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Effect of age and chronic hypoxia on oxidative status, mitochondria and apoptosis in rat liver

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Paula Moreira e da Doutora Sancha Santos (Universidade de Coimbra)

Emanuel Monteiro Candeias

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Aos meus Pais e Irmãos

À Vera

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Abbreviations

- ***•OH*** **-** hydroxyl radical
- ***8-oxo-dG*** **-** 8-oxo-2-deoxyguanosine
- ***ADP*** **-** adenosine diphosphate
- ***ALD*** **-** alcoholic liver disease
- ***ALT*** **-** alanine aminotransferase
- ***APAF1*** **-** apoptotic protease activating factor-1
- ***Asc*** **–** ascorbate
- ***AST*** **-** aspartate aminotransferase
- ***ATP*** **-** adenosine triphosphate
- ***BCA*** **–** bicinchoninic acid
- ***BSA*** **-** bovine serum albumin
- Ca^{2+} **-** calcium cation
- ***CAT*** **–** catalase
- ***CoA*** **-** coenzyme A
- ***COPD*** **-** chronic obstructive pulmonary disease
- ***COX*** **-** cytochrome c oxidase
- ***CuZnSOD*** **–** copper-zinc superoxide dismutase
- ***Cyt c*** – cytocrome c
- ***dATP*** **-** deoxyadenosine triphosphate
- ***DISC*** **-** death-inducing signaling complex
- ***DNA*** **-** desoxyribonucleic acid
- ***DTNB*** **-** 5,5'-ditiobis-2-nitrobenzoic acid
- ***eNOS*** **-** endothelial nitric oxide synthase

- ***EPO*** - erythropoietin
- ***ER*** - endoplasmic reticulum
- ***FADD*** - Fas associated death domain protein
- ***FELASA*** - Federation of Laboratory Animal Science Associations
- ***GLUT-1*** – glucose transporter 1
- ***GPx*** - glutathione peroxidase
- ***GR*** - glutathione reductase
- ***GSH*** - glutathione
- ***GSSG*** - glutathione disulfide
- H^+ - proton
- H_2O - water
- H_2O_2 - hydrogen peroxide
- ***HCT*** - hematocrit
- ***HGB*** - hemoglobin
- ***HIF-1*** - hypoxia-inducible transcription factor-1
- ***HNE*** - 4- hydroxy-2-nonenal
- ***HPLC*** – high-performance liquid chromatography
- ***HRE*** - hypoxia response element
- ***HUVECs*** - human umbilical vein endothelial cells
- ***HVA*** - homovalinic acid
- ***IAPs*** - inhibitor of apoptosis proteins
- ***Ig*** - immunoglobulin
- ***IGF*** - insulin-like growth factor
- ***KCN*** – potassium cyanide
- ***LDH-A*** - lactate dehydrogenase A

- ***LDL*** **-** low density lipoproteins
- ***MDA*** **–** malondialdehyde
- ***MnSOD*** **-** manganese superoxide dismutase
- ***mtDNA*****-** mitochondrial DNA
- N_2 **-** nitrogen
- ***NAD+*** **-** oxidized nicotinamide adenine dinucleotide
- ***NADH*** **-** reduced nicotinamide adenine dinucleotide
- $NADP^+$ **-** oxidized nicotinamide adenine dinucleotide phosphate
- ***NADPH*** **-** reduced nicotinamide adenine dinucleotide phosphate
- ***NASH*** **-** nonalcoholic steatohepatitis
- ***NBT*** **-** nitro-blue tetrazolium
- ***nDNA*** **-** nuclear DNA
- ***NEM*** **-** N-ethylmaleymide
- $NO^\bullet$ **-** nitric oxide
- O_2 **–** oxygen
- $O_2^{\bullet-}$ **-** superoxide anion
- ***ODD*** **-** oxygen dependent degradation domain
- ***OMM*** **-** outer mitochondrial membrane
- $ONOO^-$ - peroxynitrite
- ***OPT*** **–** ophthalaldehyde
- ***OSA*** **-** obstructive sleep apnea
- ***PBS*** **-** phosphate buffer saline
- ***PHDs*** **-** prolyl hydroxylase enzymes
- ***Pi*** **-** inorganic phosphate
- ***PMSF*** **-** phenylmethanesulfonylfluoride
- ***PTP*** **-** permeability transition pore

- ***PUFAs*** - polyunsaturated fatty acids
- ***PVDF*** - polyvinyl difluoride
- ***RBC*** - red blood cells
- ***ROS*** - reactive oxygen species
- ***SDS*** - sodium dodecyl sulfate
- ***SGA*** - small-for-gestational-age
- ***SH*** - thiol groups
- ***Smac*** - second mitochondrial activator of caspases
- ***SOD*** - superoxide dismutase
- ***TBA*** - thiobarbituric acid
- ***TBARS*** - thiobarbituric acid reactive substances
- ***TBS*** - tris-buffered saline
- ***TCA*** - tricarboxylic acid
- ***TCA*** - trichloroacetic acid
- ***TMPD*** - N, N, N', N'-tetrametyl-p-phenylenodiamine
- ***TNFR1***- tumor necrosis factor receptor-1
- ***TOR*** - target of rapamycin
- ***UCPs*** – uncoupling proteins
- ***UV*** – ultraviolet light
- ***v*** - velocity
- ***VEGF*** - vascular endothelial growth factor
- ***VHL*** - von Hippel–Lindau tumor suppressor protein

Abstract

Aging is a complex multifactorial process involving alterations at genetic, molecular, cellular, organ, and system levels. The "oxidative stress theory" holds that a progressive and irreversible accumulation of oxidative damage caused by mitochondrial reactive oxygen species impacts on critical aspects of the aging process and contributes to impaired physiological function, increased incidence of disease, and a reduction in life span. Additionally, periods of chronic hypoxia, which can arise from numerous disorders (e.g. chronic vascular diseases) and even aging, potentiate the development of degenerative diseases. In this study we evaluated the effects of age and chronic hypoxia in the oxidative status, mitochondrial enzymatic complexes activity and apoptotic cell death pathway of the liver. For this purpose 3- and 12-month-old male Wistar rats exposed to normoxia (21% O_2) or hypoxia (10% O_2) during 7 days were used. Several parameters were evaluated: hydrogen peroxide (H_2O_2) and thiobarbituric acid reactive substances (TBARS) levels, aconitase activity, enzymatic [manganese superoxide dismutase (MnSOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities] and non-enzymatic (glutathione and vitamin E levels) antioxidant defenses, the activities of NADH-cytochrome c reductase (mitochondrial complexes I-III), succinate-cytochrome c reductase (mitochondrial complexes II-III), cytochrome c oxidase (mitochondrial complex IV) and ATPase. The activation of caspase-9 and caspase-3 and Bax and Bcl2 protein levels were also analyzed. An age-dependent increase in H_2O_2 levels and caspases activation and a decrease in aconitase, mitochondrial enzymatic complexes, ATPase and GR activities and glutathione levels were observed. Interestingly, chronic hypoxia in young animals caused a similar pattern of oxidative imbalance and mitochondrial defects compared to that found in 12-month-

old animals. In addition, chromic hypoxia potentiated the age-dependent increase in H_2O_2 levels and decrease in glutathione levels. Curiously, hypoxia decreased caspases activation in 12-month-old animals. Altogether, these results show that age and/or chronic hypoxia enhance liver oxidative imbalance and mitochondrial damage.

Keywords: Aging, chronic hypoxia, oxidative stress and damage, liver

Resumo

O envelhecimento é um processo multifactorial complexo que envolve alterações genéticas, moleculares, celulares, nos órgãos e no organismo. A "teoria do stress oxidativo" sustenta que a acumulação progressiva de lesões oxidativas causadas pelas espécies reactivas de oxigénio produzidas pelas mitocôndrias tem um papel chave no processo de envelhecimento contribuindo para uma alteração da função fisiológica, um aumento da incidência de doenças e uma redução no tempo de vida. Além disso, períodos de hipoxia crónica associadas a diversas doenças (ex. doenças vasculares crónicas) e ao processo fisiológico do envelhecimento, potenciam o desenvolvimento de doenças degenerativas. Neste estudo foram avaliados os efeitos da idade e da hipoxia crónica no estado oxidativo, na actividade dos complexos enzimáticos mitocondriais e na via de morte celular por apoptose do fígado. Para este efeito, foram utilizados ratos Wistar de 3 e 12 meses de idade expostos a normoxia (21% O_2) ou hipoxia (10% O_2) durante 7 dias. Foram avaliados vários parâmetros: os níveis de peróxido de hidrogénio (H_2O_2) e das substâncias reactivas ao ácido tiobarbitúrico (TBARS), a actividade da aconitase, as defesas antioxidantes enzimáticas [as actividades das enzimas dismutase do superóxido de manganês (MnSOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (GR)] e não-enzimáticas (níveis de glutationa e vitamina E), as actividades dos complexos enzimáticos mitocondriais NADH- citocromo c redutase (complexos I-III), succinato-citocromo c redutase (complexos II-III), citocromo c oxidase (complexo IV) e ATPase. A activação da caspase-9 e da caspase-3 e os níveis das proteínas Bax e Bcl2 também foram analisados. A idade aumentou os níveis de H_2O_2 e a activação das caspases e diminui as actividades da aconitase, dos complexos enzimáticos mitocondriais, da ATPase e da GR e os níveis de glutationa. Curiosamente,

os animais jovens expostos a hipoxia crónica apresentaram um perfil oxidativo e uma função mitocondrial semelhante aos animais de 12 meses de idade. Além disso, a hipoxia crónica potenciou o aumento nos níveis de H_2O_2 e glutationa provocados pela idade. Curiosamente, a hipoxia crónica diminui a activação das caspases nos animais de 12 meses de idade. Estes resultados mostram que a idade e/ou a hipoxia crónica potenciam o stress oxidativo e a disfunção mitocondrial.

Palavras-chave: Envelhecimento, hipoxia crónica, stress oxidativo, lesão oxidativa, fígado

CHAPTER 1. INTRODUCTION

1.1 – Aging

Every organism has a limited time of life and suffers several physiological and biochemical changes over time (Terman *et al.*, 2007). Aging is an inevitable intrinsic biological process characterized by the accumulation of different lesions, which leads to a progressive and differential degradation of somatic cells that affects the major biological functions decreasing the ability of the organism to survive (Mármol *et al.*, 2010; Costa *et al.*, 2006; von Zglinicki *et al.*, 2001; Johnson *et al.*, 1999).

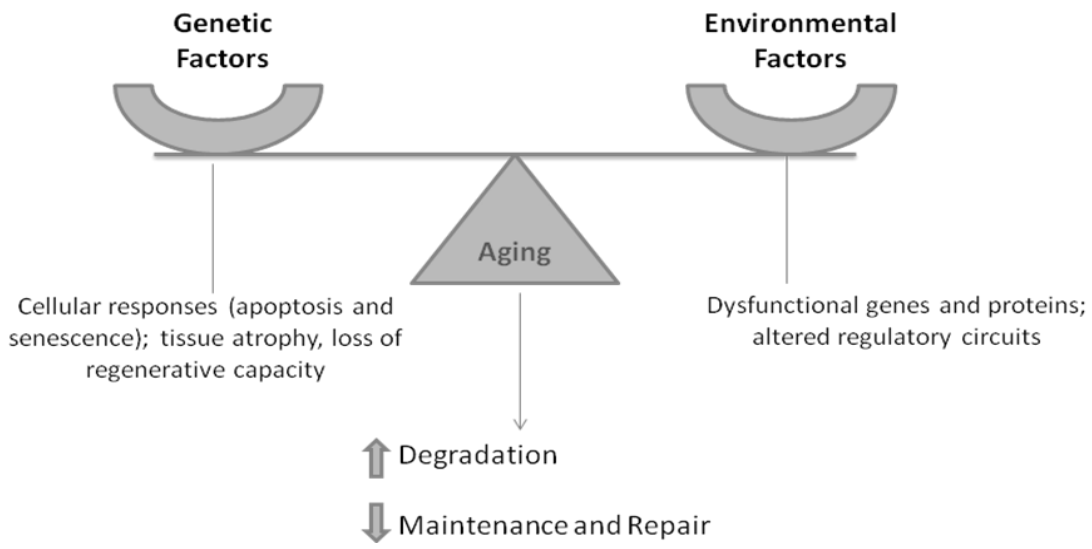


Fig. 1 – Causes of Aging. Aging results from the accumulation of lesions in the cells, resulting in deterioration and, finally, death. An aged organism is characterized by homeostatic imbalance, decreased ability to respond to stress, loss of regenerative capacity and increased risk of disease and probability of death (i.e. senescence). This irreversible series of changes inevitably ends in dysfunctional biomolecules and genes, tissue atrophy and death. Some of the causes are unavoidable such as ultraviolet radiation, free radicals, and genetic; others involve environmental and behavioral influences.

Aging is a complex and multifactorial process influenced by several conserved signalling pathways such as insulin-like growth factor (IGF) -1and target of rapamycin (TOR) signalling pathways, and by environmental factors such as nutrient availability and temperature (Vellai, 2008). Many factors contribute to cell decline during aging, but one characteristic shared by all aged cells is the intracellular accumulation of damaged biomolecules, including proteins of the membranes and organelles, particularly mitochondria (Vellai, 2008; Kirkwood 2005). A decline in the intracellular renewal and repair mechanisms, and removal of damaged macromolecules and organelles, results in the accumulation of damaged structures that interfere with cells function. The decline in the DNA repair capacity results in the accumulation of DNA damage, which is considered a main culprit of the aging process (von Zglinicki *et al.*, 2001; Johnson *et al.*, 1999). Nuclear and mitochondrial DNA (nDNA and mtDNA, respectively) are permanently exposed to exogenous and endogenous DNA-damaging agents causing an accumulation of damage in the genome leading to a situation of homeostatic imbalance of cells and tissues. Moreover, non-lethal mutations are reproduced during cell division. Non-dividing cells or long-lived post-mitotic cells, such as neurons, myocytes, hepatocytes and retinal pigment epithelial cells cannot perfectly eliminate damage, so with time they degenerate and loss their normal structure and function, which leads to the collapse of the whole organism, increasing the probability of disease and death (i.e. senescence) (Terman *et al.*, 2007; von Zglinicki *et al.*, 2001).

Aged cells are characterized by morphologic alterations including increased cell volume and size of the remaining functional structures, maybe resulting from the accumulation of "biological garbage" (Terman *et al.*, 2006; von Zglinicki *et al.*, 2001). There is also an increase in membranes fluidity, transport, permeability and response to stimuli, caused by damage of lipids and proteins (Terman *et al.*, 2007). Nuclei also

suffer age-related alterations, characterized by an increased content of heterochromatin and irregular surface, damaged nuclear proteins and DNA, among other irregularities (Hoare *et al.*, 2010; Terman *et al.*, 2007). The endoplasmic reticulum (ER), Golgi apparatus, ribosomes and other organelles decrease in number, suffer a loss of function, and present an alteration of arrangement and movements that lead to a decline in protein synthesis, these alteration being associated with injury to cytoskeleton (Terman *et al.*, 2007). The intracellular accumulation of aberrant proteins are a common characteristic in aging cells and often form indigestible aggregates associated with age-related diseases, such as Lewy bodies and neurofibrillary tangles (Terman *et al.*, 2006). Lysosomes and mitochondria seem to play a pivotal role in cellular aging. Several studies show that the autophagic process, involved in the degradation of dysfunctional organelles and protein aggregates, suffers a decline with aging (Kurz *et al.*, 2007). One of the reasons is related with the incapacity of lysosomes to exert their function resulting in the accumulation of liposfuscin (a non-degradable intralysosomal polymeric age pigment) (Kurz *et al.*, 2007; Terman *et al.*, 2007). The age-related changes in mitochondria include swelling, loss of cristae and destruction of the inner membrane, formation of amorphous electron-dense material, increased mutations in mtDNA and protein alterations. These alterations lead to a progressive decrease in the number and efficiency of mitochondria that result in lower ATP production and higher generation of reactive oxygen species (ROS) (Terman *et al.*, 2007). Because the antioxidant defense system of the cells also suffers a decline with aging, the excess of ROS is not neutralized resulting in biomolecules oxidative damage including those of mitochondria (Aydin *et al*., 2010). Indeed, it is generally agreed that the accumulation of free radicals during aging is the main driver of aging (Terman *et al.*, 2006).

1.2 - Aging, Free Radicals and Mitochondria

There are many theories that try to explain the process of aging, and several mechanisms underlying this process have been proposed, including somatic mutations, error accumulation, telomere shortening and cumulative damage by ROS (Kirkwood 2005; Johnson *et al*., 1999). One of the most plausible and accepted theory is the Mitochondrial Free Radical Theory of Aging. This theory postulates that the main driving force in the aging process and age-related diseases is the accumulation of intracellular damage, caused by mitochondrial free radicals, mainly due to the incapacity of endogenous antioxidant defenses to avoid this damage (Sanz *et al*., 2008; Gemma *et al*., 2007). In 1956, Harman was the first to suggest that aging results from cumulative damage caused by free radicals produced in normal metabolism, this idea being at the basis of the Free Radical Theory of Aging (Harman, 1956). Later in 1972, Harman expanded the original theory emphasizing the role of mitochondria as both generators and targets of ROS (Harman, 1972). Damaged mitochondria progressively become less efficient in terms of energy production and generate high levels of ROS, which potentiate mitochondrial dysfunction starting a vicious cycle that culminates in cells degeneration and, eventually, death. Since then, the Free Radical Theory of Aging has become the Mitochondrial Free Radical Theory of Aging (Harman, 1972).

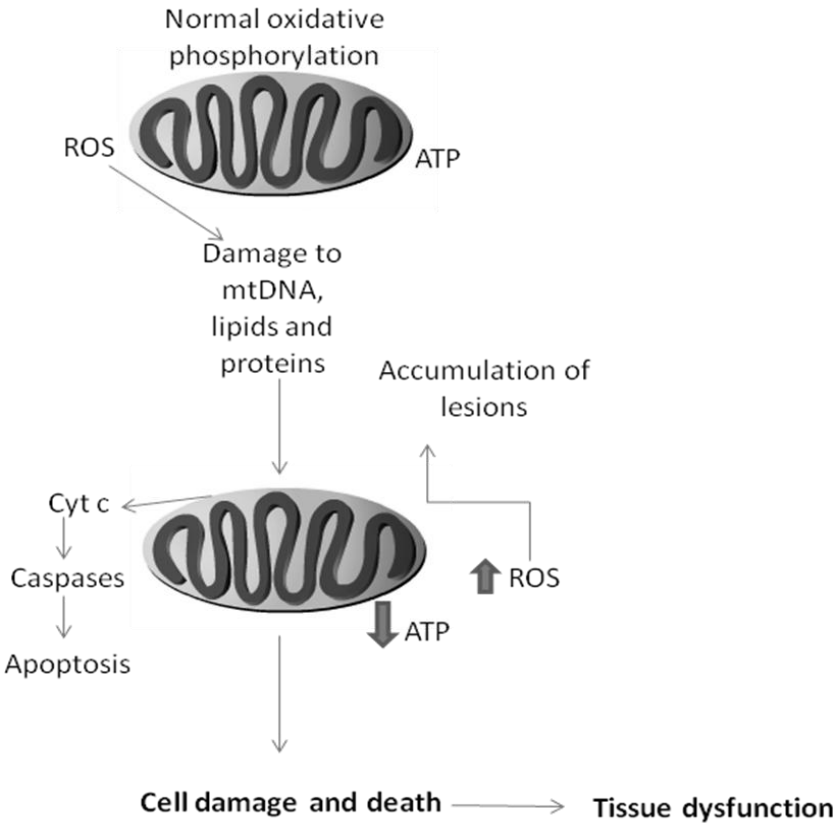


Fig. 2 - Mitochondrial Free Radical Theory of Aging. The production of energy (ATP) by mitochondria is accompanied by the production of reactive oxygen species (ROS), whose levels are tightly controlled through an intricate antioxidant defense system. During aging, the efficiency of this system decline and the levels of ROS increase, which promote oxidative damage of several biomolecules including those of mitochondria starting a vicious cycle of deleterious events. Some of these events culminate in the release of cytochrome c (Cyt c), which activates the caspase cascade culminating in apoptotic cell death. The death of cells inevitably leads to impairment of tissue function. mtDNA – mitochondrial DNA.

Mitochondria are double membrane organelles, with their own genome (mtDNA), resident in all eukaryotic cells. They play a critical role in cell life, generating energy-rich phosphate bonds in the form of adenosine triphosphate (ATP) necessary to support all cellular functions including the supervision of cellular health in

order to initiate programmed cell death if necessary. Therefore, tissues like muscles, brain, liver and heart that have high metabolic rates contain a relatively high number of mitochondria (Moreira *et al.,* 2010; Kakkar and Singh, 2007; Wallace 1999). In this process, carbon substrates, derived from the metabolism of glucose, enter the tricarboxylic acid (TCA) cycle leading to the formation of the electron donors reduced nicotinamide adenine dinucleotide (NADH) and succinate, which promote the electron flow through the respiratory chain to the final acceptor, molecular oxygen (O_2), that is reduced to water (complexes I and II – coenzyme Q – complex III – cytochrome c – complex IV – O_2). The electron transfer through the mitochondrial respiratory chain is associated with proton pumping from complexes I, III and IV to the intermembrane space and a proton gradient is formed across the mitochondrial inner membrane. This proton gradient is used by complex V (ATP synthase) to form ATP from ADP and Pi (Vendelbo and Nair, 2011; Santos *et al.,* 2010; Kakkar and Singh, 2007). For oxidative phosphorylation and ATP synthesis, mitochondria consume about 90% of a cell's O_2. However, the use of the aggressive biradical O_2 in aerobic respiration has some disadvantages; electrons derived from the respiratory chain can react with O_2 and generate free radicals. ROS formation can occur as a product of electron leak from complexes I and III where O_2 is reduced to form the superoxide anion radical ($O_2^{\bullet -}$) that, in turn, can be detoxified by the mitochondrial manganese superoxide dismutase (MnSOD) to give hydrogen peroxide (H_2O_2). H_2O_2 in the presence of transition metals, can be converted via Fenton and/or Harber-Weiss reactions to the toxic hydroxyl radical ($^{\bullet}OH$), major player in oxidative damage (Vendelbo and Nair, 2011; Santos *et al.,* 2010; Kakkar and Singh, 2007; Terman *et al.,* 2007; Moreira *et al.,* 2005). Additionally, the mitochondrial membrane potential influences the mitochondrial production of ROS. The hyperpolarization (high mitochondrial membrane potential) of mitochondria is

linked to a high flow of electrons in the respiratory chain potentiating the electron leak from mitochondrial complexes I and III. The activation of uncoupling proteins (UCPs) attenuates mitochondrial production of free radicals and protects against oxidative damage. UCPs are located in the mitochondrial inner membrane and shuttle protons from the intermembrane space to the mitochondrial matrix. This proton shuttle cause mild uncoupling that is characterized by a decrease in the protonmotive force and an increase in respiration rate, however ATP production is maintained (Vendelbo and Nair, 2011; Brand and Esteves 2005).

As previously said, free radicals produced by mitochondria are major players involved in oxidative damage associated to aging. That damage potentiates mitochondrial dysfunction favoring the aging of the cells (Sastre *et al.*, 2003). Oxidative stress damages mitochondrial biomolecules such as DNA, lipids and proteins. Compared to nDNA, mtDNA is especially susceptible to oxidative damage and mutations because it lacks protective histones and is close to the mitochondrial respiratory chain, the main source of ROS. Oxidative damage to mtDNA is inversely related to the maximum life span of mammals, whereas oxidative damage to nDNA does not correlate with maximum life span (Sanz *et al*., 2008; Sastre *et al.*, 2000). It is believed that a decrease in mtDNA turnover and increased mtDNA mutations may compromise mitochondrial functions in different ways. The impairment of mitochondria function with aging is well established and is associated with: 1) defects in the electron transport and oxidative phosphorylation that lead to a decline in ATP levels and NAD^+/NADH ratio; 2) an increase in free radicals production that will increase the occurrence of mtDNA mutations; 3) an impairment of Ca^{2+} homeostasis and 4) the induction of mitochondrial pathways of apoptosis by release of cytochrome c and other apoptotic factors. The decline in the antioxidant defense system in aged cells also

contributes to mitochondrial damage (Sastre *et al.*, 2003; Sastre *et al.*, 2000; Johnson *et al.*, 1999).

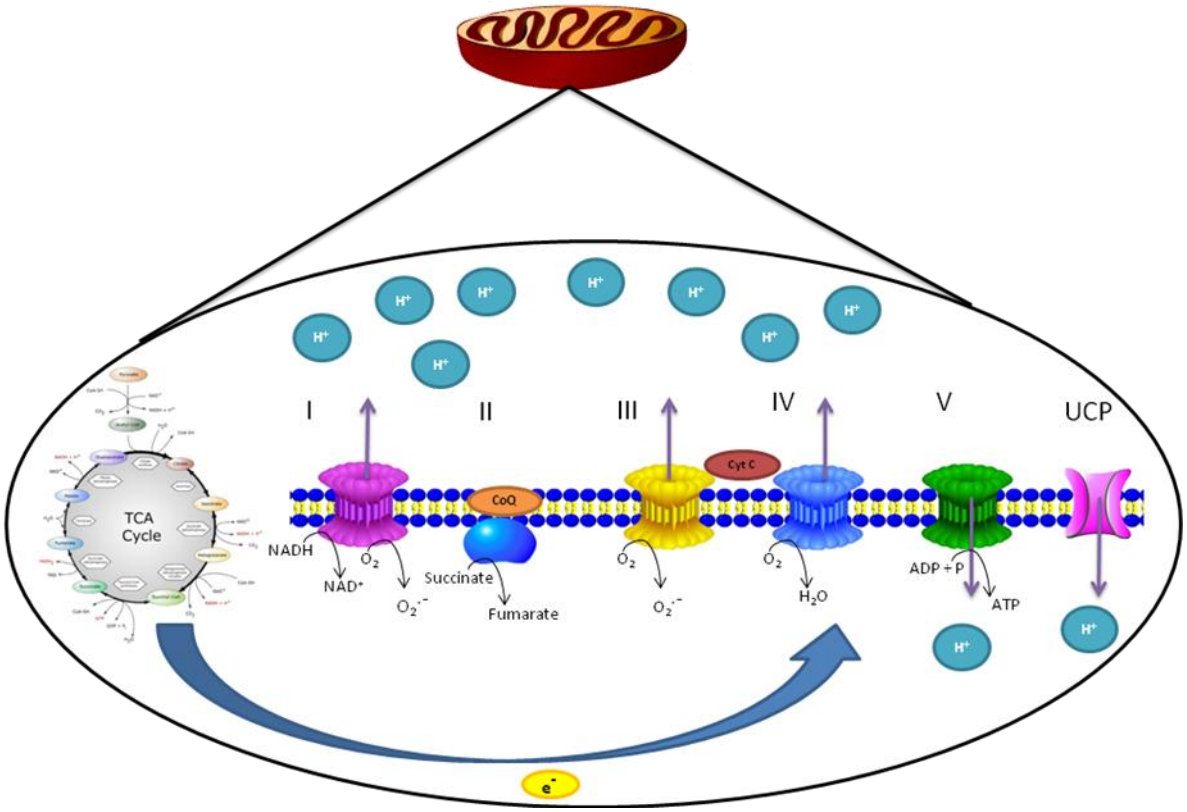


Fig. 3 – Mitochondria are the main source of reactive oxygen species. In mitochondrial oxidative phosphorylation system, the electrons donors reduced nicotinamide adenine dinucleotide (NADH) and succinate, generated from oxidation of carbon substrates that enter the tricarboxylic acid (TCA) cycle, promote the electron flow through the mitochondrial complexes of the respiratory chain (complexes I-IV) to the final acceptor, molecular oxygen (O_2). The electron flow is associated to the formation of ROS and these species, above a certain threshold, may cause mitochondrial damage, impairing organelles' function. Please see text for more information. CoQ – Coenzyme Q; Cyt – cytochrome c; UCP – uncoupling proteins

In summary, oxidative damage associated to aging leads to several mitochondrial abnormalities, including a decline in mitochondrial membrane potential, an increase in ROS production and size and heterogeneity of mitochondria and a decrease in mitochondrial protein synthesis, mitochondrial transcripts and expression of genes involved in mitochondrial turnover. There is also ample evidence that mitochondrial abnormalities play a key role in age-related diseases such as neurodegenerative diseases, cancer and type 2 diabetes (Kakkar and Singh, 2007; Sastre *et al.*, 2003; Lee and Wei, 2001).

1.3 - Oxidative Stress and Biomolecules

Free radicals are highly reactive chemical species with an unpaired electron. They are very unstable and can react with other molecules in order to capture an electron to gain stability. This reaction originates another free radical, starting a chain reaction of free radicals and leading to more and more damaging reactions. Free radicals are continuously produced in a cell and, besides the mitochondrial electron chain, other cellular sources of free radicals exist such as peroxisomes, cytochrome P-450, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Das Sarma *et al.*, 2010; Gemma *et al*., 2007).

Generally, the most harmful effects of free radicals on the cell are done by ROS, which are natural by-products formed in the cells of aerobic organisms and have important roles in cell signaling. It was estimated that 1–5% of the O_2 consumed by mitochondria is converted to ROS, therefore, it is established that the respiratory chain in mitochondria is the major producer of ROS, being responsible for inducing oxidative damage to mitochondria themselves and to other cellular compartments. Furthermore,

the decline in respiratory function during the aging process results in the increase of ROS production from mitochondria (Karthikeyan *et al.*, 2010; Das Sarma *et al.*, 2010; Lee and Wei, 2001).

In addition, there are pathways that produce ROS as their primary biological function, and apart from the destructive effects they are also responsible for some vital actions. Free radicals perform many critical functions in our bodies like destruction of bacteria, virus and other foreign agents, kill cancer cells, control blood flow and are involved in the turn on/off of important genes (Das Sarma *et al.*, 2010; Bokov *et al.*, 2004).

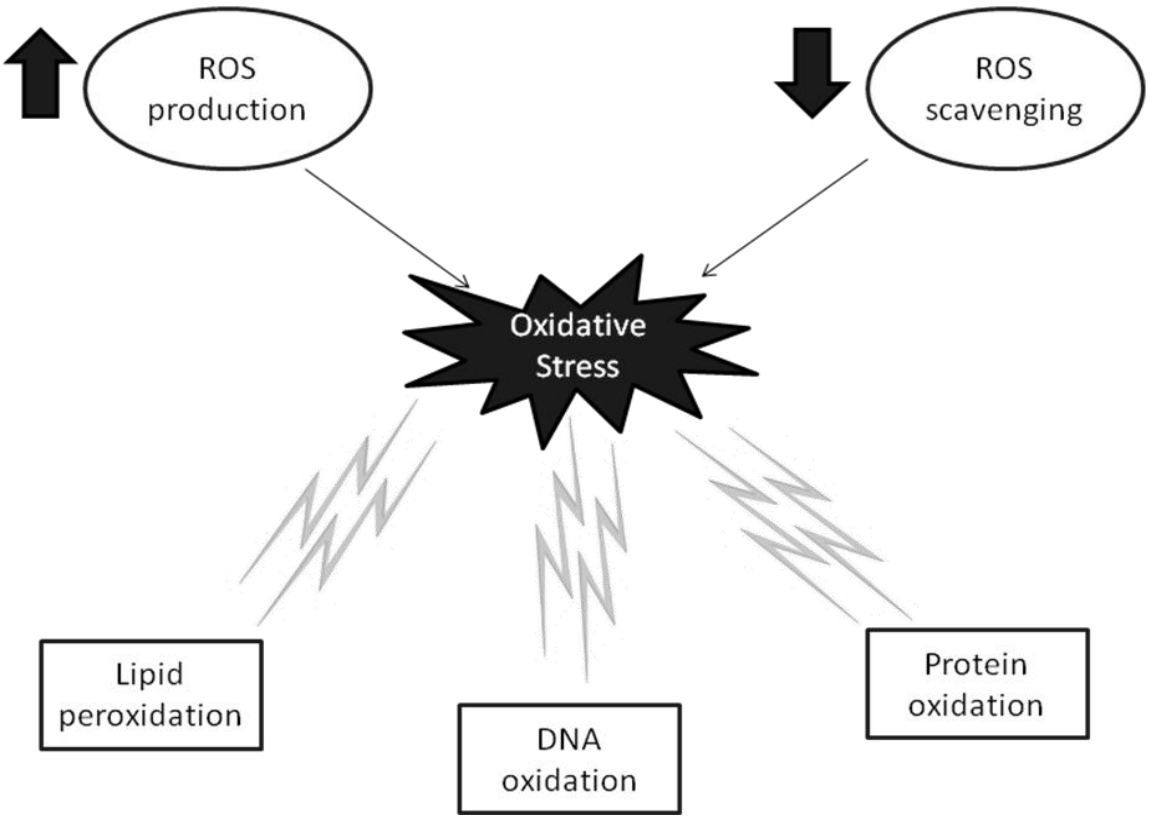


Fig. 4 - Oxidative damage of biomolecules. The extent of cellular damage and aging is related to a balance between the production of oxidants and their removal by the antioxidant defense system. The imbalance of the redox status leads to oxidative damage in lipids, proteins and DNA inducing cellular dysfunction**.**

An imbalance between the production of ROS and the capacity of antioxidant systems to detoxify those species underlies oxidative stress and damage (Lee and Wei, 2001). ROS may damage all types of biological molecules: lipids, proteins and DNA. At a molecular level, free radicals modify proteins by oxidation of amino acids, inactivate specific enzymes by oxidation of co-factors, damage DNA and the cellular transcriptional machinery and oxidize polyunsaturated fatty acids (PUFAs) (Das Sarma *et al.*, 2010; Allen, 1998).

Proteins are key targets for ROS attack due to their high overall abundance in biological systems. Susceptibility of proteins to oxidation depends on its composition, localization of amino acids and possibility of repair. Proteins can be damaged by direct free radical attack on amino acid side chains, glycation, glycoxidation or lipid oxidation products (Davies *et al.*, 1999; Dean *et al.*, 1997). As a consequence of ROS exposure, a series of chemical modifications and structure alterations occur in proteins, which can impair their biological activity. Indeed, oxidation of proteins by ROS can lead to oxidation of amino acids residues of side chains, cleavage of peptide bonds, accumulation of cross-linked proteins and formation of carbonyl groups. The formation of carbonyl groups is an indicator of the extent of proteins oxidative damage, a phenomenon that occurs during aging (Dalle-Donne *et al.*, 2003; Stadtman and Berlett, 1997; Dean *et al.*, 1997). The main consequences of amino acids oxidation are modifications of enzymatic and binding activities, increased susceptibility to proteolysis, protein solubility changes, formation of protein aggregates and altered immunogenicity. Altogether, these changes can cause metabolic dysfunction and cell death (Dalle-Donne *et al.*, 2003; Grune *et al.*, 2003).

Oxidative stress is also accompanied by changes in membrane fatty acid composition, including a decrease in the levels of PUFAs and an increase in

monounsaturated fatty acids (Gemma *et al*., 2007). Oxidative degradation of lipids is named lipid peroxidation, because most of the formed intermediates and products are lipid peroxides. This process is initiated when free radicals remove electrons from lipids generating peroxyl radicals, a highly reactive product that can combine with other PUFAs, propagating lipid peroxidation and, therefore, leading to an extensive damage to lipids. Lipid degradation may also contribute to an amplification of cellular damage, since some of the oxidized products generated, like aldehydes and alkanes, can induce the oxidation of other molecules (Kakkar and Singh, 2007; Lima and Abdalla, 2001). There are several ways through which lipid peroxidation can lead to lesions either in membranes or in extracellular lipids. Peroxidation of lipids in cell membranes can be very damaging by disrupting cells and organelles membranes fluidity and permeability, which compromises cell health and survival. Lipid peroxidation can also affect the function of membrane bound proteins such as receptors or enzymes and active transport mechanisms responsible for ionic and energetic homeostasis. Oxidation products like aldehydes are relatively stable and can diffuse within or even escape from the cell and attack targets far from the site of the original event, acting as "secondary cytotoxic messengers". In plasma, we can see the appearance of oxidized low density lipoproteins (LDL) that have cytotoxic activity and are involved in degenerative diseases as atherosclerosis (Das Sarma *et al.*, 2010; Dalle-Donne *et al.*, 2003; Beckman and Ames, 1998). The detection of lipid peroxidation is usually done by measuring the products formed during the oxidative stress-induced degradation of lipids, e.g. hydroperoxides, alkanes, aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), conjugated dienes and isoprostanes (Antunes *et al.*, 1996; Porter *et al.*, 1995).

Another target of ROS is nucleic acids. $^{\bullet}OH$ is the primary oxidant species responsible for DNA damage. Oxidative stress may cause a severe damage to DNA

through bases modification and DNA fragmentation followed by deregulation of p53, p21 and pRb, which will trigger cell cycle arrest in stressed cells (Chen et al., 2004; Remmen *et al.,* 2003; Henle and Linn 1997). These DNA modifications can be mutagenic, cytotoxic, carcinogenic or even lethal. Extensive damage can lead to death of the cell, by necrosis or apoptosis depending on the type of cellular damage (von Zglinicki *et al.,* 2001; Halliwell and Aruoma, 1991). The guanine-derived modification 8-oxo-2-deoxyguanosine (8-oxo-dG) is the major oxidative lesion that occurs in DNA bases, and their levels have been used as an indicator of oxidative stress. Much of this DNA damage can be repaired, but a decline of DNA repair mechanisms and an imbalance between ROS generation and clearance leads to an increase and accumulation of genetic damage (Moreira *et al.,* 2008; Evans *et al.,* 2004; Beckman and Ames, 1998).

Life in an O_2 environment inevitably involves the production of free radicals and other oxidants. The generation of ROS may be both valuable to cells, performing a function in cellular signaling, and harmful, damaging biomolecules. The accumulation of oxidative damage has been associated with aging, inflammation and numerous diseases such as cancer, Alzheimer's disease, arteriosclerosis and diabetes, among others (Das Sarma *et al.,* 2010; Evans *et al.,* 2004; Dalle-Donne *et al.,* 2003).

1.4 – Antioxidant defense system

O_2 is not necessary to life; there are lots of known organisms that are able to live in a complete anaerobic medium. However, for energetic reasons life began to use O_2 and had to manage the toxic risks, so all aerobic forms of life maintain elaborate anti-

free radical defense systems, also known as antioxidant defense systems (Rigoulet *et al.*, 2011; Das Sarma *et al.*, 2010).

Defense mechanisms against free radical-induced oxidative stress involve: preventive, repair and radical scavenging mechanisms, physical defenses (e.g. skin), and antioxidant defenses (Valko *et al*., 2007).

A broad network of non-enzymatic and enzymatic antioxidant defenses has evolved to protect cell components from oxidative stress and damage. Enzymatic antioxidant defenses include superoxide dismutase (SOD), thioredoxin, glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT), among others. Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, coenzyme Q10, lipoic acid, flavonoids and other antioxidants (Gemma *et al*., 2007). Under normal conditions, there is a balance, essential for the survival of organisms and their health, between the activities and the intracellular levels of these antioxidants, which can work synergistically against different types of free radicals (Gemma *et al*., 2007; Valko *et al*., 2007).

At an enzymatic level, SOD is one of the most important defense mechanisms responsible to maintain the steady-state levels of $O_2^{\bullet-}$ by capturing this free radical and metabolizing it to a much less reactive form, H_2O_2. Two isoforms of copper-zinc-containing SOD are found in mammals (Fridovich, 1995; Zelko *et al.*, 2002). One isoform is found in the intracellular space (CuZnSOD) and the other isoform is predominantly found in the extracellular matrix of most tissues (extracellular SOD) (Fattman *et al.*, 2003). MnSOD is the SOD isoform found in mitochondria (Mármol *et al.*, 2010; Aitken *et al.*, 2008). The H_2O_2 resulting from SOD activity has to be rapidly eliminated from the cell in order to prevent the induction of oxidative damage, this "elimination" being performed by CAT or GPx. CAT reacts with H_2O_2 to form H_2O and

O_2 in a reaction dependent on iron as a cofactor (Matés and Sánchez-Jiménez, 1999; Powers and Lennon, 1999). CAT is one efficient enzyme because is ubiquitous and is not saturated by H_2O_2, playing a significant role especially under conditions where H_2O_2 reaches high intracellular concentrations (Matés and Sánchez-Jiménez, 1999; Spolarics and Wu, 1997). GPx is a very important selenium-containing peroxidase that catalyzes the reduction of a variety of hydroperoxides (ROOH and H_2O_2) using GSH as a source of electrons (Matés and Sánchez-Jiménez, 1999; Powers and Lennon, 1999). Mammalian GPx has a much greater affinity for H_2O_2 compared with CAT (Powers and Lennon, 1999). In mammals, there are at least five GPx isoenzymes located in both the cytosol and mitochondria (Orrenius *et al*., 2007; Matés and Sánchez-Jiménez, 1999). The enzyme GR is also an important enzyme. Since GSH is oxidized by GPx forming glutathione disulfide (GSSG), cells require a regenerating process of GSH, which is accomplished by GR that converts GSSG back to GSH. GR has a cellular distribution similar to GPx and, although not considered a primary antioxidant enzyme, GR is essential for the normal antioxidant function of GPx (Orrenius *et al*., 2007; Matés and Sánchez-Jiménez, 1999; Powers and Lennon, 1999).

Because the extracellular medium is not rich in enzymatic antioxidants defenses, the non-enzymatic antioxidants are extremely important. They include extracellular proteins and small molecules. GSH is a ubiquitous tripeptide and is considered the major intracellular non-enzymatic antioxidant. GSH has an important role in detoxification processes and is also an immune booster (Marí *et al*., 2009). Synthesis of GSH occurs primarily in the liver and is highly abundant in the nuclei, cytosol, ER and mitochondria. GSH is a co-factor of several enzymes, like GPx and glutathione transferase, is involved in the detoxification of a variety of radicals (H_2O_2, $^{\bullet}OH$, $ONOO^-$); participates in amino acid transport through the plasma membrane and

regenerates vitamins C and E back to their active forms by reduction of semidehydroascorbate radical to ascorbate or α-tocopheroxyl radical to α-tocopherol, respectively (Valko *et al*., 2007; Powers and Lennon, 1999). The GSH/GSSG couple maintains the redox balance in the cell by interacting with most of the physiologically relevant redox couples, undergoing reversible oxidation or reduction reactions. High concentrations of GSSG may oxidatively damage many enzymes. For all these reasons, it has been demonstrated that low GSH levels lead to premature aging, disease and death (Marí *et al*., 2009; Valko *et al*., 2007).

Naturally occurring vitamin E exists in 8 different chemical forms, α-tocopherol being the most potent antioxidant (Aitken *et al.*, 2008; Burton and Ingold, 1981). Due to its high liposolubility this vitamin can be found in lipoproteins of plasma and in all cell membranes (Powers and Lennon, 1999; Burton and Ingold, 1989). Vitamin E is abundant in the inner mitochondrial membrane and is the main defense of the membranes against oxidative stress. Since the human body is unable to synthesize the fat-soluble vitamin E, it must be consumed through dietary sources. Vitamin E is an important antioxidant because can convert $O_2^{\bullet-}$, $^{\bullet}OH$ and lipid peroxyl radicals to less-reactive forms and can also break lipid peroxidation chain reactions, which occur during ROS-mediated damage to cell membranes (Das Sarma *et al.*, 2010; Powers and Lennon, 1999; Burton and Ingold, 1989). The interaction of vitamin E with ROS results in the formation of a vitamin E radical and in the reduction of functional vitamin E, which can be regenerated by other antioxidants like ascorbate and GSH. Thus, it is postulated that the function of vitamin E, during extended periods of oxidative stress, is dependent on other antioxidants that are capable of recycling this vitamin (Valko *et al*., 2007; Powers and Lennon, 1999; Janero, 1991).

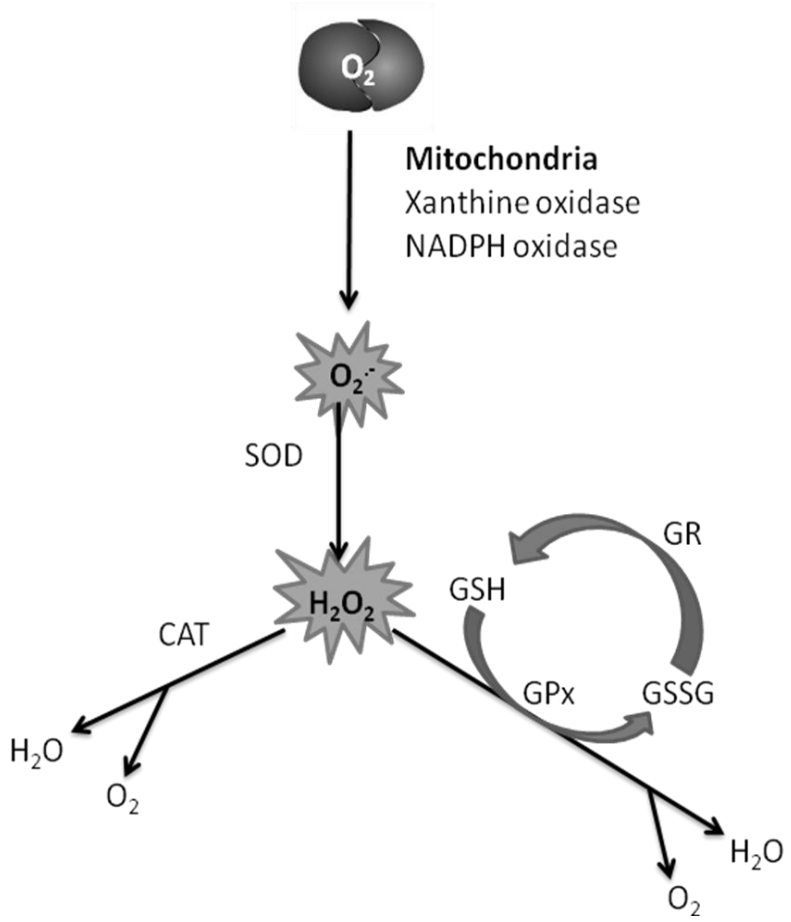


Fig. 5 - Sources of ROS and the role of antioxidants. The superoxide anion radical ($O_2^{\bullet -}$) is generated by several intracellular sources such as xanthine oxidase, or nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mitochondria. Superoxide dismutase (SOD) dismutates $O_2^{\bullet -}$ to hydrogen peroxide (H_2O_2), which can be converted to H_2O by catalase (CAT) or glutathione peroxidase (GPx). GPx requires glutathione (GSH) as electron donor converting it in glutathione disulfide (GSSG). GSSG can be reduced back to GSH by glutathione reductase (GR).

Several aging models have been used for studies on the effects of different factors modulating aging and lifespan. It has been hypothesized that lifespan can be enhanced by increasing antioxidant defenses, but very conflicting results were obtained (Gemma *et al.*, 2007). Experiments with *Drosophila melanogaster* have shown that

overexpression of MnSOD or the simultaneous overexpression of CuZnSOD and CAT increased lifespan (Sun *et al.*, 2002; Orr and Sohal, 1994). Other studies showed that in *Drosophila melanogaster* and *Caenorhabditis elegans* with the *age-1* mutation (a mutation associated with increased lifespan), present an increased activity of CuZnSOD and CAT (Hari *et al.*, 1998; Dudas and Arking 1995; Larsen, 1993). However, studies in mammals in which the levels of antioxidants are experimentally increased have shown that maximum longevity is not affected by antioxidants (Gemma *et al.*, 2007). Because this is a very controversial issue, more studies should be done to clarify the role of antioxidants in aging and lifespan extension.

1.5 - Chronic Hypoxia

As previously discussed, the maintenance of O_2 homeostasis is essential for cell survival, and for that purpose higher eukaryotes have adopted specialized mechanisms to enhance O_2 uptake and distribution (Lee *et al.*, 2004; Bruick, 2003).

Hypoxia occurs in tissues when the availability of O_2 is insufficient for cellular demand. Hypoxia can be categorized into two types: acute or transient and severe and prolonged or chronic. These hypoxic situations can result from numerous physiologic conditions (e.g. embryonic development and aging) and disorders (e.g. stroke, ischemia, vascular diseases and solid-tumor formation) (Carvalho *et al.*, 2010; Marí *et al.*, 2009; Patiar and Harris, 2006).

Mammalian cells have developed a range of adaptations to survive to a low-O_2 environment, and the adaptative response pathway is centered on the regulated expression of the transcription factor hypoxia-inducible transcription factor-1 (HIF-1) (Lee *et al.*, 2004; Bruick, 2003). HIF-1 is an ubiquitous intracellular protein, whose

levels increase in hypoxic cells and function as a master regulator of O_2 homeostasis. This heterodimeric protein is composed of two subunits, a constitutively expressed HIF-1β subunit and an inducible oxygen-sensitive HIF-1α subunit. Under normoxia, HIF-1α is hydroxylated by prolyl hydroxylase enzymes (PHDs) leading to a modification in the O_2 dependent degradation domain (ODD) within the HIF-1α protein and enables the binding to the von Hippel–Lindau tumor suppressor protein (VHL) leading to a rapid degradation of HIF-1α subunits by the ubiquitin-proteasome system (Correia and Moreira, 2010). On the other hand, under hypoxic conditions the PHDs, which are O_2 dependent, become inhibited and HIF-1α subunits are subsequently stabilized, accumulated in the cytosol and translocated to the nucleus. In the nucleus, HIF-1α binds to HIF-1β to form the active transcription factor HIF-1. The HIF-1 complex can bind to hypoxia response element (HRE) sequences in the promoter region of HIF-1 target genes to initiate gene expression (Correia and Moreira, 2010; Ratan *et al.*, 2007). Several genes regulated by HIF-1 are involved in important biological processes, such as angiogenesis, cell proliferation and survival, glucose metabolism, pH regulation and apoptosis (Sparkenbaugh *et al.*, 2011; Carroll and Ashcroft, 2005; Lee *et al.*, 2004). The severity of hypoxia determines whether cells live or die; chronic hypoxia may initiate apoptosis, whereas cells often adapt to acute and mild hypoxia and survive. If adequate compensation for hypoxia occurs pro-survival proteins are expressed and activated: 1) glucose transporter (GLUT) 1 and lactate dehydrogenase A (LDH-A), which promote cellular adaptation to reduced O_2 availability through an increase in glucose uptake and glycolysis; 2) erythropoietin (EPO) that increase O_2 transport to hypoxic tissues by promoting red blood cell maturation. By one hand, EPO production stimulates erythropoiesis activating proliferation, survival, and differentiation of the erythroid progenitor cells. On the other hand, increased expression of EPO enhance iron transport

to erythroid tissues; 3) vascular endothelial growth factor (VEGF) and endothelin-1 that are involved in angiogenesis and vasomotor control; 4) IGF-2 and IGF-binding proteins, which promote cell proliferation and survival. Failure of cells to adapt to low O_2 conditions will eventually lead to the activation of pro-apoptotic proteins such as nineteen-kilodalton interacting protein-37 (BNIP3), NIX (a pro-apoptotic BH3-only protein that belongs to the BNIP3 family) and p53–up- regulated modifier of apoptosis (PUMA) (Correia *et al.,* 2010; Agarwal and Prachal, 2008; Ratan *et al.,* 2007; Greijer and van der Wall, 2004; Lee *et al.,* 2004; Bruick, 2003).

Hypoxia is also associated with the generation and release of mitochondrial ROS that seems to be critical players involved in HIF-1α protein stabilization and activation (Correia *et al.,* 2010). It is important to note that ROS activate the redox signaling cascade and HIF-1α is only one of many target genes activated by these reactive species. Mitochondrial ROS generation was shown to be able to prevent the hydroxylation of HIF-1α, thereby stabilizing HIF-1α and allowing its translocation to the nucleus and dimerization with HIF-1β, initiating the transcription of target genes. Recent evidence demonstrated that blocking $O_2^{\bullet -}$ release by mitochondrial complex III to the intermembrane space impairs HIF-1α induction by hypoxia (Jusman *et al.,* 2010; Marí *et al*., 2009). Moreover, exogenous application of H_2O_2 can induce HIF-1α under normoxic conditions and ROS scavengers can block hypoxic induction of HIF-1. Together these findings illustrate the involvement of mitochondrial ROS in HIF-1α stabilization (Correia *et al.,* 2010; Marí *et al*., 2009).

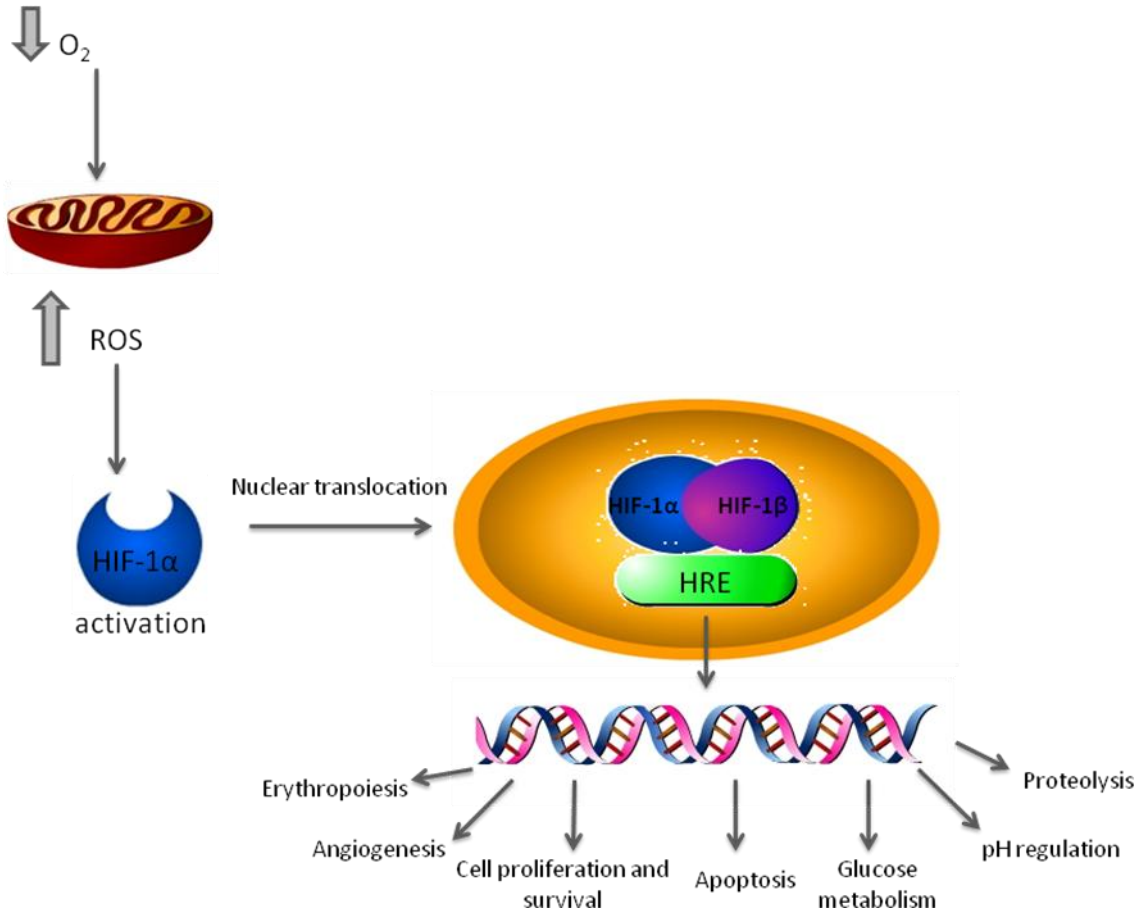


Fig. 6 - Hypoxic response pathway regulated by hypoxia inducible factor-1α (HIF-1α). Under hypoxic conditions [low levels of oxygen (O_2)], there is a burst of mitochondrial reactive oxygen species (ROS) production that inhibit prolyl hydroxylase enzymes (PHDs) activity, thus preventing HIF-1α proteasomal degradation. HIF-1α accumulates in the cytosol translocates to the nucleus and interacts with the HIF-1β subunit. The HIF-1 complex binds to hypoxia response element (HRE) sequences in the promoter region of HIF-1 target genes to initiate gene expression.

Hypoxia in solid tumors is associated with resistance to radiation therapy and chemotherapy, a phenomenon observed in many types of cancers (colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate and renal carcinomas). This resistance seems to be associated with an overexpression of HIF-1α. Indeed, several studies have reported that HIF-1α plays a key role in the adaptation of tumor cells to hypoxia, and high levels

of HIF-1α expression correlate with poor patient prognosis and increased tumor growth (Patiar and Harris, 2006; Lee *et al.*, 2004).

1.6 – Apoptosis

Cell death is an evolutionarily conserved and genetically regulated process that is important for the maintenance of homeostasis in tissues and all mammalian cells contain an intrinsic program necessary to induce cell death. Apoptosis, necrosis and autophagy are the three major and best characterized types of cell death (Kakkar and Singh, 2007; Orrenius *et al.*, 2007).

Apoptosis, also known as programmed cell death, describes a particular mode of cell death, dependent of energy (ATP), designed for the elimination of aged, damaged or cells that are no longer needed or that can be detrimental to the tissue (Kakkar and Singh, 2007; Greijer and van der Wall, 2004). Apoptosis is characterized by a series of biochemical and morphologic changes that include: chromatin condensation, membrane blebbing, phosphatidylserine exposure on the cell surface, cytoplasmic shrinkage, formation of apoptotic bodies and DNA fragmentation. Apoptotic cells are generally eliminated by phagocytes, preventing the development of an inflammatory response and tissue damage, which is often associated with necrotic cell death (Marí *et al.*, 2009; Greijer and van der Wall, 2004; Khosravi-Far and Espoti, 2004).

Apoptosis is primarily regulated by a cascade of proteins called caspases-cysteine-aspartic proteases that are essential executors of this cell death pathway. These proteins are synthesized as proenzymes in all cells and require activation. After proteolytic maturation or interaction with an allosteric activator, caspases become active and initiate a cascade of events that lead to apoptosis. There are two types of apoptotic

caspases, based on the size of the prodomain: initiator and effector caspases. Long prodomain caspases (caspase-2, -8, -9 and -10) belong to the group of initiator caspases that cleave inactive pro-forms of effector caspases, thereby activating them. Short prodomain caspases (caspase-3, -6 and -7) belong to the group of effector enzymes, which in turn, cleave other protein substrates within the cell, to trigger the apoptotic process (Orrenius *et al*., 2007; Jung and Kim, 2004; Chen and Wang, 2002). Activation of initiator caspases is mediated by various stimuli, like the tumor suppressor protein p53 that is a sensor of cellular stress and is a critical activator of the apoptotic pathway. There are two main routes that lead to apoptosis, involving either the mitochondria (the intrinsic pathway) or the activation of death receptors (the extrinsic pathway) (Marí *et al*., 2009; Orrenius *et al*., 2007; Haupt *et al*., 2003).

Briefly, the extrinsic pathway begins in the extracellular environment, when conditions are not favorable for cell survival. This pathway involves the activation of death receptors, which are cell surface receptors that activate caspases, transmitting apoptotic signals initiated by specific ligands. The best-characterized death receptors are Fas and tumor necrosis factor receptor-1 (TNFR1). Ligand binding, such as FasL, to the receptors is followed by recruitment of Fas associated death domain protein (FADD), which in turn interacts with procaspase-8, and the complex formed by Fas, FADD and procaspase-8, known as death-inducing signaling complex (DISC), is able to activate procaspase-8 (Jung and Kim, 2004; Khosravi-Far and Espoti, 2004; Pirnia *et al*., 2002). Caspase-8 can directly activate procaspase-3, which is responsible for the cleavage of target proteins, leading to apoptosis. However, in most cell types, caspase-8 first cleaves Bid, a Bcl2 family member protein, that induces the translocation, oligomerization and insertion of other family members, Bax and/or Bak, into the outer mitochondrial membrane (OMM). This is followed by permeabilization of the OMM, induction of the

mitochondrial permeability transition pore (PTP) and cytochrome c release, which binds to apoptotic protease activating factor-1 (APAF1) together with deoxyadenosine triphosphate (dATP) and procaspase-9, forming a cytosolic apoptosome complex that results in the activation of caspase-9. Caspase-9 in turn, cleaves procaspase-3 and activates caspase-3 (Marí *et al*., 2009; Khosravi-Far and Espoti, 2004; Pirnia *et al*., 2002).

The intrinsic apoptotic pathway is activated by intrinsic stressors, such as oncogenes, direct DNA damage, hypoxia, and survival factors deprivation (Orrenius *et al*., 2007; Haupt *et al.,* 2003). In the intrinsic pathway, death signals act directly or indirectly on the mitochondria, resulting in the release of several proteins such as cytochrome c and formation of the apoptosome complex. This cell death pathway is controlled by Bcl-2 family proteins (regulation of cytochrome *c* release), inhibitor of apoptosis proteins (IAPs) (inhibition of caspases), second mitochondrial activator of caspases (Smac) and HtrA2/Omi (negative regulator of IAPs) (Orrenius *et al*., 2007; Khosravi-Far and Espoti, 2004; Haupt *et al.,* 2003).

Thus, mitochondria are involved in both the extrinsic and intrinsic apoptotic pathways. The release of cytochrome c from the mitochondrial intermembrane space is decisive in this process and indicate that the two apoptotic pathways are not isolated systems but, instead, are interlinked (Marí *et al*., 2009; Kakkar and Singh, 2007; Khosravi-Far and Espoti, 2004).

1.7 – The liver: a brief overview of age-related changes

The liver is a vital organ present in vertebrates and some other animals. This organ plays a pivotal role in the metabolism of nutrients, detoxification, glycogen

storage, decomposition of red blood cells, protein synthesis, immunity, and hormone production. Thus, the liver has a major impact on health and body homeostasis. This is a highly vascular organ with a dual blood supply and a high O_2 demand. For these reasons, the liver is especially susceptible to vascular alterations and highly susceptible to oxidative stress. In healthy young livers, hepatocytes produce small amounts of ROS under basal conditions, and Kupffer cells, the resident macrophages in the liver, can release ROS in response to bacterial stimuli (Lebel *et al.*, 2011; Mármol *et al*., 2010). However, hepatocytes suffer age-related functional and morphological changes. It was shown that a significant decrease in the liver blood flow occur (at the age of 60 blood supply is reduced to 50% compared to age of 20) consequently, hepatic drug clearance, detoxification and liver's metabolic ability also decrease (Lebel *et al.*, 2011; Sabaretnam *et al.*, 2009), which reduce the regenerative capacity of this organ. Additionally, the number of liver cells reduces sharply, liver volume and weight decreases, and there is some evidence suggesting an increased variation in nuclear size associated with an increasing incidence of polyploidy of hepatocytes (Hoare *et al.*, 2010). The phagocytic function of Kupffer cells presents also an age-dependent decline, increasing the susceptibility of liver to infections in old age. The aging liver also presents cytoplasmic accumulation of highly oxidized insoluble proteins, a reduction in the expression of hepatic antioxidant enzymes and a decline in autophagy (Hoare *et al.*, 2010). Aging of the liver is also accompanied by alterations in gene expression that results in an increased susceptibility to inflammation, cellular stress, fibrosis and apoptosis (Lebel *et al.,* 2011). Several other alterations occur in the aged liver such as the suppression of genes involved in IGF-1/growth hormone pathways, carbohydrate metabolism, xenobiotic metabolism, peroxisomal biogenesis, cell-cycle control and DNA replication (Lebel *et al.,* 2011). Mitochondrial abnormalities are also key events in

liver aging, which are associated with a decrease in energy production and an increase in ROS production (Lebel *et al.*, 2011; Hoare *et al.*, 2010; Sabaretnam *et al.*, 2009).

Another factor associated with aging, constituting a major risk factor for the development of vascular diseases in the elderly, is vascular endothelial dysfunction. Endothelial dysfunction is defined by a decrease in the ability of the endothelium to dilate in response to chemical and physical stimuli, this dysfunction potentiating the occurrence of situations of hypoxia. Hypoxia, in turn, will stimulate the production of ROS and activation of factors (e.g. HIF-1) that modulate cells viability. The reduction in endothelial dilation is due to an imbalance of vasodilators and vasoconstrictors produced by the endothelium, and this imbalance is largely characterized by a progressive decrease in the bioavailability of nitric oxide ($NO^{\bullet}$) and an increased production of vasoconstrictor cyclooxygenase derivatives. These alterations are related with an increased production of ROS (Herrera *et al*., 2009; Newaz *et al*., 2006).

Due to these age-related changes, aging is associated with an increased susceptibility to develop chronic liver disorders. Recent studies have demonstrated that age is an independent risk factor for poor outcome in a variety of liver diseases such as hepatitis C virus, hepatitis B virus, primary biliary cirrhosis and autoimmune hepatitis (Hoare *et al.*, 2010).

1.8 – Objectives

As previously discussed, both aging and chronic hypoxia are closely associated with mitochondrial dysfunction and oxidative stress. In this line, we aimed to clarify the impact of age and chronic hypoxia on liver oxidative status, mitochondrial complexes activity and levels of some pro-survival/pro-death proteins. For this purpose we used 3- and 12-month-old male Wistar rats exposed to normoxia (21% O_2) or hypoxia (10% O_2, 90% N_2). Several parameters were evaluated: H_2O_2 and thiobarbituric acid reactive substances (TBARS) levels, aconitase activity, enzymatic (CAT, GPx and GR activities) and non-enzymatic (glutathione and vitamin E levels) antioxidant defenses and the activities of NADH-cytochrome c reductase (complexes I-III), succinate-cytochrome c reductase (complexes II-III), cytochrome c oxidase (complex IV) and ATPase. The protein levels of Bax and Bcl2 were also determined.

CHAPTER 2. MATERIALS AND METHODS

2.1 – Materials

Rabbit anti-Bax antibody was obtained from Cell Signalling Technology (Beverly, MA, USA), mouse anti-Bcl2 antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and mouse anti-Actin antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals were of the highest grade of purity commercially available.

2.2 – Animals

Two groups of male Wistar rats, 3-month-old (young) and 12- month-old (mature), were divided into two subgroups, control and chronic hypoxia. Animals were housed in our animal facility (Laboratory Research Center, University Hospital, Coimbra, Portugal), in a temperature and humidity controlled room on a 12 h light-dark schedule, with free access to food (powdered rodent diet: URF1; Charles River) and water. Animals were handled daily and the hypoxic group was maintained for 1 week in an O_2 controlled normobaric hypoxic chamber (Proox Model 110, Biospherix, Redfield, New York) containing mix gas of 10% O_2: 90% N_2, where the introduction of N_2 gas leads to a decrease in O_2 levels. At the end of hypoxic or normoxic period, the animals were weighed and sacrificed by cervical displacement and decapitation, adhering to procedures approved by the Federation of Laboratory Animal Science Associations (FELASA). The organs were removed and stored at -80ºC until use.

2.3 - Blood Analyses

Blood glucose levels were determined by a glucose oxidase reaction, using a commercial glucometer (Glucometer-Elite Bayer, Portugal) and compatible reactive tests (Ascencia Elite Bayer, Portugal), immediately before animals sacrifice.

After animal decapitation, total blood was collected in 1 mL tubes containing EDTA (Aquisel®) and analyzed in a Beckman/Coulter MAXM hematology analyzer, for determination of red blood cells (RBC), hemoglobin (HGB) and hematocrit (HCT). For analysis of plasma parameters, total blood was collected and centrifuged at 2500 rpm x 5 minutes (Eppendorf Centrifuge 5415C), at 4ºC. Plasma was collected and analyzed in a Beckman CX4 auto-analyzer (Beckman Synchron CX series). Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using the System SYNCHRON CX® that monitors the alterations in absorbance at 340 nm. The change in absorbance is directly proportional to ALT or AST activity in the sample and is used by the System to calculate and express the enzymes activity.

2.4 – Liver tissue homogenization and protein quantification

Defrosted liver tissue was homogenized in phosphate buffer (PBS) (150 mM NaCl; 80 mM Na_2HPO_4; 20 mM NaH_2PO_4; pH 7.4). The protein concentration was determined using the Biuret Method (Gornall *et al.*, 1949).

2.5 - Measurement of aconitase activity

Aconitase activity was determined by following the method of Krebs and Holzach (1952). Briefly, samples were diluted to 0.2 mg in 600 µL of incubation buffer (50 mM Tris-HCl; 100 mM $MnCl_2$; pH 7.4), sonicated for 5 seconds, centrifuged at 13200 rpm x 3 min (Eppendorf Centrifuge 5415C) and the supernatants were collected and stored at -80ºC until use. The aconitase activity was measured in a Jasco V560 UV/VIS Spectrophotometer by monitoring the cis-aconitase absorbance after addition of 0.5 mM isocitrate at 240 nm (37ºC). The activity of aconitase was calculated using a molar coefficient of 3.6 mM^{-1} cm^{-1} and expressed as U/mg protein/minute. One unit was defined as the amount of enzyme necessary to produce 1 µM cis-aconitate per minute.

2.6 - Determination of hydrogen peroxide production

The rate of H_2O_2 production was determined using a modification of the method described by Barja (1999). Briefly, liver homogenates were incubated, at 37ºC, with 0.5 mL of buffer solution (0.1 mM EGTA; 5 mM KH_2PO_4; 3 mM $MgCl_2$; 145 mM KCl; 30 mM Hepes; 0.1 mM homovalinic acid (HVA); 6 U/mL horseradish peroxidase; pH 7.4) during 15 min. Then, the reaction was stopped with cold stop solution (0.1 M glycine; 25 mM EDTA-NaOH; pH 12) and centrifuged during 5 min at maximum speed (Eppendorf Centrifuge 5415C). The fluorescence of supernatants was measured using 312 nm as excitation wavelength and 420 nm as emission wavelength (Spectra MAX Gemini EM, Molecular Devices). The H_2O_2 levels were calculated using a standard curve of H_2O_2.

2.7 - Determination of thiobarbituric acid reactive substances levels

The TBARS levels were determined by following a modified method described by Ernster and Nordenbrand (1967). In brief, 0.5 mL of protein was added to 1 mL of reaction medium (175 mM KCl; 10 mM Tris-HCl; pH 7.4) and incubated at 37ºC during 15 min. The reaction was stopped with 0.5 mL trichloroacetic acid (TCA) 40% and cooling in an ice bath. Then, 2 mL of thiobarbituric acid (TBA) were added to the samples and boiled during 15 min. Samples were then centrifuged at 4000 rpm x 7 min (Sigma Centrifuge 3-16K) and the absorbance was read at 530 nm (Spectronic 21, Bausch & Lomb), against a reference blank prepared in the absence of protein. The amount of TBARS formed was calculated using a molar extinction coefficient of $1.56x10^5$ mol^{-1} cm^{-1} and expressed as nmol TBARS/mg protein.

2.8 - Measurement of glutathione peroxidase (GPx) activity

GPx activity was determined by following the change in the absorbance at 340 nm (30ºC) caused by the oxidation of NADPH, according to the method of Flohé and Gunzler (1984).

In brief, 0.2 mg of each sample was incubate with 200 µL of buffer solution (0.25 M KH_2PO_4; 0.25 M K_2HPO_4; 0.5 mM EDTA; pH 7.0), 200 µL of GSH and 2.4 U/mL glutathione reductase. After 5 min of incubation (time needed for the activation of the enzyme), 200 µL of tertbutylhydroperoxide were added to the sample and reference blank. Then, the reaction was initiated by adding 200 µL of NADPH to the sample. GPx activity was assessed in a Jasco V560 UV/VIS Spectrophotometer,

calculated using the molar extinction coefficient of 6220 M^{-1} cm^{-1} and expressed as nmol/min/mg protein.

$$2GSH + H_2O_2 \rightarrow \textbf{Glutathione Peroxidase} \rightarrow GSSG + 2H_2O$$

$$NADPH + H^+ + GSSG \rightarrow \textbf{Glutathione Reductase} \rightarrow 2GSH + NADP^+$$

2.9 - Measurement of glutathione reductase (GR) activity

The activity of GR was determined by following the change in the absorbance at 340 nm (30ºC) caused by oxidation of NADPH, according to the method of Carlberg and Mannervik (1984).

In brief, 0.2 mg of each sample was incubated 1 min with 1000 µL of buffer solution (0.2 M K_2HPO_4; 2 mM EDTA; pH 7.0) and 100 µL of NADPH. Then, 100 µL of GSSG was added to the sample, but not in the reference blank. The reaction was started by adding 100 µL of GSH to the sample. GR activity was assessed in a Jasco V560 UV/VIS Spectrophotometer, calculated using the molar extinction coefficient of 6220 M^{-1} cm^{-1} and expressed as nmol/min/mg protein.

$$NADPH + H^+ + GSSG \rightarrow \textbf{Glutathione Reductase} \rightarrow 2GSH + NADP^+$$

2.10 - Measurement of manganese superoxide dismutase activity

MnSOD activity was determined based on its ability to inhibit the reduction of nitro-blue tetrazolium (NBT) by $O_2^{\bullet-}$ resulting in an increase in absorbance at 550 nm, as described by Flohé and Otting (1984).

Briefly, 0.2 mg of each sample was incubated for 2 min with 1400 µL of buffer solution (50 mM K_2HPO_4; 100 µM EDTA; pH 7.8), 200 µL hypoxanthine (except in the reference blank), 0.025% Triton X-100, 100 µL NBT, 5 mM KCN and 0.025 U/mL xanthine oxidase, which starts the reaction. MnSOD activity was assessed spectrophotometrically at 550 nm (25ºC) in a Jasco V560 UV/VIS Spectrophotometer. The activity of MnSOD was calculated using a standard curve, prepared with different concentrations of SOD commercially available.

$$\text{Hypoxanthine} + O_2 \rightarrow \textbf{Xanthine Oxidase} \rightarrow \text{uric acid} + O_2^{\bullet-}$$

$$2O_2^{\bullet-} + 2H^+ \rightarrow \textbf{SOD} \rightarrow H_2O_2 + O_2$$

2.11 - Measurement of catalase activity

CAT activity was measured spectrophotometrically, at 240 nm (25ºC), by following the decomposition of H_2O_2, as described by Beers and Sizer (1952). One unit of CAT decomposes one micromole of H_2O_2 per minute. Briefly, 25 µg of each sample were incubated for 2 min with 0.1 M potassium phosphate buffer (KH_2PO_4; pH 7) to achieve temperature equilibration. The reaction was initiated by adding 0.059 M H_2O_2 and the decrease in the absorbance was followed in a Jasco V560 UV/VIS

Spectrophotometer. CAT activity was calculated using the molar extinction coefficient of 43.6 M^{-1} cm^{-1} and expressed as U/min/mg protein.

$$2\,H_2O_2 \rightarrow \textbf{Catalase} \rightarrow 2\,H_2O + O_2$$

2.12 - Determination of glutathione and glutathione disulfide levels

GSH and GSSG levels were determined with fluorescence detection, according to Hissin and Hif (1976). In brief, 1 mg of liver homogenate was added to 1.5 mL of buffer solution (100 mM NaH_2PO_4; 5 mM EDTA; pH 8.0) and 500 μL of H_3PO_4 2.5%. Samples were sonicated twice and centrifuged at 50000 rpm x 30 min (Beckman, TL-100 Ultracentrifuge) and the supernatants frozen at -80ºC until use. For GSH determination, 100 μL of supernatant were added to 1.8 mL of buffer solution (100 mM NaH_2PO_4; 5 mM EDTA; pH 8.0) and 100 μL of ophthalaldehyde (OPT) followed by 15 min of incubation. The fluorescence was measured at 420 nm and 350 nm emission and excitation wavelengths, respectively (LS 55 Luminescence, Perkin Elmer). For GSSG determination, 250 μL of supernatant were added to 100 μL of N-ethylmaleymide (NEM) and incubated for 30 min. After the incubation period, 140 μL of sample were incubated during 15 min with 1.76 mL of NaOH (100 mM) and 100 μL of OPT. The fluorescence was measured at 420 nm and 350 nm emission and excitation wavelengths, respectively. The GSH and GSSG contents were determined from comparisons with a linear GSH and GSSG standard curve, respectively.

2.13 - Determination of Vitamin E levels

Vitamin E levels were determined according to the method described by Vatassery and Younoszai (1978). Briefly, 1 mg of liver homogenate was added to 1.5 mL sodium dodecyl sulfate **(**SDS) 10 mM and 2 mL of absolute ethanol and vortex-mixed during 1 minute. Then 2 mL of n-hexane were added to the samples and vortex-mixed during 3 minutes. The sample was centrifuged at 2000 rpm x 10 min (Sorvall RT6000 Refrigerated Centrifuge) and 1 mL of the upper phase, containing n-hexane (n-hexane layer), was recovered and evaporated to dryness under a stream of N_2 and kept at –80ºC. To determine the vitamin E levels, the extract was dissolved in n-hexane and the content was analyzed by reverse-phase HPLC. A Spherisorb S10w column (4.6 x 200 nm) was eluted with n-hexane modified with 0.9% methanol, at a flow rate of 1.5 mL/min, and detection was performed by an UV detector at 287 nm.

2.14 - Measurement of mitochondrial enzymatic activities

NADH-cytochrome c reductase (mitochondrial complexes I-III) and succinate-cytochrome c reductase (mitochondrial complexes II-III) were assayed spectrophotometrically, in a Jasco V560 UV/VIS Spectrophotometer, at 550 nm (30ºC), by following the methods of Hatefi and Rieske (1967) and King (1967), respectively. Briefly, for each assay, two identical cuvettes (sample and reference blank) were prepared with 100 mM phosphate buffer (KH_2PO_4; pH 7.4), EDTA, KCN, cytochrome c and 100 µg sample. Once a stable baseline was recorded, the reaction was initiated by the addition of 0.2 mM β-NADH (NADH-cytochrome c reductase) or 1 mM succinate (succinate-cytochrome c reductase) to the sample cuvette. The assays were left to run

for 5 min and then rotenone (complex I inhibitor) or antimycin (complex III inhibitor) was added and the assays run for more 5 min. NADH-cytochrome c reductase and succinate-cytochrome c reductase specific activities were calculated by subtracting the rate after inhibitor addition from the overall initial rate. The enzymatic complexes activities were calculated using the extinction coefficient of 19.2 mM^{-1} cm^{-1}.

Cytochrome c oxidase (COX) activity was assayed polarographically by monitoring O_2 consumption with a Clark-type oxygen electrode (YSI Model 5331, Yellow Springs Inst) connected to a suitable recorder in a 2 mL thermostated water-jacketed closed chamber under magnetic stirring at 30ºC (Estabrook, 1967). The electrode was calibrated as previously described (Rickwood et al., 1987). The reaction started when 5 mM ascorbate plus 0.25 mM N, N, N', N'-tetrametyl-p-phenylenodiamine (TMPD) were added to 1 mL of the reaction medium (130 mM sacarose; 50 mM KCl; 5 mM $MgCl_2$; 5 mM KH_2PO_4; 5 mM Hepes-Tris; pH 7.4) supplemented with 2 µM rotenone, 10 µM cytochrome and 200 µg protein, and finished with 10 µM KCN.

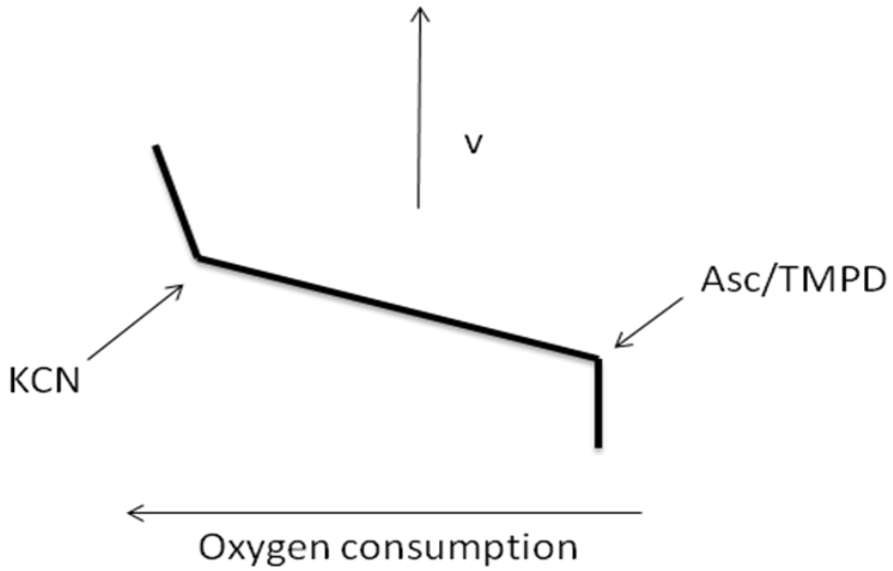


Fig. 7 – Representative trace of cytochrome c oxidase activity measurement. Scale: 1½; Velocity (v): 1cm/min. COX activity was calculated by the difference between the oxygen consumption before and after cyanide addition. KCN – potassium cyanide; Asc - ascorbate; TMPD - N, N, N', N'-tetrametyl-p-phenylenodiamine.

2.15 - Measurement of ATPase activity

ATPase activity was measured by the colorimetric determination of inorganic phosphate (Pi) hydrolyzed from ATP (ATP $\longrightarrow$ ADP+Pi) following the method of Taussky and Shorr (1953). The reaction was carried out at 37ºC in 1 mL of reaction medium (100 mM NaCl; 25 mM KCl; 5 mM $MgCl_2$; 50 mM Hepes; pH 7.4) containing 0.25 mg protein. The reaction was started by the addition of 100 mM ATP, allowed to proceed for 15 min and stopped by the addition of 500 µL of TCA 40% and cooling in an ice bath. Samples were then centrifuged at 3000 rpm x 5 min (Eppendorf Centrifuge 5415C). 1 mL of supernatant was added to 2 mL of molybdate and 2 mL of H_2O and the

reaction was left to run for 3 min at room temperature. Finally, samples absorbance was read at 660 nm (Spectronic 21, Bausch & Lomb). In the presence of ammonium heptamolybdate the Pi resulting from ATP hydrolysis forms a complex that after reduction presents a blue color. ATPase is specifically inhibited by oligomycin (200 ug/mL). ATPase activity was expressed in nmol Pi/mg protein/15 min and represents the difference between the activity in absence and presence of oligomycin. Since ATP hydrolysis releases 1 Pi ($ATP + H_2O \leftrightarrow ADP + Pi$) we can directly relate the amount of ATP that is hydrolyzed with the amount of Pi present in the solution.

2.16 - Measurement of citrate synthase activity

Citrate synthase activity was measured spectrophotometrically by following the method of Coore *et al.* (1971). This assay monitors the formation of DNTB-CoA-SH that results from the reaction of 5,5'-dithiobis-2-nitrobenzoic acid (DNTB) with the free thiol (SH) groups of coenzyme A, at 412 nm (30ºC). Briefly, 0.5 mg of sample was incubated with 200 mM Tris (pH 8), 10 mM acetyl-CoA, 10 mM DNTB, 10% Triton X-100 and the reaction was initiated by the addition of 10 mM oxaloacetate. A reference blank was prepared in the absence of oxaloacetate. The reaction was followed in a Jasco V560 UV/VIS Spectrophotometer and citrate synthase activity was determined using the molar extinction coefficient of 13.6 mM/cm and expressed as nmol/min/mg protein. The activity of this enzyme was used to normalize the previously described enzymatic activities.

acetyl-CoA + oxaloacetate + DNTB→ **Citrate synthase** → citrate + DNTB-CoA-SH

2.17 - Measurement of caspase-9 and caspase-3 activation

Caspase-9 and caspase-3 activation was measured using a modified colorimetric method (Cregan *et al.*, 1999). A small sample of liver tissue was homogenized in cold lysis buffer (25 mM HEPES; 2 mM $MgCl_2.6H_2O$; 1 mM EDTA; 1 mM EGTA) with 200 mM phenylmethanesulfonylfluoride (PMSF) and 200 mM DTT, a thiol-reducing agent. The homogenates were centrifuged for 10 min at 4 °C and maximum speed (Eppendorf Centrifuge 5415C). The resulting supernatants were stored at -80 °C until use. Protein concentrations were measured by using the Biuret Method. Samples (25 μg of protein for caspase-3 and 40 μg of protein for caspase-9) were incubated at 37 °C, for 2 h in CHAPS buffer (25 mM HEPES; 0.1% CHAPS; 10% sucrose; 10 mM DTT; pH 7.5) and caspase substrate. Caspase-like activity was determined by measuring substrate cleavage at 405 nm in a microplate reader (SpectraMax Plus 384, Molecular Devices).

2.18 - Western Blot analysis

Samples of liver tissues were homogenized in buffer solution containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% DOC and 0.1 % SDS (pH 7.4), protease inhibitors (commercial protease inhibitor cocktail from Roche), phosphatase inhibitors (commercial phosphatase inhibitor cocktail from Roche), 0.1 M PMSF (Sigma), 0.2 M DTT (Sigma). Homogenates were frozen and defrozen three times in liquid nitrogen to favor cells disruption and centrifuged 5 min at maximum speed (Eppendorf Centrifuge 5415C). The supernatant represents the cytosolic fraction and the resulting pellet the mitochondrial fraction. The BCA method was used for protein quantification. Samples

(100 μg/lane) were resolved by electrophoresis in 5-15% SDS–polyacrylamide gels and transferred to polyvinyl difluoride (PVDF) membranes. Non-specific binding was blocked by gently agitating the membranes in 5% of BSA and 0.1% Tween in Tris-buffered saline (TBS) for 1 h at room temperature. Membranes containing proteins were incubated overnight at 4ºC with gentle shaking with rabbit anti-Bax (1:1000) or mouse anti-Bcl2 (1:500). Actin (1:5000) was used as loading control. Blots were washed three times (15 min), with Tris buffer containing 0.1% non-fat milk and 0.1% Tween. Then, the membranes were incubated with anti-rabbit (1:15000) and anti-mouse (1:20000) IgG secondary antibodies, for 1h at room temperature with gentle shaking. Membranes were incubated with ECF fluorescent reagent and the immunoreactive bands were visualized by the VersaDoc Imaging System (Bio-Rad). The densities from each band were analyzed using the QuantityOne software (Bio-Rad) and the results were given as fluorescence intensity (INT)/mm^2.

2.19 - Statistic analysis

Results are presented as means ± SEM of the indicated number of experiments. Statistical significance was determined using the Kruskal-Wallis test for multiple comparisons, followed by the post hoc Mann-Whitney test. A p-value <0.05 was considered statistically significant.

CHAPTER 3. RESULTS

3.1 - Characterization of the experimental animals

Compared to their control counterparts, rats exposed to chronic hypoxia presented a significant decrease in body weight and blood glucose levels (Fig. 8).

Concerning plasma AST and ALT activities, markers of liver injury, chronic hypoxia induced a significant increase of AST and ALT activities in young rats. In 12-month-old animals, an increase in the activities of these enzymes was also observed, although not statistically significant (Fig. 9).

Total blood analyses revealed that both young and mature animals subjected to chronic hypoxia presented a significant increase in RBC (blood cells that transport O_2 to the cells), HGB (protein of RBC that transport O_2) and HCT (a measure of the % of RBC found in whole blood) (Fig. 10).

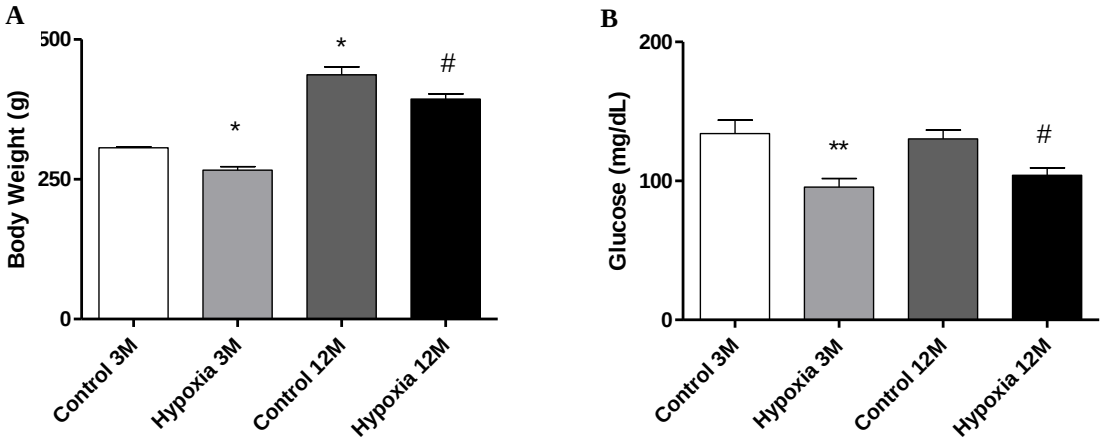


Fig. 8 – Effects of age and chronic hypoxia in body weight and blood glucose levels. Data shown represent means ± SEM from 3-6 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01 when compared with 3-month-old control animals. $^{\#}$p<0.05 when compared with 12-month-old control animals.

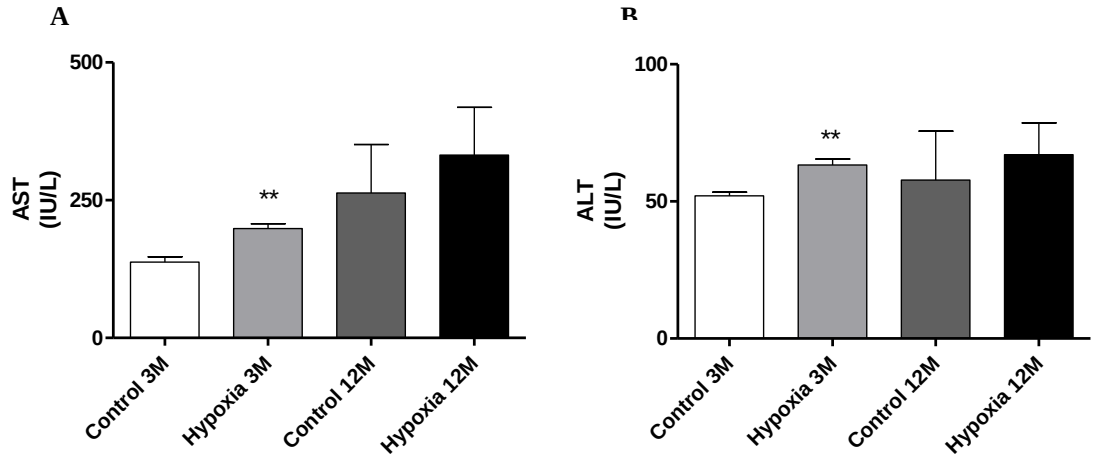
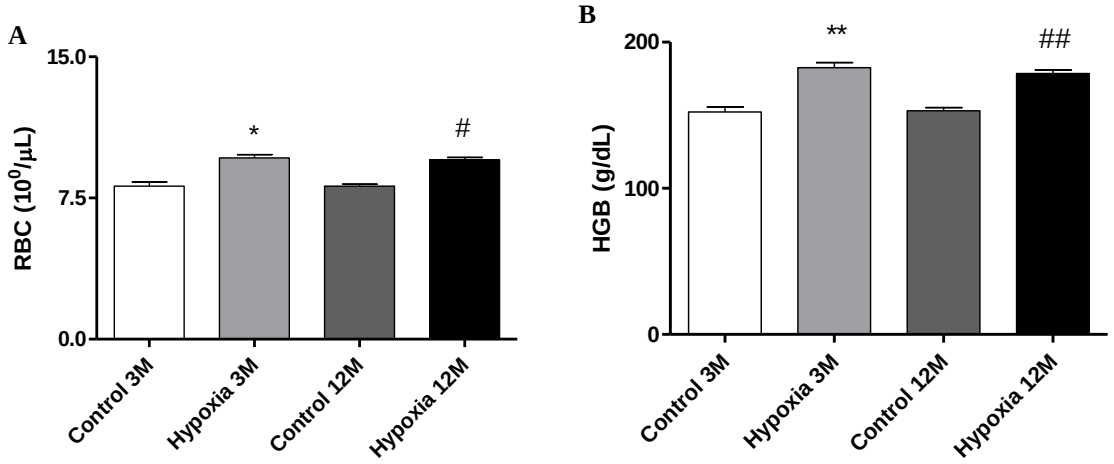


Fig. 9 – Effects of age and chronic hypoxia in plasma AST and ALT activities. Plasma aspartate aminotransferase (AST) (A) and alanine aminotransferase (ALT) (B). Data shown represent means ± SEM from 5-6 animals from each condition studied. Statistical significance: **p<0.01 when compared with 3-month-old control animals.



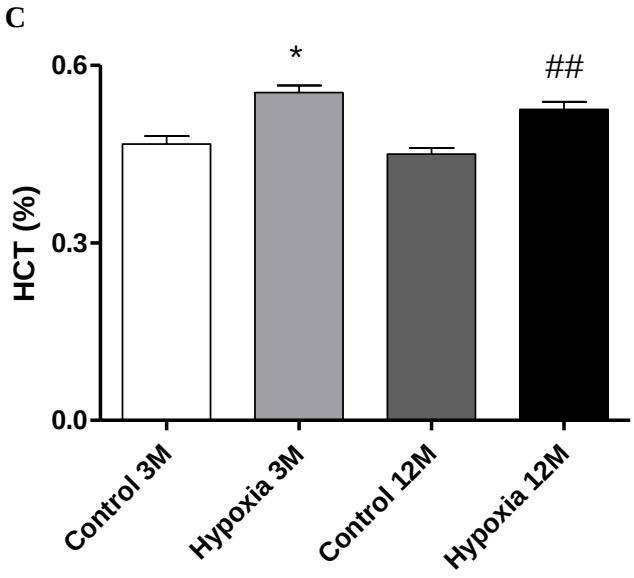


Fig. 10 – Effects of age and chronic hypoxia in blood cells. Red blood cells (RBC) (A) hemoglobin (HGB) (B) and hematocrit (HCT) (C). Data shown represent means ± SEM from 4-6 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01 when compared with 3-month-old control animals. $^{\#}$p<0.05; ##p<0.01 when compared with 12-month-old control animals.

3.2 - Age and/or chronic hypoxia increase oxidative stress levels

Aconitase activity is an important redox sensor, whose active center is highly sensitive to ROS attack. Aconitase activity presented a significant age-dependent decrease. Furthermore, a significant decrease was also observed in 3-months-old animals exposed to chronic hypoxia (Fig. 11).

H_2O_2 levels, another indicator of oxidative stress, presented a significant age-dependent increase. Animals exposed to chronic hypoxia also showed a higher H_2O_2 production when compared to control counterparts (Fig. 12).

No statistically significant alterations were observed in TBARS levels (Fig. 13).

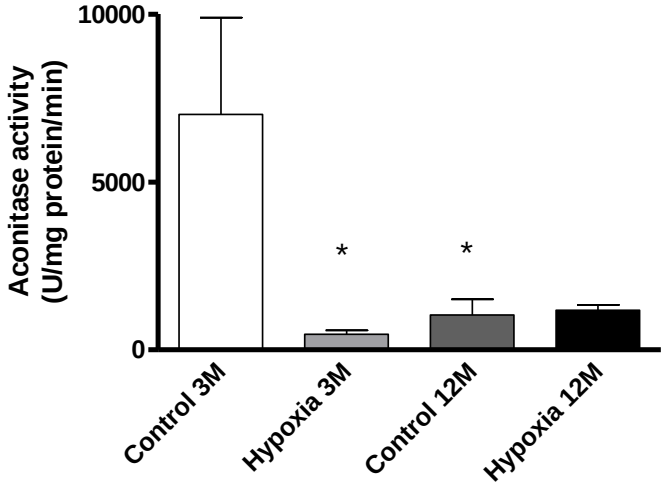


Fig. 11 - Effects of age and chronic hypoxia on aconitase activity. Aconitase activity was measured as described in the Material and methods section. Data shown represent means ± SEM from 4-6 independent experiments. Statistical significance: *p<0.05 when compared with 3-month-old control animals.

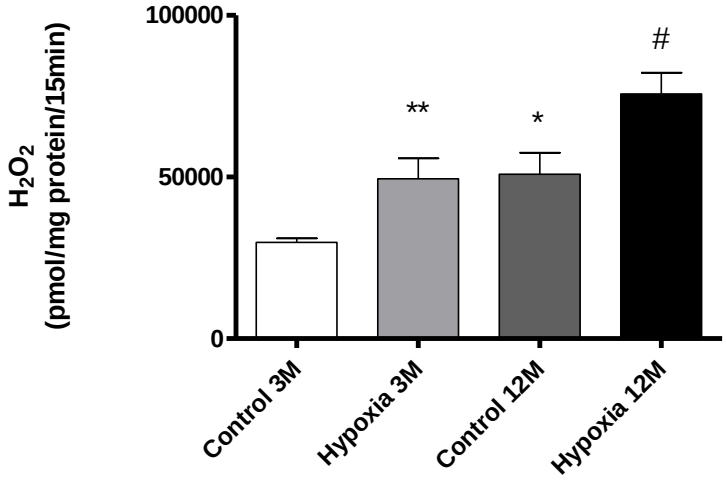


Fig. 12 - Effects of age and chronic hypoxia on hydrogen peroxide production. H_2O_2 production was measured as described in Material and methods section. Data shown represent means ± SEM of 4-6 independent experiments. Statistical significance: *p<0.05; **p<0.01 when compared with 3-month-old control animals. $^{\#}p<0.05$ when compared with 12-month- old control animals.

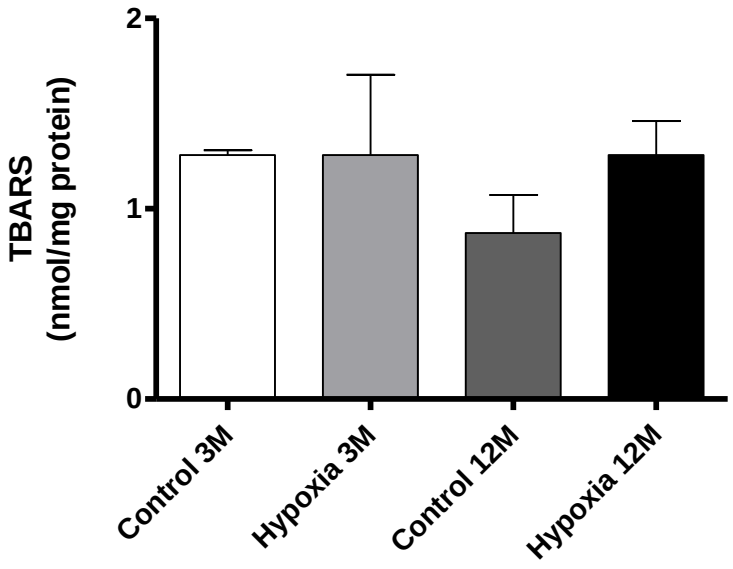


Fig. 13 - Effects of age and chronic hypoxia on lipid peroxidation. TBARS levels were measured as described in Material and methods section. Data shown represent means ± SEM from 3-6 animals from each condition studied.

3.3 - Age and/or chronic hypoxia alter enzymatic antioxidant defenses

Antioxidant enzymes are specialized defenses able to quench ROS in cells and tissues.

The activity of the mitochondrial isoform of SOD (MnSOD), an enzyme that dismutates $O_2^{\bullet -}$, increased significantly with age. However, in 12-month-old rats, chronic hypoxia decreased significantly the activity of this enzyme (Fig. 14).

GPx and CAT are involved in the reduction of H_2O_2 to H_2O. As shown in Fig. 15 chronic hypoxia induced a significant decrease in GPx activity, although no age-dependent alterations were observed. Concerning CAT activity, both age and chronic hypoxia induced a slight increase in this enzyme activity (Fig. 16).

GR is another important enzyme that reduces GSSG to the sulfhydryl form GSH. As shown in Fig. 17 an age-dependent decrease in the activity of GR was observed. Similarly, chronic hypoxia significantly decreased the activity of GR in 3-month-old animals.

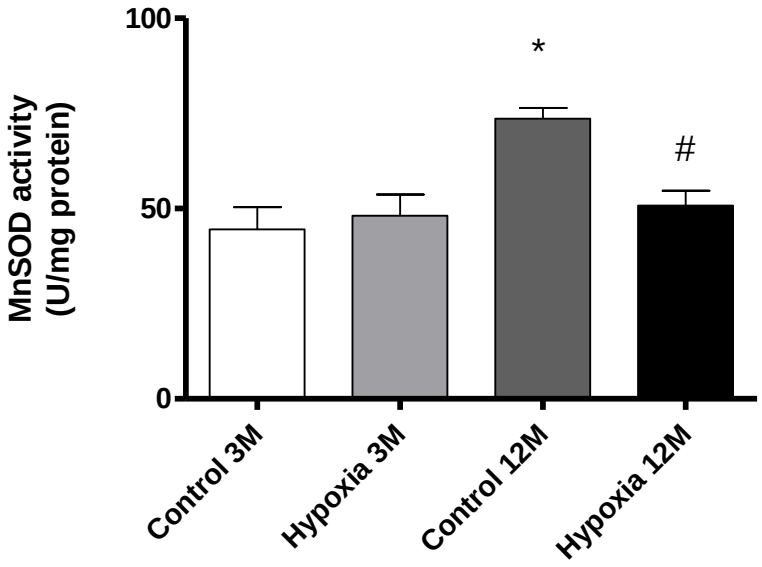


Fig. 14 - Effects of age and chronic hypoxia on manganese superoxide dismutase activity. MnSOD activity was determined as described in Material and methods section. Data shown represent means ± SEM of 4-6 independent experiments. Statistical significance: *$p<0.05$ when compared with 3-month-old control animals. $^{\#}p<0.05$ when compared with 12-month- old control animals.

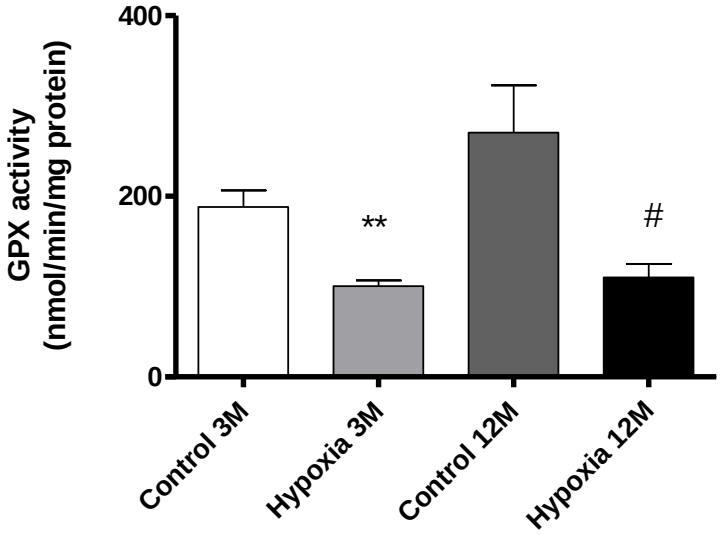


Fig. 15 - Effects of age and chronic hypoxia on glutathione peroxidase activity. GPx activity was determined as described in Material and methods section. Data shown represent means ± SEM of 5-6 independent experiments. Statistical significance: **$p<0.01$ when compared with 3-month-old control animals. $^{\#}p<0.05$ when compared with 12-month- old control animals.

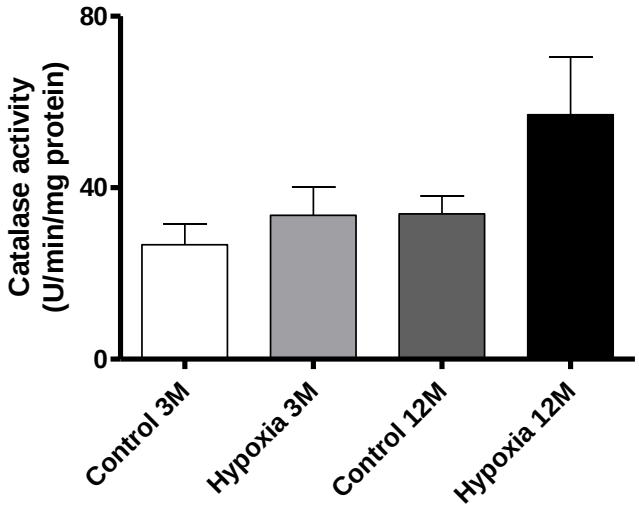


Fig. 16 - Effects of age and chronic hypoxia on catalase activity. CAT activity was determined as described in Material and methods section. Data shown represent means ± SEM of 5-6 independent experiments.

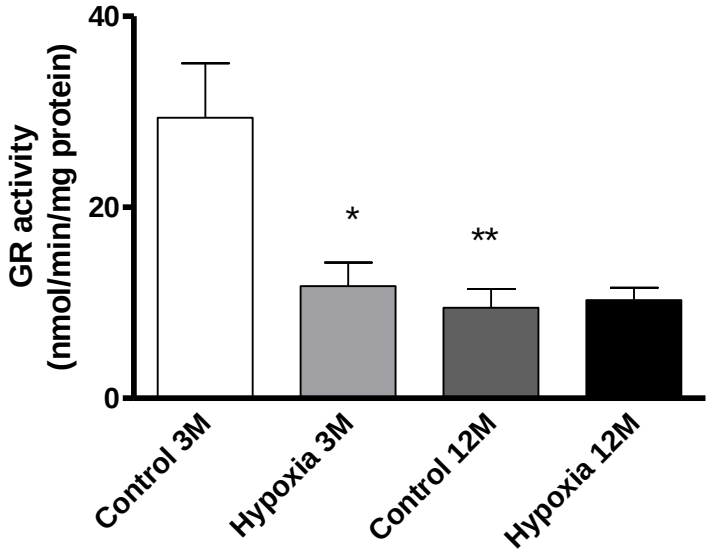


Fig. 17 - Effects of age and chronic hypoxia on glutathione reductase activity. GR activity was determined as described in Material and methods section. Data shown represent means ± SEM of 4-6 independent experiments. Statistical significance: *p<0.05; **p<0.01 when compared with 3-month-old control animals.

3.4 - Age and/or chronic hypoxia affect non-enzymatic antioxidant defenses

Extracellular proteins and small antioxidant molecules compose the non-enzymatic antioxidant defenses that act to deactivate free radicals. Glutathione and vitamin E belong to this line of defense.

Concerning vitamin E levels, a significant increase induced by chronic hypoxia in both ages was observed (Fig. 18).

Regarding GSH levels a significant age- and chronic hypoxia-dependent decrease was observed (Table I). Consequently, an age- and chronic hypoxia-dependent increase in GSSG levels occurred (Table I).

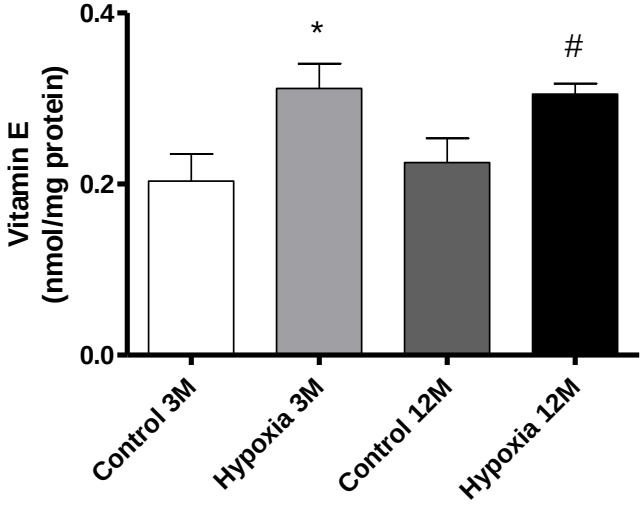


Fig. 18 - Effects of age and chronic hypoxia on vitamin E levels. Vitamin E levels were determined as described in Material and methods section. Data shown represent means ± SEM of 4-6 independent experiments. Statistical significance: *p<0.05 when compared with 3-month-old control animals. #p<0.05 when compared with 12-month- old control animals.

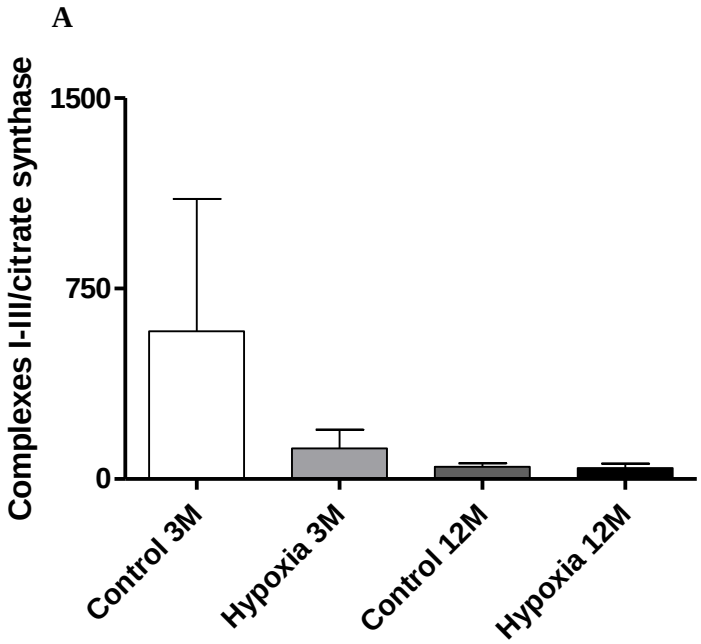
Table I - Effects of Age and/or Chronic Hypoxia on Glutathione Levels

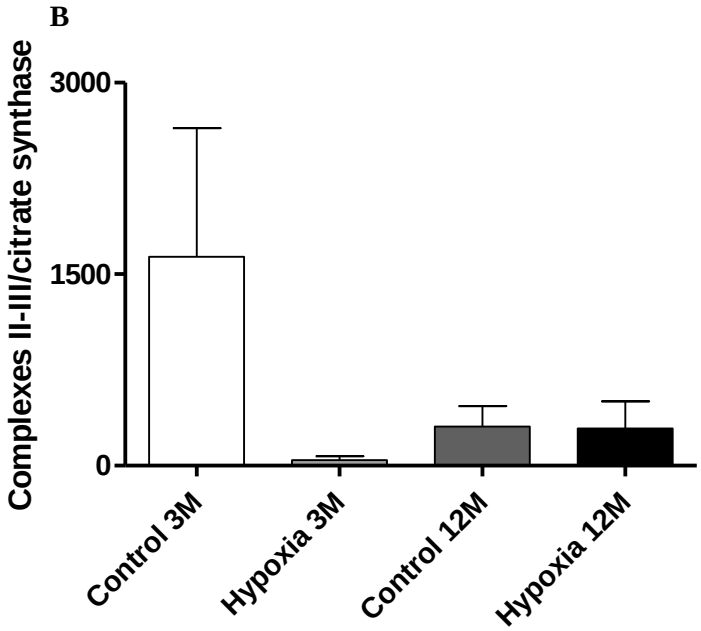
	3 months		12 months	
	Control	Hypoxia	Control	Hypoxia
GSH (nmol/mg protein)	8,819±1,153	2,222±0,08198**	4,187±0,5394*	2,048±0,2144##
GSSG (nmol/mg protein)	0,4016±0,2001	2,604±1,029*	0,8871±0,3147	4,463±1,144#

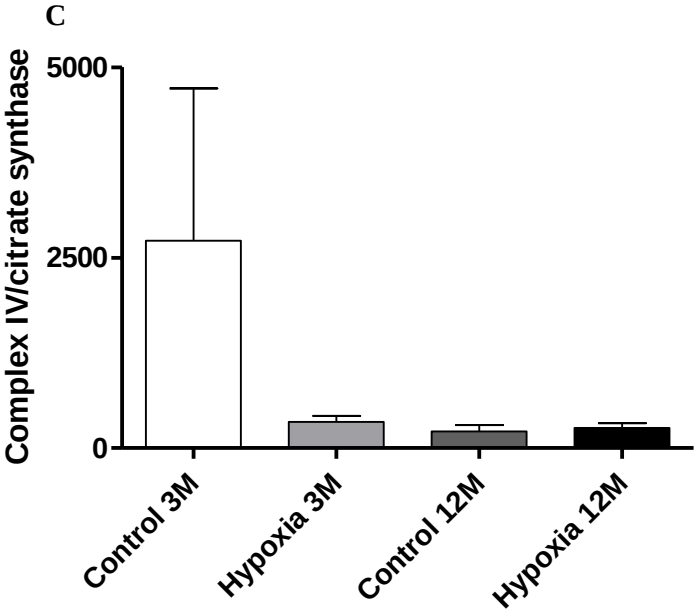
Data shown represent means ± SEM of 5-6 independent experiments. Statistical significance: *p<0.05; **p<0.01 when compared with 3-month-old control animals. #p<0.05; ##p<0.01 when compared with 12-month- old control animals.

3.5 - Age and chronic hypoxia decrease the activities of mitochondrial enzymatic complexes

NADH-cytochrome c reductase (complexes I-III), succinate- cytochrome c reductase (complexes II-III) and cytochrome c oxidase (complex IV) are crucial mitochondrial enzymatic complexes responsible for electron transfer and the formation of a H^+ gradient fundamental for the production of ATP by ATP synthase (complex V). We observed an age- and chronic hypoxia-dependent decrease in complexes I-III, complexes II-III and complex IV activity (Fig. 19A-C). A similar profile was observed in ATPase activity (Fig. 19D).




B
Complexes II-III/citrate synthase
3000
1500
0
Control 3M
Hypoxia 3M
Control 12M
Hypoxia 12M


C
Complex IV/citrate synthase
5000
2500
0
Control 3M
Hypoxia 3M
Control 12M
Hypoxia 12M

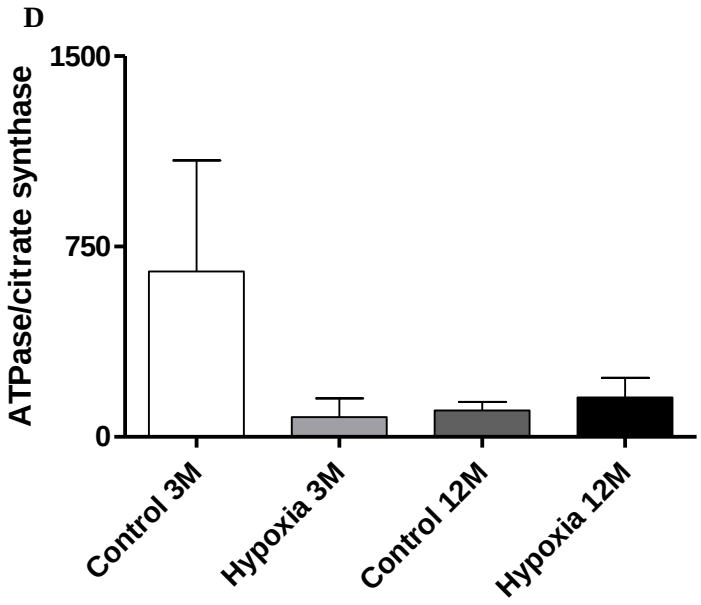


Fig. 19 - Effects of age and chronic hypoxia on mitochondrial enzymatic and ATPase activities. Complexes I-III (A) Complexes II-III (B) Complex IV (C) and ATPase (D) activities were normalized with citrate synthase activity as described in Material and methods section. Data shown represent means ± SEM from 3-6 animals from each condition studied.

3.6 - Age increases caspases activity

Caspases are a family of proteins that are one of the main executors of the apoptotic process. Caspases, like caspase-9, can activate other caspases in a cascade that eventually leads to the activation of the effectors caspases, such as caspase- 3 activating apoptotic cell death. In our study, a significant age-dependent increase in the activation of both caspase-9 and caspase-3 was observed (Fig. 20). Curiously, chronic hypoxia decreased significantly the activation of both caspases in 12-months-old rats (Fig. 20).

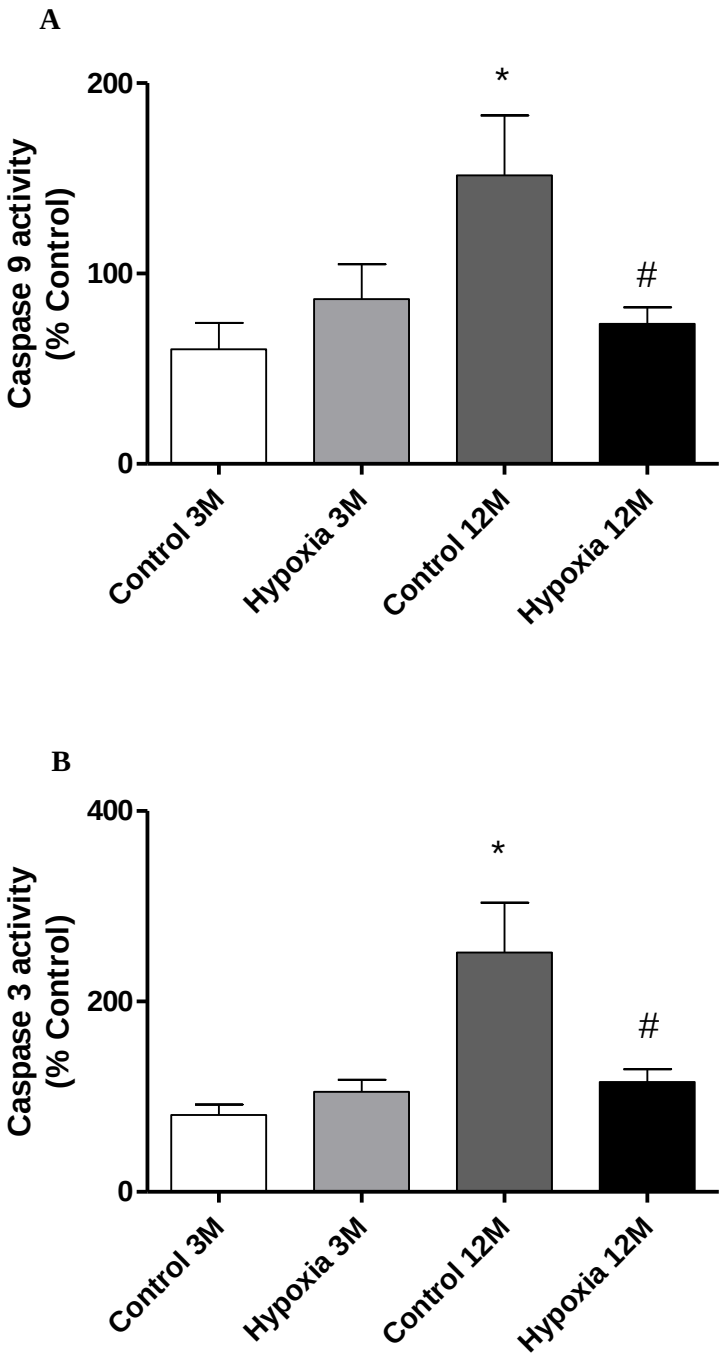


Fig. 20 - Effects of age and chronic hypoxia on caspase-9 and caspase-3 activation. Caspase-9 (A) and Caspase-3 (B) activities were measured as described in Material and methods section. Data shown represent means ± SEM from 3-6 animals from each condition studied. Statistical significance: *$p<0.05$ when compared with 3-month-old control animals. $^{\#}p<0.05$ when compared with 12-month- old control animals.

3.7 Age and/or chronic hypoxia do not change Bax and Bcl2 protein levels

The balance of protein expression is critical for the control of homeostasis and for proper function of the organism. Changes in their regulation may lead to disease and death. In our study, age and chronic hypoxia did not change significantly Bax and Bcl2 protein levels (Fig. 21).

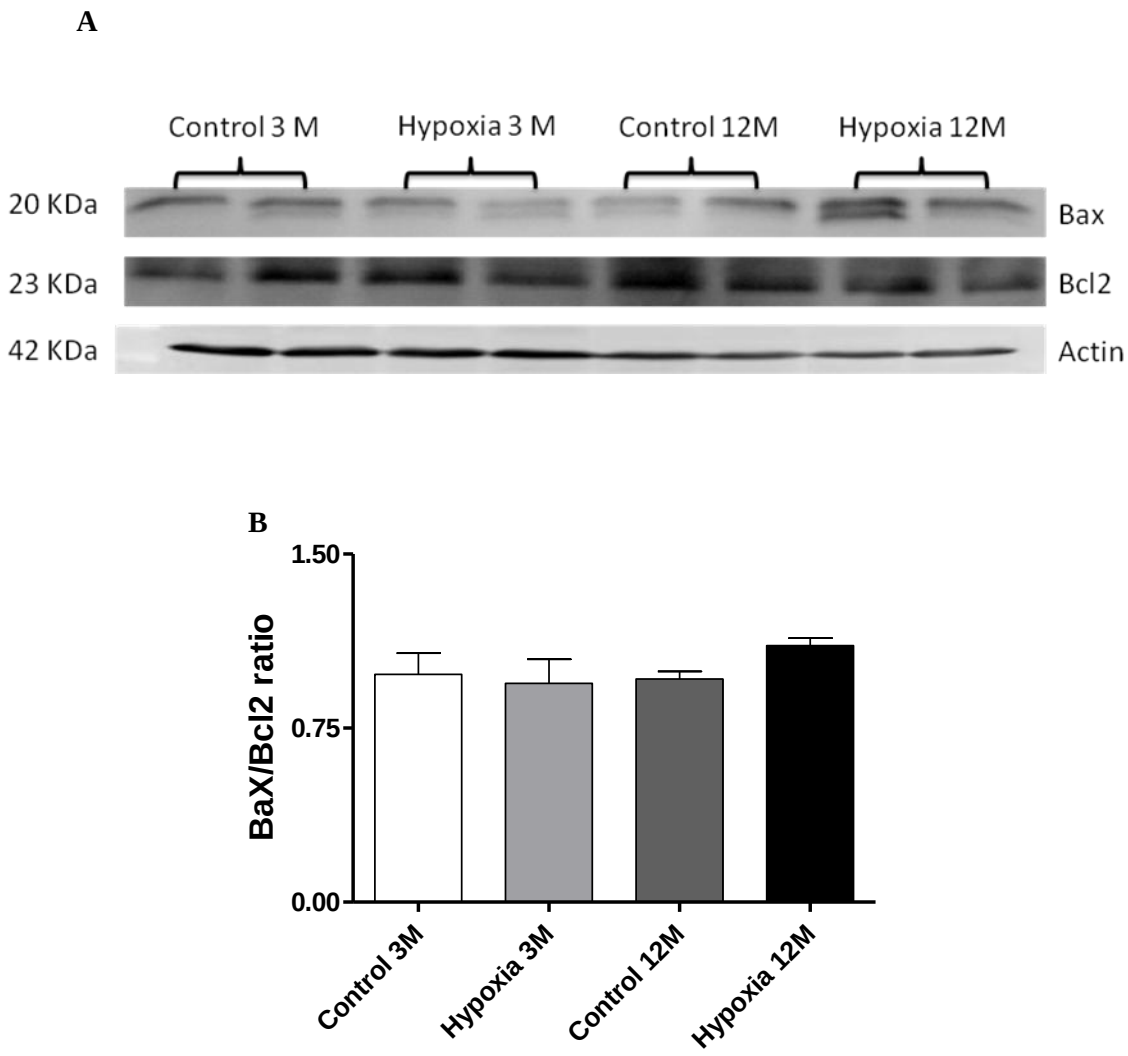


Fig. 21 - Effects of age and chronic hypoxia on Bax and Bcl2 protein levels. Representative blots of Bax and Bcl2 protein levels (A) and Bax/Bcl2 ratio (B). Data shown represent means ± SEM from 4-3 animals from each condition studied.

CHAPTER 4. DISCUSSION

4. Discussion

Increasing evidence suggests that aging affects cellular oxidative status and mitochondrial function (Serviddio *et al.,* 2011; Santos *et al.,* 2010; Amaral *et al.,* 2008; Moreira *et al.,* 2003). The aging process is accompanied by a decrease in the O_2 availability to the tissues and cells, a condition known as hypoxia (Carvalho *et al.,* 2010; Watson *et al.,* 2009). Indeed, evidence from the literature shows that aging is associated with vascular endothelial alterations in blood flow leading to a decrease in O_2 and nutrients delivery to cells (Di Giulio, 2009; Herrera *et al*., 2009; Watson *et al.,* 2009). It was also shown that chronic hypoxia *per se* promotes a remodeling of the structure and function of tissues, alterations that potentiate cell degeneration and death (Carvalho *et al.,* 2010; Watson *et al.,* 2009). Besides being associated with hypoxic episodes, aging is also the main risk factor for several age-related degenerative diseases (Carvalho *et al.,* 2010; Herrera *et al*., 2009; Di Giulio *et al.,* 2003). Furthermore, hypoxia characterizes several pathological situations such as vascular diseases, stroke, cancer, chronic obstructive pulmonary disease, obstructive sleep apnea (OSA) and a relation between hypoxia and Alzheimer's disease was also observed (Carvalho *et al.,* 2010; Marí *et al*., 2009; Savransky *et al.,* 2007; Debonneuil *et al.,* 2006). As already said, liver is a vital organ that play a critical role on health and body homeostasis. We decided to study the effects of age and/or chronic hypoxia on the liver because this is highly vascular organ and, therefore, susceptible to injury. Evidence shows that liver suffer age-related alterations characterized by a decrease in blood flow, an increase in ROS production and apoptosis, reduction in the levels of antioxidants and mitochondrial abnormalities (Lebel *et al.,* 2011; Hoare *et al.,* 2010; Sabaretnam *et al.,* 2009).

Medical advances combined with improved sanitation and better nutrition increased the average human life expectancy around the world and so populations continue to grow older (Hoare *et al.,* 2010; Burkle *et al.,* 2007). Consequently, an increase in the incidence of age-related diseases as well as healthcare resources devoted to the geriatric population occurred (Burkle *et al.,* 2007). Older individuals are more susceptible to most acquired liver disorders and more vulnerable to their complications (Hoare *et al.,* 2010). Recent evidence also suggests that age is a critical determinant of outcome for hepatitis C virus infection and liver transplantation (Hoare *et al.,* 2010; Burkle *et al.,* 2007). So it is essential to understand the mechanisms behind the aging process and identify possible therapeutic targets, in order to provide not only a longer but also a healthy life. Several pathologies such as alcoholic liver disease (ALD) (Nath *et al.,* 2011), hepatic cancers (Jeon *et al.,* 2011), hypoxic hepatitis (Ebert, 2006), congestive heart failure (Shibayama, 1987) and OSA (Savransky *et al.,* 2007) are associated with hypoxia, a condition that potentiate liver injury. So it becomes essential to gather information about the process of hypoxia in order to develop effective therapeutics for hypoxia-dependent diseases.

We characterized our experimental animals by evaluating the body weight and blood glucose levels. A significant decrease in both parameters was observed in hypoxic rats when compared with controls (Fig. 8). Similarly, Benderro and LaManna (2011) and Zhang and collaborators (2010) reported that chronic hypoxia decreases body weight in mouse and rats, respectively. Clinical studies also show that individuals suffering from COPD, a pathological situation characterized by chronic hypoxia, present a loss of body weight (Raguso and Luthy, 2011). As observed in our study, RBC, HGB and HCT levels are increased by chronic hypoxia (Fig. 10). Previous studies reported an increase in RBC, HGB and HCT levels both in human subjects under

chronic hypoxia such as small-for-gestational-age (SGA) infants and people living at high altitudes (Ozkiraz *et al.,* 2011; Heinicke *et al.,* 2003), and animal models of chronic hypoxia (Liu *et al.,* 2010; Silkin and Silkina, 2005). We also observed an age- and chronic hypoxia-dependent increase in plasma AST and ALT activities (Fig. 9), indicating liver injury. ALT and AST are members of the transaminase family of enzymes. ALT catalyzes the reversible transamination of L-alanine and α-ketoglutarate to piruvate and L-glutamate while AST catalyzes the reversible transamination of L-aspartate and α-ketoglutarate to oxaloacetate and L-glutamate. When the liver is injured or inflamed, the levels of ALT and AST in the blood rise, therefore these markers are used to check for signs of liver injury and abnormalities. ALT is a more liver-specific enzyme, since AST is found in many tissues (including heart, muscle, kidney, brain and lung) (Pratt and Kaplan, 2000; Johnston, 1999). Previous studies performed in cerebellar or lung tissues of Wistar rats (Subramanian and James, 2010; Nin *et al.*, 2008) and in liver tissue of mice and rats (Shih *et al.,* 2010; Hashimoto *et al.,* 2007) showed an increased of these enzymatic markers with age. In accordance with our results, previous studies also showed that liver of pigs and mice presented a chronic hypoxia-dependent increase in ALT and AST (Gravante *et al.,* 2010; Savransky *et al.*, 2009).

As expected an age- and chronic hypoxia-dependent increase in oxidative stress and damage was observed. The production of H_2O_2 is an indicator on the propensity of mitochondria to originate or exacerbate oxidative stress. Both age and chronic hypoxia increase the levels of H_2O_2 and the effect of age was potentiated by chronic hypoxia (Fig. 12). These results are in accordance with a previous study showing that chronic hypoxia potentiate H_2O_2 production in brain vessels of Wistar rats

(Carvalho *et al.,* 2010). Similar results were observed in aged rat mesenteric arteries (Zhou *et al.,* 2009) and hypoxic rabbit lungs (Weissmann *et al.*, 2001).

Aconitase is a mitochondrial enzyme involved in the TCA cycle and is responsible for the isomerization of citrate to iso-citrate. This enzyme is a very sensitive redox sensor due to $[4Fe\text{-}4S]^{2+}$ cluster present in its active site. Indeed, ROS, particularly $O_2^{\bullet -}$, can bind and oxidize this cluster inactivating the enzyme (Gardner, 2002; Vasquez-Vivar *et al.,* 2000; Lauble *et al.,* 1994). In this way, a decrease in the aconitase activity may be indicative of an increase in ROS levels. Indeed, we observed an age- and chronic hypoxia-dependent decrease in aconitase activity (Fig. 11), an effect that is positively correlated with the increase in H_2O_2 levels (Fig. 12). Similarly, previous studies show a decrease in the aconitase activity promoted by aging and chronic hypoxia (Alabarse *et al.,* 2011; Lustgarten *et al.,* 2011; Carvalho *et al.,* 2010; Kumar *et al.,* 2006; Peng *et al.,* 2003).

The oxidation of lipids, also called lipid peroxidation, can be analyzed by several methods including the evaluation of TBARS levels, which is the most referenced method in the literature. This method measures the levels of MDA, a naturally occurring product of lipid peroxidation (McElnea *et al.,* 2011; Rosa *et al.,* 2011; Sahreen *et al.,* 2011). In our study chronic hypoxia and age did not induce significant changes in TBARS levels (Fig. 13). Although these results seem strange at first, when we look to the levels of vitamin E (Fig. 18), the major lipid peroxidation chain breaker, we realize that a compensatory mechanism occurred, which may protect against lipid peroxidation. Our results confirm previous studies performed in livers of mice (Das *et al.,* 2010) and rats (Vannucchi *et al.,* 1997) showing a protection of vitamin E against lipid peroxidation, namely a decrease in TBARS levels. Similary,

studies done in mouse lungs (Ergonul *et al.*, 2007) and retina (Terrasa *et al.*, 2009) show that vitamin E protects against oxidative damage of lipids.

The increase in oxidative stress levels is accompanied by an antioxidant response (Aydin *et al.*, 2010). SOD is one of the most important enzymes belonging to the enzymatic antioxidant defense line and catalyzes the conversion of $O_2^{\bullet-}$ to H_2O_2 (Fridovich, 1995). We observed an age-dependent increase in the activity of MnSOD (Fig. 14), this effect being positively correlated with the age-dependent increase in H_2O_2 levels (Fig. 12). Previous studies also show an age-dependent increase in MnSOD activity (Carvalho *et al.*, 2010; Judge *et al.*, 2005; Phaneuf and Leeuwenburgh, 2001), which represent an adaptive mechanism against oxidative stress. This idea is corroborated by other studies showing that MnSOD overexpression avoid the age-dependent oxidative stress (Sarsour *et al.*, 2010; Sun *et al.*, 2002). However, chronic hypoxia decreased the activity of MnSOD in mature animals (Fig. 14). Similarly, Nozik-Grayck and collaborators (2008) found that chronic hypoxia decreased the extracellular SOD activity and protein expression in lungs. It was also reported that chronic hypoxia in the kidney decrease the mRNA levels of CuZnSOD (Son *et al.*, 2008). Catalase activity (enzyme responsible for the conversion of H_2O_2 to H_2O) did not change significantly in all groups of experimental animals (Fig. 16). It was recently shown that the activity of CAT in the liver do not significantly change with aging (Mármol *et al.*, 2010). Hussain and collaborators (1995) also reported that aging do not affect CAT activity in all brain regions, except the cerebellum. Other studies showed an increase of catalase activity with age and/or chronic hypoxia in erythrocytes (Devi *et al.*, 2007; Rauchová *et al.*, 2005) and in brain vessels of Wistar rats (Carvalho *et al.*, 2010). It was also reported that the activity and protein expression of SOD and CAT

were significantly increased in the myocardium of chronic intermittent hypobaric hypoxia guinea pigs (Guo *et al.*, 2009).

Concerning GPx and GR, it is known that these enzymes are both involved in the redox cycle of glutathione. The glutathione system is acknowledged as one of the main regulators of the intracellular redox environment, acts in the antioxidant defense system and controls the regulation of several cellular processes (Marí *et al.*, 2009). GPx catalyzes the reduction of H_2O_2 using GSH as a co-substrate that is converted to GSSG, and then GR is responsible for regenerating GSSG back to GSH (Orrenius *et al.*, 2007). In this study, chronic hypoxia decreased the activity of both GPx and GR (Figs. 15 and 17). These results are in agreement with previous studies that showed that hypoxia in the brains of Wistar rats and Sprague–Dawley rats is responsible for a reduction in GPx and GR activities (Carvalho *et al.,* 2010; Maiti *et al.,* 2006). These alterations were also observed in rat liver (Shan *et al.,* 1992; Costa, 1990). GR also presented an age-dependent decrease (Fig. 17), which corroborates the idea that aging affects the antioxidant defenses. Accordingly, studies performed by Rikans and coworkers (1992) in mitochondria from liver of aged Fischer 344 rats and of Barja de Quiroga and coworkers (1990) in liver and brain of old rats demonstrated a decrease in antioxidant enzymes, namely GR. Sahoo and coworkers (2008) also reported that age reduces GR activity in testicular mitochondria of Wistar rats. We also observed a slight age-dependent increase in the activity of GPx (Fig. 15) that can contribute for the decrease in H_2O_2 levels (Fig. 12) in mature animals. Several authors show that aging increases the GPx activity in rat inner ear (Coling *et al.,* 2009), in mice brain and liver (Sobocanec *et al.,* 2008) and in myocardium mitochondria of Fischer 344 rats (Judge *et al.,* 2005). Concerning GSH levels a chronic hypoxia- and age-dependent decline was observed (Table I), alterations that are concordant with GR and GPx activities (Figs. 15

and 17). GSH levels are a good indicator of the redox balance so, the decrease in these levels indicates that the mature animals submitted to chronic hypoxia are more susceptible to oxidative damage. In accordance, GSSG levels increase under these experimental conditions (Table I). Our results are consistent with previous studies showing that disturbances in GSH homeostasis affect the cellular antioxidant capacity and are implicated in the etiology and/or progression of a number of diseases (e.g. cancer and cardiovascular, Parkinson's and Alzheimer's diseases) and aging (Ballatori *et al.,* 2009). Concerning age-dependent decrease in GSH, our results are consistent with other studies performed in aged Sprague–Dawley rat liver (Aydin *et al.,* 2010), rat brain (Zhu *et al.,* 2006) and lungs of C57BL/6 J mice (Li *et al.,* 2001). Other studies also showed that chronic hypoxia induce a decrease in GSH levels in rat liver, lungs, brain and heart (Navarová *et al.,* 2005; Shan *et al.,* 1992).

Vitamin E, another important non-enzymatic antioxidant defense, has a crucial role in the protection against lipid peroxidation (Das Sarma *et al.,* 2010). The levels of vitamin E were increased by chronic hypoxia in both ages (Fig. 18), which may explain why chronic hypoxia did not change significantly TBARS levels (Fig. 13). The increase in vitamin E levels may represent a compensation mechanism developed by liver cells to fight against oxidative stress and damage. Indeed, numerous observations show that vitamin E protects against oxidative stress. It was observed that vitamin E reduces liver lipoperoxidation in a rodent model of nonalcoholic steatohepatitis (NASH) (Zamin Jr *et al.,* 2010). Other study show that vitamin E homologues protect against cardiolipin peroxidation (Samhan-Arias *et al.,* 2011). Clinical studies also show a protective role of vitamin E supplementation against oxidative stress in type 1 *diabetes mellitus* patients (Gupta *et al.,* 2011). The raise in vitamin E levels observed in our study may also contribute to the decrease in GSH levels (Table I), especially the decrease induced by

chronic hypoxia, since two molecules of GSH generate one molecule of vitamin E (Valko *et al*., 2007). In fact, as observed by many authors (Bautista-Ortega and Ruiz-Feria, 2010; Mach *et al.,* 2009) vitamin E seems to have a crucial role in the response to hypoxic situations.

As previously discussed mitochondria play a critical role in aging and age-related diseases (Kakkar and Singh, 2007). A natural byproduct of normal mitochondria metabolism is the generation of ROS and the redox status of mitochondria is important in combating oxidative stress (Vendelbo and Nair, 2011). However, the age-related changes in mitochondria induce lower ATP production and higher generation of ROS leading to a vicious cycle of deleterious events and mitochondrial dysfunction (Lee and Wei, 2001).

In this study an age- and chronic hypoxia-dependent decrease in the activities of mitochondrial enzymatic complexes were observed (Fig. 19). These results are in agreement with the decline in aconitase activity (Fig. 11) and with the increased levels of H_2O_2 (Fig. 12). Indeed, the decrease in the activities of mitochondrial enzymatic complexes is associated with impairment of mitochondria function that lead to a decline in ATP production and NAD^+:NADH ratio and an increase in free radicals generation (Sastre *et al.,* 2003; Johnson *et al.,* 1999). These alterations together with the reduced capacity of the antioxidant defense system will induce cell degeneration and death and tissue dysfunction. Citrate synthase activity was evaluated with the purpose to correct mitochondrial enzymes activities. However, the activity of this enzyme presents a slight age-dependent decrease and a more pronounced chronic hypoxia-dependent decrease was observed (data not shown). Since citrate synthase activity is routinely used as a marker of aerobic capacity and mitochondrial density, these results may also suggest that the number of mitochondria decrease with aging and chronic hypoxia. Previous

investigations, in several tissues, show similar findings concerning oxidative damage promoted by aging and mitochondrial decay characterized by a decrease in the activities of mitochondrial complexes (Long *et al.,* 2009; Dubessay *et al.,* 2007; Leon *et al.,* 2004; Drouet *et al.,* 1999; Feuers, 1998). A decline of mitochondria enzymatic activities was also found with chronic hypoxia in hearts of Wistar rats and human cardiomyocytes (Nouette-Gaulain *et al.,* 2005; Merante *et al.,* 1998).

Aging is associated with an increased risk of disease and probability of death, which result from several factors including the activation of mechanisms of cell death like apoptosis (Sanz *et al*., 2008). As referred before, apoptosis is primarily regulated by a cascade of caspases (Jung and Kim, 2004). Our results show a significant age-dependent increase in both caspases-9 and caspases-3 activation (Fig. 20). These results are in agreement with the oxidative imbalance (Figs. 11 and 12) and mitochondrial enzymatic complexes impairment (Fig. 19) observed in mature animals. Accordingly, several studies show an age-associated increase in the activities of caspases (Kang *et al.,* 2010; Tamilselvan *et al.,* 2007; Zheng *et al.,* 2005; Zhang *et al.,* 2002). Curiously, chronic hypoxia decreased significantly the activation of both caspases in 12-months-old rats (Fig. 20). Although some studies indicate that caspases activation increase with chronic hypoxia (Hota *et al.,* 2008; Barhwal *et al.,* 2007; Bae *et al.,* 2003; Tanaka *et al.,* 2003), others report no changes or even a decrease in caspases activation (Zamudio *et al.,* 2007; Takahashi *et al.,* 2006). One possible explanation for this observation is that chronic hypoxia may lead to a significant decrease in cell energy levels that may affect caspases activation, a phenomenon that is energy dependent. Besides caspases, Bax (a pro-apoptotic protein) and Bcl2 (a pro-survival protein) protein levels can also be used as markers of apoptosis. Khaidakov and coworkers (2011) reported an age-dependent increase in apoptosis. The authors observed a decline in Bcl2, an increase in Bax protein

levels and an increase in Bax/Bcl2 ratio in senescent primary human umbilical vein endothelial cells. Kakarla and coworkers (2010) proposed that the increase in apoptosis may be mediated by the age-related increase in ROS production. Despite the age-dependent increase in H_2O_2 levels (Fig. 12) and caspases activation (Fig. 20), no significant alterations in the Bax/Bcl2 ratio were observed (Fig. 21). Although surprising, this observation may be explained by an alteration in the levels of other pro-apoptotic (e.g. Bid) and anti-apoptotic (e.g. Bcl-x_L) proteins, which may activate the apoptotic cell death. However, future studies must be done to prove (or not) this hypothesis.

CHAPTER 5. CONCLUDING REMARKS

5. Concluding Remarks

Regardless the progress made in the aging research field and the numerous theories that have emerged to explain this process, which have brought new and complementary points of view and new questions to be answered, the phenomenon of aging is still not fully understood. The data presented in this thesis supports the Mitochondrial Free Radical Theory of Aging, since our results show the existence of an age-associated enhancement of mitochondrial defects and ROS production, and decrement in the capacity of endogenous antioxidant defenses to counteract oxidative stress and damage.

Interestingly, several age-related alterations were mimicked by chronic hypoxia in young rats suggesting that chronic hypoxia can be used as a reliable model to study some aspects of liver aging. Indeed, 3-month-old animals subjected to chronic hypoxia and 12-month-old animals present a similar pattern of oxidative imbalance and mitochondrial defects. Chronic hypoxia also potentiates age-associated oxidative imbalance, a situation that could explain why older people have a higher susceptibility to degenerative conditions associated with vascular alterations (e.g. chronic vascular diseases).

Nevertheless, further work still needs to be done to uncover the network between aging, chronic hypoxia, mitochondria, oxidative stress and apoptotic cell death. Additionally, to prove that chronic hypoxia is a reliable experimental approach to mimic the aging process, studies in several tissues must be done.

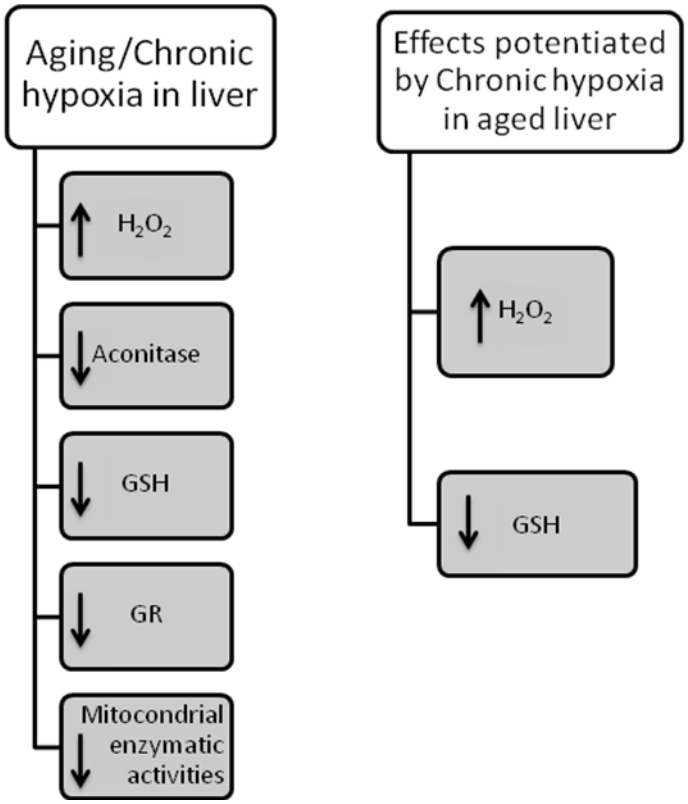


Fig. 22 – Effects of age and/or chronic hypoxia in liver. In young animals, chronic hypoxia induces a pattern of oxidative imbalance and mitochondrial defects similar to that found in mature animals. Indeed, both 3-month-old rats exposed to chronic hypoxia and 12-month-old rats present a significant increase in hydrogen peroxide (H_2O_2) levels and a decrease in glutathione levels (GSH) and aconitase, glutathione reductase (GR) and mitochondrial enzymatic complexes activities. Furthermore, chronic hypoxia potentiates the age-related increase in H_2O_2 levels and decrease in GSH.

“Aging seems to be the only available way to live a long life.”

Kitty O'Neill Collins

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