ASSESMENT OF CHRONOLOGICAL LIFE SPAN DEPENDENT MOLECULAR DAMAGES OF *S.cerevisiae* DEFICIENT IN MITOCHONDRIAL ANTIOXIDANT GENES

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ABSTRACT

ASSESMENT OF CHRONOLOGICAL LIFE SPAN DEPENDENT MOLECULAR DAMAGES OF *S.cerevisiae* DEFICIENT IN MITOCHONDRIAL ANTIOXIDANT GENES

Aging is referred as the time-dependent accumulation of biological and physiological changes in an organism. This complex process is the major factor that is associated with many diseases such as cancer, diabetes and neurodegenerative disorders. The free radical theory of aging, which states that the molecular damages formed upon free radicals lead to the aging process, is the most widely accepted aging theory. The free radicals that are primarily produced in the mitochondria upon aerobic metabolism, are known to damage the biomolecules such as DNA, proteins and lipids. However, cells have evolved different defense systems for the elimination of these molecular damages. Antioxidant defense mechanism is one these systems that play role in the repair of the molecular damages. Since mitochondria are the main sites for the free radical production, the antioxidant genes that function in mitochondria gained an importance for their roles in preventing the molecular damages in a cell. In this study, the differences in the life spans and levels of molecular damages among different mitochondrial antioxidant gene mutants of *Saccharomyces cerevisiae* were tried to be identified throughout the chronological aging process, which is the model that mimics post-mitotic cell aging in higher eukaryotes. It was shown that deletion of some mitochondrial antioxidant genes resulted in different levels of biomolecular damages as well as different sensitivities against reactive species, which may be a critical outcome for the prevention of the detrimental effects of free radicals on biomolecules formed during chronological aging.

ÖZET

MİTOKONDRİYEL ANTİOKSİDAN GENLERİ OLMAYAN *S.cerevisiae*'DA KRONOLOJİK YAŞLANMAYA BAĞLI MOLEKÜLER HASARLARIN TESPİTİ

Yaşlanma, bir organizmadaki zamana bağlı biyolojik ve fizyolojik değişimlerin birikimi olarak adlandırılır. Bu karmaşık mekanizma, kanser, diyabet ve sinir sistemi hastalıkları gibi bozuklukların ana etkenidir. Serbest radikallerin yol açtığı moleküler hasarların yaşlanmaya sebep olduğunu savunan yaşlılığın serbest radikal teorisi, en yaygın kabul görmüş yaşlılık teorisidir. Aerobik metabolizma sonucunda mitokondride oluşan serbest radikaller, DNA, protein, lipid gibi biyomoleküllere hasar verirler. Ancak, oluşan bu hasarları gidermek amacı ile canlılar bazı koruma sistemleri geliştirmişlerdir. Antioksidan savunma sistemleri, moleküler hasarların tamirinde rol oynayan bu sistemlerden bir tanesidir. Serbest radikaller birincil olarak mitokondrilerde üretildiklerinden dolayı, mitokondrilerdeki antioksidan genler, hücre içi moleküler hasarların önlenmesindeki rolleri ile ilgi odağı olmuşlardır. Bu çalışmada, *Saccharomyces cerevisiae*'nın farklı antioksidan genleri içermeyen mutantlarında, daha gelişmiş ökaryotlardaki post-mitotik hücrelerin yaşlanma modeli olan kronolojik yaşlanma sürecindeki yaşam süreleri ve moleküler hasar farklılıkları gözlemlenmeye çalışılmıştır. Farklı antioksidan genlerin hücreden çıkarılması, moleküler hasar ve reaktif türlere karşı hassasiyette farklılıklar ile sonuçlanmıştır. Elde edilen bu sonuçlar, kronolojik yaşlanma sürecinde serbest radikallerin moleküler üzerindeki zararlı etkilerinin önlenmesi yolundaki çalışmalar için önem arz etmektedir.

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CHAPTER 1

INTRODUCTION

1.1 Reactive Oxygen Species (ROS) and Oxidative Stress

Reactive oxygen species are the free radicals that are formed as by-products of the aerobic metabolism. They are important players of the redox homeostasis in a cell both by mediating the toxicity of oxygen and by acting as intracellular signaling molecules via chemical interactions with specific target proteins (D'Autreaux and Toledano 2007).

Reactive oxygen species (ROS) mainly include the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$). ROS have distinct biological properties such as lipid solubility and chemical reactivity. Chemical reactivity is the property that distinguishes ROS from other signaling molecules (D'Autreaux and Toledano 2007). Superoxide anion has high atomic reactivity with [Fe-S] clusters while hydrogen peroxide has high reactivity with cystein residues. Hydroxyl radical is considered to be the most harmful reactive oxygen species since it has indiscriminate reactivity towards biological molecules (D'Autreaux and Toledano 2007).

Mitochondria are the sites of energy production for the biological processes of aerobic organisms, while also being the main sites for ROS production (Murphy 2009). Oxidative phosphorylation leads to formation of an electron flux through the respiratory chain during mitochondrial respiration, which subsequently results in the formation of proton gradient across the inner mitochondrial membrane (Giorgio, et al. 2007). Normally, electrons are transferred to the O_2 through the electron transport chain (ETC) and O_2 is reduced into H_2O completely via the cytochrome c oxidase (complex IV) of the ETC without any radical formation (Giorgio, et al. 2007). However, upon electron leakages throughout ETC, if O_2 reduction takes place upstream of the complex IV, such as at complex I and III, $O_2^{\cdot-}$ production takes place.

Superoxide anion ($O_2^{\cdot-}$) is the primarily formed reactive oxygen species in the mitochondria due to an electron gain of O_2 (Murphy 2009). $O_2^{\cdot-}$ can either react with other electron acceptors or be dismutated into H_2O_2 in the mitochondria with the

function of MnSOD. H_2O_2 is membrane permeable, longer-lived and less reactive when compared to other reactive species, which makes it a more suitable signaling molecule (Giorgio, et al. 2007). It has been shown that H_2O_2 plays an important role in physiological and oncogenic growth and angiogenic signaling (Giorgio, et al. 2007). The toxicity of H_2O_2 results from its conversion into hydroxyl radical ($OH^{\cdot}$) in the presence of redox active metals through Fenton reactions (D'Autreaux and Toledano 2007). Normally, iron regulation in a cell prevents the formation of free iron molecules in a cell. However, under stress conditions, excessive superoxide radicals result in release of 'free iron' from iron-containing compounds (Valko, et al. 2006). The released free iron (Fe (II)) can react with H_2O_2 and result in the formation of highly reactive $OH^{\cdot}$ via the Fenton reactions. Copper (Cu) is another redox active metal that results in $OH^{\cdot}$ production upon its breakdown by H_2O_2. Superoxide also participates in the Haber-Weiss reaction in which it reacts with H_2O_2 and results in the formation of $OH^{\cdot}$ (Valko, et al. 2006). $OH^{\cdot}$ is the most harmful radical since it has indiscriminate reactivity towards many biological molecules, such as DNA, lipids and amino acids (D'Autreaux and Toledano 2007).

Oxidative stress results from the excessive ROS production and insufficient removal or repair of the ROS-dependent damaged molecules (Chen, et al. 2005). When ROS synthesis exceeds a certain threshold level, cellular defense systems have difficulties to remove them from the cellular environment. Therefore, the increased amount of ROS production over the primary defense systems in a cell results in biomolecular damages upon the reactions of ROS with target molecules, such as DNA, proteins, and lipids (Squier 2001). Biomolecular damages are shown to be related with many disorders such as cancer, and neurodegenerative diseases (Klaunig and Kamendulis 2004). Therefore, oxidative stress is accepted to play a crucial role in cell dysfunction (Chen, et al. 2005).

1.2 Biomolecular Damages and Antioxidant Defense Mechanisms

Reactive oxygen species react with biological targets due to their intrinsic chemical properties. The reactivity of ROS with their target molecules enhances their toxicity effect while decreasing the signaling properties (D'Autreaux and Toledano 2007). Most of the cellular components are damaged upon oxidative stress, such as

proteins, lipids and DNA. These biomolecular oxidation events can lead either reversible or irreversible changes (Poppek and Grune 2005). The irreversible changes are eliminated from the cell through degradation systems whereas the reversible modifications can be repaired by the cell via enzymatic or non-enzymatic repair systems (Stadtman and Levine 2000).

1.2.1 DNA Oxidation

Nuclear DNA undergoes oxidative modifications continuously due to their high susceptibility for ROS attack. These modifications lead to formation of DNA strand breaks or damaged DNA bases (Chen, et al. 2005). Therefore, upon DNA oxidation, the genome stability can said to be decreased. Formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 2,6-diamino-4-hydroxy-5-formanidopyrimidine (Fapy-guanine) are among the mostly seen DNA oxidation products due to ROS attack (Chen, et al. 2005) and they are used as general markers for detection of DNA damages upon oxidative stress as well as the other techniques used for detection of spontaneous DNA mutation rates, such as canavanine and erythromycin resistance assays used in yeast.

1.2.2 Protein Oxidation

Proteins can either be modified via direct oxidation of ROS or via compounds resulting from oxidation processes that take place in other parts of the cell (Poppek and Grune 2005). ROS reactions with proteins lead to oxidation of various amino acid side chains and structural modifications that subsequently lead to loss of protein function (Poppek and Grune 2005).

The basic protein oxidation mechanisms were clarified by the earlier studies of Garrison, where they exposed free amino acids and proteins to ionizing irradiation under conditions where $OH^{\cdot}$, $O2^{\cdot-}$, or their mixture is formed (Garrison, et al. 1962). It was demonstrated that a carbon-centered radical is formed by abstraction of a hydrogen atom from the protein polypeptide backbone upon reaction with $OH^{\cdot}$. Under aerobic conditions, this carbon-centered radical reacts with free oxygen to form protein peroxyl radical derivative, which can readily be converted into protein peroxide either via its reaction with the protonated form of superoxide anion or via abstraction of a hydrogen

atom from another molecule to form another radical derivative. The alkoxyl radical is formed when protein peroxide reacts with another $HO_2^{\cdot-}$ and upon its further reaction with $HO_2^{\cdot-}$, a stable protein hydroxyl derivative can be formed. The alkoxyl radical may also undergo peptide bond cleavage by diamide or α-amidation pathways (Banerjee, et al. 2007).

One of the outcomes of protein oxidation is the formation of the protein-protein cross-linked derivatives. There are several mechanisms in which protein-protein cross-linked derivatives are formed. Among these mechanisms are; cystein sulfhydryl group oxidation into disulfide derivatives, reaction of 2 carbon-centered radicals with each other, and bridging of 2 tyrosine residues after they are oxidized into carbon-centered radicals (Banerjee, et al. 2007).

Almost all amino acids are prone to attack by ROS, but sulfur-containing amino acids (methionine and cystein) are more susceptible to ROS oxidation. Cystein oxidation results in formation of disulfide bridges, mixed disulfides, cystein-sulfenic, cystein-sulfinic, and cystein-sulfonic acids. Methionine oxidation results in methionine sulfoxide formation which in turn leads to methionine sulfone upon further oxidation (Mary, et al. 2004). Some of the amino acid modifications are reversible, such as thiolation and disulfide bond formation, whereas, others are irreversible, such as methionine sulfone formation.

Amino acid side-chain modifications other than cystein and methionine results mainly in hydroxyl and carbonyl group formation (Mary, et al. 2004). Oxidation of aliphatic and aromatic amino acids, which lead to ketoacid and hydroxyl derivative formation, respectively and protein oxidation by the oxidation products of poly-unsaturated fatty acids which result in formation of aldehydes, are among these protein modifications (Poppek and Grune 2005).

Formation of carbonyl derivatives constitutes an important part in protein oxidation since they are formed by multiple ROS related reactions and are extremely stable (Chen, et al. 2005). Among the mechanisms in which protein carbonyl groups are formed, are; metal catalyzed oxidation of arginine, proline, and lysine residues, cleavage of alkoxyl radicals by α-amidation pathway, oxidation of glutamyl residues, reactions of the lysyl amino group of a protein with one of the aldehyde groups of melandialdehyde and products of glycation reactions (Banerjee, et al. 2007). Formation of carbonyl groups is a general and most common measure of the ROS mediated protein

oxidation since these derivatives are extremely stable and are therefore good indicators of the oxidative stress (Dalle-Donne, et al. 2003).

1.2.3 Lipid Oxidation

Lipids are major components of living organisms. Therefore, they are one of the easy targets for the ROS attack. Polyunsaturated fatty acids (PUFAs) have the highest sensitivity against free radical mediated attack, since the sensitivity against attack increases proportionally with the number of double bonds in a molecule (Pratico 2002).

Upon a free radical attack to the fatty acid, a hydrogen atom is abstracted from the fatty acid and in the presence of oxygen, lipid peroxyradical is formed. Further reaction of the lipid peroxyradical with fatty acids leads to formation of lipid hydroperoxides, which can subsequently undergo fragmentation and form reactive intermediates (Pratico 2002). The reactive intermediates can be classified into three groups, which are 2-alkenals, 4-hydroxy-2-alkenals (4-HNE), and ketoaldehydes (Chen, et al. 2005). Ketoaldehydes are the predominant reactive intermediates of lipid peroxidation, with melondialdehyde (MDA) being one of the best characterized one. However, in means of effects on protein modifications, 2 alkenals and ketoaldehydes are shown to be less reactive when compared to Hydroxyalkenals (HNE). Therefore HNE took a great attention due to its reactivity that results in protein modifications. HNE reacts with cystein, histidine, and lysine residues and lead to cellular cytotoxicity via formation of protein aggregates, loss of enzymatic function, and inhibition of proteasome mediated protein degradation (Chen, et al. 2005). Therefore, due to their best characterized nature and functions, Melondialdehyde (MDA) and 4-hydroxy-2-alkenals are the two major lipid peroxidation products that are used as general lipid peroxidation measurements.

1.2.4 Antioxidant Defense Mechanisms

Cells have evolved various defense mechanisms for the removal or repair of oxidative stress damages. The irreversible damages on molecules are eliminated via degradation systems, such as proteosomes and lysosomes, whereas the reversible ones

are repaired by general repair systems (Poppek and Grune 2005). Antioxidant defense mechanism is one of these primary defense mechanisms for damage repair.

Antioxidant defense mechanism can be divided into two parts named as enzymatic and non-enzymatic mechanisms. Enzymatic defense systems can either directly act as ROS detoxifiers, such as superoxide dismutases (SOD) and catalases, or can act as redox regulators to maintain the redox balance in a cell, such as thioredoxin and glutaredoxin systems (Herrero, et al. 2008).

The two different enzymatic defense systems against the oxidatively modified cystein residues are; *Thioredoxin / Thioredoxin reductase* system and *Glutaredoxin / Glutathione / Glutathione reductase* system (Mary, et al. 2004). Both systems are found in the cytosol and the mitochondria of the cell and they act as antioxidant defense systems via regulation of the redox homeostasis in a cell (Petropoulos and Friguet 2006).

Both thioredoxin and glutaredoxin belong to the thiol/disulfide oxidoreductase family of proteins and both are small ubiquitous proteins (Petropoulos and Friguet 2006). They consist of a redox active center with two cysteines, which upon oxidation can form a disulfide bridge. Thioredoxin reductase and Glutathione, subsequently reduce the oxidized thioredoxin and glutaredoxin, respectively, in a NADPH-dependent manner (Petropoulos and Friguet 2006); since the reduction of the oxidized thioredoxin and glutaredoxin is necessary for their further function.

In the Thioredoxin (TRX) system, thioredoxin with two –SH groups, transfers the hydrogen atom to the protein containing the disulfide (S-S) bridge. The protein is then reduced to its normal form with a -SH group and thioredoxin is oxidized with a subsequent disulfide bridge formation on it. For the reduction of the oxidized thioredoxin, thioredoxin reductase transfers the electrons from NADPH by the help of the flavin and thioredoxin function is restored (Poppek and Grune 2005).

In the Glutathione system, glutaredoxin (GRX) reduces the oxidized protein and then two glutathione molecules transfer the hydrogen atom from NADPH and reduce the oxidized glutaredoxin non-enzymatically. The oxidized glutathione is further reduced by the action of the glutathione reductase (Poppek and Grune 2005). Instead of glutathione reductase, another protein called thiol transferase can also act for the reduction of disulfide bridges (Poppek and Grune 2005). The GSH-dependent cytosolic thiol transferase dethiolates the protein-S-S-glutathione and restores the free thiol groups which are necessary for the normal protein function (Poppek and Grune 2005).

This enzyme has also shown to be highly resistant to oxidation via hydrogen peroxide, whereas glutathione reductase is severely inactivated with hydrogen peroxide (Poppek and Grune 2005).

There also exist enzymatic antioxidant defense systems that act as direct detoxifiers instead of acting through redox regulation. Superoxide dismutases (SOD), catalases, and peroxidases are the main enzymatic ROS detoxifiers. SODs act as antioxidants via converting superoxide anion into hydrogen peroxide and they require redox active metals for their activity (Herrero, et al. 2008) similar to catalases, which act in reduction of hydrogen peroxide. Unlike SODs and catalases, peroxidases reduce peroxides into alcohol using active site cystein thiols (Herrero, et al. 2008).

As being the primary sites of ROS production, mitochondria are also the major sites of ROS attack. Therefore, there exists specific antioxidant defense genes in mitochondria against ROS dependent damages (Unlu and Koc 2007). There exists ten specific genes whose functions are known to be involved in mitochondrial antioxidant defense system. These are superoxide dismutases (SOD1 and SOD2), copper chaperone protein (CCS1), glutaredoxins (GRX2 and GRX5), cytochrome c peroxidase (CCP1), glyoxylase II (GLO4), thioredoxins and their reductases (TRX3 and TRR2), and peroxiredoxins (PRX1) (Unlu and Koc 2007).

1.2.4.1 Mitochondrial Antioxidant Genes and Their Functions

Superoxide dismutases (Sods) function as direct detoxifiers of superoxide radicals via converting them into H_2O_2 since H_2O_2 is less toxic than $O_2^{\cdot -}$. There are two main superoxide dismutases in mitochondria of S.cerevisiae, which are Sod1 and Sod2 (Herrero, et al. 2008). Sod1 is a Cu-Zn dependent enzyme which is localized both in the cytosol and at the mitochondrial intermembrane space, whereas, Sod2 is a Mn-dependent enzyme that is localized in the mitochondrial matrix. The copper chaperone protein Ccs1 is an essential protein for Sod1 function since it carries the copper ions to Sod1, which is necessary for its proper function, and plays role in the maturation of Sod1 (Herrero, et al. 2008). Ccs1 is located both in the mitochondrial inner membrane space and in the cytosol.

Mitochondrial glutaredoxins, Grx2 and Grx5, play role in maintaining the redox state of a cell via reducing the protein disulphides and glutathione-protein mixed

disulfides. Grx5 also participates to the Fe/S cluster biosynthesis of the respiratory complexes (Unlu and Koc 2007). Grx2 proteins, which are located in the mitochondrion as well as in the cytosol, have two cystein motifs in their active sites, while Grx5 proteins that are located in the mitochondrial matrix, have only one cystein motif (Unlu and Koc 2007).

Cytochrome c peroxidase (Ccp1), which is localized in mitochondrial intermembrane space, acts as antioxidant defense enzyme via converting the H_2O_2 to water under aerobic conditions (Unlu and Koc 2007). Glyoxylase-II (Glo4) functions in the hydrolysis of S-D lactoylglutathione into glutathione and D-lactatase and therefore acts as an antioxidant enzyme of mitochondria (Unlu and Koc 2007).

Thioredoxin system in the mitochondria has two important components, which are thioredoxin (Trx3) and thioredoxin reductase (Trr2). They regulate the redox homeostasisand are both located in the mitochondrion. Trx3 reduces the oxidized thiol groups of proteins and is reduced by Trr2 for its proper re-function (Herrero, et al. 2008).

Peroxiredoxins, which are ubiquitous peroxidases located in the mitochondrion, reduce hydroperoxides (Chang, et al. 2004). They control cytokine-induced peroxide levels and regulate the signal transduction in mammalian cells. Peroxiredoxins (Prx) are divided into three groups: typical 2-Cys peroxiredoxins, atypical 2-Cys peroxiredoxins and 1-Cys peroxiredoxins. All proxiredoxins exist as homodimers and contain a conserved cystein residue in their N-terminal region (Woo, et al. 2005). They only differ in that typical 2-Cys peroxiredoxins, which are Prx I, II, III and IV, have an additional conserved cystein residue in their C terminal region, whereas, atypical 2-Cys (Prx V) and 1-Cys peroxiredoxins do not (Chang, et al. 2004).

1.3 Aging

Aging is a complex process that is generally characterized by accumulation of some metabolic products due to excessive molecular oxidation and to the decline in physiological functions of an organism throughout its life (Longo, et al. 2005). The free radical theory of aging, which is first proposed by Denham Harman in 1956, is the most widely accepted theory of aging (Wickens 2001). Upon aerobic metabolism, approximately 2% of the consumed oxygen is converted into free radicals. From this

point, Harman proposed that the damages caused on biomolecules by the free radicals can be the pioneer of the aging process and age-related disorders (Wickens 2001).

There are different mechanisms associated with the aging and age-related disorders, such as telomere shortenening, and differences in gene expression patterns (e.g. oncogenes). The free radical theory is one of them and the formation of biomolecular damages upon free radical attack is shown to induce the aging process in many studies.

Protein oxidation is shown to take place during aging process and upon this oxidation the induction of tertiary structural changes in proteins result in the formation of protein aggregates (Squier 2001) that block the removal of these unfunctional proteins from the cell. Protein repair systems are also shown to be impaired with age upon excessive oxidation of repair system proteins (Carrard, et al. 2002). Both the increase in the number of oxidized, unfunctional proteins and the decrease in the ability of cells to degrade and remove them, lead to the impairment of the redox balance in a cell and result in aging of cell and subsequent cell death.

The cell membrane is mainly composed of lipids. Therefore, lipids are one of the easy targets for the ROS attack. Oxidation of lipid molecules is also shown to increase with age, which is an indicator that lipid peroxidation is also a contributor of the aging process (Pratico 2002). Lipid peroxidation is especially important for the post-mitotic cells, since in these cells the lipid turnover rate is lower than the mitotic cells (Pratico 2002). The levels of lipid peroxidation products, such as melandialdehyde (MDA), and hydroxyalkenals (HNE) were shown to increase with age especially in post-mitotic cells, such as brain, heart, and skeletal muscle cells (Pratico 2002).

Among different model organisms to study aging and understand its basic mechanims, yeast S.cerevisiae has been a good model because of its wide characterized genes that shows a great homology with higher eukaryotes, short life-span, and easy manipulation (Bitterman, et al. 2003). Yeast and human aging have the same fundamental biology (Bitterman, et al. 2003). Therefore yeast aging models are good mimickers of human aging. There are two aging patterns in yeast; the replicative life-span and the chronological life-span. Replicative life-span, which is the aging model of mitotic cells in higher eukaryotes, is referred as the number of divisions that a yeast cell undergoes, and the chronological life-span, which is the aging model for the post-mitotic cells in higher eukaryotes, is described as the time period that a yeast cell remain viable in a non-dividing state (Bitterman, et al. 2003). Chronological life span is

an interesting model to study aging, since its high metabolic state is thought to resemble more to the metabolic activities of post-mitotic cells of higher organisms. The molecular mechanisms of chronological aging is also important for understanding the age related disorders, such as neurodegenerative disorders.

It has been shown in many studies that molecular damage accumulation is greater in post-mitotic cells when compared to the actively dividing, mitotic cells (Pratico 2002). Protein oxidation and lipid peroxidation are shown to be related with many age related disorders (Poppek and Grune 2005). Since ROS are primarily formed in the mitochondria, understanding the specific roles of mitochondrial antioxidant genes in defense against oxidative stress is important for the assesment of their roles in the aging and age-related disorders of eukaryotic cells.

Up to date, only superoxide dismutases SOD1 and SOD2 were shown to have effects on both replicative and chronological life span of yeast model via acting against oxidative stress defense (Longo, et al. 1996). Copper chaperone protein Ccs1 was also shown to have effect only in replicative life span in budding yeast S. cerevisiae (Unlu and Koc 2007). The effects of deletion of other mitochondrial antioxidant genes were only shown during replicative life span of yeast in the study of Unlu and Koc. However, there is no data exists to date that shows the effects of deleting these antioxidant genes on chronological aging model of yeast. Therefore, with this study, we aimed to see the effects of these mitochondrial antioxidant genes on chronological life-span and to observe the differences in biomolecular damages upon chronological aging among different mitochondrial antioxidant gene deletions.

CHAPTER 2

MATERIALS AND METHODS

2.1 Methods

The detailed experimental procedures used in this study are indicated below and each experiment is repeated for at least two times.

2.1.1 Yeast Strains

The BY4741 strain of the budding yeast *Saccharomyces cerevisiae* is used throughout this study. The wild type strain (BY4741) and the ten isogenic deletion mutants are all haploids with the mating type 'a' (having the MATa locus) and are exempted from the genes that synthesize the amino acids *his3, leu2, met15*, and *ura3 (*MATa, *his3, leu2, met15, ura3)*. The ten antioxidant gene deletion mutants also do not contain the specific gene that is deleted and instead of the deleted gene, they contain a kanamycin resistance gene (MATa, *his3,leu2, met15, ura3*, Deleted geneΔ : KAN^R).

2.1.2 Mitochondrial Antioxidant Gene Selection

The mitochondrial antioxidant genes were identified by using Mitochondrial Proteome Database, which is an internet based software (Andreoli et al. 2004).

2.1.3 Selection of Young and Old Yeast Cells

The cells were inoculated in 5 ml YPD media and incubated at 30°C at 180 rpm overnight. The overnight grown cells were subsequently transferred into fresh YPD media and incubated for 3 hours at 30°C. The cells are then washed with dH_2O for two times and pelleted. This procedure is also followed for the cell growth in YPG media,

except that the cells were incubated in fresh media for 25 hours. These cells were considered as 'Young cells'.

The cells that are considered as 'Old cells' are grown in either YPD or YPG media for 15 days after transferring the overnight YPD grown cells into fresh media following the 2X dH_2O wash.

2.1.4. Colony Formation Unit (CFU) Assay

The chronological aging pattern of yeast cells are identified by using the colony formation unit (CFU) assay (Parrella and Longo 2008).

Yeast strains were grown in 5 ml YPD media overnight at 30°C at 180 rpm. 2 ml of overnight grown cell cultures are washed with 2X dH_2O and suspended in 2 ml YPD. 23 ml of fresh YPD media is added on cell suspension and the cultures are transferred into 250 ml flasks with the volume/medium ratio of 1:10. The cells were incubated at 30°C at 180 rpm for 15 days. Starting from the 3rd day, 100µl cell sample is taken and diluted for the 'Thoma Lam' counting. According to the Thoma Lam counts, approximately 1000 cells were strewed onto YPD agar plates and incubated at 30°C for 2 days. The grown colonies are counted and the ability of cells to form a colony is calculated by the formula below;

CFU % = (100 X counted colony number) / (strewed cell number)

This survival is monitored repeatedly in every 72 hrs for each strain until the 15th day of the starting culture is reached. CFU assay is done both in YPD media and YPG media in order to monitor the chronological aging pattern differences in different carbon sources.

2.1.5. Determination of Protein Carbonylation and Lipid peroxidation Levels

2.1.5.1. Cellular Extraction

15 ml of the cells that were grown in either YPD or YPG media, were pelleted and washed with dH_2O for two times. 1 ml of PBS, 500µl Glass beads, 50 µl of 100mM PMSF (phenylmethylsulfonylfluoride) and 10 µl of 0.5M BHT were added on cell

pellets and vortex is done for 15 min while keeping the cell pellets cold. The cell suspensions were centrifuged at 3000 rpm for 3 min. The supernatants were stored at -80°C in order to use in protein carbonylation and lipid peroxidation assays.

2.1.5.2 Protein Carbonylation Assay

For the protein carbonylation assay, DNPH (2,4-dinitrophenylhydrazine) assay is used. In this assay, DNPH reacts with carbonyl groups of proteins, namely with aldehydes and ketones, and lead to the formation of the stable 2,4-dinitrophenyl (DNP) hydrazone product. Since DNP group itself can absorb UV light, the carbonyl contents can be determined via spectrophotometric measurements.

During DNPH assay, 1 ml of cell extracts of each strain is taken and divided equally into two eppendorf tubes, which one is marked as control and the other is marked as test sampes. 500 µl of 40% TCA is added on cell extracts to reach the final TCA concentration of 20%. Following a gentle mix, centrifuge is done at 5000 rpm for 5 min. The supernatant parts are discarded and 400 µl 2M HCl is added on control samples while 400 µl 10mM DNPH dissolved in 2M HCl is added on test samples. The samples are incubated at dark for 1 hour at room temperature with a gentle shake in every 10 min. 400 µl of 40% TCA is added on samples with the final TCA concentration of 20% and vortex is done. Subsequently, centrifuge is done at 10000 rpm for 5 min and the supernatants are discarded. The pellets were washed with 20% TCA, followed by a 2X wash with 500 µl of ethanol:ethyl acetate (v/v: 1/1) . The pellets are then suspended in 1 ml of 6M Guanidine HCl and incubated ate 37°C for 15 min. The spectrophotometric read is done at 366nm by using 6M Guanidine HCl as a blank. After the normalization of the results, the carbonyl contents of each strain were graphed.

2.1.5.3 Lipid Peroxidation Assay

The BIOXYTECH® LPO-586™ colorimetric assay of OxisResearch™ is used for the determination of the lipid peroxidation levels of the yeast strains. This assay is based on the reaction of N-methyl-2-phenylindole with Malondialdehyde (MDA) and 4-hydroxyalkenals at 45°C. One molecule of either MDA or 4-hydroxyalkenal reacts with

2 molecules of N-methyl-2-phenylindole and yield a stable chromophore with the maximum absorbance at 586nm.

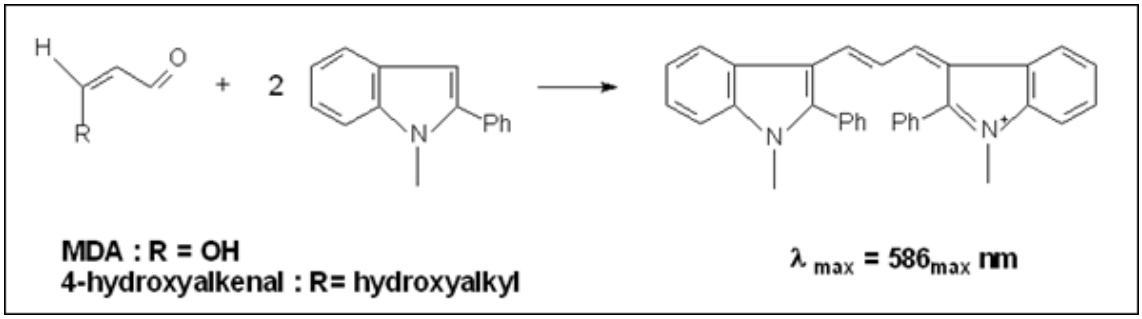

Figure 2.1. The reaction of either MDA or 4-hydroxyalkenal with 2 molecules of N-methyl-2-phenylindole that results in the formation of a stable chromophore.

The standard curve for the MDA levels are prepared by using the acetal Malondialdehyde (TMOP) since the aldehyde is not stable itself. The 10mM stock solution of TMOP standard is diluted 1/500 (v/v) in deionized water to yield a 20µM stock solution for use in the assay. The standards with different concentrations were prepared as shown in Table 2.1.

Table 2.1. Standard curve dilution volumes

Target concentration of standard in reaction mixture, µM	**0**	**1.00**	**2.00**	**4.00**
Volume of 20 µM standard to add (ml)	0	50	100	200
Volume of dH2O used for diluting the sample to add (ml)	200	150	100	0

Methansulfonic acid (MSA) is used for the simultaneous determination of both MDA and 4-hydroxyalkenal, while HCl is used to determine only the MDA levels in the cell extracts since 4-hydroxyalkenals do not form a chromophorewith N-methyl-2-phenylindole in the presence of HCl. In this study we used the HCl procedure to determine only the MDA levels in cell extracts.

The standards were prepared according to the Table 2.2 and run at triplicates to form the MDA standard curve. 200 µl of the cell extracts were put into eppendorf tubes and 650 µl N-methyl-2-phenylindole (R1 reagent) is added. The samples were vortexed gently and 150 µl 12N HCl is added. The samples were vortexed and incubated at 45°C for 60 min. The samples are then centrifuged for 10 min at 15000 x g and the supernatants were measured for MDA levels by spectrophotometric measurement at 586 nm. 325 µl (75% acetonitrile+ 25% diluent) is used instead of R1 reagent as the blank of the assay and whole the assay procedure is applied further on.

The MDA concentrations of the strains were calculated by the following formula;

$$[MDA] = a[MDA] + b \quad (2.1)$$

$$[\text{MDA}] = ((A_{586}\text{-b})/a) \text{ x df} \quad (2.2)$$

where; A_{586} is the net absorbance of the sample at 586nm, [MDA] is the µM concentration of MDA in the sample, a is the 'regression coefficient (slope)', b is the 'intercept', and df is the 'dilution factor'.

2.1.5.4. Normalization of the Protein Carbonylation and Lipid Peroxidation Measurements

The normalizations of the protein carbonylation and lipid peroxidation measurements were done according to the protein contents of the strains. Bradford Assay is used for the protein content measurements. 1µl of protein extracts were added on 99 µl of dH_2O and 100 µl of Commassie blue dye. After 5 min incubation at room temperature at dark, spectrophotometric measurement is done at 595nm. The standard curve that is used for the protein concentration calculations is obtained from the spectrophotometric measurements of the BSA samples with the concentrations 1, 2, 4, 6, 8, 10, and 20µg/ml at 595nm. The protein carbonylation and lipid peroxidation levels were then divided to the protein concentrations for normalization.

2.1.6 Determination of Genomic Instability

2.1.6.1 Determination of spontaneous Nuclear DNA mutation rates

The spontaneous genomic mutation rates were determined by using the Canavanine resistance assay. The cells were grown in 5 ml YPD at 30°C at 180 rpm overnight and transferred into a fresh YPD media after 2XdH_2O wash. The cells were grown in fresh YPD media for 3 hours and washed with dH_2O for two times and suspended in 500 µl dH_2O. The samples were diluted and Thoma Lam counting was applied. For each strain, 10^7 cells were strewed on two YNB-Arg + Can plates, which include 60mg/L Canavanine. The same amount of cells is also strewed on YPD plates as experimental controls. The cells were incubated at 30°C for 4 days and the colonies were counted. Same procedure is followed for the YPG grown cells, except that the cells were incubated in fresh YPG media for 25 hours instead of 6 hours.

2.1.6.2. Determination of Spontaneous Mitochondrial DNA Mutation Rates

The mitochondrial DNA mutation frequencies were determined via Erythromycin assay. The overnight YPD grown cells were taken into fresh media and incubated for 3 hours in fresh YPD media. 10^4 cells were strewed on YPEG agar plates (including 3%EtOH and 3% glycerol) with the final erythromycin concentration of 2mg/ml. YPD agar plates are used as control plates and 10^2 cells were seeded on the control plates. The plates were incubated at 30°C for 15 days and observed for their colony formation ability. The same procedure was followed for the YPG plates, except that the cells were incubated in fresh YPG media for 25 hours before strewing on agar plates.

2.1.7 Measurement of Superoxide Production Rates

The superoxide production rates of the yeast strains were measured via fluorometric dye MitoSOX^{TM} Red from Molecular Probes^{TM}. MitoSOX Red is designed

for live-cell imaging and is selectively targeted to the mitochondria and oxidized by the superoxide in the mitochondria and subsequently exhibits a red fluorescence at Ex/Em: 510/580.

Cells were incubated in 5 ml YPD at 30°C at 180 rpm overnight. The OD_{600} values of the overnight grown samples were adjusted to approximately 0.2 with fresh media. The cells were incubated for 2 hours at 30° C at 180 rpm. 10^7 cells were taken and 250 µl of YPD containing 5µM of MitoSOX Red dye is added on each test samples and 250µl of YPD is added on each control samples. After 1 hour of incubation at 30°C, the samples were washed with PBS for two times and fluorometric detection is done at Ex/Em: 510/580 with flow cytometry. The same procedure is followed on for YPG grown cell, except that cells were grown in fresh YPG media for 24 hours and in YPG containing 5µM of MitoSOX Red dye for 1 hour.

2.1.8 Determination of Oxidative Stress Tolerances

The oxidative stress tolerances of yeast strains were examined by using three different oxidative stress generating agents; which are hydrogen peroxide (H_2O_2), diamide and menadione.

2.1.8.1 HALO Assay (H_2O_2 resistance)

Halo assay is used to test the sensitivities of different stress against hydrogen peroxide. The cells were grown overnight in YPD and taken into fresh YPD where they are incubated for 3 hours at 30°C at 180 rpm. The OD_{600} values of the cells were adjusted to 0.2 and 50.10^6 cells were strewed on YPD plates. After 30 min incubation, 5µl of 8.8M H_2O_2 was dropped into the center of the YPD plate and the plates were incubated at 30°C for 2 days. The diameter of the hole at the center of the plate was measured to determine the H_2O_2 sensitivity of the mutants and the wild type strain.

2.1.8.2 Diamide Resistance

Diamide oxidizes the small thiols and decreases the glutathione pool in a cell rapidly (Thorpe et al. 2004). Therefore it is considered as an oxidative stress generating agent.

Spotting assay is done to observe the diamide sensitivities of the strains. The overnight grown cell cultures are diluted to OD_{600} value of 0.2. Then a dilution series is formed with OD_{600} values of $2x10^{-1}$, $2x10^{-2}$, $2x10^{-3}$, and $2x10^{-4}$. 5µl of each dilution is dropped on YPD plates containing 1,5mM. 2,0 mM and 2,5mM diamide. YPD plates with no diamide are used as control plates. After 2 days of 30C incubation, the sensitivies of strains against different concentrations of diamide were observed and compared to each other. Same procedure is followed for YPG grown cells, except the agar plates were YPG plates instead of YPD.

2.1.8.3 Menadione Resistance

Menadione is a polycyclic aromatic ketone, which increases the intracellular superoxide levels by distrupting the GSH-GSSH redox balance in a cell and facilitating the intracellular O_2^{2-} accumulation (Pocsi et al. 2005).

The same procedure is followed as in diamide resistance assay, except that the menadione concentrations were 0,1mM, 0,25mM and 0,5mM for YPD grown cells while 0,025mM, 0,05mM and 0,1mM for YPG grown cells. The menadione concentrations were adjusted by using 100% EtOH and the control plates were containing 100% EtOH only.

CHAPTER 3

RESULTS

3.1 Chronological Aging Patterns

For the chronological life span patterns of different mitochondrial antioxidant gene mutants, colony formation assay was applied. The mutant strains were both grown in YPD media, which is a glycolytic carbon source and in YPG media, which is a respiratory carbon source.

In YPD media, almost all mutant strains have shown stable aging patterns, in which they showed a stable decrease in their colony formation abilities. The colony formation unit (CFU) percentages of *grx2*Δ, *ccp1*Δ, *sod1*Δ, *trr2*Δ, *trx3*Δ, and *sod2*Δ showed 7%, 3%, 5%, 6%, 5% and 7% decrease, respectively, at day 15 of chronological aging when compared to the WT strain (Figure 3.1).

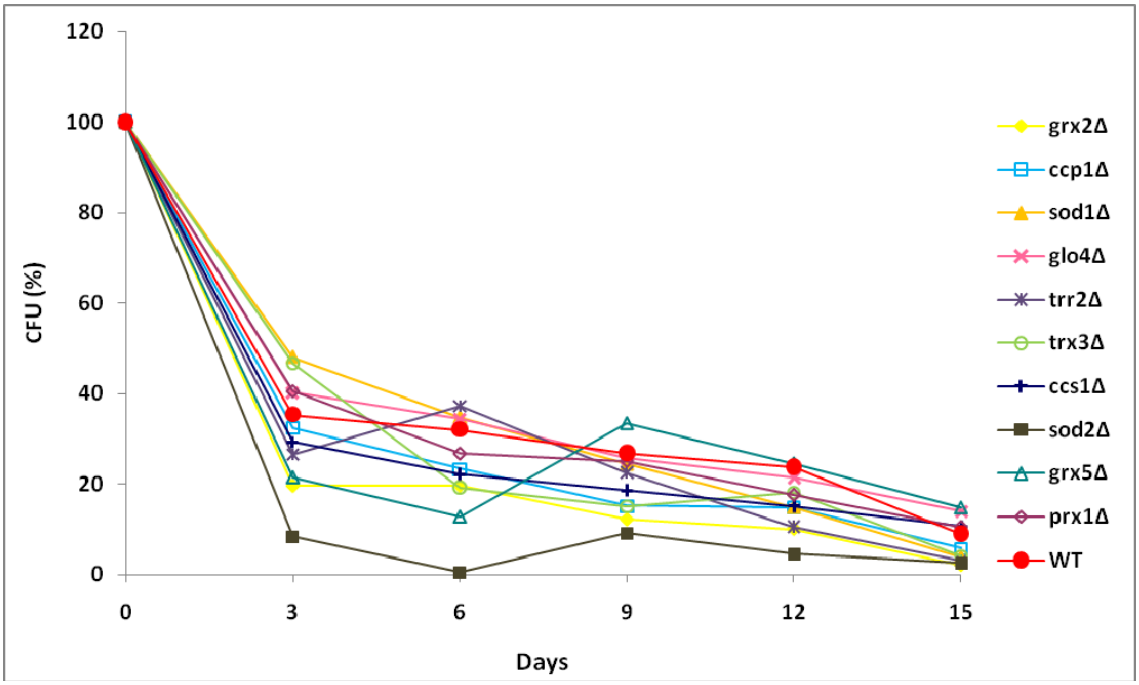

Figure 3.1. Colony formation abilities of antioxidant gene mutants and the wild type strain (BY4741) of S.cerevisiae in YPD media.

In YPG media, where strains undergo mitochondrial respiration in order to survive, *grx2*Δ and *glo4*Δ showed stable aging pattern, in which they showed a stable decrease in their colony formation ability. *trr2*Δ, *ccs1*Δ, *grx5*Δ, and *prx1*Δ entered in stationary phase at day6. *trr2*Δ and *ccp1*Δ showed a diauxic shift at day12 of their incubation, while *grx5*Δ and *trx3*Δ showed at day 15 as shown in Figure 3.2.

The colony formation unit (CFU) percentages were lower in *ccs1*Δ and *glo4*Δ mutant strains. Their CFU percentages were 20% and 11% lower, respectively, when compared to the WT strain percentages.

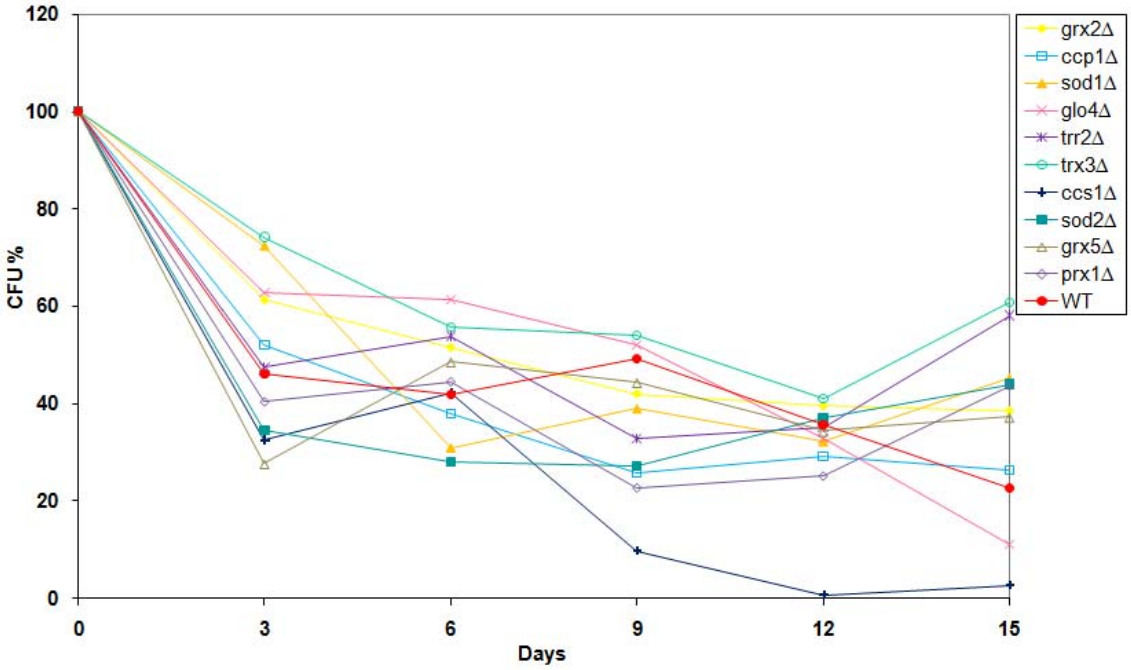

Figure 3.2. Colony formation abilities of antioxidant gene mutants and the wild type strain (BY4741) of S.cerevisiae in YPG media.

The colony sizes also differed among different mutants. *ccs1*Δ, *sod1*Δ and *sod2*Δ mutant colonies were smaller in size when compared to other mutant colonies and WT.

3.2 Biomolecular Damages

3.2.1. Protein Carbonylation

For protein carbonylation, DNPH assay is used. As shown in Figure 3.3 When young YPD grown young mutants were compared among themselves, *grx2*Δ, *ccp1*Δ,

*sod1*Δ and *glo4*Δ mutants showed 25%, 19%, 11%, and 13% increase in their protein carbonyl contents when compared to the WT young strain, while *ccs1*Δ, *sod2*Δ, and *grx5*Δ showed 57%, 58%, and 53% decrease. When comparison of chronologically aged YPD grown mutants with the chronologically aged WT strain were examined, it is seen that *ccs1*Δ and *grx5*Δ mutants showed 57% and 48% increase in their protein carbonyl contents, respectively. Chronologically aged *grx2*Δ and *ccp1*Δ mutants also showed 24% and 18% increase in protein carbonylation levels, respectively. When YPD grown young and chronologically aged mutant populations were compared among themselves, the protein carbonylation was shown to increase significantly in chronologically aged populations (p value = 0,041 by using Mann Whitney-U test).

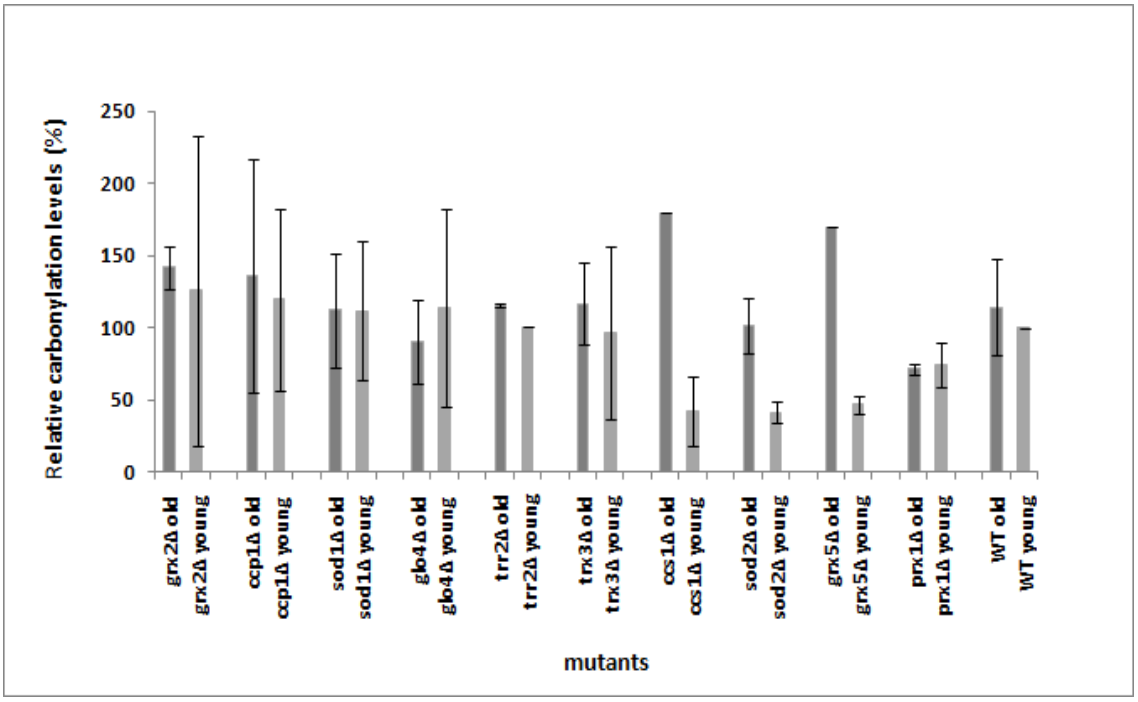

Figure 3.3. Cellular protein carbonylation levels in WT and mitochondrial antioxidant gene mutants that were grown in YPD media

When cells are grown in YPG media, chronologically aged cells again showed higher protein carbonyl contents, except *ccp1*Δ, in which the protein carbonylation level was higher in *ccp1*Δ young cells.

When young strains are compared among themselves, *grx2*Δ, *ccs1*Δ, and *ccp1*Δ showed 2-folds, 1.5-folds, and 3-folds increase in their protein carbonyl contents, respectively, when compared to the young WT strain, while *sod2*Δ, and *grx5*Δ strains showed decreased levels of protein carbonyl content.

The old strains showed relatively higher levels of protein carbonylation to their young strains. When compared to the old WT strain, *sod1*Δ, *grx5*Δ, ccs1Δ mutants showed a 2-folds increase in protein carbonyl contents. *glo4*Δ, *trr2*Δ and *grx2* showed, a 1.5-fold, 3-folds, and 4-folds increase in protein carbonyl content,respectively, as shown in Figure 3.4. When YPG grown young and chronologically aged mutant populations were compared, the lipid peroxidation levels were shown to be significantly increased upon chronological aging (p value= 0,005 by using Mann Whitney-U test).

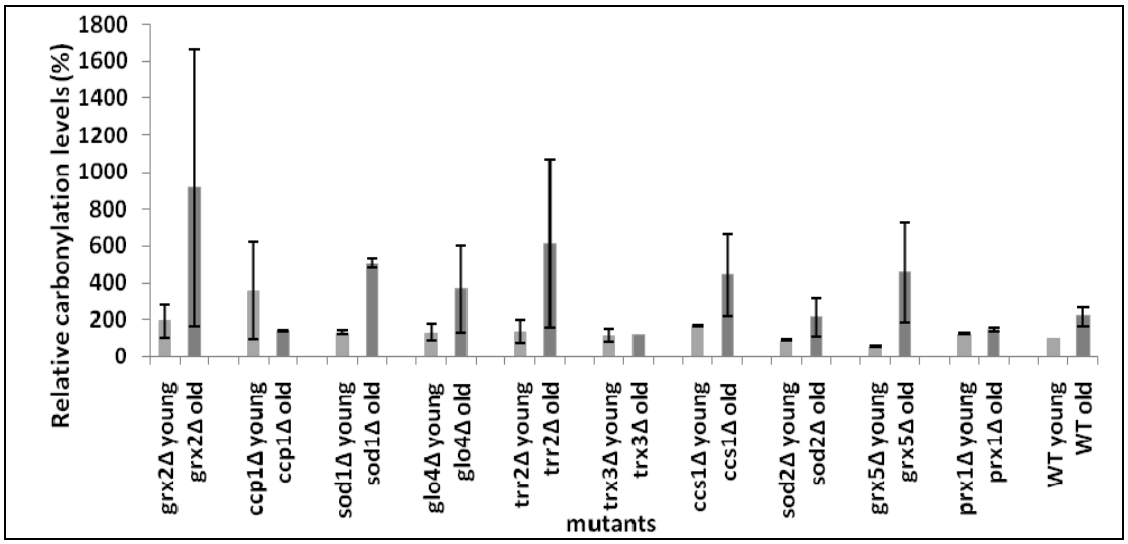

Figure 3.4. Cellular protein carbonylation levels in WT and mitochondrial antioxidant gene mutants that are grown in YPG media

3.2.2. Lipid Peroxidation

The lipid oxidation of different mitochondrial antioxidant gene mutants and WT strains were measured via measuring the MDA levels in these cells.

When YPD grown young mutants were compared with the young WT strain, *grx2*Δ, *ccp1*Δ, and *sod1*Δ showed 81%, 10%, and 34% increase in MDA levels. However, in YPD grown chronologically aged mutants, *grx2*Δ, *ccp1*Δ, *sod1*Δ, *trr2*Δ, and *ccs1*Δ showed 69%, 38%, 46%, 82% and 81% increase in lipid peroxidation levels, respectively, when compared to the chronologically aged WT cells. When YPD grown young and chronologically aged populations were compared, the lipid peroxidation levels were shown to be significantly higher in chronologically aged population as shown in Figure 3.5 (p value = 0,000 by using Mann Whitney-U test).

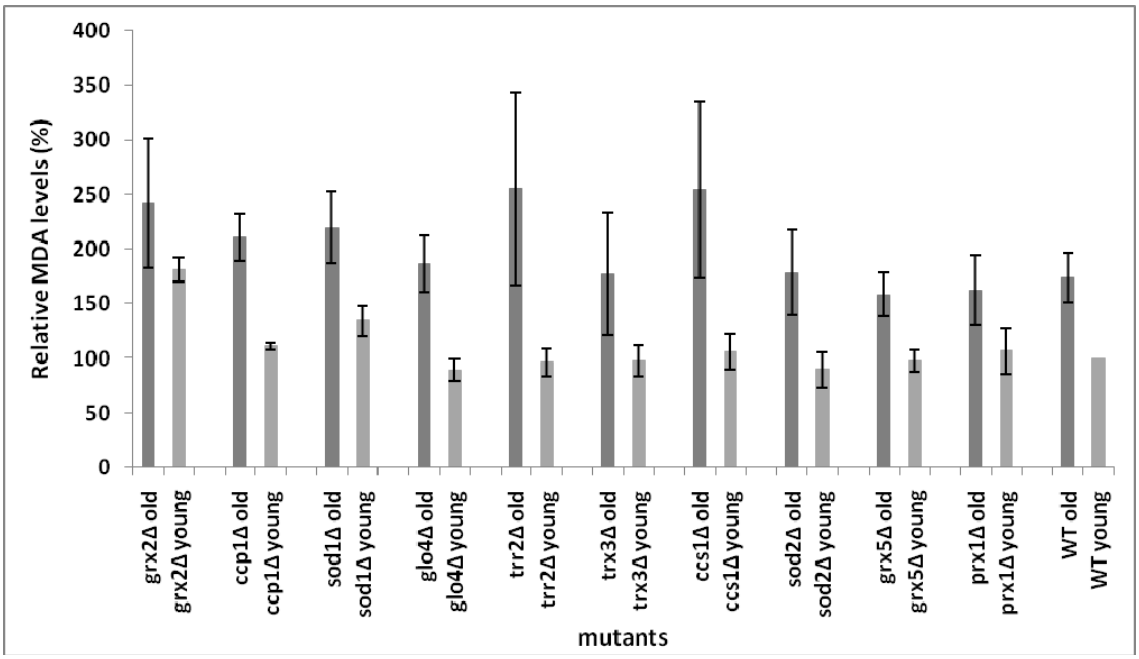

Figure 3.5. Relative cellular lipid peroxidation levels of WT and mutant cells grown in YPD media

When cells were grown in YPG media, the lipid peroxidation level were generally higher in old cells, except *trx3*Δ *and sod2*Δ strains, as shown in Figure 3.6. When young strains are compared among themselves, only *sod2*Δ*, ccs1*Δ*,* and *trx3*Δ strains showed higher levels of MDA when compared to the young WT strain. The MDA levels were lower than the WT strain in other mutants. When old strains were compared among each other, all mutant strains showed relatively higher MDA levels compared to old WT strain, except *glo4*Δ mutant. The old *ccs1*Δ, *prx1*Δ*, grx5*Δ*,* and *ccp1*Δ mutants showed 7-folds, 2-folds, 3-folds, 2-folds increase in MDA levels, respectively, when compared to old WT strain.

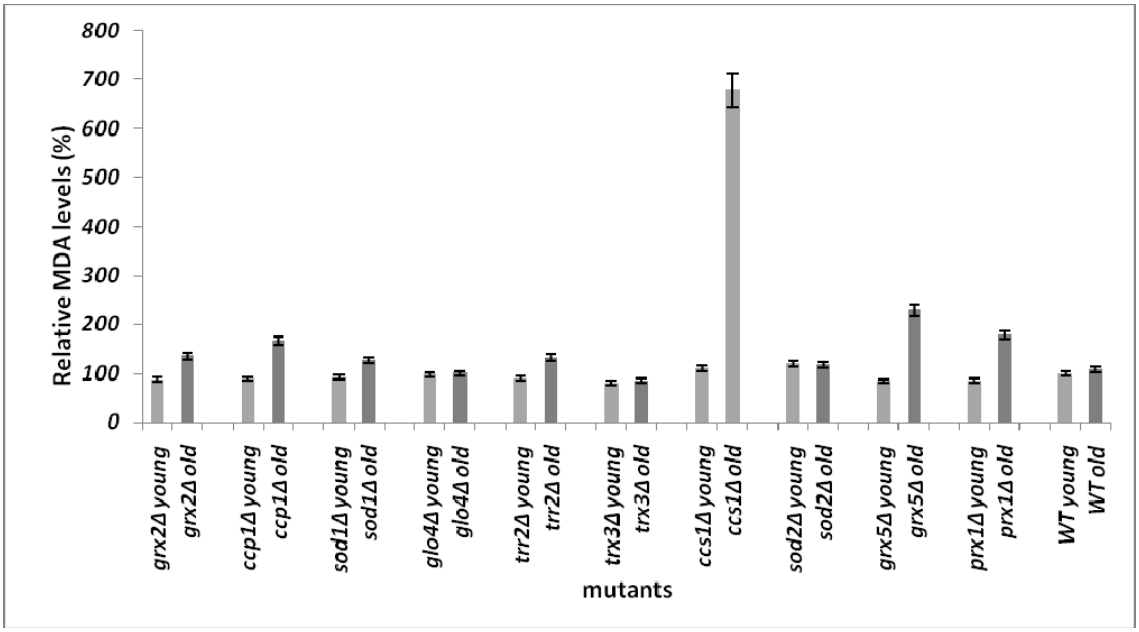

Figure 3.6. Relative cellular lipid peroxidation levels of WT and mutant cells grown in YPG media

3.2.3. Genomic Instability- Spontaneous Mutation Rates

The genomic instability of the mutants and the WT strain was measured via canavanine resistance assay. Canavanine is a competitive inhibitor of the arginine permease that is encoded from the CAN1 locus. Canavanine inhibits arginine and stops translation process. Therefore, cells cannot grow if CAN1 gene is normally functional. However, due to spontaneous mutations, if CAN1 locus is mutated, cells do not uptake canavanine and can continue to survive.

As it is shown in Figure 3.7, when grown in YPD media, *glo4*Δ*, ccs1*Δ*,* and *sod1*Δ mutant strains showed significant increase in spontaneous mutation rates. The increase in mutation rates is shown to be increased in chronologically aged cells.

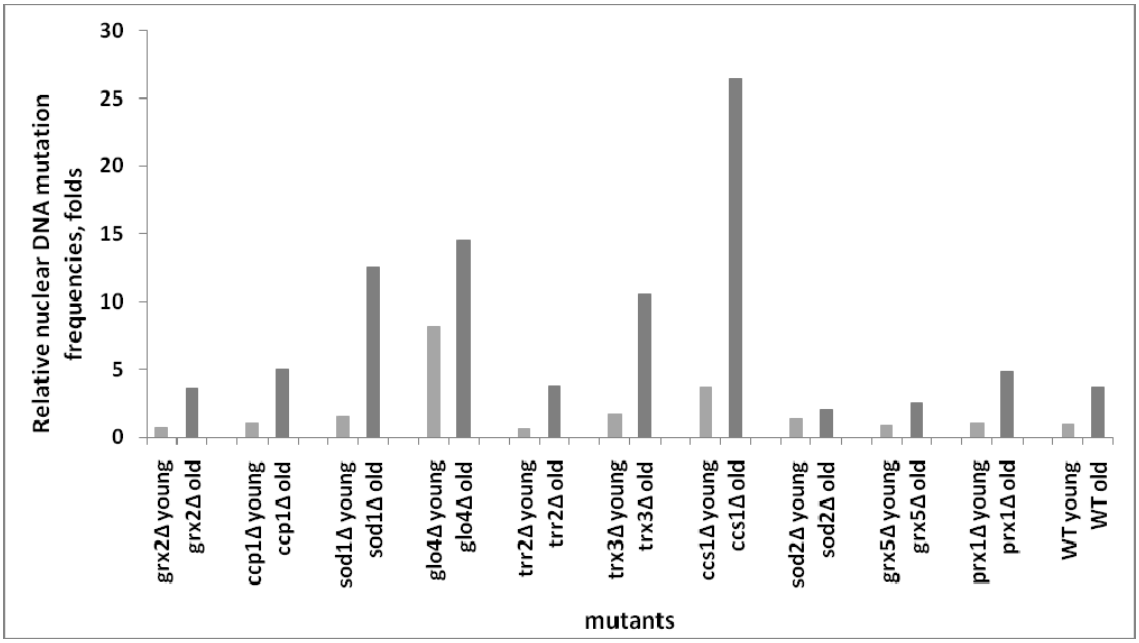

Figure 3.7. Relative spontaneous mutation rates of WT and mutant strains grown in YPD media

In YPG grown strains; *sod1*Δ*, glo4*Δ*,* ccs1Δ and *sod2*Δ mutants showed increased spontaneous mutation rates when compared to WT strain. The mutation rates is also shown to be increased in the chronologically aged cells as shown in Figure 3.8.

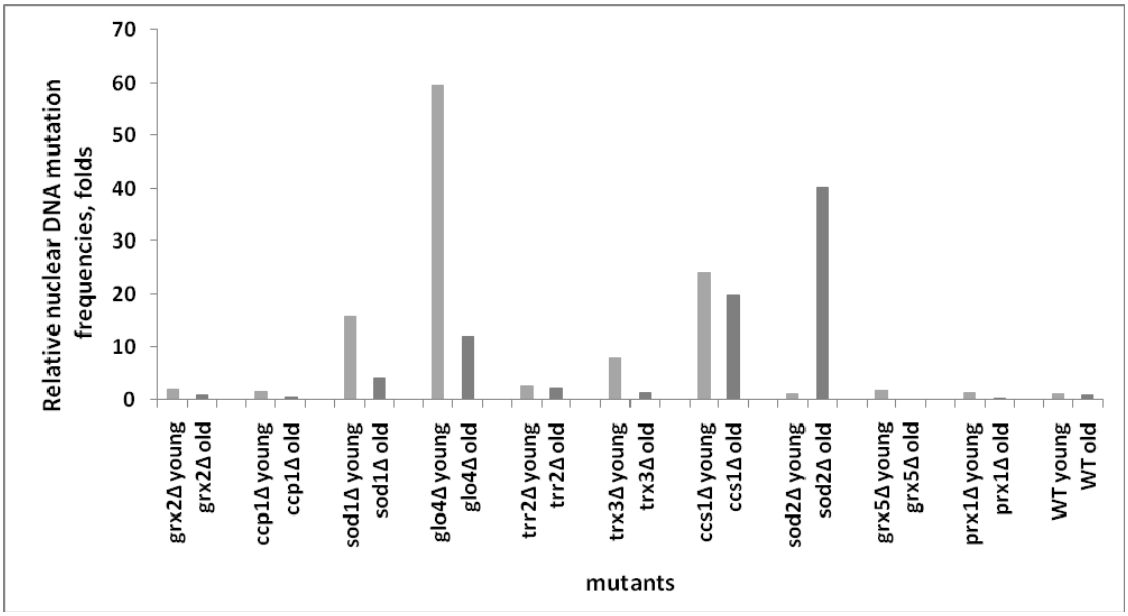

Figure 3.8. Relative spontaneous mutation rates of WT and mutant strain grown in YPG media

The mitochondrial mutation rates were measured by using erythromycin assay. Erythromycin is an antibotic that inhibits mitochondrial translation. Point mutaiotns in the mitochondrial large ribozomal RNA genes, rib2 and rib3, result in drug resistance. So erythromycin assay is a direct measurement of mitohondrial DNA point mutations. In YPD grown cells, grx2Δ, ccp1Δ, and glo4Δ mutants showed 311, 632 and 275 fold increased mitochondrial DNA mutation rates, respectively. Figure 3.9 shows the relative erythromycin resistance among mutant strains that were grown in YPD media.

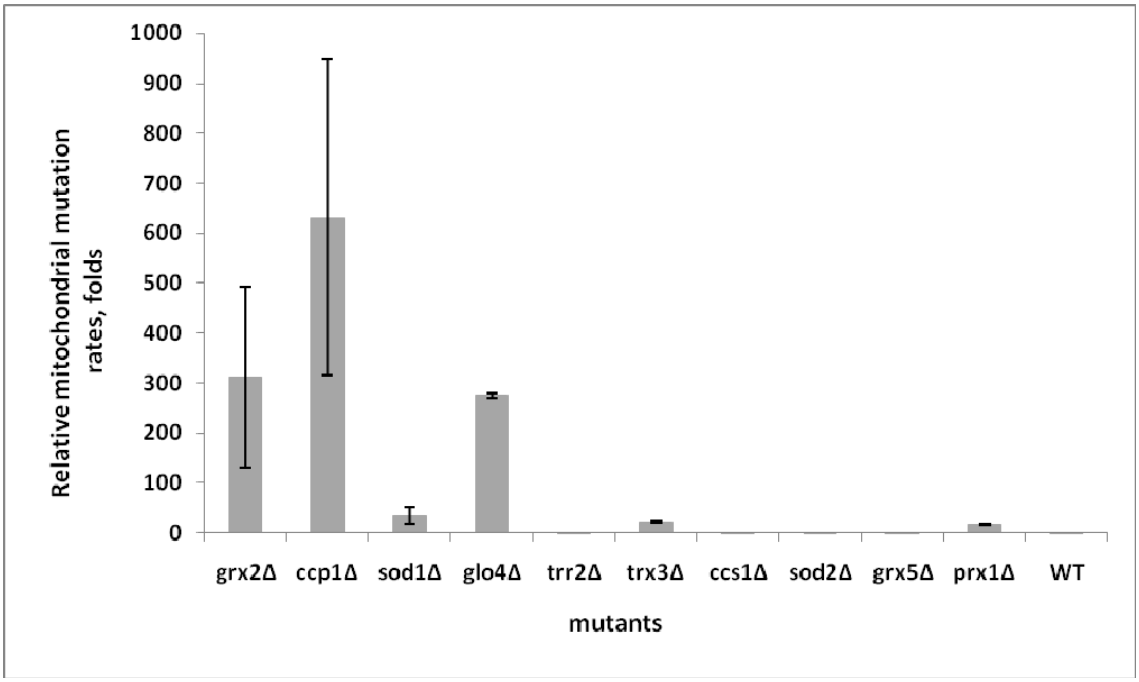

Figure 3.9. Relative mitochondrial mutation rates of WT and mutant strains

3.3 Superoxide Production Rates

The superoxide levels in different mitochondrial antioxidant gene mutants and their comparison with WT strain is shown in Figrure 3.10. Chronologically aged cells showed greater superoxide levels. Only the old Δtrr2 strain showed greater superoxide levels when compared to old WT strain. However, when different strains are compared among themselves, *trr2*Δ, *ccs1*Δ, *sod2*Δ showed greater superoxide accumulation in their mitochondria.

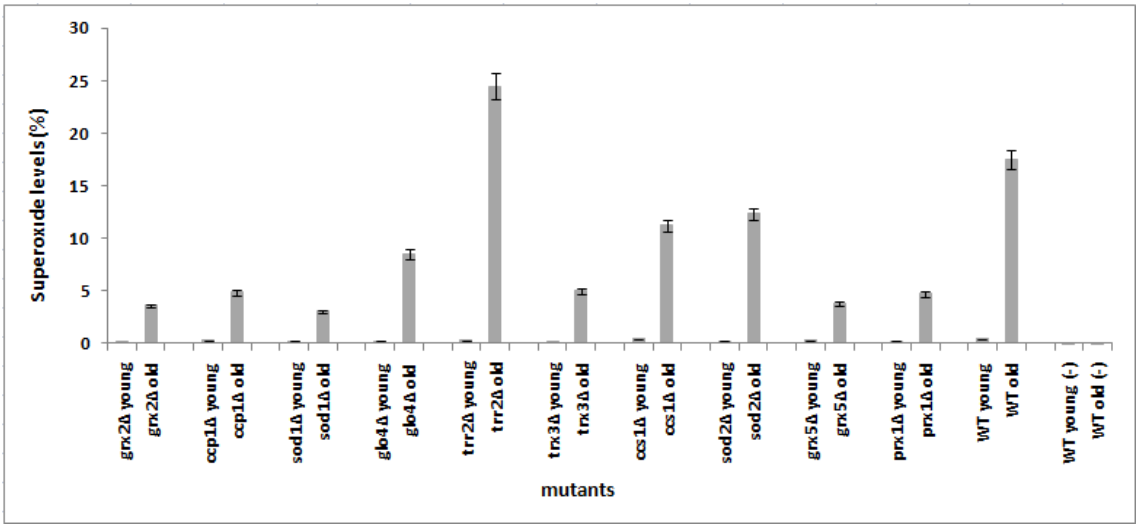

Figure 3.10. Superoxide levels in the mitochondria of WT strain and the mitochondrial antioxidant gene mutants grown in YPD media

When cells were grown in YPG, the superoxide accumulations were greater than the cells grown in YPD media. All aged mutants showed relatively low superoxide accumulation compared to old WT, in YPG. *ccs1*Δ, *ccp1*Δ, *sod1*Δ and *trr2*Δ were among the mutants that accumulated superoxide in their mitochondria higher than other mutants as shown in Figure 3.11. The levels of superoxide accumulation in mutants were higher in YPG media when compared to mutants grown in YPD media.

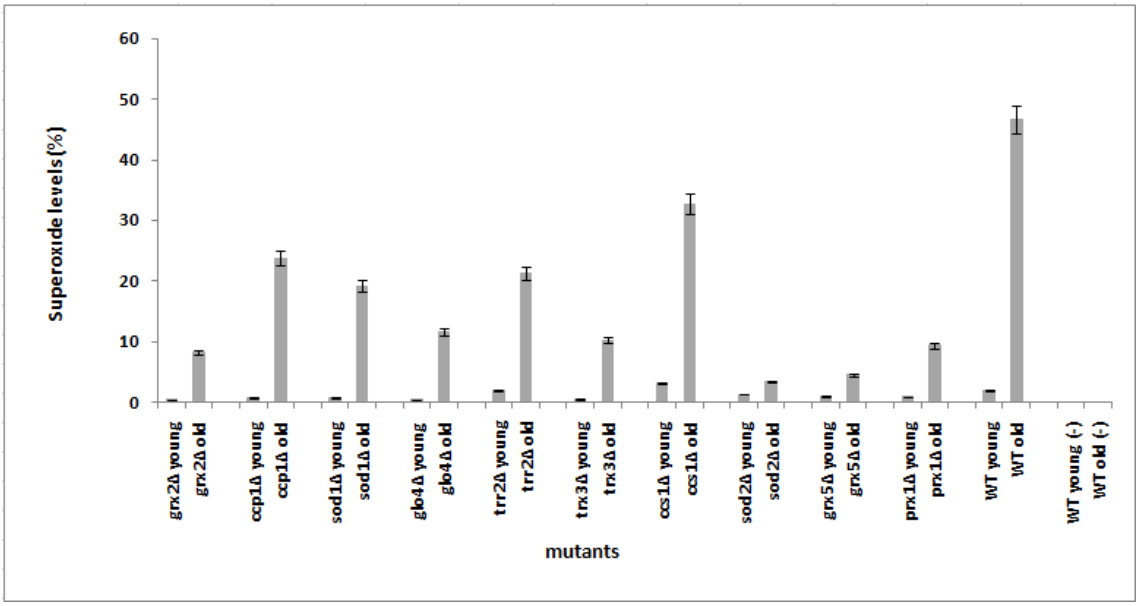

Figure 3.11. Superoxide levels in the mitochondria of WT strain and the mitochondrial antioxidant gene mutants grown in YPG media

3.4 Oxidative Stress Tolerance

3.4.1 H_2O_2 Sensitivities

To measure the hydrogen peroxide sensitivities of the mutant strains, Halo Assay was applied. The radius of the H_2O_2 hole determines the sensitivity of the cells against H_2O_2. The sensitivity increases proportionally with the radius. When cells are grown in YPD media, the mutants were less sensitive to H_2O_2 when compared to WT strain, as shown in Figure3.11. However, upon growth in YPG media, *grx2*Δ, *ccp1*Δ, *sod1*Δ, *ccs1*Δ, and *sod2*Δ mutants showed a greater sensitivity against H_2O_2 when compared to the WT. *ccs1*Δ showed the greatest sensitivity among other mutants. The results of the Halo assay can be found in Figure 3.12.

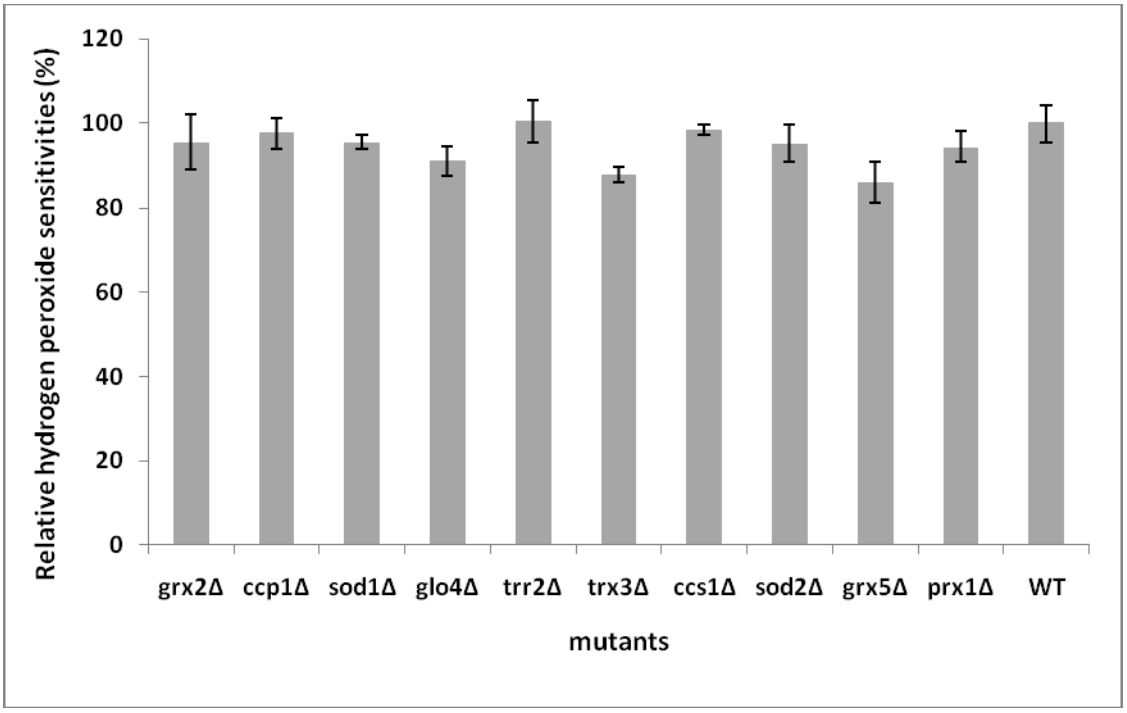

Figure 3.12. The Halo assay. Hydrogen peroxide hole radius measurements of YPD grown WT and mutant strains.

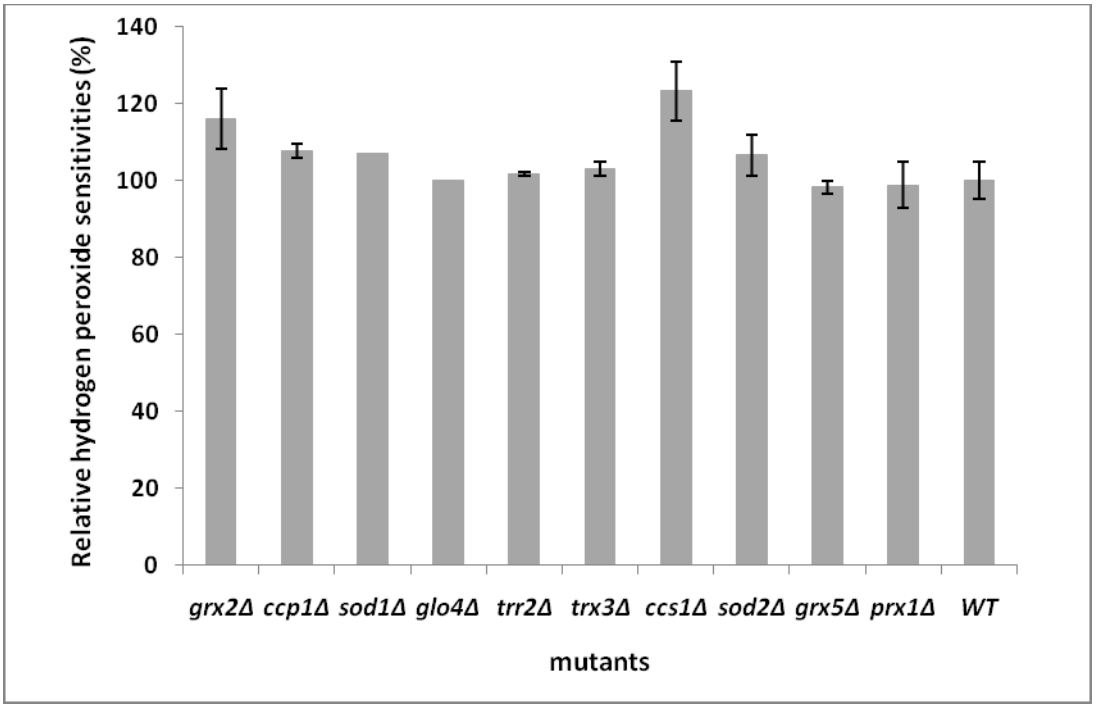

Figure 3.13. The Halo assay. Hydrogen peroxide hole radius measurements of YPG grown WT and mutant strains.

3.4.2 Menadione Sensitivities

The superoxide sensitivities of the strains were measured with menadione resistance assay. There different concentrations were tried in YPD grown cells, which were, 0.1mM, 0.25mM and 0.5mM. *ccs1*Δ and *sod2*Δ were more sensitive to superoxide relative to other mutant strains, while other mutant strains showed a similar sensitivity pattern as WT strain as shown in Figure 3.14.

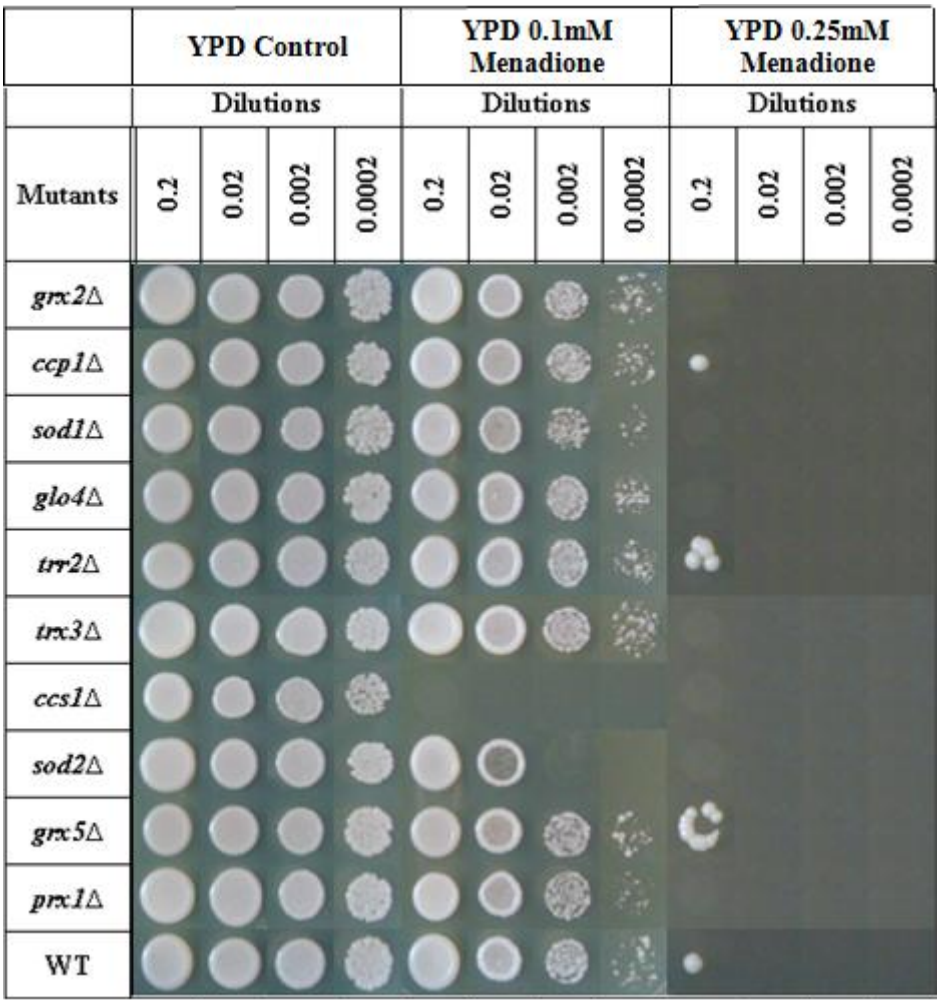

Figure 3.14. Menadione sensitivities of YPD grown strains

However in YPG grown cells, the same menadione concentrations were too much for cells to survive so that no growth was observed at those concentrations (data not shown). Therefore, more diluted menadione concentrations were used, which were 0,025mM, 0.05mM, and 0.1mM, fort he YPG grown cells. *ccs1*Δ and *sod2*Δ mutants were again more sensitive than the other mutant strains. These mutants could not even grow at all in 0,025mM. The other mutants showed almost a similar sensitivity pattern as the WT strain as shown in Figure 3.15.

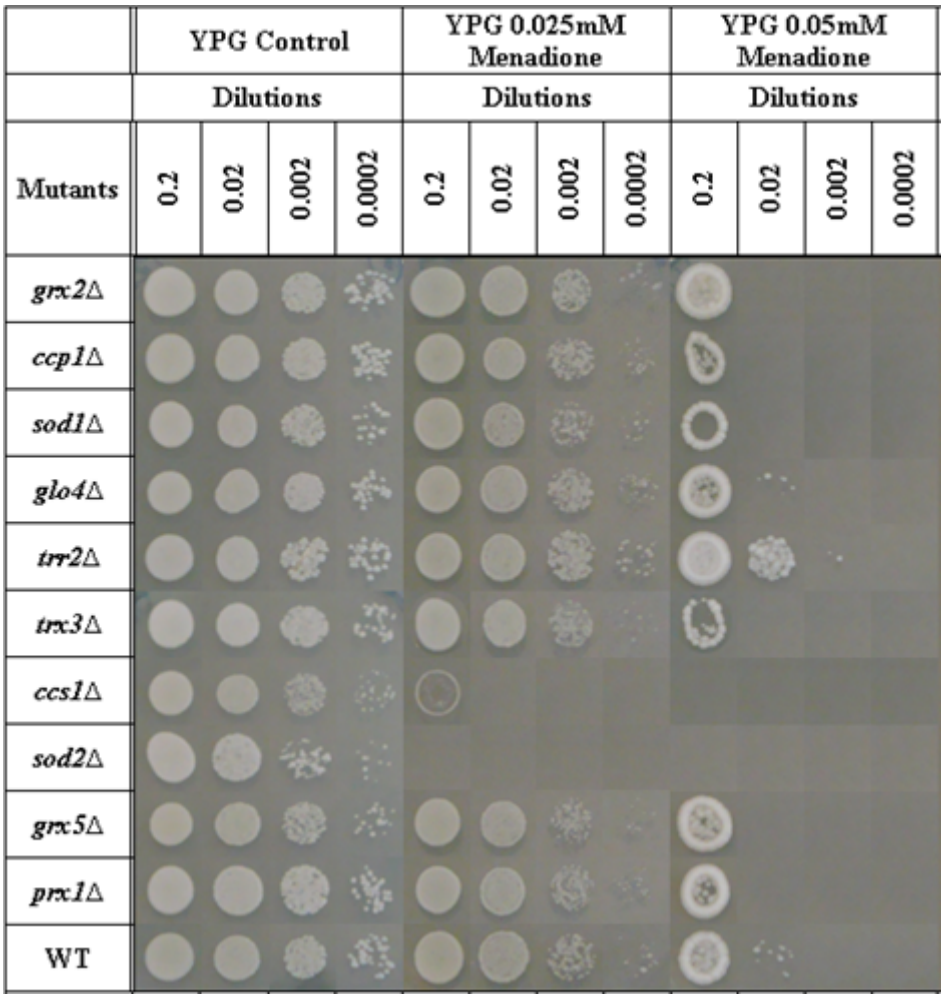

Figure 3.15. Menadione sensitivities of YPG grown strains

3.4.3 Diamide Sensitivities

Diamide resistance were observed in YPD grown cells. 1.5mM, 2.0mM, and 2.5mM diamide concentrations were tried and *ccs1*Δ and *sod2*Δ were again shown to be the most sensitive strains to diamide toxicity. *prx1*Δ,*grx5*Δ,*trx3*Δ, and *ccp1*Δ mutants showed a similar sensitivity pattern and these mutants were also more sensitive to diamide toxicity than the WT strains. *grx2*Δ, *glo4*Δ, and *trr2*Δ showed a similar sensitivity pattern as the WT strain as shown in Figure3.16.

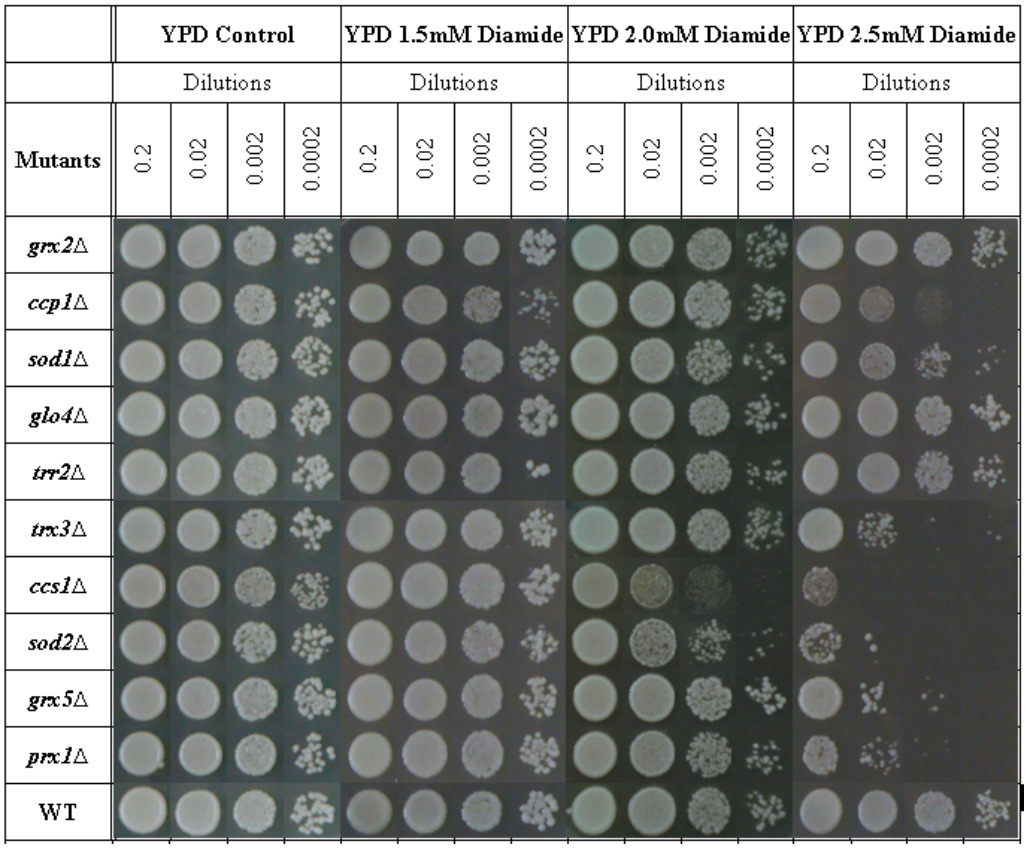

Figure 3.16. Diamide sensitivities of YPD grown strains

CHAPTER 4

DISCUSSION

When the chronological aging patterns were compared among different mitochondrial antioxidant gene mutants and the WT (BY4741) strain, it was shown that half of the mutant strains had lower CFU percentages. In YPD media, *grx2*Δ, *ccp1*Δ, *sod1*Δ, *trr2*Δ, *trx3*Δ, and *sod2*Δ showed relatively lower CFU percentages compared to WT. However, unexpectedly *glo4*Δ and *grx5*Δ mutants showed higher CFU percentages at day 15 of the aging process. In YPG media, when compared to WT, *ccs1*Δ and *glo4*Δ mutant strains showed a significant decrease in CFU percentages, which are 20% and 11% respectively, at day 15.

It was shown before that YPD, as a glucose media, was not suitable for the chronological aging studies, since yeast strains showed unstable viabilities on YPD media when compared to SDC (synthetic dextrose complete) media. SDC media is the mostly used media in chronological aging studies since cells maintain high metabolism rates through their life spans (Fabrizio and Longo 2008). Instead of SDC media, glycerol media (YPG) and glucose media (YPD) are used in this study in order to see the effects of different carbon sources on chronological aging pattern. As being a respiratory growth source, more accurate CFU patterns were expected to be observed in YPG media when compared to YPD media.

Earlier studies showed that CCS1 gene is essential for cells to maintain at stationary phase (Longo, Gralla, and Valentine 1996), which is consistent with our recent data obtained from YPG growth. The *ccs1*Δ colonies were also smaller when compared to other mutant strains and these cells had difficulties in growth when compared to other mutant strains. However, even it was shown before that SOD mutants had a shorter chronological life spans, only a decrease in *sod1*Δ was observed in YPD media. Even deletion of SOD2 gene did not have any significant effect in shortening the life span, the colonies formed by this mutant were smaller in size and more apoptotic bodies were observed in this mutant strain under the microscope (data not shown).

The reason of such chronological aging pattern differences between the same mutants in different media probably arises from the carbon source differences. The

strains were shown to enter altruistic aging in glycerol media (YPG), in which they show diauxic shifts via living on the remnants of the dead cells. The CFU percentages of the strains were reached approximately 10% at the end of 15th day of the chronological life span in YPD media so that the cells were collected at that time period and in order to observe the differences between the glycolytic and respiratory carbon sources, the cells were also chronologically aged in YPG for 15 days. Probably longer time periods than 15 days for chronological aging studies is required in the YPG media. Unexpected longer chronological life span patterns of different mutants when compared to the WT strain, can also be due to the activation of alternative oxidative stress defense mechanisms upon the deletion of specific antioxidant genes.

The biomolecular damage levels in chronologically aged and young yeast strains were generally higher in old mutants as expected. The protein carbonylation levels were mainly higher in old strains when compared to the young strains, which supports the data to date that protein oxidation increases with age. In YPD media, when compared to the WT strain, *grx2*Δ*, ccp1*Δ*, ccs1*Δ*,* and *grx5*Δ old strains showed 27%, 22%, 66%, and 56% increase in protein carbonyl content, respectively. However, in *prx1*Δ and *sod2*Δ old mutants, 43% and 13% decrease in protein carbonylation levels were observed. The increase in protein carbonyl content in *ccs1*Δ was higher as expected, however, the SOD gene deletions did not show any increase in carbonylation levels, instead *sod2*Δ showed a 13% decrease. This may be either due to the decrease in protein content of the mutants upon aging or due to activation of other antioxidant defense mechanisms in order to compensate the absence of the mutant gene. When YPD grown young strains were compared, *ccs1*Δ*, sod2*Δ*, grx5*Δ*, prx1*Δ young mutant strains showed 58%, 59%, 56%, and 26% decrease in protein carbonylation compared to the WT strain, respectively. This may explain that other antioxidant defense mechanisms are activated due to the absence of one antioxidant gene and more ROS detoxification takes place in young cells. Probably, the proteosomal system as well as other protein degradation systems may also function more actively. In YPG media, aged strains showed higher protein carbonyl content, except *ccp1*Δ. Having a higher protein oxidation in young mutants may be explained either by having more active mitochondria in young cells when compared to old ones, so that ROS production is more active in these cells, or by the decrease of protein content upon aging. In YPG grown old cells, *sod1*Δ*, grx5*Δ, ccs1Δ mutants showed almost a 2 fold increase in protein carbonyl content when compared to old WT cells, which is consistent with the earlier studies, while *glo4*Δ*, trr2*Δ and *grx2*

showed, a 1.5-fold, 3-folds, and 4-folds increase in protein carbonyl content, respectively. The protein carbonyl contents were higher in in YPG grown cells when compared to YPD grown cells.

In means of lipid oxidation, aged cells showed again higher levels when compared to young ones. In YPD, *grx2*Δ, *ccp1*Δ, and *sod1*Δ young mutants showed increased MDA levels when compared with the young WT strain. In YPD grown chronologically aged mutants, *grx2*Δ, *ccp1*Δ, *sod1*Δ, *trr2*Δ, and *ccs1*Δ showed higher lipid peroxidation levels when compared to WT, as expected. Chronologically aged *ccs1*Δ and *trr2*Δ showed the highest significant increase among other mutants, which are 81% and 82% respectively. In YPG, young *sod2*Δ, *ccs1*Δ, and *trx3*Δ strains showed higher levels of MDA when compared to the young WT strain and among old cells, all mutant strains showed relatively higher MDA levels compared to old WT strain, except Δglo4 mutant. This may lead to a decision that the glyoxylase-II (Glo4) does not play an important role in defense against lipid oxidation.

However, when random DNA mutation rates are compared, *glo4*Δ, *ccs1*Δ, and *sod1*Δ strains showed significantly higher mutation rates when compared to WT strain. In glucose media, this increase was 17-folds, 8-folds, and 2.5-folds for *glo4*Δ, *ccs1*Δ, and *sod1*Δ respectively, while in glycerol media, it was 31-folds, 26-folds, and 3.3-folds respectively. Also, in YPD media, the *prx1*Δ strain and in YPG media, *grx2*Δ strain showed 2-fold increase in random DNA mutation rates when compared to WT strain. In YPG media, *sod2*Δ mutant also showed higher DNA mutation rates, which is consistent with the earlier data. The effects of SOD1 and CCS1 gene deletions were as expected since these mutants are known to be sensitive to oxidative stress, however the increase in DNA instability due to deletion of glo4 gene was unexpected and this gene seems to be important for the maintenance of DNA stability during chronological aging process. The effect of GLO4 deletion on chronological aging has not been studied before and with this study it can be proposed that GLO4 gene is important for the protection of DNA against ROS attack.

When the mitochondrial DNA mutation rates were compared grx2Δ, ccp1Δ, glo4Δ, trx3Δ and prx1Δ mutant strains showed higher mtDNA point mutation rates in YPD grown cells. Unexpectedly, no colonies were observed in YPG grown strains, which may be an indicator of the increased redox control over the mitochondria upon antioxidant gene deletions in order to overcome the absence of the deleted antioxidant genes and maintain the mitochondrial homeostasis.

The chronologically aged strains also showed higher superoxide production rates in mitochondria when compared to young strains. This result also supports the data that as the cells age, ROS production increases and the ability of defense systems to remove them decreases. In YPD media, *trr2*Δ*, ccs1*Δ*, sod2*Δ old cells showed the highest superoxide accumulations among other mutant strains. However, only *trr2*Δ superoxide levels were higher than the old WT strain. In YPG, *ccs1*Δ*, ccp1*Δ*, sod1*Δ and *trr2*Δ showed relatively higher superoxide accumulation in mitochondria when compared to other mutant strains. However, the superoxide levels of these mutants were less than the WT old strain.

The ROS sensitivities are also measured for these antioxidant gene mutants and the WT strain. In YPD media, there were no significant differences in sensitivities against H_2O_2. However, in YPG media, *grx2*Δ*, ccp1*Δ*, sod1*Δ*, ccs1*Δ*,* and *sod2*Δ mutants showed a greater sensitivity against H_2O_2 when compared to the WT, and Δccs1 showed the greatest sensitivity among other mutants. *sod1*Δ*, ccs1*Δ*,* and *sod2*Δ mutants were also shown to be more sensitive to diamide and menadione induced stresses than WT and other mutant strains. The mutants were more sensitive to these stresses when they were grown in YPG media in which they used mitochondrial respiration in order to survive.

In this study, total protein and total lipid damages upon mitochondrial antioxidant gene deletions are observed. The absence of a specific antioxidant defense gene may trigger the activation of alternative defense meachanisms in order to keep the redox homeostasis constant in a cell. Therefore, the unexpected reduction of molecular damages in different mitochondrial antioxidant gene mutants relative to the WT strain can be an outcome of the increased activation of other antioxidant defense mechanisms in order to compensate the absence of the specific antioxidant gene.

CHAPTER 5

CONCLUSION

The free radical theory of aging is the most widely accepted theory of aging. Therefore, a great attention was paid by the scientists for the effects of free radical dependent molecular damages in aging process and the mechanisms that cells evolved to defense against them. Antioxidant defense mechanism is one of the defense mechanisms that cells evolved against the detrimental effects of reactive species.

Chronological aging model of *S.cerevisiae* is a good model to study aging in post-mitotic cells. Understanding the biochemical and genetic alterations that take place during post-mitotic cell aging is important to understand the biochemical and molecular basis that lay behind the abnormalities observed due to aging process.

The aim of this study was to show the comparable effects of different mitochondrial antioxidant genes on chronological life span and chronological age dependent damages in yeast *S.cerevisiae.* It was shown that the molecular damages are increased upon aging process. Among ten mitochondrial antioxidant gene mutants, ccs1 deletion showed a stable pattern, in which the deletion of this gene resulted in significant increase in protein carbonylation, lipid peroxidation and genomic instability as well as more sensitivity against reactive oxygen species. These data indicate that copper chaperone protein, Ccs1, can have a major role in chronological aging process and can be an important player in prevention of post mitotic cell disorders such as neurodegenerative diseases. As well as CCS1, deletion of GLO4 gene also resulted in increased amounts of canavanine resistance as well as decreased CFU percentages, which indicates that glyoxylase-II gene can be important to maintain the genome stability against ROS attacks on genomic DNA. Future studies can be done on the protein expression patterns among different antioxidant gene mutant strains in order to observe if there is any increase in protein expression of antioxidant genes other than the mutated gene in each mutant strain.

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