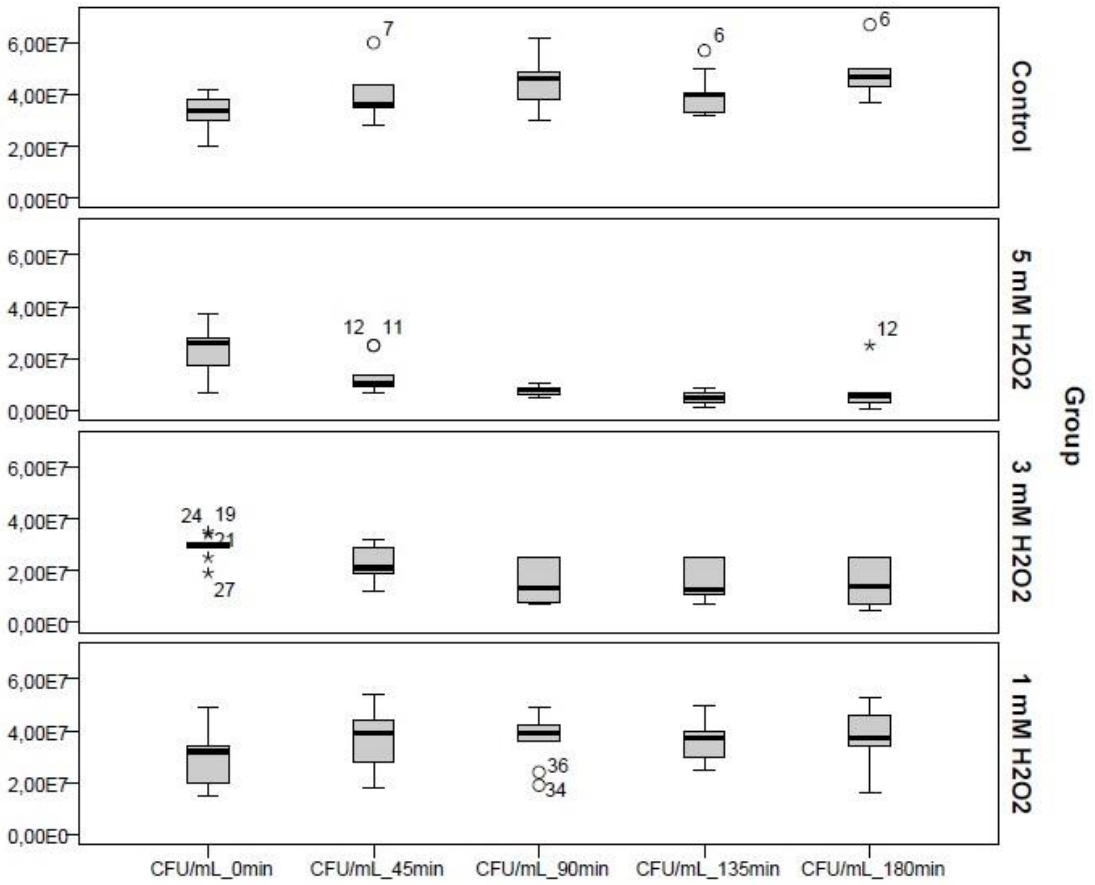
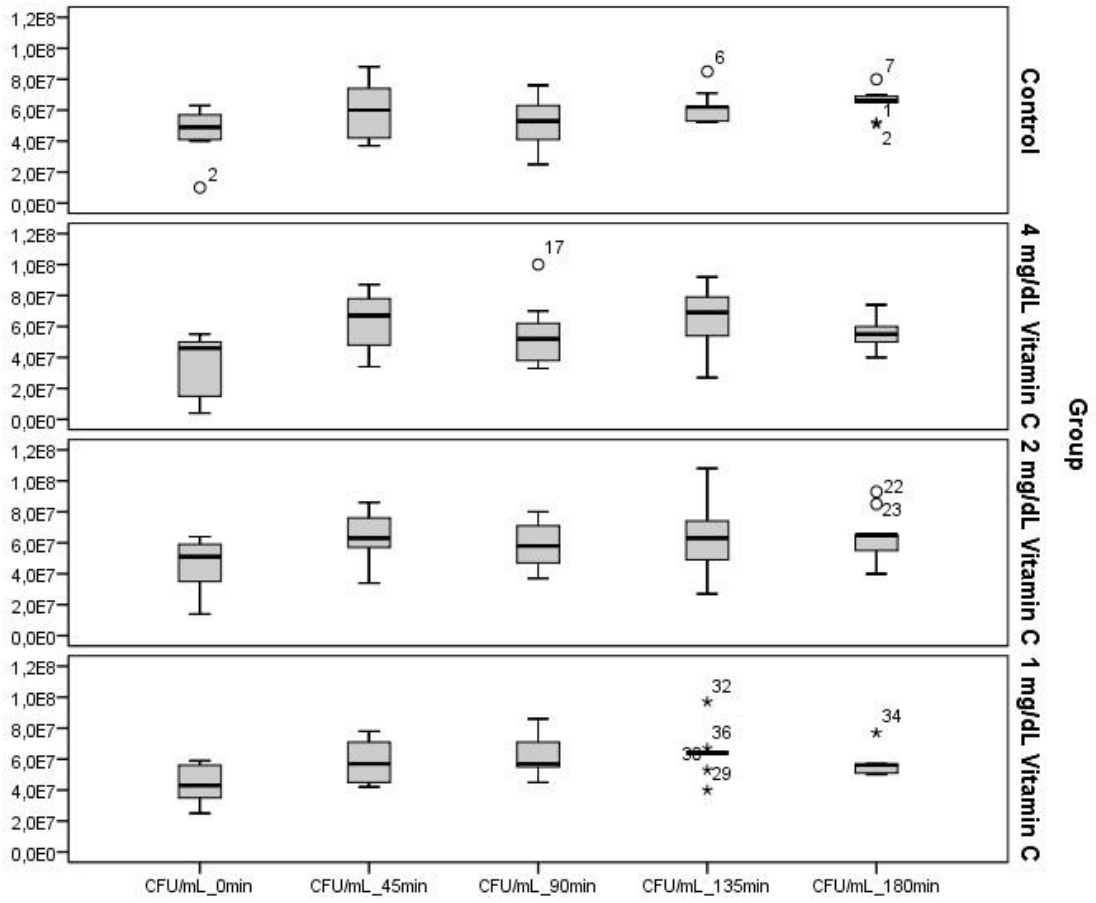
Appendix

Appendix I



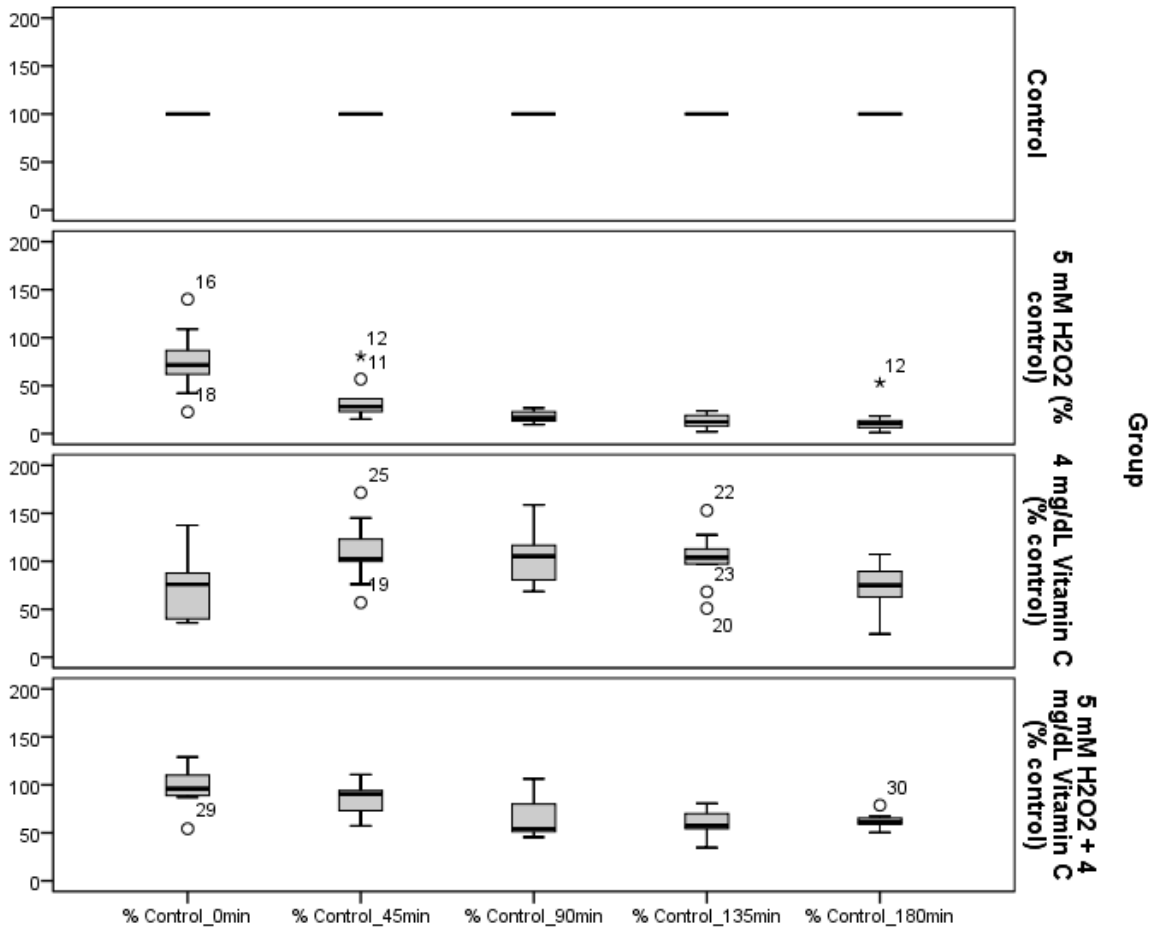
Graphic I | Boxplot representing the effects of different concentrations of H_2O_2 on cell viability across the time.

Appendix II



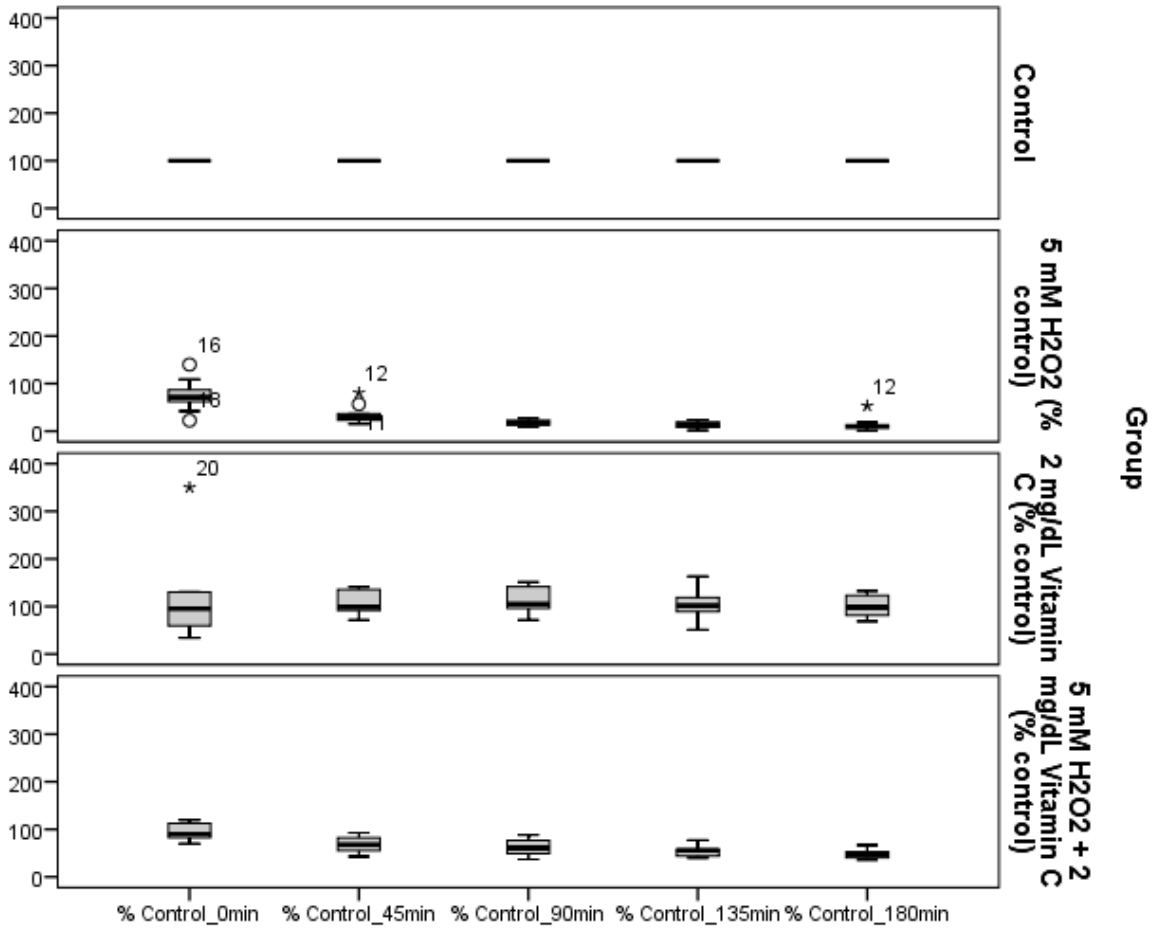
Graphic II | Boxplot representing the effects of different concentrations of vitamin C on cell viability across the time.

Appendix III (A)



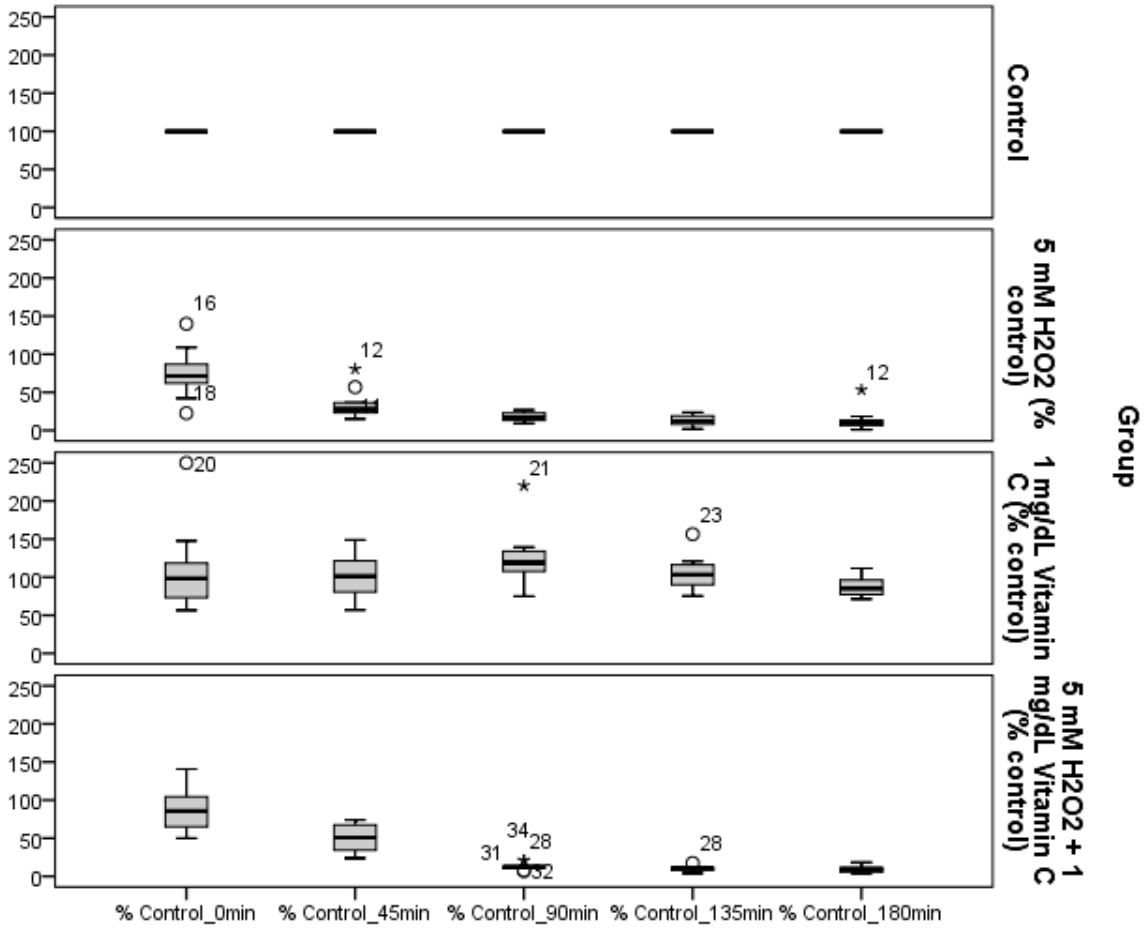
Graphic III | Boxplot representing the effects of 5 mM H_2O_2, 4 mg/dL vitamin C and the combined effect of 5 mM H_2O_2 + 4 mg/dL vitamin C on cell viability across the time.

Appendix III (B)



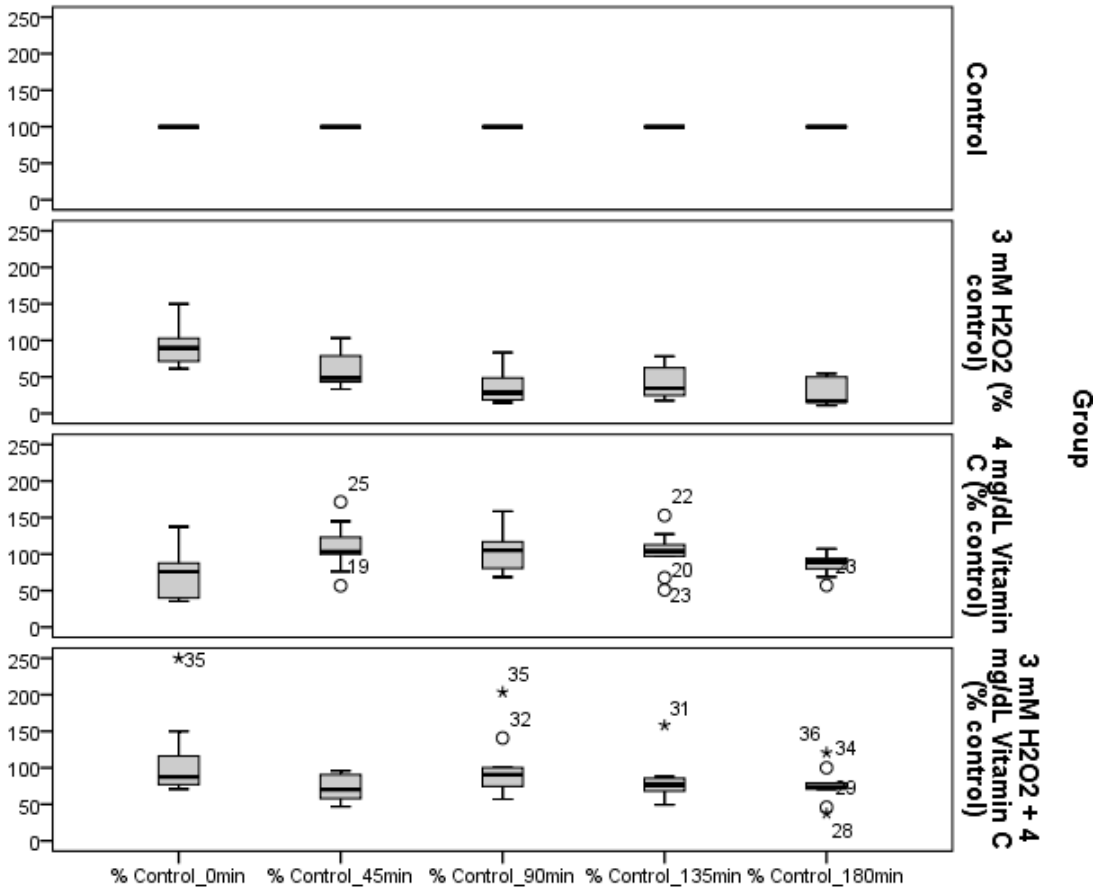
Graphic IV | Boxplot representing the effects of 5 mM H_2O_2, 2 mg/dL vitamin C and the combined effect of 5 mM H_2O_2 + 2 mg/dL vitamin C on cell viability across the time.

Appendix III (C)



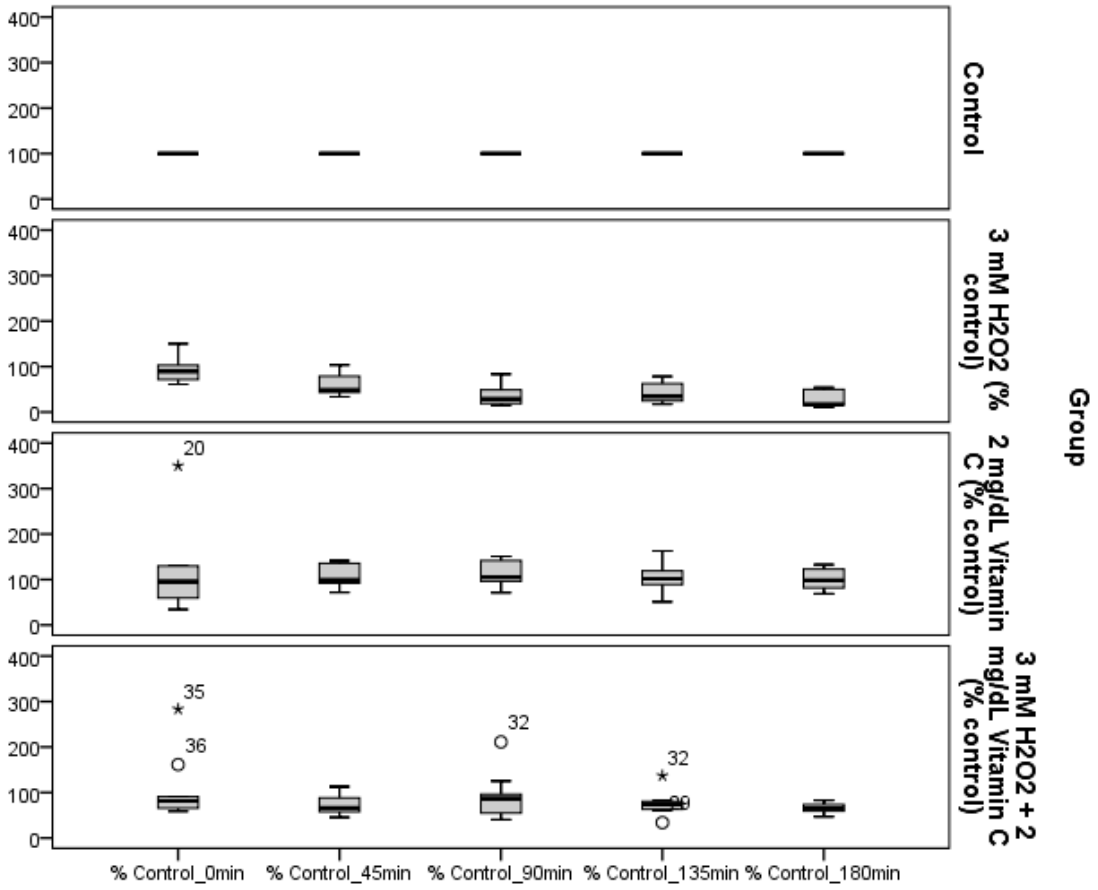
Graphic V | Boxplot representing the effects of 5 mM H_2O_2, 1 mg/dL vitamin C and the combined effect of 5 mM H_2O_2 + 1 mg/dL vitamin C on cell viability across the time.

Appendix IV (A)



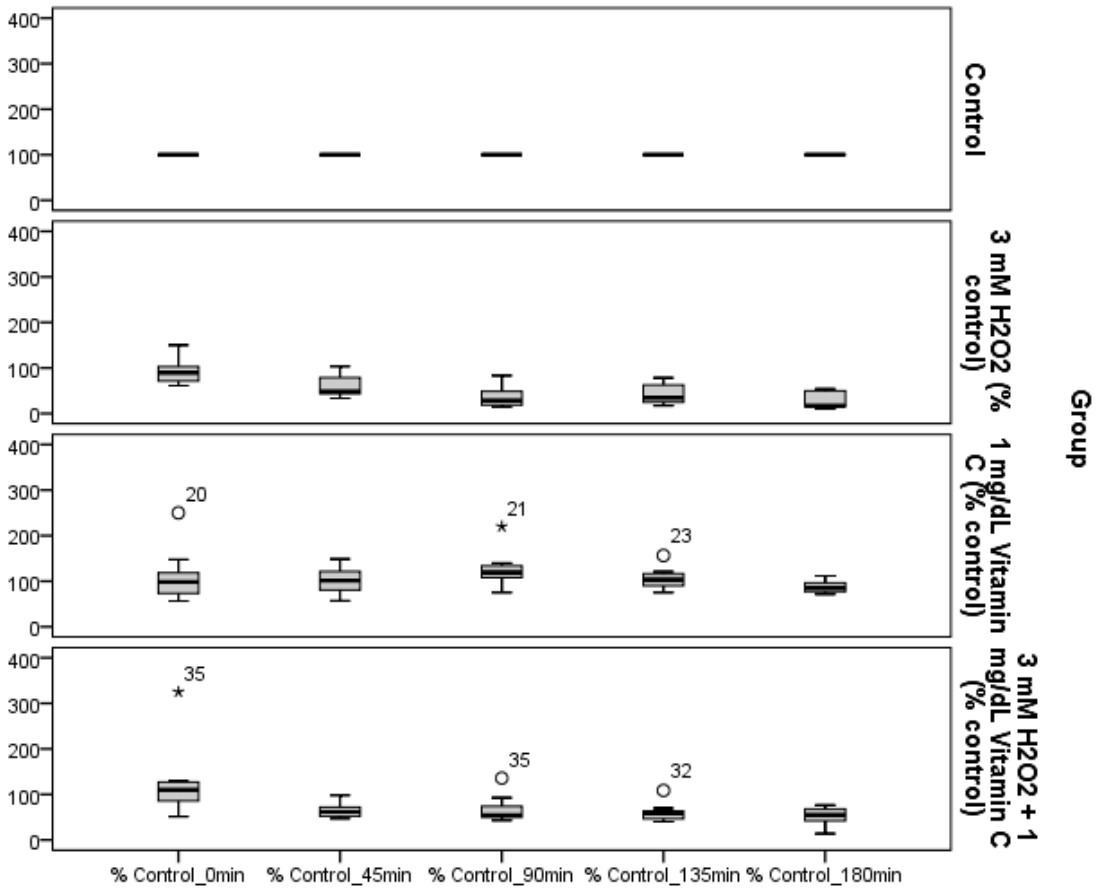
Graphic VI | Boxplot representing the effects of 3 mM H_2O_2, 4 mg/dL vitamin C and the combined effect of 3 mM H_2O_2 + 4 mg/dL vitamin C on cell viability across the time.

Appendix IV (B)



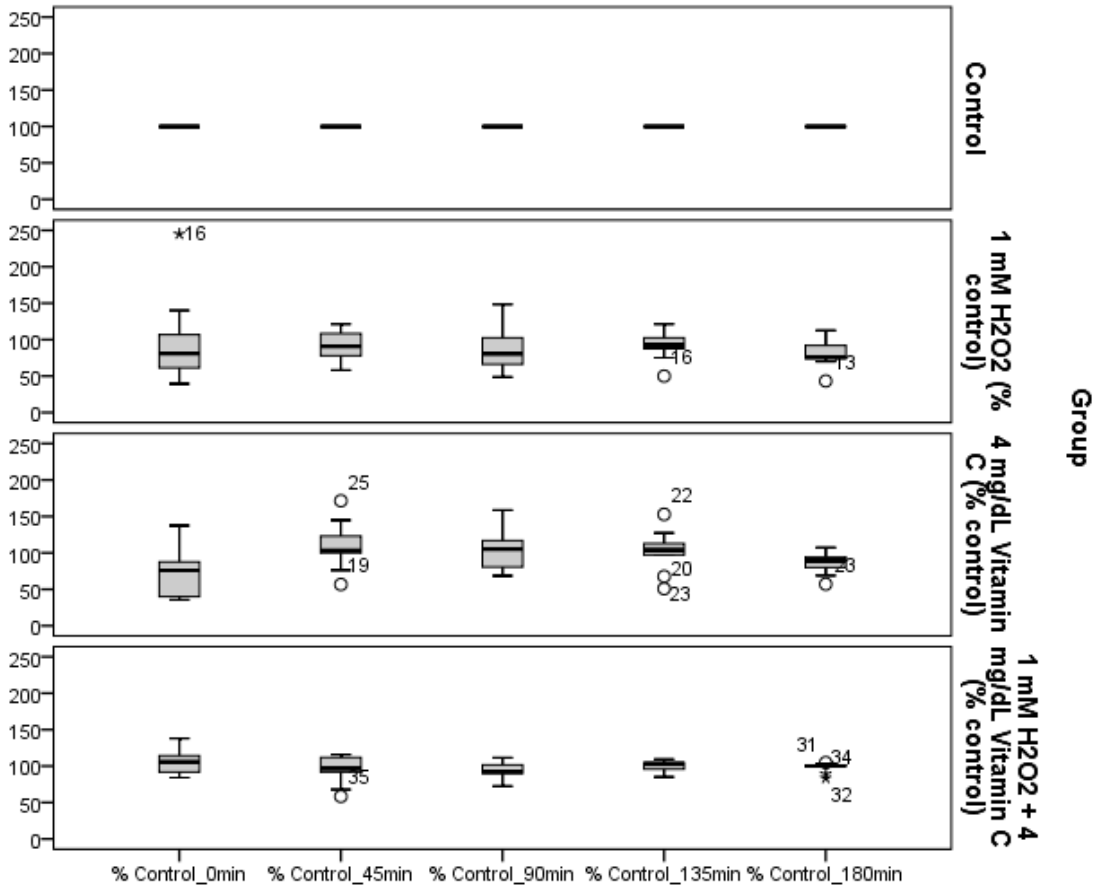
Graphic VII | Boxplot representing the effects of 3 mM H_2O_2, 2 mg/dL vitamin C and the combined effect of 3 mM H_2O_2 + 2 mg/dL vitamin C on cell viability across the time.

Appendix IV (C)



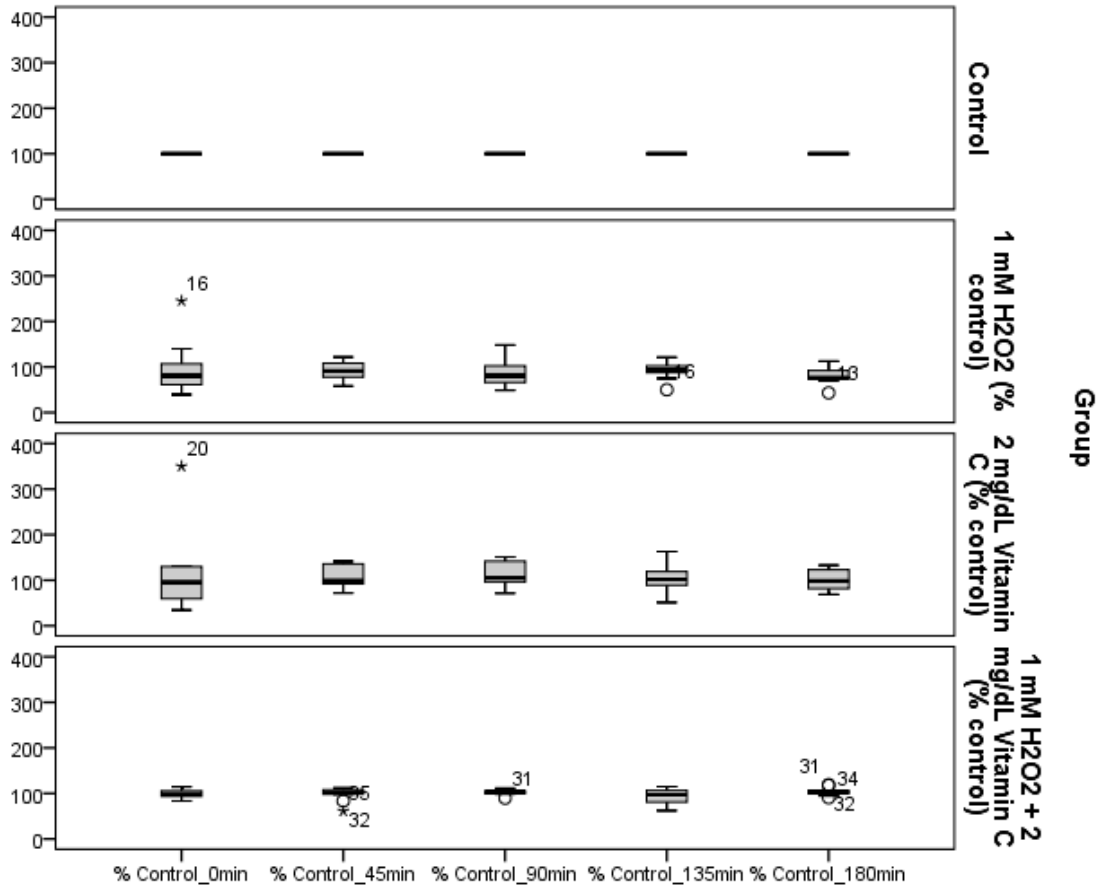
Graphic VIII | Boxplot representing the effects of 3 mM H_2O_2, 1 mg/dL vitamin C and the combined effect of 3 mM H_2O_2 + 1 mg/dL vitamin C on cell viability across the time.

Appendix V (A)



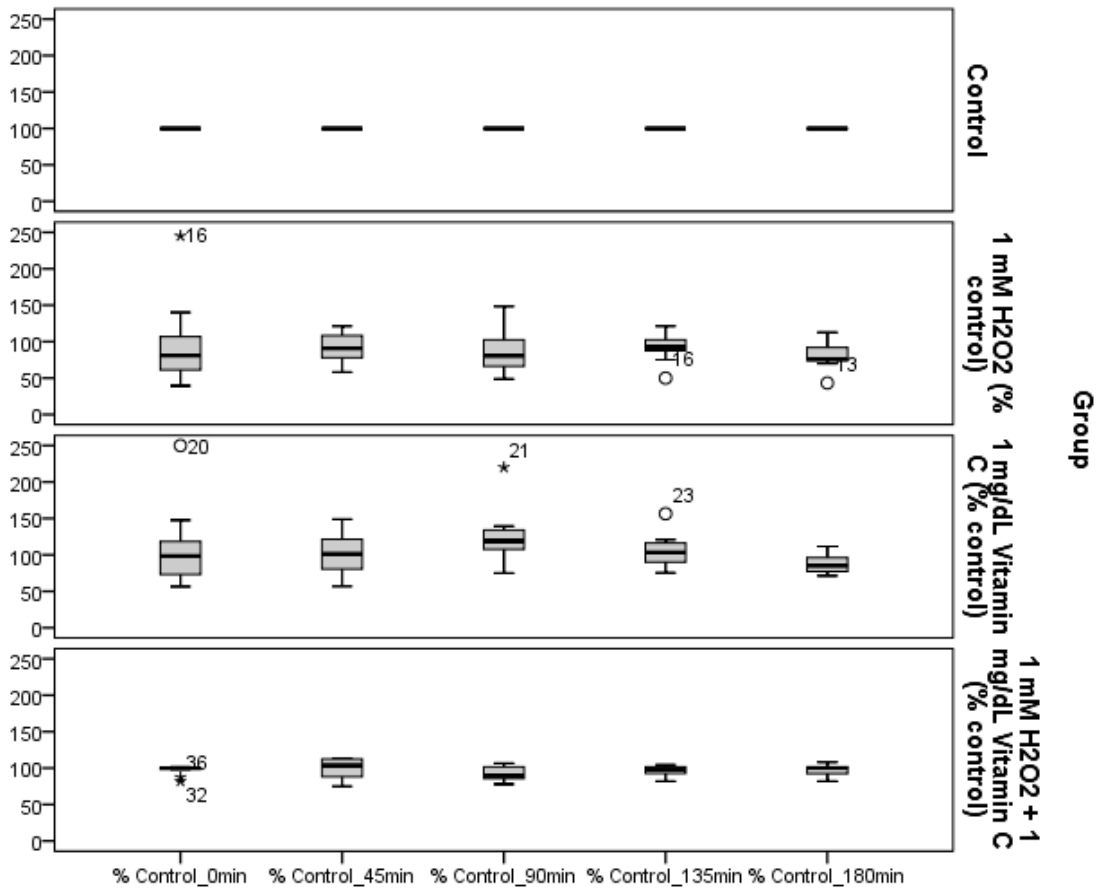
Graphic IX | Boxplot representing the effects of 1 mM H_2O_2, 4 mg/dL vitamin C and the combined effect of 1 mM H_2O_2 + 4 mg/dL vitamin C on cell viability across the time.

Appendix V (B)



Graphic X | Boxplot representing the effects of 1 mM H_2O_2, 2 mg/dL vitamin C and the combined effect of 1 mM H_2O_2 + 2 mg/dL vitamin C on cell viability across the time.

Appendix V (C)



Graphic XI | Boxplot representing the effects of 1 mM H_2O_2, 1 mg/dL vitamin C and the combined effect of 1 mM H_2O_2 + 1 mg/dL vitamin C on cell viability across the time.