DOKUZ EYLUL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE INVESTIGATION OF THE CLOTRIMAZOLE INDUCED APOPTOSIS MECHANISM IN *SACCHAROMYCES CEREVISIAE*

by
Berna KAVAKCIOĞLU YARDIMCI

November, 2017
İZMİR

THE INVESTIGATION OF THE CLOTRIMAZOLE INDUCED APOPTOSIS MECHANISM IN *SACCHAROMYCES CEREVISIAE*

A Thesis Submitted to the
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by
Berna KAVAKCIOĞLU YARDIMCI

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Ph.D. THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"THE INVESTIGATION OF THE CLOTRIMAZOLE INDUCED APOPTOSIS MECHANISM IN SACCHAROMYCES CEREVISIAE"** completed by **BERNA KAVAKCIOĞLU YARDIMCI** under supervision of **PROF. DR. LEMAN TARHAN** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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THE INVESTIGATION OF THE CLOTRIMAZOLE INDUCED APOPTOSIS MECHANISM IN *SACCHAROMYCES CEREVISIAE*

ABSTRACT

Clotrimazole is an antifungal drug whose potential antineoplastic usage has been taken into consideration recently. In the scope of this thesis, it was primarily aimed to highlight the cytotoxic and apoptotic effects of clotrimazole on eukaryotic model *Saccharomyces cerevisiae* BY4742 yeast cells. This organism has been gradually become a preferred and popular research tool, especially in exploring human disease over the years because it can be easily handled and genetically modified.

To examine cytotoxic effect of clotrimazole on yeast cells, FUN-1 viability test was used. The apoptotic character of death was determined by Annexin V – PI and DAPI - TUNEL double stainings which indicate phosphatidylserine externalization and DNA fragmentation, respectively. The variations in mitochondrial membrane potentials in BY4742 cells treated with clotrimazole were investigated in a concentration and time dependent manner. The dependency of clotrimazole induced apoptotic death to certain mitochondrial proteins including yeast caspase, apoptosis inducing factor, endonuclease G and HtrA-like serine protease Nma111p was highlighted with the use of knockout mutant strains.

Oxidant and antioxidant status together with the resulting damage in *S. cerevisiae* BY4742 yeast cells exposed to clotrimazole were researched. For this aim, the variations in intracellular reactive nitrogen and reactive oxygen levels and the activities of NADH and NADPH oxidases were measured. Among antioxidant defense system, the activities of superoxide dismutase, catalase, and glutathione related enzymes and the levels of reduced – oxidized glutathione were determined. Finally, as markers of clotrimazole induced oxidative stress conditions, malondialdehyde and protein carbonyl levels were investigated.

These findings can be summarized as clotrimazole induced apoptotic cell death in yeast mainly depends on metacaspase acivity. On the other hand, although significant inductions have been observed in antioxidant defence system, it could not protect cells from clotrimazole based stress conditions.

Keywords: Clotrimazole, *Saccharomyces cerevisiae*, apoptosis, reactive species, antioxidant system, damage in biological molecules

SACCHAROMYCES CEREVISIAE'DA KLOTRİMAZOL İNDÜKLÜ APOPTOZİS MEKANİZMASININ AYDINLATILMASI

ÖZ

Klotrimazol, son zamanlarda antineoplastic ajan olarak kullanımı araştırılmakta olan antifungal bir ilaçtır. Sunulan tez kapsamında, öncelikli hedef klotrimazolün ökaryotik model *Saccharomyces cerevisiae* BY4742 maya hücresi üzerindeki sitotoksik ve apoptotic etkilerinin aydınlatılmasıdır. *S. cerevisiae*, kolay kültürlenebilmesi ve genetic olarak modifiye edilebilmesi nedeniyle özellikle insan hastalıklarının aydınlatılmasında giderek tercih edilen ve popular bir araç haline gelmiştir.

Klotrimazolün, maya hücreleri üzerindeki sitotoksik etkisinin belirlenebilmesi amacıyla FUN-1 canlılık testinden yararlanılmıştır. İndüklenen ölümün apoptotik karakteri sırasıyla fosfatidil serin eksternalizasyonu ve DNA fragmentasyonunu gösteren Annexin V – PI ve DAPI – TUNEL ikili boyamaları ile belirlenmiştir. Klotrimazol ile muamele edilen BY4742 hücrelerinde mitokondriyal membran potansiyelinde meydana gelen değişimler, ilaç derişimi ve muamale süresine bağımlı olarak araştırılmıştır. Klotrimazol indüklü apoptotik hücre ölümünün maya kaspazı, apoptoz indükleyici faktör, endonukleaz G ve HtrA-like serine proteaz Nma111 olmak üzere mitokondriyal proteinlere bağımlılığı, ilgili gen bölgesinin çıkarıldığı mutant suşlar kullanılarak araştırılmıştır.

Klotrimazole maruz bırakılan *S. cerevisiae* BY4742 maya hücrelerinin oksidan – antioksidan dengeleri meydana gelen hasarlarla beraber araştırılmıştır. Bu amaçla, hücre içi reaktif azot ve reaktif oksijen seviyeleri ile NADH ve NADPH oksidaz aktiviteleri belirlenmiştir. Antioksidan savunma sistemlerinden, superoksit dismutaz, katalaz ve glutatyon ilişkili enzim aktiviteleri ve ayrıca indirgenmiş – yükseltgenmiş glutatyon seviyeleri irdelenmiştir. Son olarak, klotrimazol indüklü oksidatif stres koşullarında önemli birer belirteç olan malondialdehid ve protein karbonil seviyeleri ölçülmüştür.

Klotrimazol indüklü maya apoptozunun önemli oranda metakaspaz aktivitesi üzerinden yürüdüğü belirlenmiştir. Öte yandan, antioksidan savunma sisteminde anlamlı indüksiyonlar olmakla birlikte, maya hücresinin klotrimazol indüklü stres koşullarından korunmasında yeterli olmadığı görülmüştür.

Anahtar kelimeler: Klotrimazol, *Saccharomyces cerevisiae*, apoptozis, reaktif türler, antioksidan sistem, biyolojik moleküllerde hasar

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CHAPTER ONE
INTRODUCTION

1.1 General Overview of Oxidative Stress and Biological Consequences

In the development of complex life on Earth, oxygen molecule (O_2) has known with its central function. This is predominantly due to the fact that biochemical symmetry of oxygenic photosynthesis and aerobic respiration can maintain homeostasis within the biosphere of Earth. In other words, O_2 utilization ability of aerobic creatures to control oxidation of carbon-containing molecules which in turn leads to energy production makes this molecule essential for normal metabolism and life (Tvrda, Knazicka, Bardos, Massanyi, & Lukac, 2011). On the other hand, the levels of exposed O_2 can be a source of stress. It should be noted that although atmospheric O_2 in its ground-state has two unpaired electrons having spin in the same direction and is a biradical, it is not reactive and not very dangerous for cells. But, by products generated from O_2 known as reactive oxygen species (ROS) are destructive for cells at their high concentrations (Tremellen, 2008).

Basically, ROS can be defined as a subset of free radicals that involve oxygen or unstable reactive molecules which have one or more unpaired electron(s). ROS are the most typical oxido-reductants together with other free radical species of nitrogen, sulfur and halogens. These species are able to oxidize nearby biomolecules to gain an electron to enter their ground states and a cycle that results in the destruction of neighboring cells and tissues is perpetuated by these oxidized molecules which have become free radical themselves. However, at physiological levels, ROS are beneficial and assist in the maintenance of normal cell function. They play a key role in the regulation of vital cellular processes such as proliferation and programmed cell death (PCD), intracellular transport, cellular motility, membrane integrity and immune response. One example, people suffering from granulomatous disease, a primary immunodeficiency in which ROS production is reduced or absent, become more susceptible to infection by a range of pathogenic bacterial species. Another,

cytosolic ROS produced in response to stimulation by growth factors are involved in regulating the proliferative response (Finkel, 1998).

ROS encompass a variety of diverse chemical species include lipid peroxides, nitric oxide (NO), singlet oxygen ($^1O_2$), ozone (O_3), and hypochlorous acid (HOCl); however, superoxide anions ($O_2^{\cdot -}$), hydroxyl radicals (HO·), and hydrogen peroxide (H_2O_2) are the three most important ROS playing dual roles in biological systems. While $O_2^{\cdot -}$ and HO·are extremely unstable, H_2O_2 is freely diffusible and relatively long-lived. Also these three ROS are interconvertible. O_2 can be converted to $O_2^{\cdot -}$ by accepting an electron. In turn, the dismutation reaction by antioxidant enzyme superoxide dismutase (SOD) can convert $O_2^{\cdot -}$ to H_2O_2, and this finally may be partially reduced to HO·or fully reduced to water.

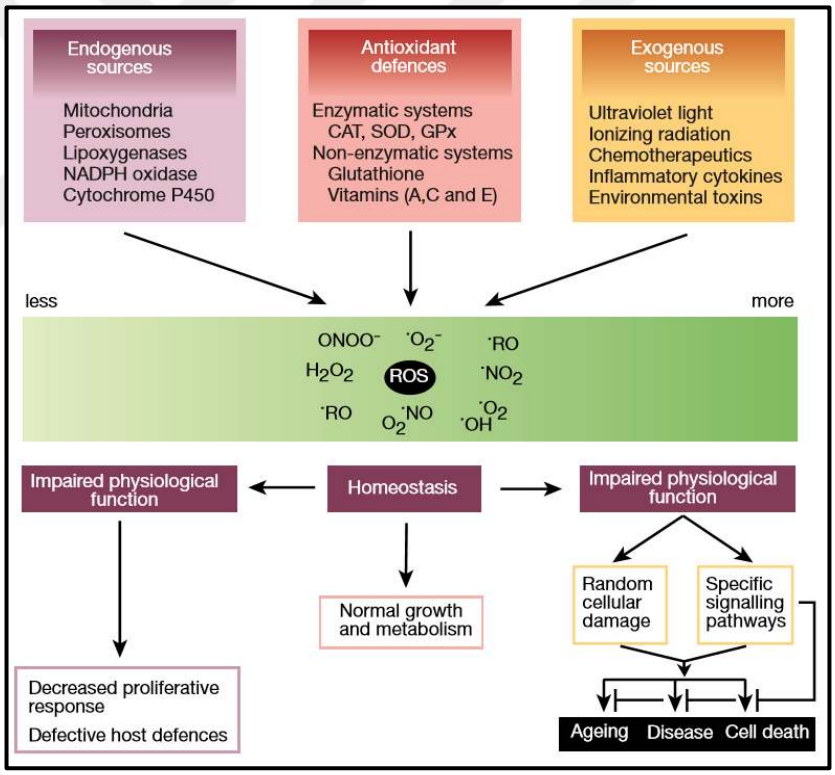


Figure 1.1 The sources and cellular responses to reactive oxygen species (ROS) (Finkel & Holbrook, 2000)

The sources of ROS can be classified as endogenous and exogenous (Figure 1.1). They are endogenously produced by various enzyme reactions in the systems such as those carried out by xanthine oxidase, cytochrome P450 and also NADPH oxidase

(NOX) complex in the cell membrane, mitochondria, peroxisomes, and endoplasmic reticulum. In addition, autoxidation of endogenously produced small molecules such as catecholamine, which function as neurotransmitters and hormones, results with ROS production. On the other hand, the majority of endogenous ROS is derived from the electron transport chain in mitochondria as a result of leakage of electrons. The production of mitochondrial $O_2^{\cdot -}$ radicals occur primarily at two discrete points in the electron transport chain, namely at complex I (NADH dehydrogenase) and at complex III (ubiquinone–cytochrome c reductase) that is the main site of ROS production under normal metabolic conditions (Turrens, 1997) (Figure 1.2).

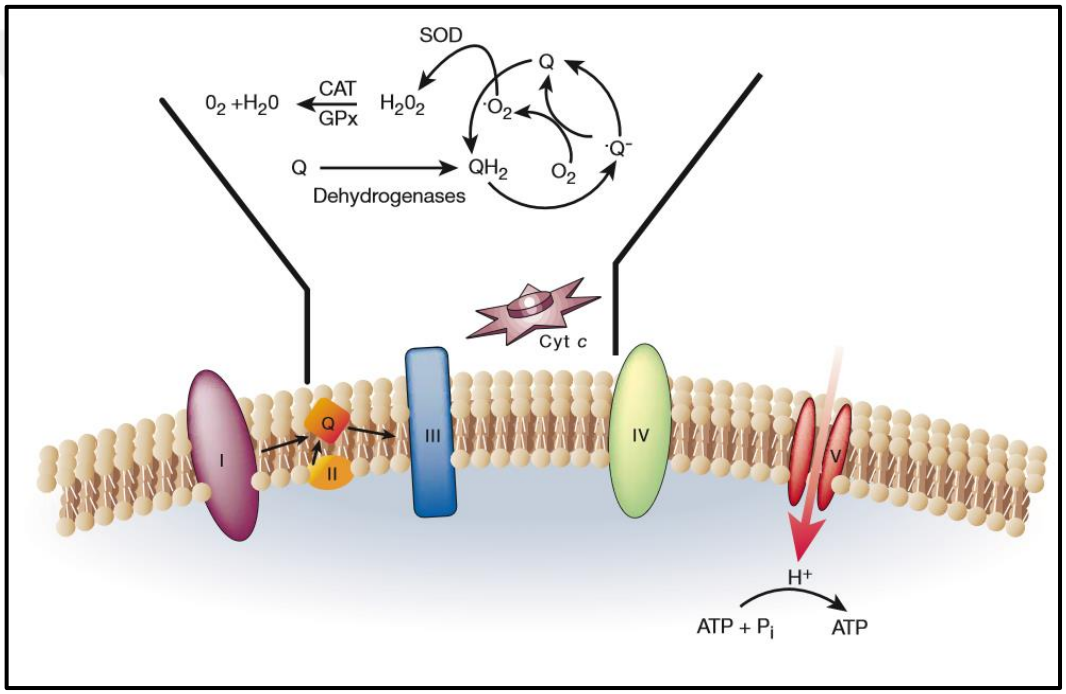


Figure 1.2 Superoxide radical productions through mitochondrial electron transport chain (Finkel & Holbrook, 2000)

Along with endogenous sources, a number of exogenous agents playing roles in ROS production include xenobiotics, pollutants, drugs, smoke, tobacco, and radiation. Industrial, agricultural, pharmacological, and lifestyle applications have increased the types and amounts of exogenous agents to which humans are exposed. For instance, the people living in cadmium polluted regions may accumulate this heavy metal up to several hundred micrograms. The routes of cadmium intake involve the lungs, intestines and skin (Jomova & Valko, 2011). Although this metal

is itself unable to generate free radicals directly, it can replace iron and copper in various cytoplasmic and membrane proteins such as ferritin and apoferritin and participate ROS generation via Fenton reactions indirectly (Price & Joshi, 1983). Occupational exposure of workers to naturally occuring asbestos which contains high amounts of iron has been related with increased risk of mutagenesis and cancer due to the iron ability to catalyze the formation of HO·radicals (Stayner, Dankovic, & Lemen, 1996). Carbon tetrachloride (CCl_4) is a manufactured chemical that is used especially in cleaning industry. It is extremely toxic when metabolized in the liver. As shown in Figure 1.3, the toxic metabolite is formed when CCl_4 binds to the ferrous-heme iron of cytochrome P450, which removes one electron by a complex reaction and releases the trichloromethyl ($^{\bullet}CCl_3$) which is a carbon-centered radical (Kalyanaraman et al., 1979). The $^{\bullet}CCl_3$ radical might combine directly with biological molecules, causing covalent modification as well as abstracting hydrogen from membrane lipids, thereby initiating a chain reaction of lipid peroxidation where oxygen-centered radicals are formed. In general, many chemical agents and drugs can give rise to the formation of superoxide radicals.

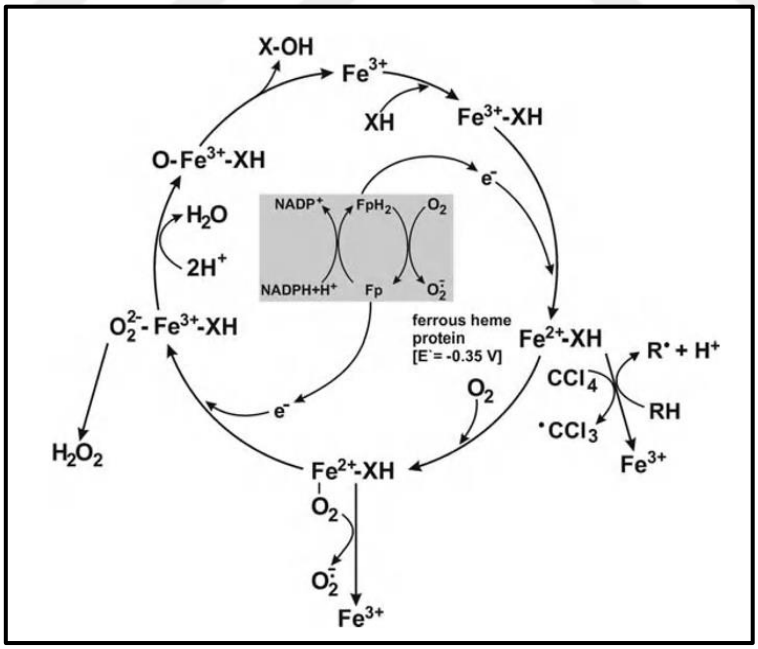


Figure 1.3 Catalytic cycle of the microsomal cytochrome P450/cytochrome b5 system that is involved in the metabolism of endogenous and exogenous substrates (Nohl, Kozlov, Gille, & Staniek, 2004)

Living systems have a number of defence mechanisms that predominantly involve enzymatic/non-enzymatic molecules whose functions are to scavenge excessive and unwanted ROS, and detoxify both endogenous and exogenous chemicals in the cells. ROS scavenging enzymes include SOD, catalase (CAT) and glutathione peroxidase (GSHPx). Whereas SOD directs the conversion of $O_2^{\cdot -}$ to H_2O_2, CAT and GSHPx convert H_2O_2 to water. In addition to these well characterized antioxidant enzymes, at least five members of a family of peroxide scavengers termed as peroxiredoxins have been isolated (Chae, Kim, Kang, & Rhee, 1999). A variety of other non-enzymatic, low molecular mass molecules are important in scavenging ROS. These include ascorbic acid, α-tocopherol or vitamin E, pyruvate, flavonoids, carotenoids and perhaps most importantly, glutathione (GSH), which is present in millimolar concentrations within cells. On the other hand, the detoxification enzymes, also known as Phase I and Phase II drug-metabolizing enzymes, including cytochrome P-450 system, glutathione S-transferases, UDP-glucuronyltransferases, and sulfotransferases which are responsible from biotransformation of toxicants and other chemicals play vital roles in determining the relative susceptibilities of different tissues to toxic chemicals together with their antioxidant defence capacities.

The imbalance between the free radicals and antioxidant systems in favor of the former can result in oxidative stress. This term was previously defined as a situation of unbalance with either an excessive production of oxidants or a decrease in antioxidants that lead to cellular damage (Sies, 1985). After the recognition that significant changes occur in the redox state of intracellular thiols in the oxidative stress situations (Jones, 2008), the definition incorporated the concept of the redox state of intracellular thiols (-SH) and disulfides (-SS-). Prolonged oxidative stress which is defined as an imbalance between reactive species and antioxidants leading to a disruption of regulation of redox signaling and molecular damage is responsible for the pathogenesis of various diseases such as cancer, neurodegeneration, cardiovascular, and pulmonary diseases.

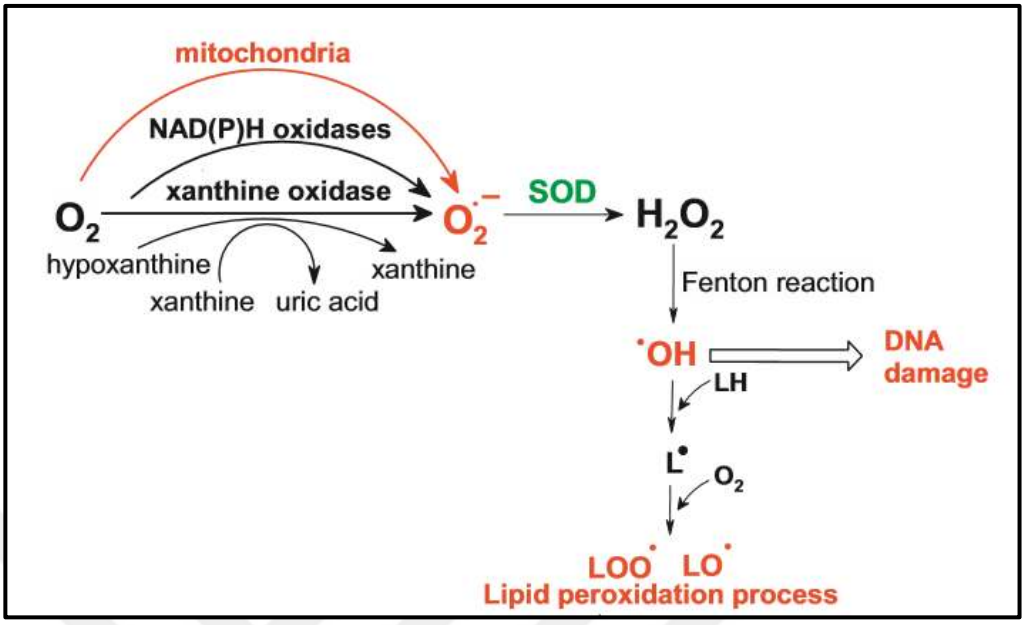


Figure 1.4 ROS production and damages in cells (Ahmad, 2016)

In the cells under oxidative stress conditions, ROS cause damages to biological systems, including DNA strand breaks, base modifications leading to mutations, inhibition of RNA and protein synthesis, protein damage including disruption of amino acid bonds and also their cross-linking, oxidation of membrane phospholipids, lipid peroxidation, disruption of membrane ion gradients, and depletion of cellular levels of ATP leading to cellular dysfunction (Figure 1.4). HO·is the most reactive species and can immediately interact with any molecule in its vicinity and can remove electron, turning that molecule into a free radical, giving rise to chain reactions. HO·specifically induces hydroxylation of deoxyguanosine in DNA forming 8-hydroxyguanine (8-OH-dG), which can be a site for mutagenesis and, possibly, cancer. $O_2^{\cdot -}$ in comparison to HO·is not that much reactive by itself but can initiate lipid peroxidation in its protonated form or can inactivate certain specific enzymes. H_2O_2 has low reactivity, is more stable, and hence can travel into the nucleus and can react with important components such as nucleic acids and nuclear proteins besides other cellular components such as lipids. Of course, the effects exerted by ROS are dependent on the type and amount of ROS involved (Aitken,

1995; Griveau, Dumont, Renard, Callegari, & Le Lannou, 1995) and the length and the moment of exposure to ROS (De Lamirande & Gagnon, 1992).

Aerobic cells are continually confronted by an "oxygen paradox" (Sies, 1993), necessitating the need for the regulation of these molecules at a homeostatic level by defence system. Therefore, "Reactive Oxygen and Nitrogen Species", "Antioxidant Systems", "Possible Damage Effects" and "Repair Mechanisms" that cover highly complex areas of biochemical metabolism are emerging as important parameters that determine the future of the cell.

1.2 Reactive Oxygen and Nitrogen Species

1.2.1 The Chemistry of Oxygen (O_2)

The atomic oxygen atom has eight electrons and forms the diatomic oxygen molecule (O_2). O_2 has a biradical nature, because two last electrons are located in different molecular orbitals with parallel spin (Figure 1.5).

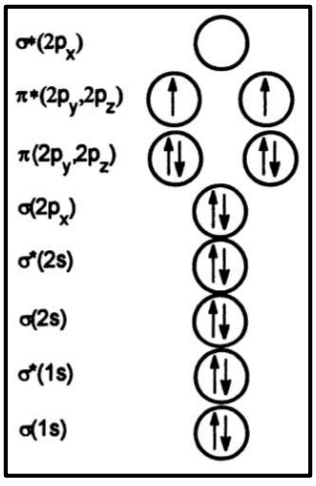


Figure 1.5 Oxygen molecule in the ground state (Frederick, 2013)

The O_2 in its ground state is rather a weak oxidant since the parallel electron spin arrangement prevents the direct addition of the electron pair to a molecule. In order to form a bond, a spin inversion should occur which is slow in comparison to the lifetime of collisional complexes. Simultaneous two-electron reactions with organic

molecules which are in the singlet state require a spin conversion. As a consequence most of the reactions involving the O_2 in the ground state are one-electron reactions. In contrast to the four-electron reduction of oxygen catalyzed by cytochrome oxidase in the last step of the mitochondrial electron transport chain, the nonenzymatic monovalent pathway for oxygen reduction results in the formation of several reactive intermediates. Therefore, it is clear that the radicalic structure of O_2 is a consequence of the oxidation/reduction chemistry (Danilewicz, 2003). Four sequential single electron (e^-) reduction that is the main factor in the toxic effect of O_2 was shown in Figure 1.6.

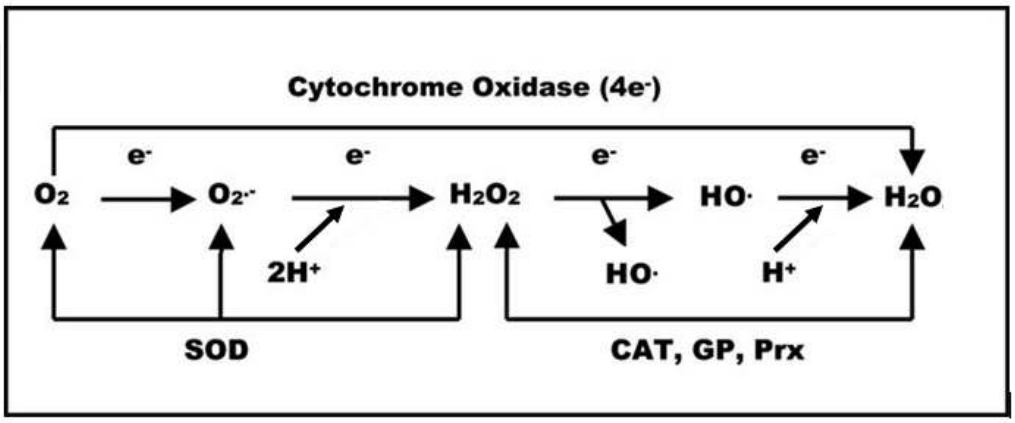


Figure 1.6 A monovalent pathway for oxygen reduction (Galaris, Barbouti, & Korantzopoulos, 2006).

As can be seen from Figure 1.6, the initial step is a reduction of O_2 by the univalent pathway resulting in the formation of $O_2^{\cdot-}$. The addition of a second e^- and two protons (H^+) to the $O_2^{\cdot-}$ radical forms to H_2O_2, a highly reactive species but does not exhibit radical character due to the lack of unpaired e- in its last orbital. In the third reduction step, a highly reactive HO·radical is produced and a hydroxide ion (OH^-) is released. Finally, in the fourth reduction step, it forms a non-toxic water molecule (H_2O).

1.2.2 Singlet Oxygen ($^1O_2$)

Singlet oxygen ($^1O_2$) is a highly reactive, short-lived intermediate which readily oxidizes a variety of biological molecules. It can be also considered as diamagnetic and less stable form of O_2 (Figure 1.7).

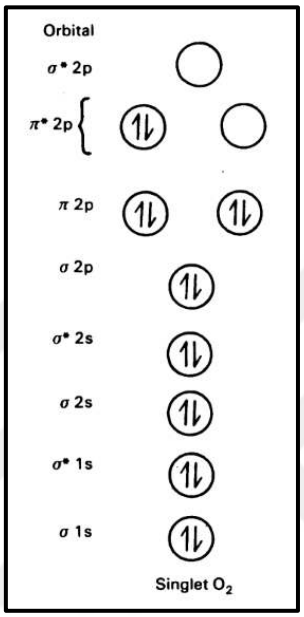


Figure 1.7 Singlet oxygen ($^1O_2$) (Frederick, 2013)

Several model biochemical systems have been shown to produce $^1O_2$ including the decomposition of alpha-linolenic acid hydroperoxide by cytochrome c and lactoperoxidase (Sun, Bao, Ma, Zhang, & Zheng, 2007), metabolism of indole-3-acetic acid by horseradish peroxidase and neutrophils (Escobar, Vasquez-Vivar, & Cilento, 1992), oxidation of NADPH by liver microsomes (King, Lai, & McCay, 1975), from myeloperoxidase-H_2O_2-chloride system (Kiryu, Makiuchi, Miyazaki, Fujinaga, & Kakinuma, 1999) or from horseradish peroxidase-H_2O_2-GSH system (Medeiros, Wefers, & Sies, 1987). $^1O_2$ has also been shown to be chemically produced from H_2O_2 and hypochlorite ion (ClO^-), potassium superoxide (KO_2) reaction with water, and thermal decomposition of aryl peroxides (Wilkinson, Helman, & Ross, 1995). $^1O_2$ is also produced by the absorption of electromagnetic energy that temporarily reverses the spin of one of the two unpaired electrons of O_2, which both spins are anti-parallel orientation (Davies, 1995). O_2 cannot accept two

electrons directly because the addition of a pair of parallel-electrons is restricted to the spin-parallel state of electrons. However, there are anti-parallel electron spins in $^1O_2$ and two electron-rich oxides which are very suitable for many biomolecules since there is no spin restriction. On the other hand, in order to produce $^1O_2$, a large amount of electromagnetic energy must be absorbed by O_2, which is largely confined to photochemical events.

The rate constants for reaction of $^1O_2$ vary significantly between biomolecules with greatest reactivity towards amino acid side chains. The most frequent type of reaction initiated by this species is addition across unsaturated bonds yielding a cyclised product (Davies, 2003).

1.2.3 Superoxide Anion Radical ($O_2^{\cdot-}$)

Superoxide ($O_2^{\cdot-}$) is the main precursor of the most highly oxidizing or reducing species in biological system. The one electron reduction of triplet dioxygen forms $O_2^{\cdot-}$ (Frederick, 2013). The molecular orbital of $O_2^{\cdot-}$ shows one unpaired electron in the antibonding orbital (Figure 1.8)

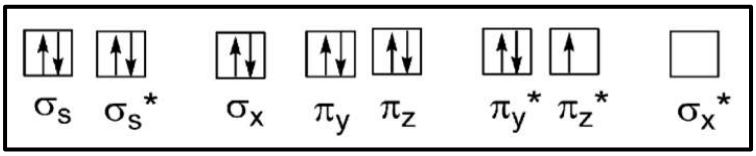


Figure 1.8 Molecular orbital diagram of $O_2^{\cdot-}$ (Frederick, 2013)

Enzymes including xanthine oxidase (Fridovich, 1970), aldehyde oxidase (Massey et al., 1969), dihydroorotic dehydrogenase and a number of flavoprotein hydroxylases, oxidases and dehydrogenases are responsible from biological production of $O_2^{\cdot-}$. Mitochondrial and microsomal electron chains are the other sources of $O_2^{\cdot-}$ (Kovacic, Pozos, Somanathan, Shangari, & O'Brien, 2005; Valko, Izakovic, Mazur, Rhodes, & Telser, 2004). It should be stated that about 75% of all the $O_2^{\cdot-}$ production in a cell originates just from the mitochondrial membrane (Cadenas & Sies, 1998). In addition, oxidation of hemoglobin to methemoglobin,

autoxidation and oxidation of biogenic catecholamines and synthesis of prostaglandins and tromboxans are the other biological sources of $O_2^{\cdot-}$.

$O_2^{\cdot-}$ can either undergo oxidation or reduction to form dioxygen or hydrogen peroxide, respectively (Halliwell & Gutteridge, 1999).

$$O_2^{\bullet-} \rightarrow O_2 + e^- \quad \text{(oxidation)}$$
$$O_2^{\bullet-} + e^- + 2H^+ \rightarrow H_2O_2 \quad \text{(reduction)}$$

Thereby allowing $O_2^{\cdot-}$ to dismutate to H_2O_2 and O_2 according to equation below.

$$2O_2^{\bullet-} + H^+ \rightleftharpoons H_2O_2 + O_2$$

The $O_2^{\cdot-}$ radical can function as both a reducing and also an oxidizing agent in the cells. For instance, while cytochrome c and plastocyanin are reduced, ascorbic acid (AH_2) is oxidized by this species. $O_2^{\cdot-}$ radical does not oxidize NADH or NADPH in measurable levels. However, it may interact with the active site of lactate dehydrogenase which contains NADH, to form the NAD• radical.

In general, the reactivity of the $O_2^{\cdot-}$ radical at pH 7.4 is very low. The direct biological damage caused by the $O_2^{\cdot-}$ radical is very limited and requires interaction with other components, such as NO- radicals or iron ions present in iron-sulfur proteins. In biological system, iron–sulfur clusters are known as significant cofactors. These clusters serve as active sites in various metalloproteins catalyzing electron-transfer reactions and play a role in other biological functions. The interaction of $O_2^{\cdot-}$ radicals with these clusters is especially important for organisms. For example, hydrolyase enzymes such as dihydroxyacid dehydratase, fumarase A and B and aconitase can be inactivated by $O_2^{\cdot-}$.

$O_2^{\cdot-}$ can reduce Fe (III) as well as oxidize Fe (II). These reactions may proceed through intermediate metabolites such as perferrile:

$$Fe(III) + O_2^{\bullet-} \rightleftharpoons [\ Fe(II)\text{-}O_2 \longleftrightarrow Fe(III)\text{-}O_2^{\bullet}\ ] \rightleftharpoons Fe(II) + O_2$$

Reduction of Fe (III) with $O_2^{\cdot-}$ accelerates the Fenton reaction, one of the reactions responsible for the hydroxyl radical generation:

$$Fe(II) + H_2O_2 \longrightarrow OH^{\bullet} + OH^{-} + Fe(III)$$

$$Fe(III) + O_2^{\bullet-} \longrightarrow Fe(II) + O_2$$

$$H_2O_2 + O_2^{\bullet-} \longrightarrow OH^{\bullet} + OH^{-} + O_2$$

1.2.4 Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is perhaps one of the most ubiquitous ROS present in biological systems. This is due to the fact that it has relative stability with an oxidation potential of 1.8 V. H_2O_2 is the protonated form of the two-electron reduction product of O_2 and is a nonradical ROS with all the antibonding orbitals occupied by paired electrons (Figure 1.9).

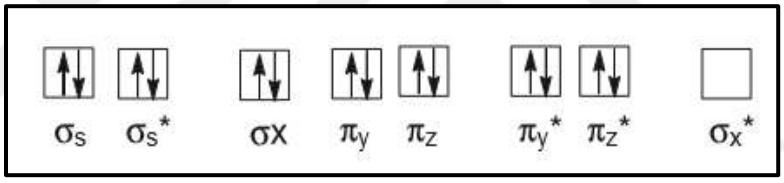


Figure 1.9 Molecular orbital diagram of H_2O_2 (Frederick, 2013)

H_2O_2 is not one of the radicalic ROSs, but easily diffuses into the cell with high concentrations and causes the oxidation of cellular biomolecules. Many endogenous enzymes such as xanthine, urate and D-amino acid oxidases can generate H_2O_2 (Allegra et al., 2003). In addition, H_2O_2 is produced by $O_2^{\cdot-}$ dismutation in many biological systems. H_2O_2 is a weak oxidant and a reducing agent. On the other hand, it is known that H_2O_2 has the ability to directly neutralize enzymes by causing the oxidation of basic thiol groups in the active sites of these enzymes. A known example of this is the inactivation of chloroplast fructose biphosphatase in H_2O_2 treated cells (Charles & Halliwell, 1980). Also, H_2O_2 can react with Cu and Fe ions in the cells to generate HO·with Fenton and Haber-weiss reactions and indirectly damages biomolecules (Spencer et al., 1995).

1.2.5 Hydroxyl Radical (HO•)

Hydroxyl radical (HO•) originates from the three-electron reduction of O_2. Among all the ROS, HO·perhaps is the most reactive and short-lived species. It plays a direct role in the initiation of oxidative damage to macromolecules in biological systems. Unlike $O_2^{\cdot-}$ and H_2O_2 whose reactions are limited due to their lower oxidizing ability, HO·can practically react with almost every organic molecules via H-atom abstraction, electrophilic addition, or radical–radical reactions, to name a few. The half-life of HO·is $\sim 10^{-9}$ s compared to $\sim 10^{-5}$ s and ~60 s for $O_2^{\cdot-}$ and H_2O_2, respectively.

In biological systems, HO·radicals can be produced by many reactions. One of these is the Fenton reaction catalyzed by transition metals. In fact, iron ions in organisms are not free but under stress conditions, $O_2^{\cdot-}$ radical breaks iron from iron-containing molecules (Valko et al, 2007). The liberated Fe^{+2} ions participate in the Fenton reaction.

$$Fe^{3+} + O_2^{\cdot-} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \quad \text{Fenton reaction}$$

$$O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + OH^{\cdot} + OH^{-} \quad \text{Haber-Weiss reaction}$$

The $O_2^{\cdot-}$ radical also participates in the Haber-Weiss reaction, resulting in HO·radical production. In the sun-exposed skin, HO·is produced by the hemolytic fission of the O-O bond in the UV-induced H_2O_2. In addition, HO·can also be produced during the decomposition of ozone and peroxynitrite acid and also ethanol metabolism. Other HO·sources include ionizing radiation, reaction between hypochlorous acid and superoxide anion radical, ultrasonography and lithotripsy (Halliwell & Gutteridge, 1999).

1.2.6 Hydroperoxyl Radical ($HO_2^{\bullet}$)

Peroxyl (ROO•) radical is another oxygen-derived radical. The simplest peroxyl radical, hydroperoxyl ($HO_2^{\bullet}$) is formed by the protonation of $O_2^{\cdot-}$ radical.

$$O_2^{\bullet-} + HO_2^{\bullet} \rightarrow O_2 + HO_2^{-}$$

The concentration of $HO_2^{\bullet}$ is a hundred times smaller than that of $O_2^{\cdot-}$ in biological pH. On the other hand, the presence of small equilibrium concentration of $HO_2^{\bullet}$ can contribute to the $O_2^{\cdot-}$ instability in neutral pH due to dismutation reaction shown below.

In addition, in the presence of strong or weak acids, electrochemical reduction of O_2 generates $HO_2^{\bullet}$. This radical is also generated when lipid peroxidation chain reactions begin. Due to its neutral charge, it is capable of penetrating the lipid bilayer. Therefore, it has been suggested that $HO_2^{\bullet}$ is capable of H-atom abstraction from polyunsaturated fatty acids (PUFAs) or from the lipids present in low-density lipoproteins.

1.2.7 Nitric Oxide (NO or NO$^{\bullet}$)

Nitric oxide is a paramagnetic molecule whose unpaired electron occupies an antibonding orbital.

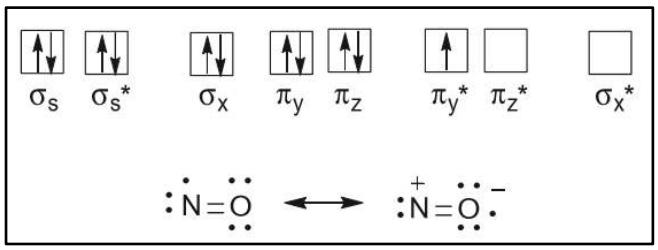
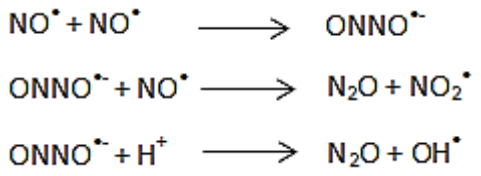


Figure 1.10 Molecular orbital diagram of NO (Frederick, 2013)

Nitric oxide (NO) functions as an intracellular signaling molecule and is the main precursor of highly oxidizing reactive nitrogen species (RNS) in cells. The toxicity of NO is generally limited to its reaction or oxidation to form the more highly reactive species such as peroxynitrite (ONOO−) and nitrogen dioxide radical ($\bullet NO_2$).

NO is capable of reacting with radicals or transition metals to form complexes. On the other hand, it is relatively stable and unreactive to non-radical species. That NO

reacts with lipid alkoxyl (LO•) and peroxyl (LOO•) radicals are relevant in the termination of lipid peroxidation processes since NO, being more soluble in nonpolar solvents, can concentrate in lipid bilayers, and therefore, can play a role in the regulation of lipid peroxidation. In addition, certain nitric oxide synthases (NOS) metabolize arginine to sitrulin, leading to the formation of NO• (Ghafourifar & Cadenas, 2005). Since NO• radical contains an unpaired electron, it can easily diffuse into the cell. An electron reduction causes the formation of the nitroxyl anion, ONNO•$^-$. Nitroxyl is a short-lived species that reacts with NO• to give nitrogen oxides, N_2O and HO• radicals.



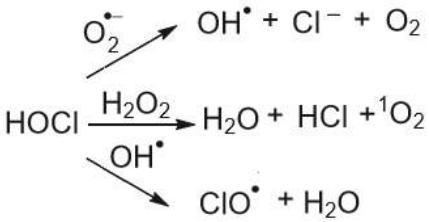
During inflammatory processes, cells that are responsible for the immune system produce $O_2^{\cdot-}$ and NO• radicals. Under these conditions, $O_2^{\cdot-}$ and NO• can react to produce ONOO-, which has relatively high reactivity and can cause DNA fragmentation and lipid peroxidation (Carr & Frei, 2000).

1.2.8 Hypochlorous Acid (HClO)

Hypochlorous acid (HClO) is non-radicalic ROS. HClO is usually formed from the reaction of Cl_2 gas with water, however in biological systems, their formation have been mediated by a secreted heme protein, myeloperoxidase (MPO), which can convert H_2O_2 to HOCl in the presence of chloride ion (Cl^-) according to equation below (Weiss, 1989).

$$H_2O_2 + Cl^- + H^+ + MPO \rightarrow HOCl + H_2O$$

HClO reacts with various ROS including H_2O_2 to generate stoichiometric amounts of O_2, with $O_2^{\cdot-}$ to generate HO• and with HO·to form ClO•



HOCl can damage biomolecules directly and through chlorine. In addition, HOCl can oxidize thiols, mercury, NAD(P)H and can lead to the chlorination of DNA bases, especially pyrimidines and tyrosine units in proteins.

1.3 Defense Mechanisms in Aerobic Organisms

Free radicals can cause oxidative damage to biomolecules and lead to cell and tissue injury. On the other hand, endogenous defence mechanisms, namely antioxidant systems, have been developed to counteract the detrimental effects of ROS and related reactive species (Cadenas, 1997).

The term antioxidant can be defined as any substance that can prevent, reduce, or repair the ROS-induced damage of a target biomolecule (Halliwell, 2007). Antioxidants protect ROS and other related reactive species induced damage by three known mechanisms including inhibition of ROS generation, scavenging of ROS already formed and repair of ROS-induced damage. Antioxidant defense systems include enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidases, which catalytically scavenge ROS and other free radicals; transferrin, which interact with pro-oxidants such as iron ions and haptoglobins, non-enzymatic proteins such as hemopexin and metallothioin, heat shock proteins, low molecular weight ROS scavengers such as glutathione, α-tocopherol and ascorbic acid (Halliwell & Gutteridge, 1999).

1.3.1 Enzymatic Antioxidant System

1.3.1.1 Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) that is a metalloenzyme, catalyze the dismutation of $O_2^{\cdot-}$ to form H_2O_2 and O_2.

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

In mammals, SOD is found as three different isoforms: (1) copper, zinc superoxide dismutase (CuZnSOD or SOD1), (2) manganese superoxide dismutase (MnSOD or SOD2), and finally (3) extracellular superoxide dismutase (ECSOD or SOD3). CuZnSOD mainly is localized in cytosol, but was also detected in lysosomes, peroxisomes, nucleus, and intermembrane space of mitochondria (Keller, Warner, Steimer, & Hallewell, 1991; Crapo, Oury, Rabouille, Slot, & Chang, 1992). MnSOD is present in mitochondrial matrix and ECSOD is associated with plas ma membrane or present in extracellular space.

Escherichia coli cytoplasm contains MnSOD, iron superoxide dismutase (FeSOD), and a hybrid enzyme composed of MnSOD and FeSOD subunits (Fridovich, 1995). It is known that while eukaryotic MnSODs are tetrameric, mostly bacterial enzymes are dimeric.

Some bacteria species such as *Bacillus cereus* and *Streptococcus sanguis* contain only FeSOD (Halliwell & Gutteridge, 1999). FeSODs occurs also in plants mainly in the chloroplasts and are common in protozoa. Some anaerobic bacteria including *Bacteroides fragilis, Bacteroides thetaiotaomicron, Bacteroides gingivalis, Streptococus mutans, Propionibacterium shermanii* also contains SODs. A different type of SOD, a Ni-containing enzyme, was found in the filamentous Gram-positive bacterium *Streptomyces* ssp (Kim, Kim, Hah, & Roe, 1996).

In the case of yeast *Saccharomyces cerevisiae*, it has been found that there are two SOD genes, SOD1 and SOD2. While SOD1 encodes Cu/ZnSOD, SOD2 gene is

responsible from the production of MnSOD. As in most other eukaryotes, Cu/ZnSOD is localized in cytoplasm while MnSOD in the mitochondrion (Gralla & Kosman, 1992). It has been shown that a fraction of the CuZnSOD and its metallo chaperone CCS are co-localised to the mitochondrial intermembrane space, and that this form of the enzyme helps protect the mitochondrion against oxidative damage in yeast.

Mammalian CuZnSOD is known for its high stability. It shows resistance to heating, proteolysis, and denaturing agents such as sodium dodecyl sulfate, guanidinium chloride and urea. It is not inhibited in a broad range of pH. In contrast to mammalian CuZnSOD, bacterial enzyme is extremely sensitive against proteases (Imlay & Imlay, 1996). This enzyme is inhibited by the agents including azide, cyanide, diethyldithiocarbamate and H_2O_2. ECSOD has also high stability and shows considerable resistance to high temperatures, pH extremes, and high urea concentrations (Tibell, Skarfstad, & Jonsson, 1996). Similarly to CuZnSOD, it is also inhibited by azide, cyanide, diethyldithiocarbamate and H_2O_2 (Marklund, 1984). MnSODs and FeSODs are known with their lower stability in response to chemicals. When compared with CuZnSOD, MnSOD is more susceptible than to heat and detergents but is not inhibited by cyanide, diethyldithiocarbamate or H_2O_2. FeSOD, unlike MnSOD, is inhibited by H_2O_2.

There are numerous studies that inform about the biological importance of SODs. Kobayashi and colleagues remarked the possible clinical application of SOD in the use of spermatozoa for in-vitro fertilization (IVE) or artificial insemination (Kobayashi, Miyazaki, Natori, & Nozawa, 1991). Satomi et al. showed that SOD activity in the colon and rectum cancers increased with progression of the stage of the diseases. They also indicated that SOD activity was associated with venous invasion, cell proliferation, and the degree of malignancy of colorectal cancer, which point the necessity of the elucidation of the antioxidant system in cancer tissues (Satomi et al., 1995). In the study conducted with human lens epithelial B-3 cells, protective effect of SOD against H_2O_2 induced oxidative damage, ROS accumulation and cell apoptosis was observed in the cells treated with resveratrol (Zheng et al.,

2010). It was shown that ECSOD plays a role not only as a ROS scavenger, but also as a pro-apoptotic factor via cyclooxygenase-2 (COX-2)/galectin-7 pathways in the epidermis (Lee et al., 2011). High expression of ECSOD contributes to the suppression of atherosclerosis by scavenging $O_2^{\cdot -}$ (Makino, Kamiya, Hara, & Adachi, 2012).

In yeast, it was found that the levels of CuZnSOD expressed by SOD1 increased as a response of oxygen, paraquat treatment, and growth on the non-fermentable carbon sources including acetate and lactate (Lee & Hassan, 1985; Gralla & Kosman, 1992). Copper ion was determined as another factor that triggered enzyme activity in yeast cells (Lee and Hassan, 1985; Greco, Hrab, Magner, William, & Kosman, 1990). Deletion mutants of SOD1 show elevated carbonylation in mitochondrial proteins (Sturtz, Diekert, Jensen, Lill, & Culotta, 2001). According to the researches, similar to CuZnSOD, mitochondrial MnSOD encoded by SOD2 gene was induced by the treatment of the yeast cells with paraquat and growth in oxygen, or on non-fermentable carbon sources (Lowry & Zitomer, 1984; Westerbeek-Marres, Moore, & Anne, 1988; Galiazzo & LabbeBois, 1993; Pinkham, Wang, & Alsina, 1997). It is known that the major defence against $O_2^{\cdot -}$ toxicity is MnSOD in *S. cerevisiae* (Longo, Liou, Valentine, & Gralla, 1999). One of the significant roles of the MnSOD is to protect mitochondrial Fe–S enzymes, such as aconitase, from superoxide-induced damage. On the other hand, it has been thought that CuZnSOD may assist reconstitution of Fe–S cluster-containing enzymes (Wallace et al., 2004). In the same study, it has been also shown that although both genes are not essential for growth, sod1 mutants have reduced rates of aerobic growth in synthetic glucose medium and reduced ability to grow on non-fermentable substrates. *S. cerevisiae* mutants with deletions of both SOD1 and SOD2 genes cannot synthesise methionine and lysine, are defective in sporulation and have an increased mutation rate.

1.3.1.2 Catalase (CAT)

Catalase (CAT) is a water-soluble and heme-containing enzyme. It is best known for its ability to catalyze the decomposition of H_2O_2, a product of SOD enzymatic

activity, to form water and molecular oxygen (Alfonso-Prieto, Biarnés, Vidossich, & Rovira, 2009).

$$2H_2O_2 \xrightarrow{CAT} H_2O + O_2$$

CAT also possesses peroxidase and oxidase activities toward a number of substrates, including alcohols. Peroxidase activity of CAT in converting alcohol (AH_2) to aldehyde (A) is shown below:

$$H_2O_2 + AH_2 \xrightarrow{CAT} A + 2H_2O$$

CAT shows diversity and can be classified into three different groups according to their physical and biochemical properties; (1) Catalase-peroxidases with enhanced peroxidative activity, (2) Non-heme catalases with manganese in their active center and (3) monofunctional heme catalases. While catalase-peroxidases are widely distributed in procaryotes and are also found in lower eukaryotes, only in fungi, non-heme catalases (Mn-catalases) were found only in prokaryotic cells (Bartosz, 2005). On the other hand, almost all eukaryotes and many prokaryotes have monofunctional heme catalases.

The molecular weight of catalase-peroxidases ranges from 120 to 340 kDa. Both homodimer and also homotetramer forms were reported for catalase-peroxidases. Mn catalases sometimes referred to as pseudocatalases have molecular weights of about 170 to 210 kDa. They may form unusual oligomeric structures like homopentamers and homohexamers (Zámocký & Koller, 1999). Monofunctional heme catalases are usually homotetramers, of molecular weight 200–340kDa.

Catalase-peroxidases are vulnerable to inactivation by high temperatures, pH, and H_2O_2. They are not inhibited by aminotriazole but their heme iron is readily reduced by sodium dithionite. While monofunctional heme catalases are irreversibly inhibited by a suicide inhibitor, 3-amino-1,2,4-triazole, Mn catalases are not inhibited by CN^- or N_3^-. Among others, monofunctional heme catalases are very efficient as catalases (k_{cat}=4x10^7 M^{-1} s^{-1} for human erythrocyte catalase). They can also catalyze 2-

electron oxidation of short-chain aliphatic alcohols at reasonable rates (Sichak & Dounce, 1986).

S. cerevisiae has two haemoprotein catalases which can dismutate H_2O_2 into water and oxygen. One, catalase A encoded by the CTA1 gene (Cohen, Fessl, Traczyk, Rytka, & Ruis, 1985) is located in the peroxisome (Seah & Kaplan, 1973), and the other encoded by CTT1 is cytosolic (Spevak, Hartig, Meindl, & Ruis, 1986; Ruis & Hamilton, 1992). Neither of these catalases has the ability to react with larger hydroperoxides such as tert-butyl hydroperoxide. The catalases in yeast may not be important in growing cells in scavenging of endogenous H_2O_2 that is produced during respiration or β-oxidation, and that this function is performed by cytochrome c peroxidase, or glutathione peroxidases. This is consistent with the demonstration that in exponentially growing cells, glutathione has a more important role than the catalases in the cellular response to H_2O_2 (Grant et al., 1996). The catalases have, however, an important role in removal of H_2O_2 during stationary phase, and may play some part in the adaptive response to oxidative stress (Izawa, Inoue, & Kimura, 1996). Disruption of the CTT1 gene has been reported to lead to sensitivity to hydrogen peroxide and to exposure to elevated temperatures (Wieser et al., 1991). It should be stated that disruption of CAT expressing genes in yeast or bacteria has less dramatic effects on normal growth as compared with disruption to SOD genes (Halliwell & Gutteridge, 1999). On the other hand, it was shown that hyperosmotic stress in *S. cerevisiae* increased significantly CAT activity besides CuZnSOD (Garay et al., 2004).

CATs are believed to play a important role in plant defense in combination with salicylic acid. In healthy tissues having low concentrations of H_2O_2, CAT is inhibited by H_2O_2. This situation allows increase of H_2O_2 acting as a second messenger to activate expression of defense-related genes. Conversely, in tissues exposed to high H_2O_2 concentrations, higher concentrations of salicylic acid protect CAT (Durner & Klessig, 1996). In mammalians, CATs are thought to protect SODs against higher levels of H_2O_2 (Fridovich, 1995). It is due to the low affinity and high K_m value of CAT for the substrate when compared with glutathione peroxidase (GSHPx). The

peroxidative activity of CAT in an important source of acetaldehyde in the brain where no other enzyme is known to convert significant amounts of ethanol to acetaldehyde (Zimatkin, Liopo, & Deitrich, 1998).

1.3.1.3 Glutathione Peroxidase (GSHPx)

Glutathione peroxidase (GSHPx) refers to a family of multiple isozymes. This family is the unique mitochondrial enzyme that uses H_2O_2 in the mitochondria of most mammalian organs except heart (Itoh et al., 1997). In mammalian tissues, there are six GSHPx isozymes, namely, GSHPx1, 2, 3, 4, 5, and 6 (Margis, Dunand, Teixeira, & Margis-Pinheiro, 2008). GSHPx1, 2, 3, and 4 are selenoproteins. GSHPx consists of four protein subunits, each of which contains one atom of selenium at its active site, probably incorporated into molecules of cysteine, replacing their sulfur atoms. It is not usually found in developed plants and in bacteria, its presence is reported only for a few algae and fungi.

GSHPx catalyze the reduction of H_2O_2 or organic hydroperoxides (LOOH) to water or corresponding alcohols (LOH) using glutathione (GSH) as an electron donor. During the reactions, GSH is oxidized to glutathione disulfide (GSSG).

$$H_2O_2 + 2GSH \xrightarrow{GPx} 2H_2O + GSSG$$
$$LOOH + 2GSH \xrightarrow{GPx} LOH + GSSG + H_2O$$

The activity of GSHPx and the concentration of GSH provide a maximal mitochondrial rate of H_2O_2 utilization of 2.4 mMs^{-1} (Boveris & Cadenas, 1997). This rate of H_2O_2 utilization, which accounts for about 60% of the rate of H_2O_2 production, appears to indicate both a role of H_2O_2 as messenger and a vital function of GSHPx in the reduction of mitochondrial hydroperoxides. GSH deficiency is associated with widespread mitochondrial dysfunction that leads to other types of cell damage (Abraham & Kappas, 2008). GSHPx requires a continuous supply of GSH, since this reductant becomes oxidized during H_2O_2 and hydroperoxide reduction.

The biological activities of individual GSHPx isozymes have been studied in transgenic overexpression and gene knockout animal models. For example, GSHPx1 knockout mice are more sensitive to tissue injury induced by redox cycling chemicals, including paraquat and doxorubicin. Knockout of GSHPx1 gene also sensitizes the mice to tissue ischemia-reperfusion injury, angiotensin II-induced vascular oxidative stress, and development of atherosclerosis (Shiomi et al., 2004; Chrissobolis et al., 2008; Torzewski et al., 2007). Mice with overexpression of GSHPx1 are more resistant to oxidative tissue injury induced by redox cycling chemicals. GSHPx3 may act as one of the major scavenger of ROS in plasma. Mice overexpressing GSHPx3 are resistant to lipopolysaccharide-induced endotoxemia, infl ammation, and oxidative stress (Mirochnitchenko et al., 2000). The GSHPx3-overexpressing mice manifest better control of ROS levels under high body temperatures when compared with wild-type mice. In addition, GSHPx3 plays a role in regulating the bioavailability of vascular nitric oxide, a critical antioxidative and antiinflammatory molecule in the cardiovascular system (Loscalzo, 2001). Taken together, extensive experimental evidence demonstrates an important role for GSHPx isozymes in protecting tissues from oxidative stress injury.

S. cerevisiae has been known to contain GSHPx activity and three genes encoding GSHPx 1–3 were identified from the genome sequencing data (Inoue, Matsuda, Sugiyama, Izawa, & Kimura, 1999). Avery & Avery (2001) have shown that yeast is unusual in that these probably encode phospholipid hydroperoxide peroxidases which differ from more widespread glutathione peroxidases in being monomeric, partly membrane-associated, and capable of reducing lipid hydroperoxides esterified to membranes (Avery & Avery, 2001). Among these three deletants, only the GSHPx3 was sensitive to peroxides. While GSHPx1 gene was induced by glucose starvation, the GSHPx2 gene by oxidative stress. On the other hand, it has been known that GSHPx 3 was reported to be basically expressed (Inoue, Matsuda, Sugiyama, Izawa, & Kimura, 1999). GSHPx1p and GSHPx2p contribute to the adaptive response to lipid peroxide based stress, while GSHPx3p provides the greatest protection to hydroperoxide stress (Izawa, Inoue, & Kimura, 1995).

1.3.1.4 Glutathione Reductase (GR)

As mentioned previously above, GSH is oxidized to GSSG upon reaction with ROS. On the other hand, in mammalian cells, the ratios of intracellular GSH to GSSG are in the range of 10:1 to 100:1. Protection of such high ratios of intracellular GSH to GSSG is essential for normal cellular activities as redox signaling. Glutathione reductase (GR) is the enzyme that is responsible for the regeneration of GSH from its oxidized form GSSG.

The enzyme uses NADPH as a cofactor:

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

GR has significant function in terms of both in detoxification of ROS and in the regulation of cellular redox homeostasis. In the research conducted with Drosophila as a model organism, overexpression of GR increased the resistance of flies exposed to hyperoxic conditions and extended life span under such conditions, although it does not affect life span of flies maintained in normal air GR deficient mice are shown to be more susceptible to ROS-induced tissue injury. Inhibition of GR activity in animals or cultured cells by chemical inhibitors or small interfering RNA techniques results in increased sensitivity to oxidative injury (Mockett, Sohal, & Orr, 1999). Increased expression of GR in macrophages also decreases atherosclerotic lesion formation and vascular oxidative stress in low-density lipoprotein receptor deficient mice (Qiao et al., 2007).

The regeneration of GSH by the reaction with NADPH catalysed GR encoded by GLR1 gene in *S. cerevisiae*. The disruption of the GLR1 gene does not lead to impairment of growth of yeast under normal aerobic conditions, but does cause them to become sensitive to a range of oxidants (Collinson & Dawes, 1995; Grant, MacIver, & Dawes, 1996). GLR1 mutants have a higher level of GSSG than the wild type, but only a slightly higher than normal level of total glutathione, indicating that the cells can synthesise reduced glutathione to maintain an appropriate concentration of GSH as it becomes oxidise. In addition to Grant's research, Muller determined

that the GLR1 delta mutant showed increased sensitivity to the thiol oxidant diamide (Muller, 1996).

1.3.1.4 Glutathione-S-Transferase (GST)

Glutathione-S-transferase (GST) is a general name for the description of enzyme superfamily which catalyze the conjugation of GSH to a wide variety of xenobiotics (Hayes, Flanagan, & Jowsey, 2005). This superfamily catalyzes the conjugation reactions of GSH with a number of electrophilic xenobiotics, including reactive aldehydes and quinone compounds to form less reactive conjugates.

$$\text{Xenobiotic} + \text{GSH} \xrightarrow{\text{GST}} \text{Xenobiotic-GS}$$

In addition, certain GST isozymes can exhibit GSHPx activity and catalyze the reduction of organic hydroperoxide (LOOH) to form alcohol (LOH).

$$\text{LOOH} + 2\text{GSH} \xrightarrow{\text{GST}} \text{LOH} + \text{GSSG} + \text{H}_2\text{O}$$

Some GST isozymes also regulate various cellular processes through nonenzymatic reactions. This enzyme superfamily consists of three major families widely distributed in mammalian tissues, namely (1) cytosolic GSTs, (2) mitochondrial GSTs, and (3) microsomal GSTs. Among these families, cytosolic GSTs are the most extensively studied enzymes involved in detoxification of xenobiotics and ROS, and play important roles in protecting mammalian cells and tissues from electrophilic and oxidative stress.

It is known that this superfamily is ubiquitously distributed in nature, being found in organisms as diverse as microorganisms, insects, plants and fish besides mammalians. On the other hand, there is no detailed knowledge about GST activity in yeast. However, Ure2, that is a nitrogen catabolite repression transcriptional regulator, apparently serves as a GST and protects *S. cerevisiae* from heavy metal ion

and oxidant toxicity (Rai & Cooper, 2005). The enzymatic antioxidant system as a whole was shown in Figure 1.11.

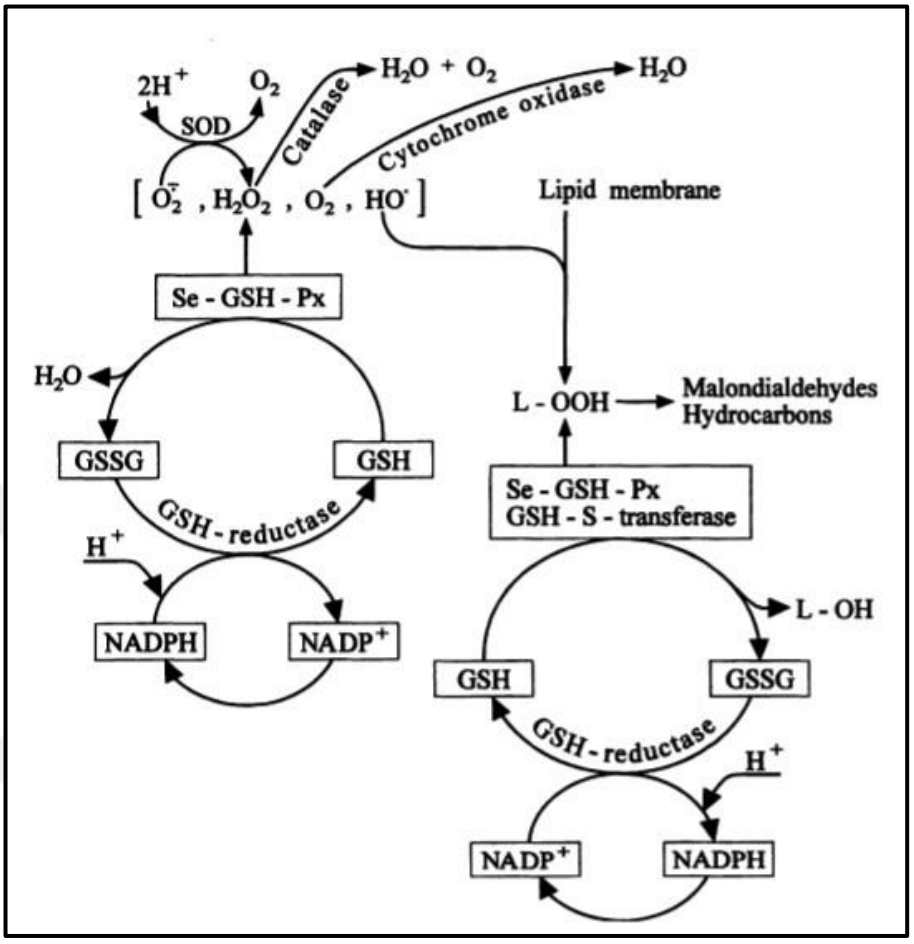


Figure 1.11 The schematic representation of enzymatic antioxidant system (Hutzinger, Beek, & Metzler, 2013)

1.3.2 Non-Enzymatic Antioxidant System

1.3.2.1 Glutathione (GSH)

Glutathione (GSH) is the most abundant non-protein thiol in cells (Jocelyn, 1975; (Masella, Benedetto, Vari, Filesi, & Giovannini, 2005). Similar to glucose levels, the intracellular GSH concentration is around 5 mmol per gram of tissue (Vina, Jose, Hems, Reginald, & Krebs, 1978).

GSH is a tripeptide (γ -glutamylcysteinylglycine) synthesized from three amino acids through two enzymatic reactions in cytoplasm (Lu, 2009). The first step involves a combination of cysteine and glutamate to produce γ -glutamylcysteine. This reaction is catalyzed by GCL, also known as γ -glutamylcysteine synthetase.

$$\text{Cysteine} + \text{Glutamate} + \text{ATP} \xrightarrow{\text{GCL}} \gamma\text{-Glutamylcysteine} + \text{ADP} + \text{Pi}$$

The next step involves the enzyme g lutathione synthetase (GS) that catalyzes the addition of glycine to the dipeptide to form γ-glutamylcysteinylglycine (GSH).

$$\gamma\text{-Glutamylcysteine} + \text{Glycine} + \text{ATP} \xrightarrow{\text{GS}} \gamma\text{-Glutamylcysteinylglycine} + \text{ADP} + \text{Pi}$$

Both steps require coupled ATP hydrolysis. In GSH biosynthesis, GCL is the rate-limiting enzyme which consists of two subunits, a heavy catalytic subunit (73 kDa) and a light regulatory subunit (30 kDa), which are encoded by different genes both in rats and in humans (Gipp, Chang, & Mulcahy, 1992). The catalytic subunit modulates the activity of GCL by GSH feedback inhibition (Seelig, Simondsen, & Meister, 1984). In addition, the physiological regulation of GCL activity relies on the availability of L-cysteine (Tateishi, Higashi, Shinya, Naruse, & Sakamoto, 1974). In other words, the availability of intracellular cysteine determines the rate of GSH synthesis under physiological conditions in mammalians.

In mammalian cells and tissues, GSH is mainly involved in four types of biochemical reactions including reaction with ROS, reaction with electrophiles, reaction with other nonenzymatic antioxidants, and protein deglutathionylation (Mieyal, Gallogly, Qanungo, Sabens, & Shelton, 2008).

The biological activities of GSH have been investigated by modulating its levels in cells or tissues. Increasing cellular or tissue GSH affords protection against ROS- and electrophile-elicited injury in various disease models. Elevation of tissue GSH levels also leads to protection against experimental carcinogenesis. In contrast, depletion of cellular or tissue GSH sensitizes animals to various pathophysiological

processes involving oxidative and electrophilic stress (Meister, 1991). These include neurodegeneration, tissue ischemia-reperfusion injury, and xenobiotic/drug-induced toxicity.

In the yeast, GSH cycle is presented in Figure 1.12. As in the case of mammalian, GSH is synthesized from its constituent amino acids via two ATP-dependent steps. While γ-glutamylcysteine synthetase is encoded by GSH1, glutathione synthetase is encoded by GSH2 gene. The factors that control the degradation of GSH/GSSG are unknown in yeast (Grant, 2001).

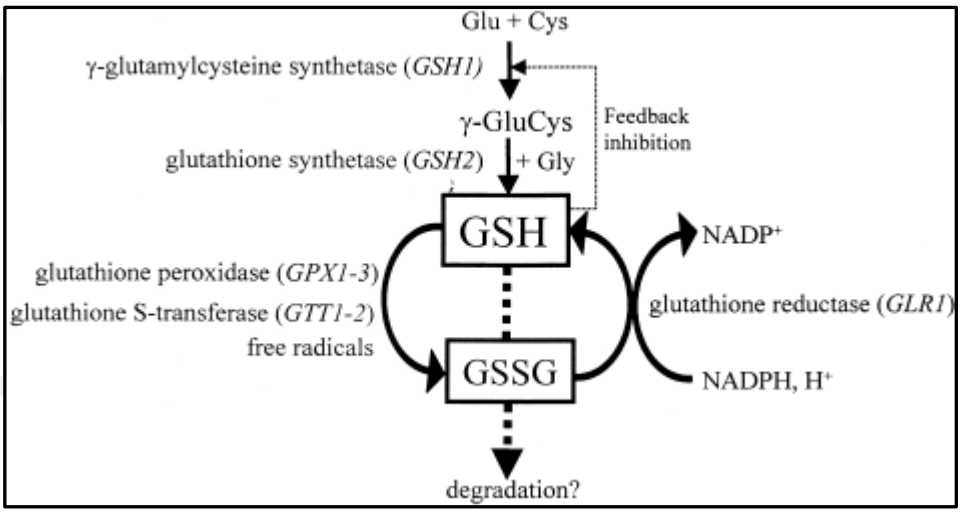


Figure 1.12 GSH cycle in yeast (Grant, 2001)

S. cerevisiae strain YPH98gsh1 which lacks GSH due to a deletion of the GSH1 gene died after 3 day on glutathione-free media (Brendel et al., 1998). In another research, intracellular GSH content of stationary phase *S. cerevisiae* S288C cells, that is known with higher resistance against several environmental stresses compared to cells in other growth phases, was found about 3-fold higher than the ones in log phase (Izawa, Inoue, & Kimura, 1995). In the same study, it was also found that depletion of cellular GSH or inhibition GSH-1 enhanced the sensitivity and suppressed the adaptation to H_2O_2. Similarly, *S. cerevisiae* CY8 (gsh1::LEU2) and CY10 (yap1::HIS3) mutants which are unable to synthesis GSH due to a gene disruption in GSH1, require exogenous GSH for growth under non-stress conditions (Grant, MacIver, & Dawes, 1996). Madeo et al. observed that apoptotic cell death

was induced by growing a gsh1 deletion mutant in the absence of GSH (Madeo et al., 1999). Besides GSH, glutaredoxin and thioredoxin systems have been well studied in yeast Draculic, Dawes, & Grant, 2000). Glutaredoxins and thioredoxins are small redox proteins. They have important functions together with GSH in reduction of disulfide bonds in biomolecules.

Yeast contains two gene pairs encoding cytoplasmic glutaredoxins (GRX1, GRX2) and thioredoxins (TRX1, TRX2). The oxidized disulphide form of thioredoxin is reduced directly by NADPH and thioredoxin reductase (Trr1). In contrast, oxidized glutaredoxin is reduced by GSH, and oxidized GSSG is in turn reduced in an NADPH-dependent reaction catalysed by GR (Glr1). The redox status of GSH may provide a functional link between the GSH/glutaredoxin and thioredoxin systems, as thioredoxins function along with GR to maintain the high intracellular GSH–GSSG ratio. However, it is not known whether the redox status of the GSH/glutaredoxin system affects the thioredoxin system (Grant, 2001).

The known antioxidant defence mechanisms in yeast are presented in Table 1.1 together with related gene(s), their locations and also functions.

1.3.3 Other Non-Enzymatic Antioxidant System

1.3.3.1 Vitamins

Vitamin C is one of the important and powerful antioxidants. In addition to working with antioxidant enzymes, it works with Vitamin E (α-tocopherol) and carotenoids. Vitamin C can regenerate α-tocopherol by transferring hydrogen to α-chromanoxyl radical (Kojo, 2004; Carr & Frei, 1999).

Vitamin C has been reported to reduce gastric cancer risk (Knekt et al., 1991). The positive effect of vitamin C is thought to be due to the its inhibitory effects in the formation of N-nitroso compounds. It has also been reported that it protects the organism against lung and colorectal cancer (Golde, 2003). In contrast to in vivo

studies, vitamin C does not affect lipid peroxidation in vitro. In addition, under physiological conditions, vitamin C can lead to hydroxyl radical formation (Smith, Harris, Sayre, Beckman, & Perry, 1997).

Vitamin E is an oil-soluble antioxidant and has eight isoforms. α-tocopherol is the active form and is known as the most basic membrane-bound antioxidant (Burton & Ingold, 1989). Vitamin E often protects the cells from lipid peroxidation (Pryor, 2000). It works together with Vitamin C in the cyclic type process. While α-tocopherol functions as an antioxidant, lipid or lipid peroxyl radical convertes α-tocopherol to radical with its unstable hydrogen. As stated above, the role of vitamin C in this process is regeneration of α-tocopherol (Kojo, 2004).

1.3.3.2 Carotenoids

Carotenoids are a group of produced colored pigments that are common in plants. They are also found in some animals and bacteria. Nutrient-derived carotenoids are found in human tissues and in some other mammals, but they are not absorbed by most of animals. In humans, carotenoids are the most abundant in fat and in the liver. In plants, carotenoids act as antioxidants and helps to prevent the formation of ROS, especially $^1O_2$, during photosynthesis. β-carotene is also protective against light-induced skin damage in porphyry patients.

In vitro studies have shown that β-carotene inhibits lipid peroxidation in systems at low O_2 concentrations but it is not successful at high O_2 concentrations. However, studies with Low Density Lipoprotein (LDL) have shown that β-carotene does not protect LDL against peroxidation, regardless of its O_2 concentration. (Halliwell & Gutteridge, 1999).

1.3.3.3 Transferrin

Transferrin binds free metal ions without participating in free radical reactions (Gutteridge, Quinlan, & Evans, 1994). Transition metals are required for numerous

biological processes such as DNA, RNA or protein synthesis due to the fact that they are cofactors for many enzymes. When the levels of these metals fall, the metabolic processes will deteriorate. However, excessive accumulation of these metals in the tissues is cytotoxic for the cells. At this point, the antioxidant properties of transferrin are prominent. By binding metals that accumulate in the cell, they prevent cells from radical-producing reactions such as Fenton and Haber-Weiss.

Table 1.1 Important antioxidant defence systems in *S. cerevisiae* (Perrone, Tan, & Dawes, 2008)

Name	Gene(s)	Localization	Function
Antioxidant metabolites			
Glutathione		General	Aqueous redox buffer, excretion of xenobiotics
Ubiquinone		Mit, ER, Mem	Lipid soluble antioxidant, respiratory chain component
D-erythroascorbate		Cyt?	Water soluble antioxidant
Enzymes			
Catalase	CTT1; CTA1	Cyt; Per	Dismutation of H_2O_2
Superoxide dismutase	SOD1; SOD2	Cyt, Mit, Nuc?; Mit	Dismutation of $O_2^{\cdot -}$
SOD chaperone	CCS1	Cyt	Loading copper into Sod1p
Thioredoxins	TRX1, TRX2	Cyt	Deoxyribonucleotide synthesis, sulphate assimilation, cofactor for some peroxidases, redox control
Dithiol glutaredoxins	GRX1, GRX2	Cyt, Nuc	Overlap with thioredoxins in many enzymatic functions, have glutathione peroxidase activity
Monothiol glutaredoxins	GRX3, GRX4, GRX5	Nuc, Mit matrix	Protect cells from redox damage Fe/S cluster assembly
Peroxiredoxins	AHP1, PRX1	Cyt, Mit	Reduction of alkyl peroxides
Thioredoxin peroxidase	TSA1	Cyt	Peroxiredoxin activity, thioredoxin peroxidase activity
Sulfiredoxin	SRX1	Cyt, Nuc	Reduces cysteine-sulfinic acid residues
Glutathione peroxidases	GPX1, GPX2	Cyt/Mem	Reduction of oxidized lipid hydroperoxides
Phospholipid hydroperoxide	GPX3/ORP1	Cyt	Thioredoxin peroxidase activity
Cytochrome c peroxidase	CCP1	Mit inner membrane space	Reduction of H_2O_2 in mitochondrion
Glutathione synthesis	GSH1, GSH2	Cyt	Stepwise synthesis of GSH
Glutathione reductase	GLR1	Cyt	Reduction of GSSG to GSH
Pentose phosphate pathway enzymes	ZWF1, GND1, TAL1, TKL1,2, RPE1, ALO1, ARA2	Cyt Mit Outer Membrane?	Generation of NADPH for recycling of oxidized glutathione, glutaredoxins and thioredoxins D-erythroascorbate synthesis
Metal binding proteins			
Metallothioneins	CUP1, CRS5	Cyt	Copper binding protein, also binds cadmium ions
Transcription factors, regulators			
Yap1p	YAP1	Cyt/Nuc	Oxidative stress, resistance to xenobiotics, cadmium
Skn7p	SKN7	Nuc	Auxiliary transcription factor, functions with Yap1p for some oxidative stress, also acts in osmoregulatin
Msn2/4	MSN2, MSN4	Cyt/Nuc	Responsive to heat, starvation, osmotic and oxidative stress
Yap1p-binding protein	YBP1	Cyt	Protein involved in one mechanism for nuclear localization of Yap1p
Haem activated protein	HAP1,2,3,4,5	Nuc	Regulation of respiratory functions
Metal-binding activator	MAC1	Nuc	Regulator of genes involved in copper ion homeostasis and H_2O_2-induction of CTT1
Cup2p	ACE1/CUP2	Nuc	Cu-binding transcription factor

1.4 Biological Damages Caused by Reactive Species

As mentioned previously, reactive species can also cause extensive damage towards enzymes, membranes, nucleic acids or polysaccharides, besides playing important physiological functions. Especially HO·and $^1O_2$ are involved in a majority of human diseases. The damage may be a result of the direct action of the reactive species with biomolecules, or from the action of toxic products of reactions initiated by these species.

1.4.1 Damage in Lipids

Lipids are essential components of cell membranes that maintain structure and control the function of cells. They are primary targets of the attack by free radicals, especially HO·and $^1O_2$, and the oxidation of lipid is associated with various pathological states (Esterbauer, 1993). Lipid peroxidation has been implicated in several human diseases such as atherosclerosis (Berliner & Heinecke, 1996), cancer (Hammad et al., 2009), diabetes (Silverstein & Febbraio, 2009), acute lung injury (Imai et al., 2008; Nonas et al., 2006) as well as the neurodegenerative disorders including Alzheimer's and Parkinson's diseases (Simonian & Coyle, 1996; Montine, Li, Perl, & Galasko, 2005). This process also worsens the initial tissue damage caused by ischemic or traumatic brain injury (Yoshida et al., 1980). Therefore, the increase in end-product concentrations of lipid peroxidation is a clear proof of participation of reactive species to the disease formation.

While saturated and monounsaturated fatty acids do not undergo peroxidation during physiologically relevant conditions, polyunsaturated fatty acids (PUFAs) readily undergo peroxidation. The potential energy for abstraction of hydrogen from an alkane chain is rather high. On the other hand, the potential energy for abstraction of allylic hydrogen from a monounsaturated fatty acid is still too great for abstraction to occur physiologically. Conversely, the potential energy for abstraction of the bis-allylic hydrogen of a PUFA is as low as possible for generation a lipid radical. The greater the number of double bonds in a fatty acid side chain, the easier is the

removal of a hydrogen atom. The first initiation step of lipid peroxidation is the abstraction of hydrogen from an acyl chain to form a lipid radical (Figure 1.13).

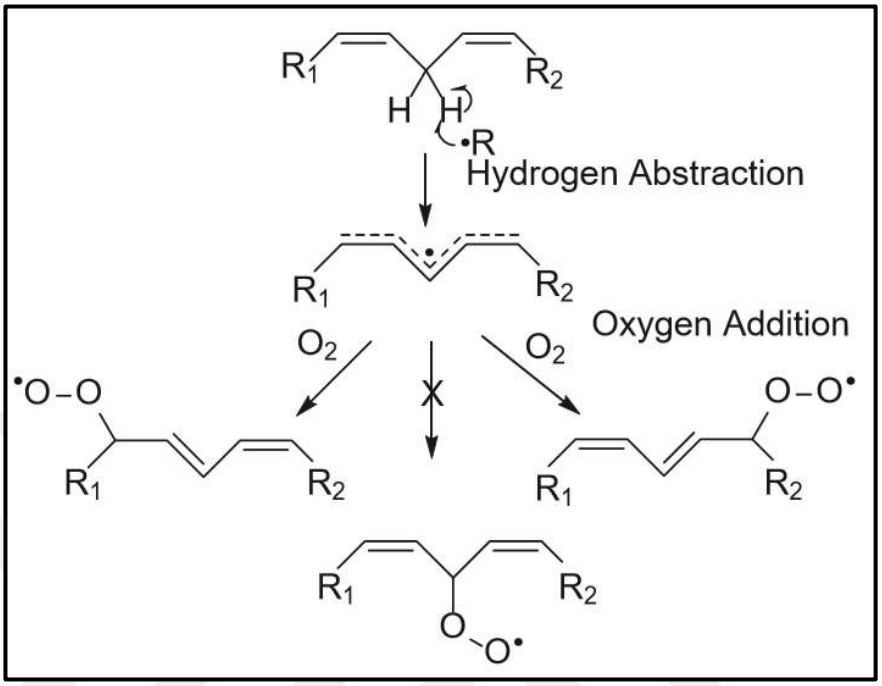


Figure 1.13 Abstraction of hydrogen by free radical and addition of oxygen forms lipid peroxyl radical (Davies & Guo, 2013)

C-centered radical is generated by the abstraction of a hydrogen atom from a fatty acid and by molecular arrangement. Then the generated radical interacts with O_2 to produce the peroxyl radical. One critical point is that O_2 is added to the carbon where the hydrogen abstraction did not occur and is the most energetically favorable positions. Once formed, lipid peroxyl radical spontaneously can abstract the hydrogen atom from other fatty acid depending on the specific PUFA being oxidized and so they are capable of propagating the chain reaction of lipid peroxidation. Peroxidation may last until the substrate is finished if the chain interrupting antioxidants do not interact with the reaction. In the termination step, the chain reaction is terminated by the reaction of peroxyl with another radical or an antioxidant and quenching of peroxyl.

1.4.1.1 Mechanism of Lipid Peroxidation

PUFAs can be oxidized by non-enzymatic and enzymatic pathways. As stated above, the peroxyl radical can abstract hydrogen from one of these adjacent PUFA or other hydrogen donor such as alpha-tocopherol to form hydroperoxy fatty acids. As a model substrate, if the peroxidized lipid is linoleic acid (LA), then the name of the resulting product will be hydroperoxy octadecadienoic acid (HpODE). In addition to non-enzymatic free radical mechanisms, HpODEs can be generated by various lipoxygenases. It was found that the genetic ablation of these iron-containing oxidoreductases has lowered the level of other peroxidation products in vivo (Cyrus et al., 2001). Iron and copper metals can reduce hydroperoxy fatty acids to their hydroxy fatty acids through a mechanism of two-step. Firstly, alkoxyl radical is formed by cleavage of oxygen–oxygen bond of the peroxyl and then generated the alkoxyl radical abstracts hydrogen from another PUFA molecule. Hydroxy fatty acids derived from LA are termed as hydroxyoctadecaenoic acids (HODEs). HODEs are biologically active metabolites (Yu et al., 1995; Naruhn et al., 2010; Kliewer et al., 1997; Shappell et al., 2001) serve as intermediates for the generation of other biologically active lipids including B4-isoleukotrienes.

For PUFAs that have more than three double bonds, cyclization of the peroxyl radical occurs (Porter, Caldwell, & Mills, 1995). Isoprostanes may well be the most studied of the cyclic endoperoxide products of lipid peroxidation. In addition to oxygenation and cyclization reactions, peroxidation results in fragmentation of acyl chains. Fragmentation products include some of the earliest and most well-studied products of lipid peroxidation, such as malondialdehyde (MDA), acrolein, and 4-hydroxy-2-nonenal, as well as highly potent ligands for receptors such as oxidatively fragmented phospholipids. Among these fragmentation products, especially MDA has been widely used for many years as a suitable biomarker for lipid peroxidation of fatty acids and so oxidative stress due to reaction with thiobarbituric acid (TBA) (Abuja & Albertini, 2001). The TBA test is based on the reactivity of the TBA against MDA and the chromogenic product giving a red light dependent on the MDA level. This test was first used by food chemists to determine the autoxidative

degradation of oils and fats (Claxson et al., 1994). However, the substances that react with thiobarbituric acid (TBARS) test are not very specific. In recent years, attempts have been made to develop GC-MS/MS, LC-MS/MS and various derivation based strategies to determine free and total MDA levels (Giera, Lingeman, & Niessen, 2012).

1.4.1.2 In Vivo Origin of MDA

The peroxidation of polyunsaturated fatty acids with two or more methylene-interrupted double bonds is the main source of MDA in biological samples (Rio, Stewart, & Pellegrini, 2005). There are different hypotheses that describe in vivo formation of MDA. According to the Pryor and Stanley, MDA is originated from precursor as a bicyclic endoperoxide under stressed conditions which is generated by the conversion of methyl linoleate (Pryor & Stanley, 1975). The other two mechanisms that were postulated by Esterbauer and colleagues are summarized in Figure 1.14 (Esterbauer, Schaur, & Zollner, 1991). They are based on hydroperoxide formations and β-cleavage of the fatty acid chain to give a hydroperoxyaldehyde. MDA is then generated by β-scission or by reaction of the final acrolein radical with a hydroxyl radical. Finally, MDA can be also formed in vivo by enzymatic processes from various prostaglandins (Hecker & Ullrich, 1989). The researchers have showed that the biosynthesis of thromboxane A2 (TXA2) leads to MDA and 12(S)-hydroxy-8,10(E,E)-heptadecadienoic acid (HHT) with a high yield as secondary products.

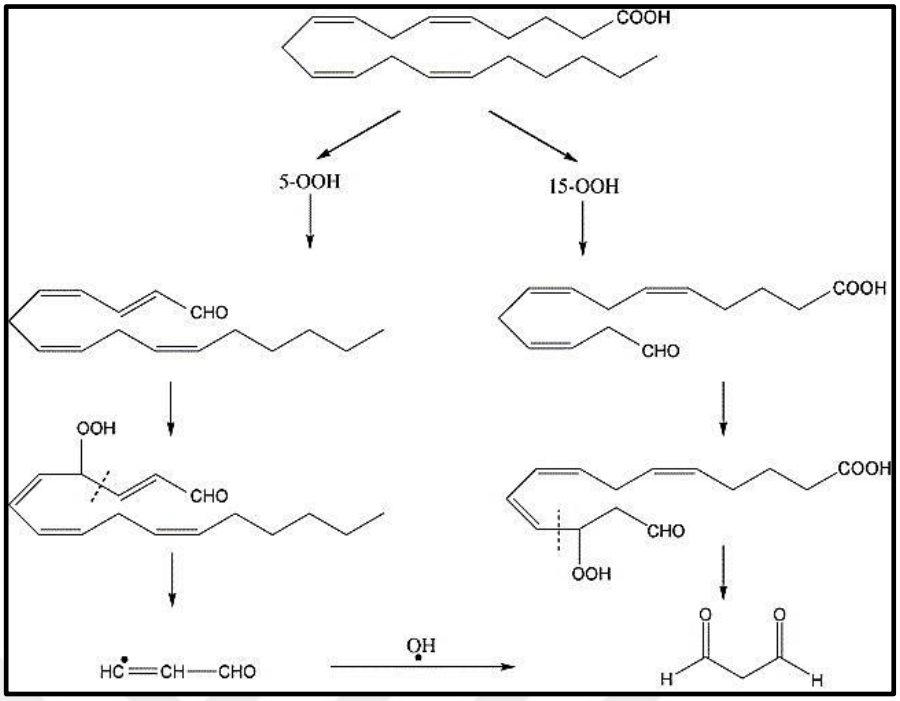


Figure 1.14 The mechanisms of MDA generation (Del Rio, Stewart, & Pellegrini, 2005)

1.4.1.3 Reactivity and Toxicity of MDA

MDA is present as an enolate anion with low reactivity in its physiological state, at neutral pH (Esterbauer et al., 1991). However, this anion can interact with nucleic acid bases and form several adducts, the main of which is pyrimido-[1,2-α]purin-10(3H)-one deoxyribose abbreviated as M1dG, M1GdR or M1G. This product is able to trigger sequence-dependent frameshift mutations and base-pair substitutions in bacteria and mammalian cells (VanderVeen, Hashim, Shyr, & Marnett, 2003). The alternative mechanism responsible from the genotoxicity of MDA is the creation of interstrand cross-links in DNA (Figure 1.15) (Niedernhofer, Daniels, Rouzer, Greene, & Marnett, 2003).

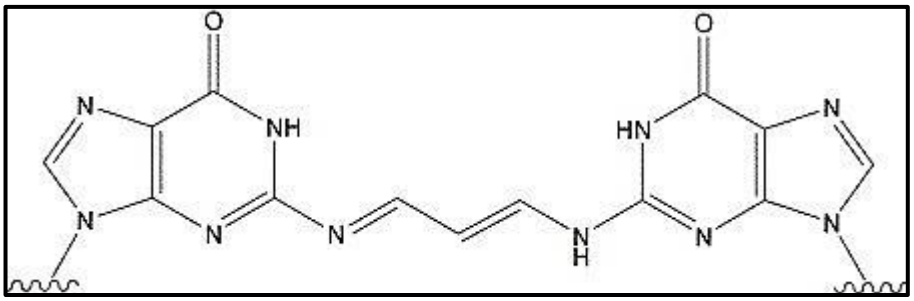


Figure 1.15 N^2,N^2-Malondialdehyde-interstrand cross-link structure as proposed (Niedernhofer, Daniels, Rouzer, Greene, & Marnett, 2003)

Voitkun and Zhitkovich reported that MDA can form DNA–histone cross-links (Voitkun & Zhitkovich, 1999). According to the researchers, MDA first reacts with the protein fraction and then forms the cross-link. On the other hand, if MDA reacts first with DNA, no cross-link is formed. Another probable toxic action of MDA involves collagen. Even if the nature of the cross-link is not yet determined in detail, the inter-molecular cross-linking of collagen through MDA may significantly contribute to the stiffening of cardiovascular tissue (Slatter, Bolton, & Bailey, 2000). MDA is able to impair several physiological mechanisms of the human body through its ability to react with molecules such as DNA and proteins. It is therefore useful to consider this molecule as something more than a lipid peroxidation product.

1.4.2 Damage in Proteins

Proteins that constitute more than 50% of the dry weight of cells have vital functions in the vast majority of biological processes including catalysis, ligand binding, signaling, etc. These functions are transduced via molecular interactions involving amino acid side chains. These side chains present a wide variety of functional groups, from simple hydrocarbons to thiols, alcohols or amines. While some of these side chains are largely unaffected by oxidative stress, a subset of amino acids can be rapidly modified by free radicals. This situation depends on the species of protein and oxidant besides whether or not the oxidant can gain access to susceptible amino acid residues within that protein. Furthermore, oxidative stress leads to the formation of electrophilic molecules that can subsequently react with amino acids bearing nucleophilic side chains. Through these two pathways, oxidative

stress can result in a variety of posttranslational modifications which alter structure and function of a protein. Therefore, oxidative stress related posttranslational modifications of proteins can be classified as “oxidation reactions” and “modifications by oxidation-produced electrophiles”.

1.4.2.1 Oxidation Reactions

Under oxidative stress conditions, especially sulfur-containing amino acids cysteine and methionine participate in oxidation reactions because of their low oxidation potentials.

In many proteins, cysteine residues serve as members of a complex redox signaling network that regulates enzymatic and protein activity via reversible cysteine modifications under physiological conditions (Paulsen & Carroll, 2013). However, in the presence of excessive reactive species, this amino acid can undergo both one-electron and two-electron oxidation. While one-electron oxidation leads to formation of the thiyl radical (RS•), two-electron oxidation generally involves the formation of thiolate anion and nucleophilic attack of the thiolate (RS^-) on the electrophilic center in the oxidant (Figure 1.16).

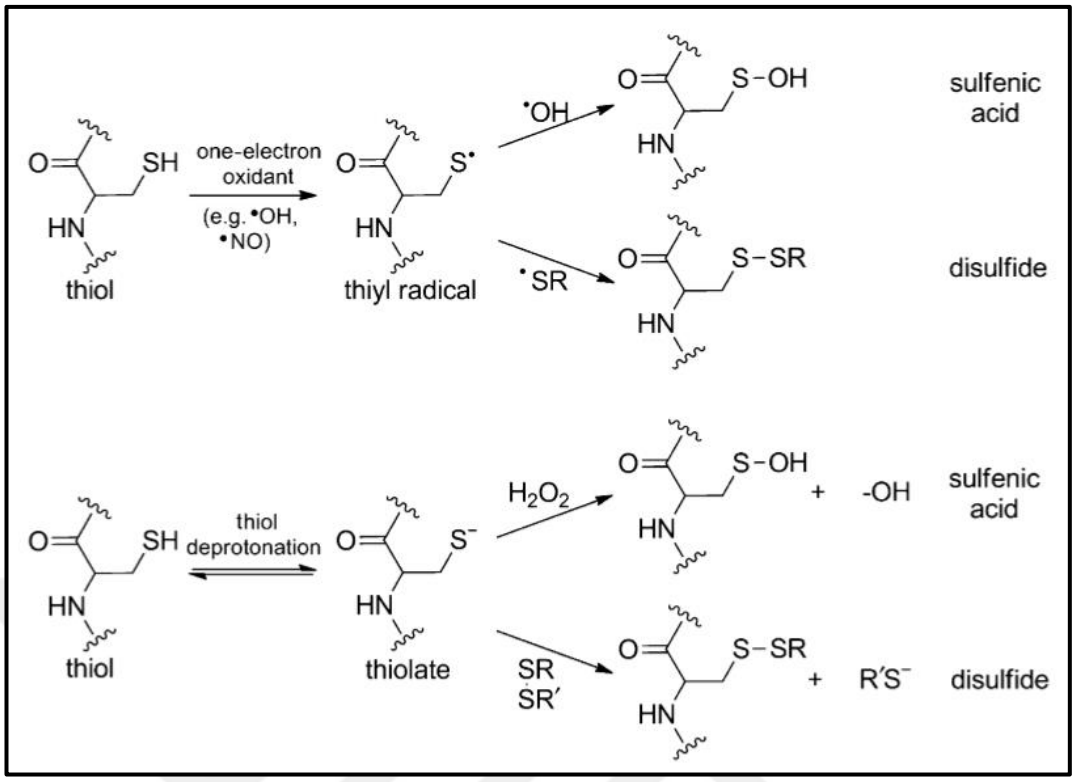


Figure 1.16 Cysteine oxidation pathways in the presence of reactive oxygen and nitrogen species (Hougland, Darling, & Flynn, 2013)

Following initial oxidation by these reactive species, oxidized cysteines can then participate in a variety of reactions as formation of sulfenic, sulfinic, sulfonic acids, S-nitrosothiols, sulfonamides, disulfides and S-glutathionylation.

After cysteine, methionine is the most susceptible amino acid to modifications caused by oxidative stress due to its easily oxidized thioether side chain. The methionine thioether may react with reactive species to reversibly generate a sulfoxide at the thioether sulfur atom, which yields methionine-S-oxide (Vogt, 1995; Bonvini, Bougnoux, Stevenson, Miller, & Hoffman, 1984). Methionine can be further irreversible oxidized to the sulfone under nonbiologically relevant oxidation conditions (Figure 1.17) (Arterburn & Nelson, 1996).

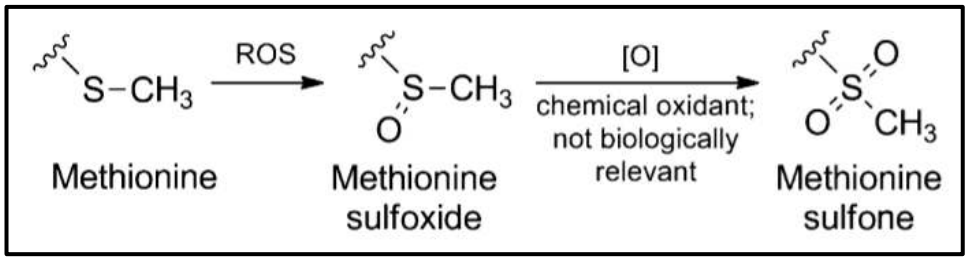


Figure 1.17 Irreversible oxidation of methionine to sulfone (Hougland, Darling, & Flynn, 2013)

In addition to cysteine and methionine, aromatic amino acids easily undergo oxidative additions in the presence of reactive species. Among aromatic amino acids, tyrosine is the most susceptible against oxidative additions. In the case of oxidative stress, a hydrogen atom can be abstracted from the tyrosine ring to form the tyrosinyl radical (Pacher, Beckman, & Liaudet, 2007; Gunaydin & Houk, 2009).

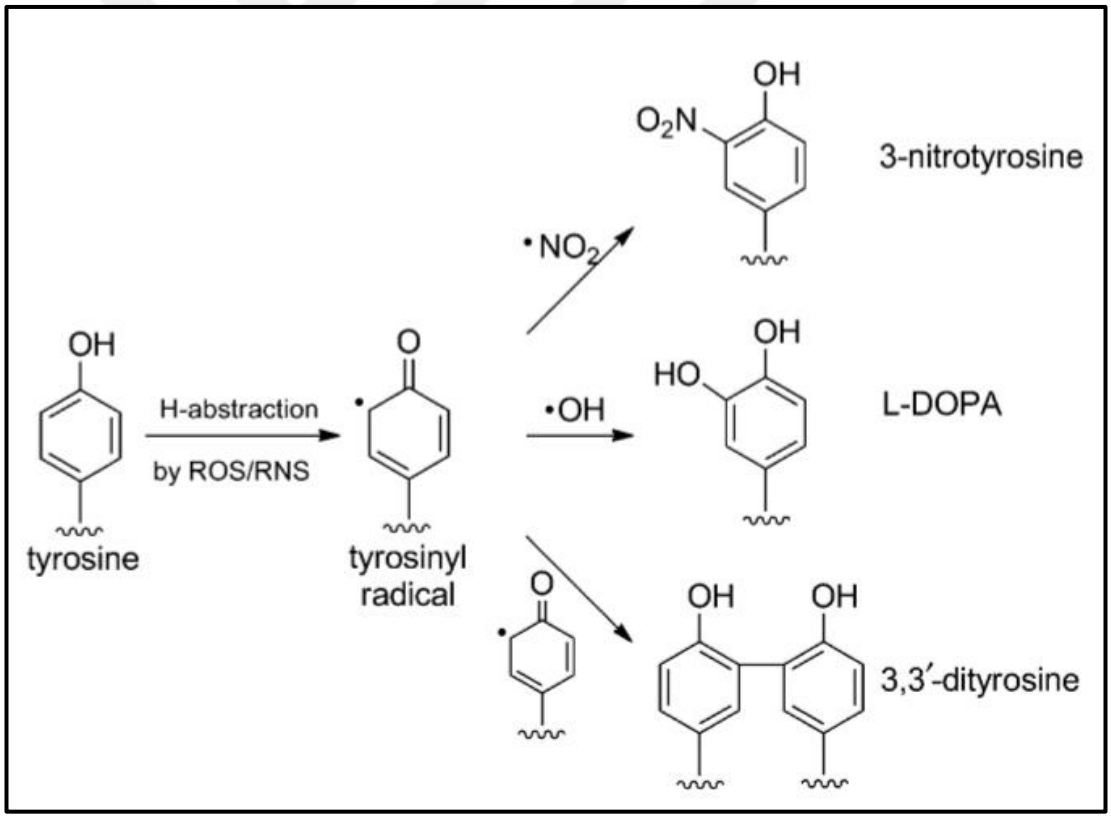


Figure 1.18 The products generated by tyrosine oxidation (Hougland, Darling, & Flynn, 2013)

As seen from Figure1.18, tyrosine oxidation leads to multiple products. Further reaction of tyrosinyl radical with hydroxyl yields the neurotransmitter precursor L-3,4 dihydroxyphenylalanine (L-DOPA) (Maskos, Rush, & Koppenol, 1992; Fitzpatrick, 1991). On the other hand, tyrosinyl radical may react with nitrogen dioxide and form 3-nitrotyrosine (Lymar, Jiang, & Hurst, 1996). Finally, tyrosinyl radicals can also dimerize to form 3,3′-dityrosine. Importantly, all these modifications are biologically irreversible and therefore tyrosine oxidation products are characterized as biomarkers for oxidative stress (DiMarco & Giulivi, 2007).

Amino acids with aliphatic side chains are also susceptible to oxidative damage, but these modifications are relatively rare when compared to those of cysteine, methionine, and the aromatic amino acids. These modifications are often metal- or enzyme-catalyzed reactions, as opposed to the uncatalyzed reactions with reactive species possible in the case of cysteine, methionine, and the aromatic amino acids.

1.4.2.2 Oxidation-Produced Electrophiles

Under oxidative stress conditions, carbonyl groups including ketones and aldehydes may be introduced into proteins by side chain oxidation or oxidative backbone cleavage. The carbonyls can serve as electrophiles in subsequent reactions. These reactive electrophiles can then react with nucleophilic amino acid side chains, resulting in protein carbonylation. Protein carbonylation is non-enzymatic and biologically irreversible modification that requires more severe oxidative conditions than cysteine or methionine oxidation. Therefore it has been proposed as a biomarker for oxidative stress (Yan, 2009). Many human diseases, such as Alzheimer's disease, cystic fibrosis, and chronic obstructive pulmonary disease are associated with protein carbonylation (Dalle-Donne, Rossi, Giustarini, Milzani, & Colombo, 2003).

There are various oxidative pathways by which carbonyl groups are introduced into proteins (Figure 1.19).

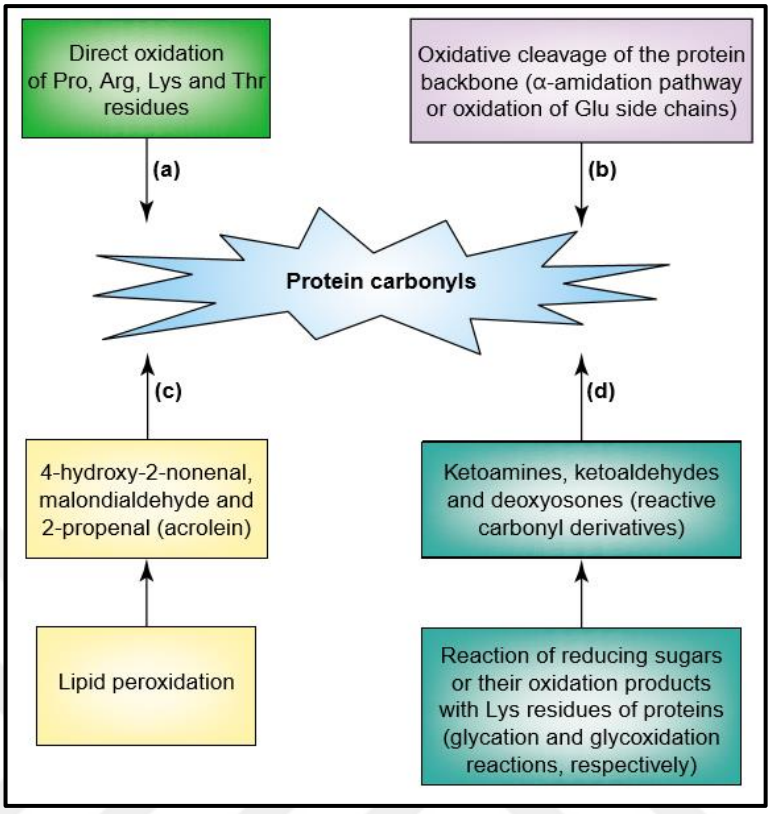


Figure 1.19 Oxidative pathways by which carbonyl groups are introduced into proteins (Dalle-Donne, Rossi, Giustarini, Milzani, & Colombo, 2003)

Reactive species may react directly with the amino acid residues of protein (a) or direct oxidation of proteins by these species can also yield highly reactive carbonyl derivatives that result either from oxidation of the side chains of lysine, arginine, proline, and threonine residues, particularly via metal-catalysed oxidation or the cleavage of peptide bonds by the α-amidation pathway or by oxidation of glutamyl residues (b). One other pathway is the introduction of carbonyl groups by adduction of carbonyl-containing oxidized lipids derived from the metal-catalysed oxidation of polyunsaturated fatty acids (Esterbauer, Schaur, & Zollner, 1991; Uchida & Stadtman, 1992; Refsgaard, Tsai, & Stadtman, 2000). These include MDA reacting with lysine residues and α,β-unsaturated aldehydes including 4-hydroxy-2-nonenal (HNE) and acrolein (c). Finally, carbonyl groups can also be generated by secondary reaction of the primary amino group of lysine residues with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones), produced by the reaction of

reducing sugars or their oxidation products with lysine residues of proteins (glycation/glycoxidation reactions) (d).

1.4.3 Damage in DNA

Cellular DNA is continuously damaged by endogenous and exogenous reactive species. DNA lesions can lead to inhibition of DNA replication or induce mutagenesis, the conditions which are associated with cell death and cancers or several other disease states, respectively (Forbes, Coughlan, & Cooper, 2008; Martin & Barrett, 2002; Sallmyr et al., 2008).

It should be stated that DNA is a relatively poor substrate for oxidation in the context of an entire cell. One of the main reasons for this situation is rather higher oxidation potential of bases required for the generation of radicals compared to proteins and lipids. Secondly, while proteins comprise more than 50% of the nonwater biomaterial in a cell, DNA comprises a relatively minor portion, approximately 10%, which means it is less likely for a radical to react with DNA than a protein. Thirdly, nuclear and mitochondrial DNAs are packaged by histone proteins or histone-like mitochondrial transcription factor. Finally, transport into the nucleus is actively controlled and certain small, charged, reactive molecules have problems passing through the membrane.

It is important to note that, besides these factors that obstruct DNA oxidation, cells possess several DNA repair pathways that combat the damage from oxidative stress (Barzilai & Yamamoto, 2004). In other words, to prevent the time-dependent erosion of genes due to undesired but irresistible accumulation of DNA injury one of the most important systems is DNA repair. Nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and double strand break repair (DSBR) are the major DNA repair mechanisms.

Reactive species react with different components of DNA to produce different types of DNA lesions. These reactive species can change the structure of bases, cause

chain breakages and DNA-protein cross-linking. On the other hand, most common oxidative lesion products in DNA can be examined under three different categories as 8-Oxo-7,8-Dihydro-2′-Deoxyguanosine, apurinic or apyrimidinic Sites and oxidative cross-links.

1.4.3.1 8-Oxo-7,8-Dihydro-2'-Deoxyguanosine

Among all DNA bases, guanine has the lowest oxidation potential and therefore it is exposed to continuous attack of different reactive species. The structure of specific guanine lesion is listed in Figure 1.20. 8-oxo-7,8-dihydro-2′-deoxyguanosine, or 8O-dG is one of the most well-studied guanine oxidation products. It has been determined that concentration of this product increase by Fenton-type reagents, cigarette smoke, tar, asbestos, gamma rays, and under many other conditions that leads to oxidative stress (Valavanidis, Vlachogianni, & Fiotakis, 2009; Oikawa & Kawanishi, 1998; Pilger et al., 2001; Floyd, West, Eneff, & Schneider, 1989). Reaction of HO·with guanine leads to addition at C8 to generate a C4 radical (Pratviel & Meunier, 2006). This radical can then lose an electron and a proton to form 8O-dG, or it can rearrange to form a derivative known as formamidopyrimidine. An alternative pathway is the addition of $O_2^{\cdot -}$ to an already formed guanine radical or guanine radical cation. Because 8O-dG formation occurs in various oxidative conditions, it is used as a general marker of oxidative stress (Cadet, Douki, & Ravanat, 2010).

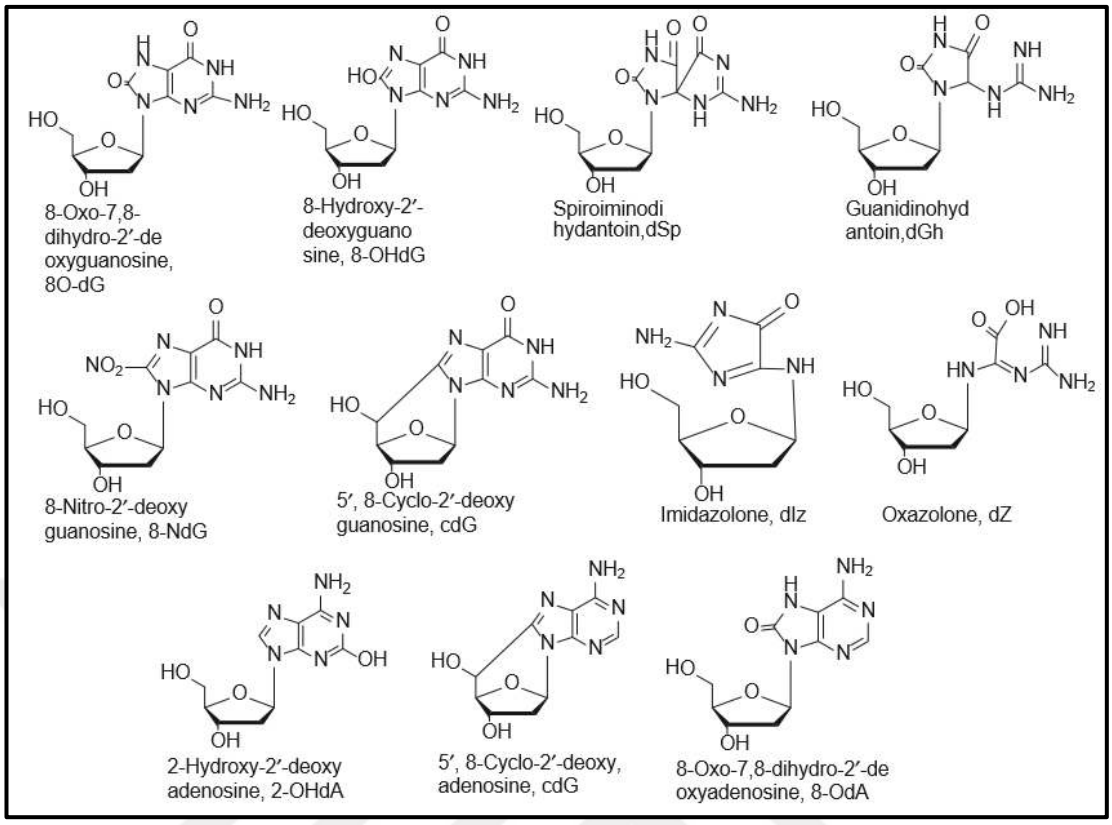


Figure 1.20 Various modified nucleosides (Nemera, Jones, & Merino, 2013)

There are several researches dealing with the chemistry of 8O-dG. This lesion is stable but its formation is highly influenced by pH. In the study conducted by Tsou et al. it was showed that the highest level of 8O-dG in calf thymus DNA treated with a chromium-based oxidant was observed at pH 7–8 and development of the lesion was unfavorable at pH < 6 (Tsou, Chen, Liu, & Yang, 1996). Another important feature is the easy oxidization with a potential of ~ 0.8 V of 8O-dG that is more favorable than that of guanine base. This potential is also slightly more favorable than the oxidation of tyrosine and close to the oxidation of a –SH to a disulfide (Stubbe, Nocera, Yee, & Chang, 2003; Stover, Ciobanu, Cliffel, & Rizzo, 2007; Hall, Holmlin, & Barton, 1996). Oxidation of 8O-dG leads to the formation of a variety of lesions, including guanidinohydantoin, spiroiminodihydantoin, oxazolone, and several others (Slade, Priestley, & Sugden, 2007).

8O-dG is a strong mutagen that plays central role in DNA oxidation. Therefore, it is strongly correlated with several pathogenic and diseased states (Svilar, Goellner,

Almeida, & Sobol, 2011). 8O-dG has been found to increase in concentration in a variety of diseases, including several types of cancers and other chronic diseases such as diabetes, heart disease, and neurodegenerative diseases (Valavanidis, Vlachogianni, & Fiotakis, 2009; Evans, Dizdaroglu, & Cooke, 2004).

1.4.3.2 Apurinic or Apyrimidinic Sites

Apurinic/apyrimidinic (AP) sites are expected to be one of the most frequent lesions in DNA. AP sites can be formed by spontaneous hydrolysis of the N-glycosylic bond. Additionally, they can be formed as a consequence of the removal of damaged or inappropriate bases by DNA N-glycosylases.

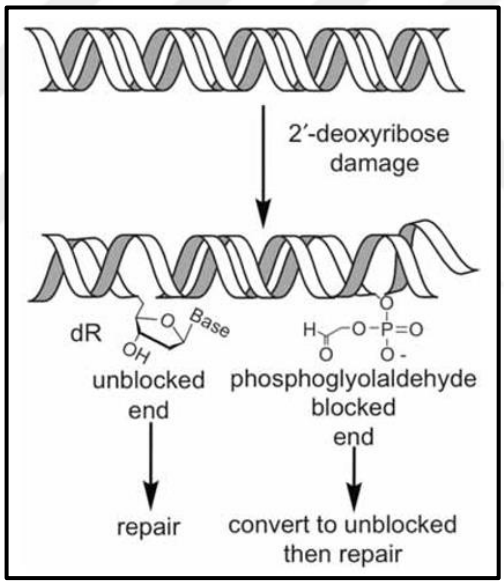


Figure 1.21 The presentation of unblocked and blocked ends in DNA (Nemera, Jones, & Merino, 2013)

These AP sites are also processed by endonucleases generating “unblocked” single-strand breaks (SSBs) which possess no modifications. Conversely, oxidation at dR can lead to fragmentation and the formation of “blocked” lesions (Nilsen, Forstrøm, Bjørås, & Alseth, 2011) (Figure 1.21). While unblocked SSBs can be directly repaired, blocked ends require conversion of the DNA strand to an unblocked site by endonucleases. AP sites are cytotoxic by blocking DNA

replication and transcription. AP sites are also mutagenic yielding mainly single base-pair substitution in *Escherichia coli* and yeast cells.

1.4.3.3 Oxidative Cross-Links

Interactions between DNA and proteins are essential for proper cellular function. On the other hand, cross-links are of intense interest since they are present at high levels when several repair pathways are compromised, as occurs in Fanconi anemia (Niedernhofer, Lalai, & Hoeijmakers, 2005). In addition, environmental toxins that generate ROS significantly elevate the level of cross-linking (Macfie, Hagan, & Zhitkovich, 2010).

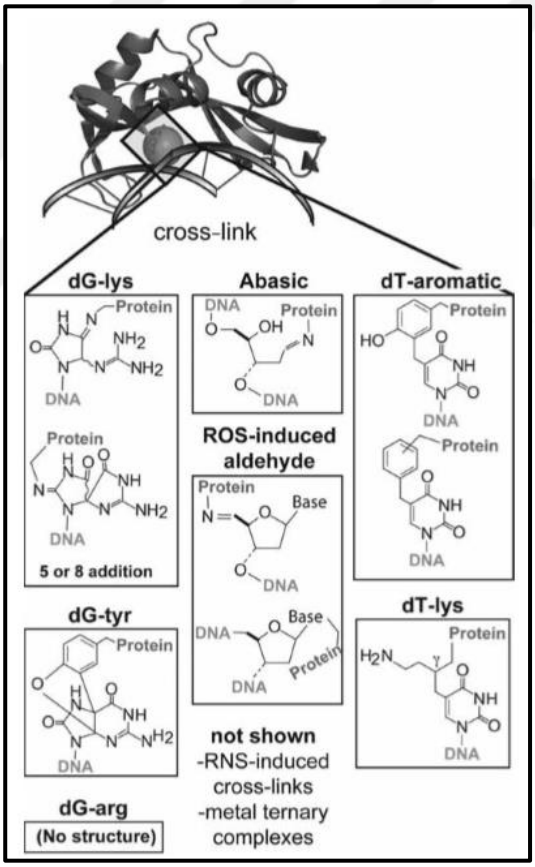


Figure 1.22 Representative cross-link adducts (Nemera, Jones, & Merino, 2013)

The formation of DNA–protein cross-links is common because both nuclear and mitochondrial DNA are closely associated with proteins such as histones, single-stranded DNA binding proteins, transcription factors, and other DNA binding

proteins. The close association means the effective concentration of protein is very high relative to water. Thus, cross-linking is an outcome when reactive oxygen is present (VanderVeen, Harris, Jen-Jacobson, & Marnett, 2008). Cross-links arrest replication and require repair due to their large size (Ide, Shoulkamy, Nakano, Miyamoto-Matsubara, & Salem, 2011). The cross-link structures determined to date have been shown in Figure 1.22.

1.5 Programmed Cell Death

Cell death which interacts with cell division to generate and maintain appropriate numbers and types of cells is a part of normal development and maturation cycle (Ellis, Yuan, & Horvitz, 1991). It is also the component of many response patterns of living tissues to exogenous factors, such as xenobiotic agents and to endogenous modulations, such as inflammation and disturbed blood supply (Clavien, Yadav, Sindram, & Bentley, 2000). The view that some cells can naturally die as a normal part of both development and homeostasis dates back to 1842, when it was firstly shown by the fact that the notochord of the midwife toad is removed during metamorphosis, the process which facilitates the formation of vertebra (Peter, Heufelder, & Hengartner, 1997). On the other hand, the term programmed cell death (PCD) was put forward in the mid 1960s based on the observation that developmental cell death depends on the expression of endogenous genes (Lockshin & Williams, 1964) and further understood with the electron microscopy studies carried out by Kerr, Wyllie and Currie in the 1970s (Kerr, Wyllie, & Currie, 1972).

Programmed cell death (PCD) is a common phenomenon in developmental processes or in normal physiological conditions, where the old or damaged cells have to be eliminated (Sreedhar & Csermely, 2004). On the other hand, it should be noted that PCD can be induced by a variety of exogenous stimuli which will be discussed in detail later as well as normal conditions. There are three known types of PCD including Apoptosis, Autophagy and Regulated necrosis (Figure 1.23). Apoptosis is the principal mechanism by which cells are physiologically eliminated in vertebrates. During apoptotic death, cells are properly hydrolized by caspases and packaged into

apoptotic bodies as a mechanism to avoid immune activation. Recently, necrosis, once thought of as simply a passive, unorganized way to die, has emerged as an alternate form of programmed cell death whose activation might have important biological consequences, including the induction of an inflammatory response. Autophagy has also been suggested as a possible mechanism for non-apoptotic death despite evidence from many species that autophagy represents a survival strategy in times of stress.

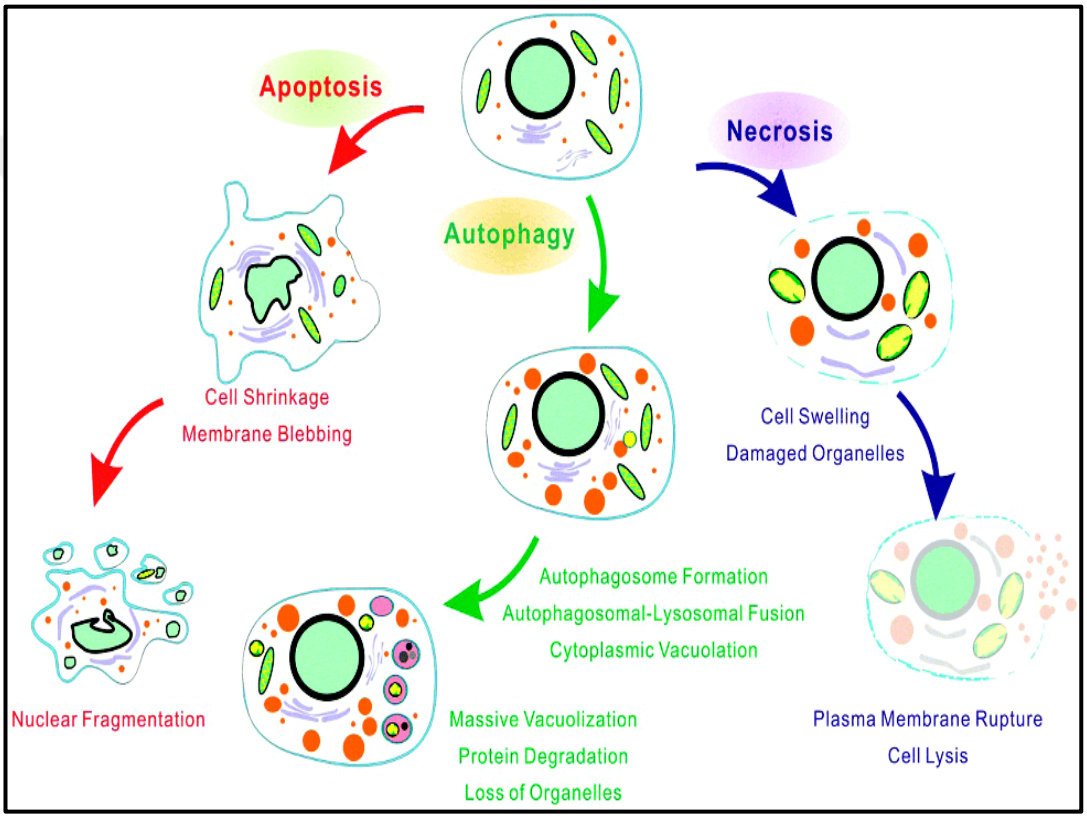


Figure 1.23 The schematic representation of the difference between apoptotic, autophagic and necrotic cells (Tan, Lu, Ji, & Mao, 2014)

1.5.1 Apoptosis (Type I PCD)

Apoptosis or Type I PCD can be defined as a genetically programmed mechanism allowing the damaged cells to commit suicide for not to interfere with normal functioning cells. Apoptotic cell death has a vital function in tissue homeostasis and in the elimination of damaged cells. Both increased and insufficient rates of

apoptosis can lead to human diseases including neurodegenerative diseases and cancer, respectively (Danial & Korsmeyer, 2004).

It was determined by light and electron microscopy studies that various morphological changes occur during mammalian apoptotic cell death such as cell shrinkage, pyknosis, densed cytoplasm, tightly packed organelles, chromatin condensation, and loss of cell surface microvilli (Figure 1.24) (Häcker, 2000).

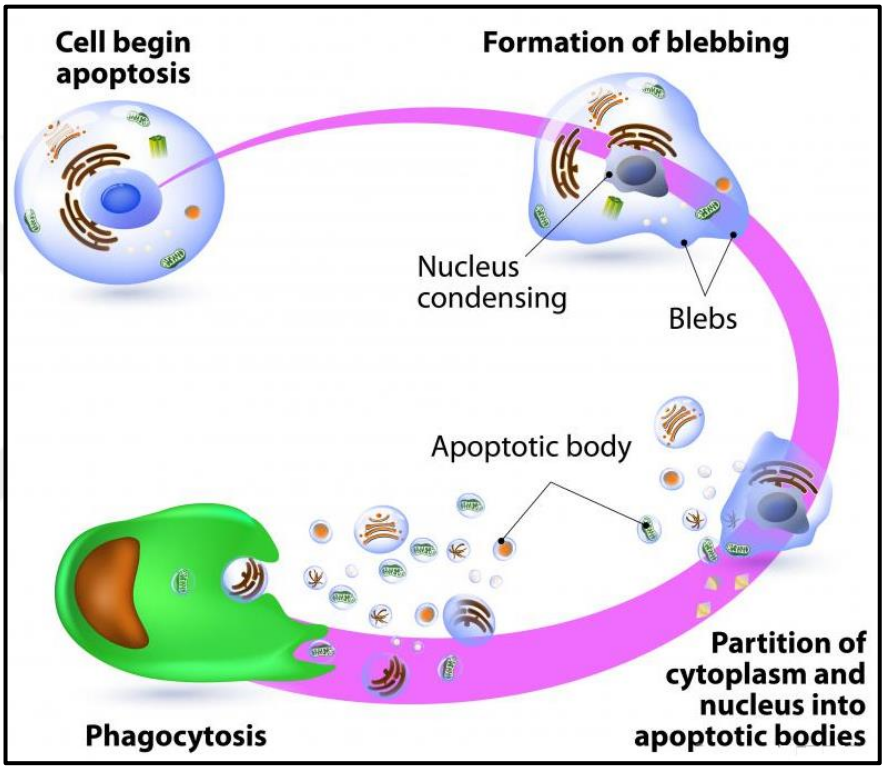


Figure 1.24 Schematic representation of a cell dying by apoptotic cell death at various stages

During the early stage of apoptotic cell death, cell shrinkage and picnosis become visible by light microscopy (Kerr, Wyllie, & Currie, 1972). When the cells shrink, their sizes decrease, the cytoplasm becomes more intense and organelles become packed more tightly. Pyknosis is a consequence of chromatin condensation and is the most characteristic feature of apoptotic cell death. Histological examinations which carried out with the dyes as haematoxylin and eosin showed that apoptotic cell death involves single cells or small cell clusters. Apoptotic cells appear as a round/oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin

fragments. Electron microscopy describes better subcellular changes. In the early phase of chromatin condensation, the electronically dense nucleus material characteristically aggregates under the nuclear envelope. On the other hand, homogeneous dense nuclei may also be present. Protrusions that involve the extension of cellular membranes are formed and this process is followed by karyorrhexis that is destructive fragmentation of the nucleus and degradation of cell fragments to the apoptotic bodies called as budding. Apoptotic bodies originate from the cytoplasm covered by an intact plasma membrane with tightly packed organelles that contain or not a nuclear fragment. These bodies are then phagocytized by macrophages, parenchymal cells or neoplastic cells and degraded in phagolysosomes. Macrophages that encapsulate and digest apoptotic cells are called "sticky body macrophages". During apoptosis and elimination of apoptotic cells, no inflammation is observed because apoptotic cells do not release their cellular contents into the surrounding tissues, they are very rapidly phagocytized, and the immune cells do not produce cytokines.

The apoptotic cell death mechanism is a complex and complicated, energy-dependent cascade. Investigations show that there are two main pathways termed as extrinsic or death receptors and intrinsic or mitochondrial pathways in apoptotic cell death in mammalians (Figure 1.25). It must be stated that these two pathways are linked to each other and a molecule in one pathway can affect the other (Igney & Krammer, 2002). In addition to extrinsic and intrinsic pathways, another pathway is perforin/granzyme pathway. All these pathways merge at death pathway. The death pathway begins with the activation of the enzyme called caspase-3 and ends with DNA fragmentation, cytoskeletal and nuclear protein degradation, cross-linking of proteins, formation of apoptotic bodies, expression of phagocytic cell receptor ligands and retrieval of apoptotic bodies by phagocytic cells (Martinvalet, Zhu, & Lieberman, 2005).

The extrinsic pathway is initiated through a subset of the Tumor Necrosis Factor receptors including TNFR, Fas and TRAIL. Activation of these death receptors allows the recruitment and activation of initiator caspases such as caspases- 8 and -

10. This process includes the formation and activation of the complexes as “Death inducing signaling complex, DISC”. The DISC activates the effector caspases, mainly caspase 3 and the active caspase-3 is responsible for the hydrolysis of a large number of substrates. Recent findings indicate that the extrinsic pathway is much more complex and diverse and also interacts with especially intrinsic apoptosis as well as necrosis (Thorburn, 2004). On the other hand, the intrinsic pathway is largely regulated by mitochondria. The most studied and known form of intrinsic apoptosis is the cascade that is mediated by the release of cytochrome c from mitochondria and formation of apoptosome in the event of stress. In this cascade, apoptosome then activates the initiator caspases that activates effectors such as caspase-9. In response to any apoptotic stimulation, pro-apoptotic members of the Bcl-2 protein family (Bax and Bak) are activated and they act on mitochondria to induce cytochrome c release. In addition to cytochrome c, other pro-apoptotic proteins such as Smac/Diablo (Second Mitochondrial derived activator of Caspase/Direct IAP-Binding protein with low pI), the serine protease Omi/HtrA2, endonuclease G (EndoG) and apoptosis inducing factor (AIF) are also released into the cytosol from the mitochondria.

The proteins that play crucial roles in extrinsic and intrinsic pathways of human apoptotic cell death, their abbreviations and alternate nomenclatures are presented in Table 1.2a and 1.2b, respectively.

Table 1.2a The extrinsic pathway proteins, abbreviations and alternate nomenclatures (Elmore, 2007)

Abbreviation	Protein Name	Alternate Nomenclature
TNF-α	Tumor necrosis factor alpha	TNF ligand, TNFA, cachectin
TNFR1	Tumor necrosis factor receptor 1	TNF receptor, TNFRSF1A, p55 TNFR, CD120a
FasL	Fatty acid synthetase ligand	Fas ligand, TNFSF6, Apo1, apoptosis antigen ligand 1, CD95L, CD178, APT1LG1
FasR	Fatty acid synthetase receptor	Fas receptor, TNFRSF6, APT1, CD95
Apo3L	Apo3 ligand	TNFSF12, Apo3 ligand, TWEAK, DR3LG
DR3	Death receptor 3	TNFRSF12, Apo3, WSL-1, TRAMP, LARD, DDR3
Apo2L	Apo2 ligand	TNFSF10, TRAIL, TNF-related apoptosis inducing ligand
DR4	Death receptor 4	TNFRSF10A, TRAILR1, APO2
DR5	Death receptor 5	TNFRS10B, TRAIL-R2, TRICK2, KILLER, ZTNFR9
FADD	Fas-associated death domain	MORT1
TRADD	TNF receptor-associated death domain	TNFRSF1A associated via death domain
RIP	Receptor-interacting protein	RIPK1
DED	Death effector domain	Apoptosis antagonizing transcription factor, CHE1
Caspase-8	Cysteinyl aspartic acid-protease 8	FLICE, FADD-like Ice, Mach-1, Mch5
c-FLIP	FLICE-inhibitory protein	Casper, I-FLICE, FLAME-1, CASH, CLARP, MRIT

Table 1.2b The intrinsic pathway proteins, abbreviations and alternate nomenclatures (Elmore, 2007)

Abbreviation	Protein Name	Alternate Nomenclature
Smac/DIABLO	Second mitochondrial activator of caspases/direct IAP binding protein with low pI	None
HtrA2/Omi	High-temperature requirement	Omi stress regulated endoprotease, serine protease Omi protein A2
IAP	Inhibitor of Apoptosis Proteins	XIAP, API3, ILP, HILP, HIAP2, cIAP1, API1, MIHB, NFR2-TRAF signaling complex protein
Apaf-1	Apoptotic protease activating factor	APAF1
Caspase-9	Cysteinyl aspartic acid-protease-9	ICE-LAP6, Mch6, Apaf-3
AIF	Apoptosis Inducing Factor	Programmed cell death protein 8, mitochondrial
CAD	Caspase-Activated DNAse	CAD/CPAN/DFF40
Bcl-2	B-cell lymphoma protein 2	Apoptosis regulator Bcl-2
Bcl-x	BCL2 like 1	BCL2 related protein
Bcl-XL	BCL2 related protein, long isoform	BCL2L protein, long form of Bcl-x
Bcl-XS	BCL2 related protein, short isoform	
Bcl-w	BCL2 like 2 protein	Apoptosis regulator BclW
BAG	BCL2 associated athanogene	BAG family molecular chaperone regulator
Bcl-10	B-cell lymphoma protein 10	mE10, CARMEN, CLAP, CIPER
BAX	BCL2 associated X protein	Apoptosis regulator BAX
BAK	BCL2 antagonist killer 1	BCL2L7, cell death inhibitor 1
BID	BH3 interacting domain death agonist	p22 BID
BAD	BCL2 antagonist of cell death	BCL2 binding protein, BCL2L8, BCL2 binding component 6, BBC6, Bcl-XL/Bcl-2 associated death promoter
BIM	BCL2 interacting protein BIM	BCL2 like 11
BIK	BCL2 interacting killer	NBK, BP4, BIP1, apoptosis inducing NBK
Puma	BCL2 binding component 3	JFY1, PUMA/JFY1, p53 up-regulated modulator of apoptosis
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1	PMA induced protein 1, APR
Myc	Oncogene Myc	c-myc, Myc proto-oncogene protein

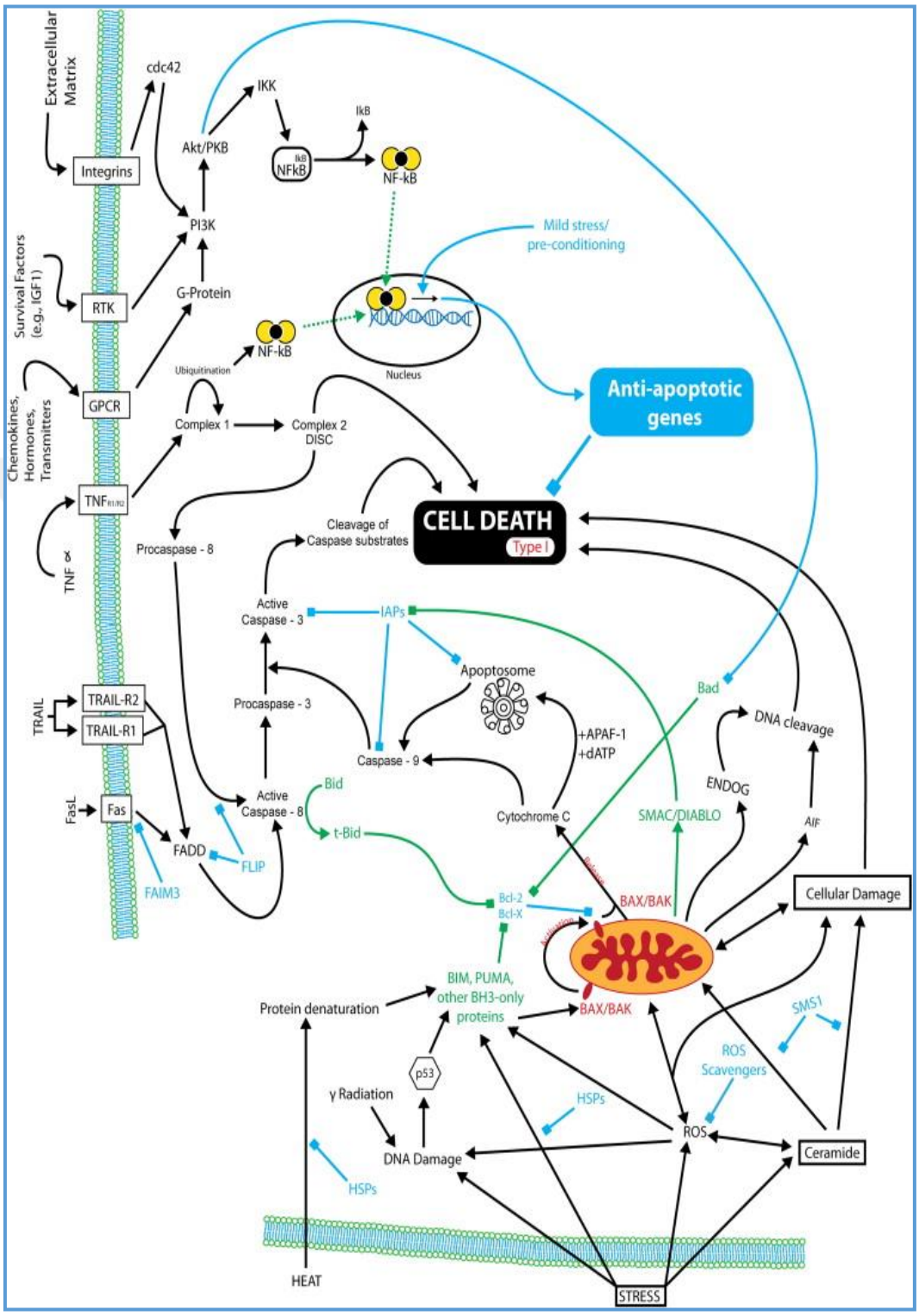


Figure 1.25 Schematic representation of the cellular pathways of apoptosis (Portt, Norman, Clapp, Greenwood, & Greenwood, 2011)

1.5.1.1 Yeast Apoptosis

Yeast is a common term for bringing together single-cell ascomycetous and basidiomycetous fungi. Most yeast cells (such as *Saccharomyces spp*.) bud and grow gradually as a small "daughter cells" growing from the "mother cells". Some yeast such as *Schizosaccharomyces spp*. proliferate via fission and form a septum, which allows the cell to divide into two equal sized cells. On the other hand, most of the yeast cells proliferate through meiosis division.

Saccharomyces cerevisiae is budding yeast that is best researched and probably the best known eukaryotic organism. This organism can be found both as haploid (16 chromosomes) and diploid (32 chromosomes). When adequate nutrient intake, constantly repeated circular protrusions form via the vegetative growth and mitosis in both cell types. The growth and development can happen in many different ways and the signals needed for all these mechanisms except reproduction are related to nutrition. Haploid species may be of the cell type having any of two different mating species called a (a pheromone producers) and α (α pheromone producers). Each cell type has a surface receptor that recognizes the pheromone produced by the cell with the opposite mating type. a factor causes to arrest cell cycle of the α haploid in the G1 phase and the α factor in the same way arrests a haploid in the G1 phase. Thus, in the presence of both cell types, cell proliferation is stopped and protrusions that is oriented towards each other. The resulting structure is called “shmoo” and the diploid structure arises as a result of fusion with the cellular contact (Figure 1.26).

When nutrients are consumed, both haploid and diploid cells are arrested at the stationary phase. Stationary phase cells are morphologically and biochemically different from proliferating cells. In these cells, the buds do not observed, they are rounded and shiny, containing trehalose and glycogen storage carbohydrates in higher amounts than proliferating cells. Moreover, stationary phase cells are much more resistant to many different stresses and adverse environmental conditions than proliferating yeast cells (Werner-Washburne, Braun, Johnston, & Singer, 1993).

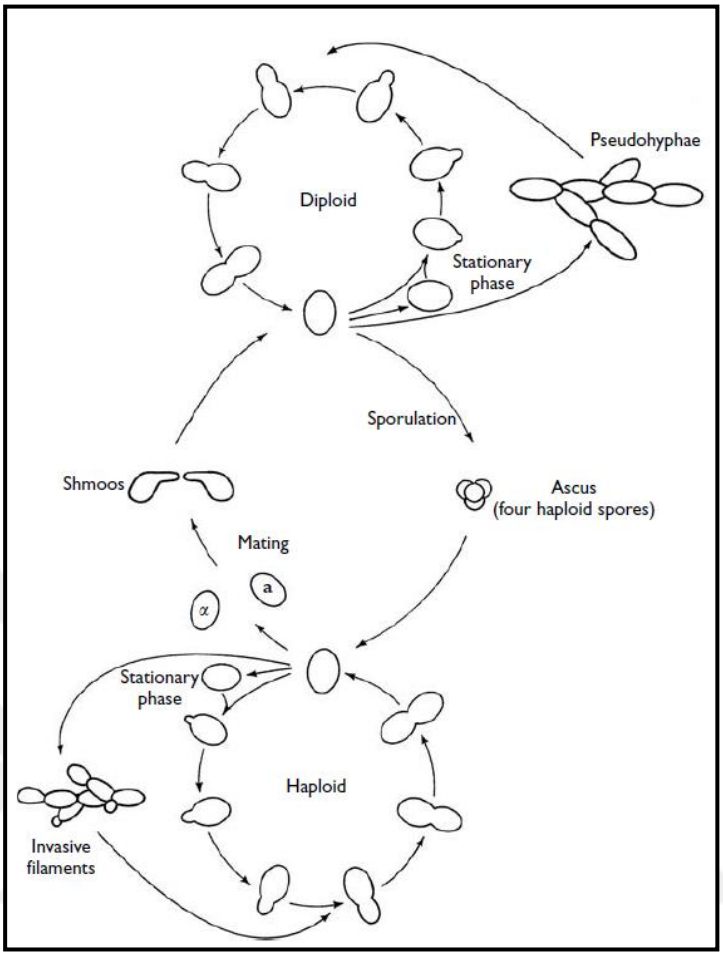


Figure 1.26 The life cyclus of *S. cerevisiae* (Dickinson, 1999)

S. cerevisiae has been gradually became a preferred and popular research tool over years because it can be easily handle and genetically modified. In 1996, the gene coding of this organism was fully clarified. As well as the advantages of yeast genetics, high degree of conservation of basic cellular processes between yeast and higher organisms makes yeast cell a suitable model organism to investigate human diseases. The discovery of yeast apoptosis dates back to 1997 to the study revealed that *S. cerevisiae* mutant in cell division cycle gene CDC48 shows typical markers of apoptosis (Madeo, Fröhlich, & Fröhlich, 1997). In the last decades, compelling and significant evidences accumulated showed that yeasts are valuable for apoptotic cell death research because of their ability to undergo intrinsic apoptotic responses that show a certain degree of conservation with the ones of higher eukaryotes (Madeo et al., 2004). Specifically, an apoptosis-inducing factor (AIF1), cytochrome c (cyt c)

and HtrA/Omi, that play an important role in the intrinsic pathway of yeast and mammalian systems have been identified (Gourlay & Ayscough, 2006) (Figure 1.27).

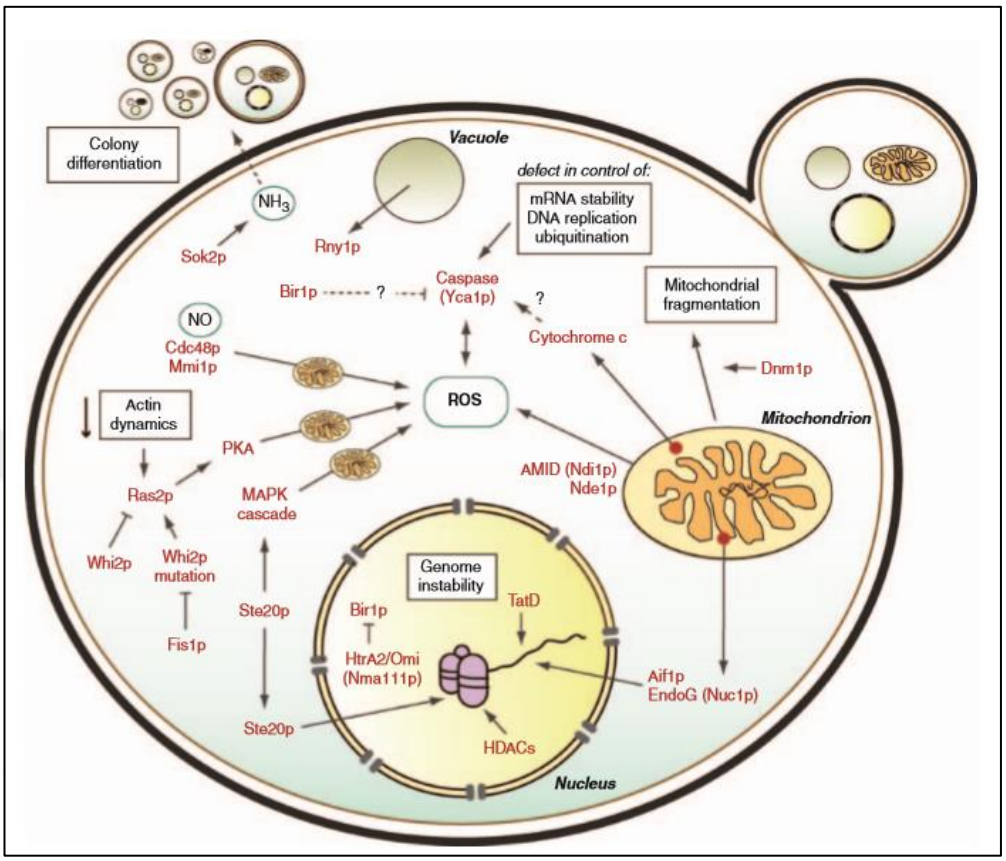


Figure 1.27 Proteins and pathways in yeast apoptosis (Carmona-Gutierrez et al., 2010)

On the other hand, no components of the extrinsic apoptotic regulatory pathway such as death receptors and their ligands have been described, showing that yeast cells do not completely recapitulate the mammalian apoptotic system. The yeast orthologs of the human apoptotic proteins and their functions are presented in Table 1.3.

Table 1.3 The yeast orthologs of the human apoptotic proteins and their functions

Human	***S. cerevisiae***	**Function (www.yeastgenome.org)**
VCP	CDC48/YDL126C	ATPase, a component of several multiprotein ATPase complexes involved in the release of polyubiquitinated proteins; part of the ER-associated ubiquitin-dependent protein degradation system, also plays a role in mitotic spindle disassembly, autophagy, ER membrane fusion, retrograde protein transport; localized throughout the cell.
Cytochrome c (Cyt c)	CYC1/YJR048W	Mitochondrial release of cytochrome c initiates apoptosis by triggering caspase activation
Endonuclease G (Endo G)	NUC1/YJL208C	Major mitochondrial nuclease; has RNAse and DNA endo- and exonucleolytic activities; roles in mitochondrial recombination, apoptosis and maintenance of polyploidy; involved in fragmentation of genomic DNA during PND (programmed nuclear destruction); encodes ortholog of mammalian EndoG.
Apoptosis Inducing Factor (AIF)	AIF1/YNR074C	Mitochondrial cell death effector; translocates to the nucleus in response to apoptotic stimuli, homolog of mammalian Apoptosis-Inducing Factor, putative reductase.
Inhibitors of Apoptosis Proteins (IAP)	BIR1/YJR089W	Bir1p contains three baculovirus IAP repeat domains, a protein motif which is generally found in inhibitor-of-apoptosis proteins, and Bir1p appears to play independent roles in chromosome stability and apoptosis.
The mitochondrial serine protease (HtrA2/Omi)	NMA111/YNL123W	Serine protease and general molecular chaperone; involved in response to heat stress and promotion of apoptosis; may contribute to lipid homeostasis; sequence similarity to the mammalian Omi/HtrA2 family of serine proteases.
Apoptosis-Inducing Factor (AIF)-Homologous and Mitochondria-Associated Protein	NDI1/YML120C	NADH:ubiquinone oxidoreductase; transfers electrons from NADH to ubiquinone in respiratory chain but does not pump protons, in contrast to higher eukaryotic multisubunit respiratory complex I; upon apoptotic stress, is activated in mitochondria by N-terminal cleavage, then translocates to cytoplasm to induce apoptosis.
Caspases	MCA1/YOR197W	Ca^{2+}-dependent cysteine protease; may cleave specific substrates during the stress response; regulates apoptosis upon H_2O_2 treatment; required for clearance of insoluble protein aggregates during normal growth; implicated in cell cycle dynamics and lifespan extension; undergoes autocatalytic processing; similar to mammalian metacaspases.

1.5.1.1.1 Cell Division Control Protein 48 (CDC48/YDL126C). Regulation of intracellular proteolysis is significant and especially necessary in terms of the evaluation of the protein quality, the elimination of mis-folded and damaged proteins and the control of metabolic pathways, signal transduction and cell division through the degradation of key enzymes. In the eukaryotic systems, cells carry out this proteolysis by two major systems: (1) the proteasome (generally soluble proteins) and (2) lysosomal compartment (insoluble protein aggregates) (Buchberger, 2013).

Cdc48 that is known as transitional endoplasmic reticulum ATPase (TER94) in *Drosophila* and as valosin-containing protein (VCP) or p97 in vertebrates is a significant protein in proteasomal degradation pathways. This protein is the member of the ATPase associated with various cellular activities (AAA) protein family (Figure 1.28) (Hanson & Whiteheart, 2005). Because the enzyme segragates the ubiquitylated substrate proteins from stable ones, it facilitates the degragation of proteins by the proteasome (Bays & Hampton, 2002). In addition, it must be stated that it is significant for apoptosis, autophagy and endolysosomal protein degradation (Madeo, Fröhlich, & Fröhlich, 1997).

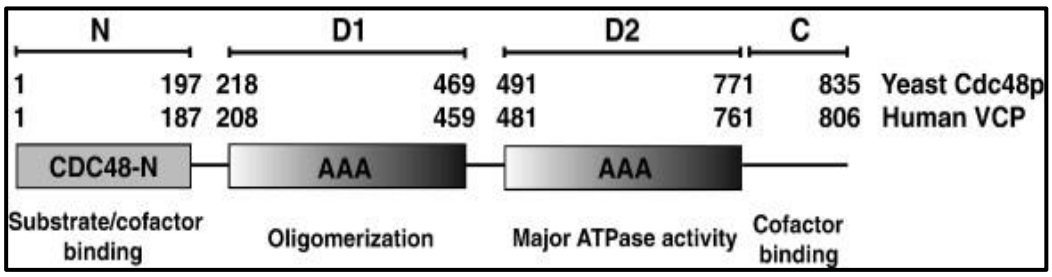


Figure 1.28 Domain structure of Cdc48/VCP. The numbers refer to the amino acid positions within the protein in yeast Cdc48p and human VCP, respectively (Braun & Zischka, 2008)

Cdc48p was the first apoptotic intermediate found in *S. cerevisiae* (Braun and Zischka, 2008). In the study conducted by Madeo et al. (1197), it was firstly shown that replacement of a conserved serine residue in the second ATPase domain (D2) of yeast CDC48 to glycine (serine 565 to glycine, S565G) results in cells with increased susceptibility to undergo death and typical markers of apoptosis characterized by membrane staining with annexin V that indicates an exposure of phosphatidylserine

at the outer layer of the cytoplasmic membrane and intense staining by using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling method that indicates DNA fragmentation, chromatin condensation and fragmentation (Madeo, Fröhlich, & Fröhlich, 1997). In another research, it was also determined that the same $CDC48^{S565G}$ mutant produces reactive oxygen species (ROS) during apoptotic cell death and inhibition of protein synthesis by applying the specific ribosome inhibitor cycloheximide prevented the emergence of apoptotic features in mutant yeast cells. In addition, it was also found in the same research that depletion of ROS with the addition of various radical scavengers to the growth media prevented cells to undergo apoptosis even under stress conditions such as heat shock (Madeo et al., 1999). In another study that aimed to explore whether mitochondria are affected at the molecular and functional level and participate yeast apoptosis upon the same CDC48 mutation, qualitative and quantitative protein alterations, mitochondrial enlargement, facilitated release of cytochrome c into the cytosol and accumulation of ROS were observed (Braun et al., 2006). On the other hand, it must be stated that the hallmarks of apoptosis could not be observed for all known CDC48 mutations, seem rather specific for the $cdc48^{S565G}$ allele. The Exchange of serine 565 to glycine is localized in the highly conserved D2 domain responsible for the major ATPase activity of Cdc48p, which indicates that ATPase-deficiency of Cdc48p-S565G may be important for apoptotic cell death (Braun & Zischka, 2008). In the view of all these data, it is thought that CDC48 mutation (S565G) may induce apoptotic cell death in yeast by generation of radicals (Figure 1.29) (Madeo et al., 2002).

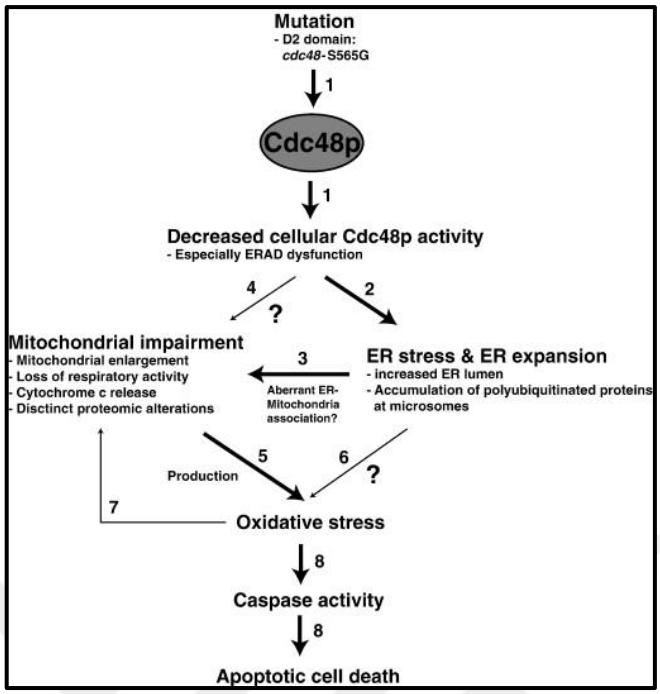


Figure 1.29 The possible apoptotic cell death mechanism in cdc48^{S565G} yeast (Braun and Zischka, 2008)

1.5.1.1.2 Cytochrome c (CYC1/YJR048W). Cytochrome c is a protein that is highly conserved with 12 kDa molecular weight. This protein consists of a single 104 amino acid peptide with a single heme group covalently attached to Cys14 and Cys17. It is known as an electron-carrying mitochondrial protein and the primary function is to transport electrons from cytochrome c reductase to cytochrome oxidase. More recently, cytochrome c has been identified as an important mediator in apoptotic pathways. Cytochrome c is involved in the best known form of intrinsic apoptosis. Under normal conditions, cytochrome c is localized in the space between the mitochondrial outer and inner membranes where it participates to oxidative phosphorylation process. In this form of apoptosis, upon the exposure of apoptotic stimuli, cytochrome c is released from the mitochondria to the cytosol that results in the formation of the apoptosome and activation of initiator caspase, typically caspase 9 that then leads to the activation of the effector caspase-3.

Biochemical assays revealed the presence of two cytosolic proteins in vertebrates that activate caspase-3 by creating a complex with cytochrome c, Apaf-1 and Apaf-3.

The CARD motif in Apaf-1 interacts with the caspase-9 prodomain. In the presence of cytochrome c and ATP or ADP, the complex induces autocatalytic activation of caspase-9 and then the effector caspases such as caspase-2, -3, -6, -7, -8 and -10. Apaf-1 dimerization is suppressed by its C-terminus, which contains WD-40 repeats (WD-40 repeats are short 40-amino acid repeats and usually terminate with Trp-Asp). It's thought that a conformational change is induced and this conformational change allows Apaf-1 to oligomerization by binding of cytochrome c to WD repeats. Then oligomerized Apaf-1 activates caspase-9 by clustering this enzyme (Figure 1.30).

When considered in terms of yeast cells, it can be said that cytochrome c plays an important role in intrinsic apoptosis of *S. cerevisiae*. Acetic acid, amiodarone and H_2O_2 are some of the compounds which trigger cytochrome c release associated apoptosis in yeast (Ludovico, Sousa, Silva, Leao, & Corte-Real, 2001; Carmona-Gutierrez et al., 2010; Madeo et al., 2002). On the other hand, it is not clear whether cytochrome c causes apoptosome-like structure formation and then caspase activation as in mammalian cells (Giannattasio et al., 2008).

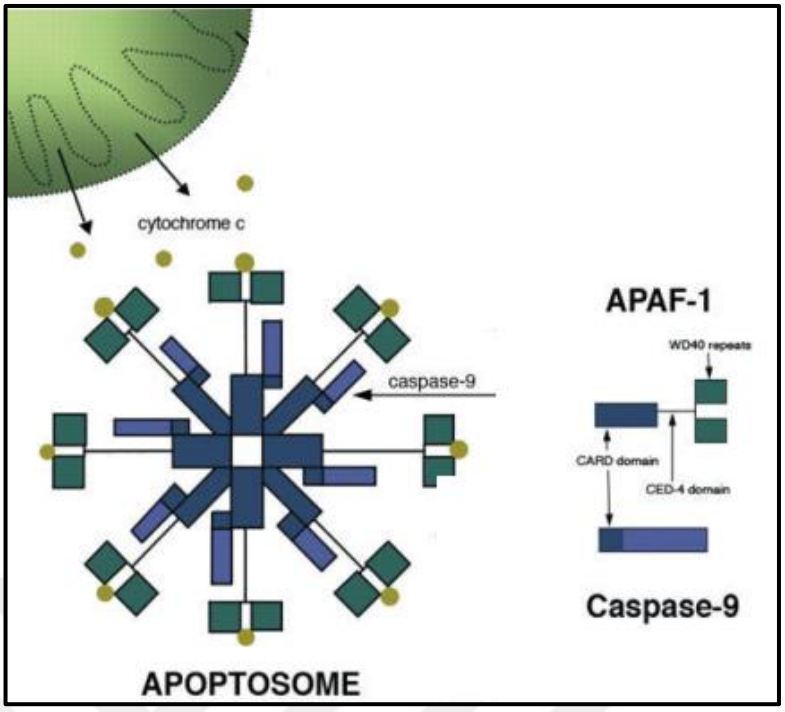


Figure 1.30 The formation of apoptosome complex (Lindholm & Arumäe, 2004)

In addition, no homologs or functionally related proteins of apoptosome-associated factor Apaf could have been detected in the yeast apoptosis until now. However, cytochrome c can be described as the pro-death factor that triggers cell death as a consequence of its release to cytosol followed by mitochondrial respiratory chain breakdown and ROS accumulation.

1.5.1.1.3 Endonuclease G (NUC1/YJL208C. Endonuclease G (Endo G) is a nuclease which involved in apoptosis in an independently manner from the caspases. It is a mitochondrial enzyme thought to participate physiologically in the replication of mitochondrial DNA (Ruiz-Carrillo & Renaud, 1987). Endo G participates to apoptotic process with the change in its intracellular localization. While this enzyme is found in the space between mitochondrial membranes in healthy cells, it is released to cytosol from mitochondria upon apoptotic induction and causes oligonucleosomal DNA fragmentation by translocating to the nucleus.

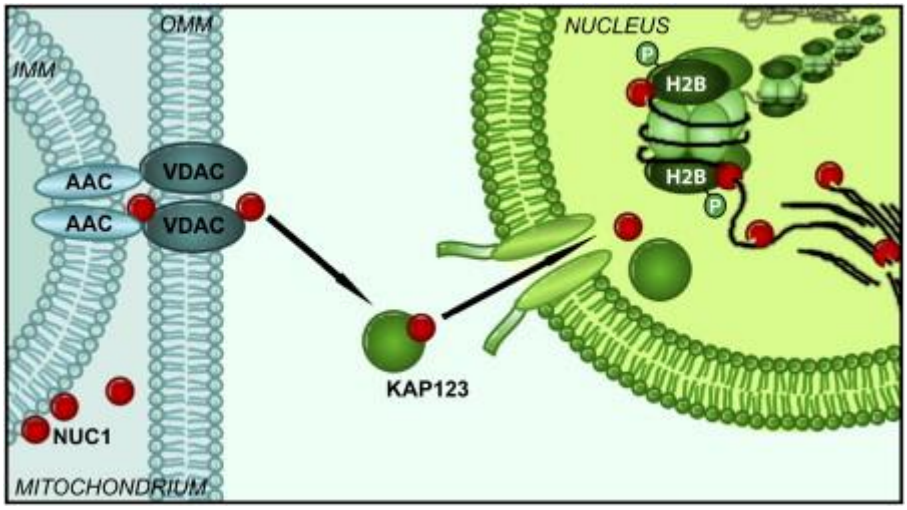


Figure 1.31 A Potential Pathway for Yeast EndoG-Mediated Death (Büttner et al., 2007)

The in vivo roles of EndoG in mammalian development and apoptosis were investigated in mouse cells without expression of EndoG (Zhang et al., 2003). It was shown that EndoG homozygous mutation cause to early embryo death. EndoG heterozygous mutant mouse cells showed resistance to death induced by both TNF-α and staurosporin. In addition, DNA fragmentation upon activation of apoptosis has been reduced in mutant thymocytes and splenocytes as compared to wild-type cells. These findings showed that EndoG plays an critical role during early embryogenesis and apoptosis.

With the researches dealing with yeasts, the valuable findings into how EndoG regulates cell death have been provided. In these studies, it was found that yeast Nuc1p that most probably contains mitochondrial localization signal (MLS) is the homologous of mammalian EndoG. Until today, Nuc1p is the best conserved yeast homologous among the regulatory protein of mammalian apoptosis. It was observed that upon treatment both acetic acid (Sousa et al., 2013) and H_2O_2 (Falcone & Mazzoni, 2016) the overexpression of yeast Nuc1p caused apoptotic cell death compared to the control group and its nuclease activity and MLS were found responsible from this apoptotic induction. In addition, it was shown that the mitochondrial proteins Aac2p and Por1p, the karyopherin Kap123p, and histones H2B and H4 copurified with FLAG-tagged Nuc1p in yeast. Therefore it was thought that mitochondrial adenine nucleotide translocator AAC, the karyopherin KAP123,

and phosphorylation of histone H2B interact with and are necessary for Nuc1p-mediated cell death (Büttner et al., 2007). All the process carries out independent manner from caspases as in mammalians.

1.5.1.1.4 Apoptosis Inducing Factor (AIF1/YNR074C). AIF is encoded as a 67 kDa protein containing the MLS at the N-terminus. When taken inside the mitochondrion, the MLS is cleaved by mitochondrial peptidases and the 62 kDa mature protein occurs (Otera, Ohsakaya, Nagaura, Ishihara, & Mihara, 2005). AIF was previously seen as a soluble protein localized in the internal membrane space (Susin et al., 1999). On the other hand, later studies have shown that the N-terminal of AIF is bound to the inner mitochondrial membrane where it exhibits NADH oxidase activity (Miramar et al., 2001). The crystal structures of mouse and human AIFs which have 92% similarity have shown that the protein is oxidoreductase type (Maté et al., 2002; Ye et al., 2002).

According to the studies using model cells produced by gene silencing and gene deletion techniques, it is proposed that AIF plays a role in oxidative phosphorylation by modulating the structure and function of respiratory chain enzymes, mainly complex I (Vahsen et al., 2004; Klein et al., 2002). In mice, muscle and liver-specific AIF deficiency resulted in insufficient oxidative phosphorylation (Pospisilik et al., 2007). Genetic inactivation of AIF has damaged cavitation which is a necessary process for early embryonic morphogenesis regulated by apoptotic cell death (Joza et al., 2001). For this reason, it is embryonic death that the organism is completely devoid of AIF. In Harlequin (Hq) model mice, AIF expression was suppressed by 10-20% compared to normal levels, due to retroviral insertions into the gene region of interest. These mice are alive compared to embryos that do not contain AIF protein, on the other hand blindness, ataxia, advanced loss in retinal and cerebellar neurons are observed. The susceptibility of Hq mice to oxidative stress suggests that AIF functions as a free radical scavengers (Klein et al., 2002; Lipton & Bossy-Wetzel, 2002).

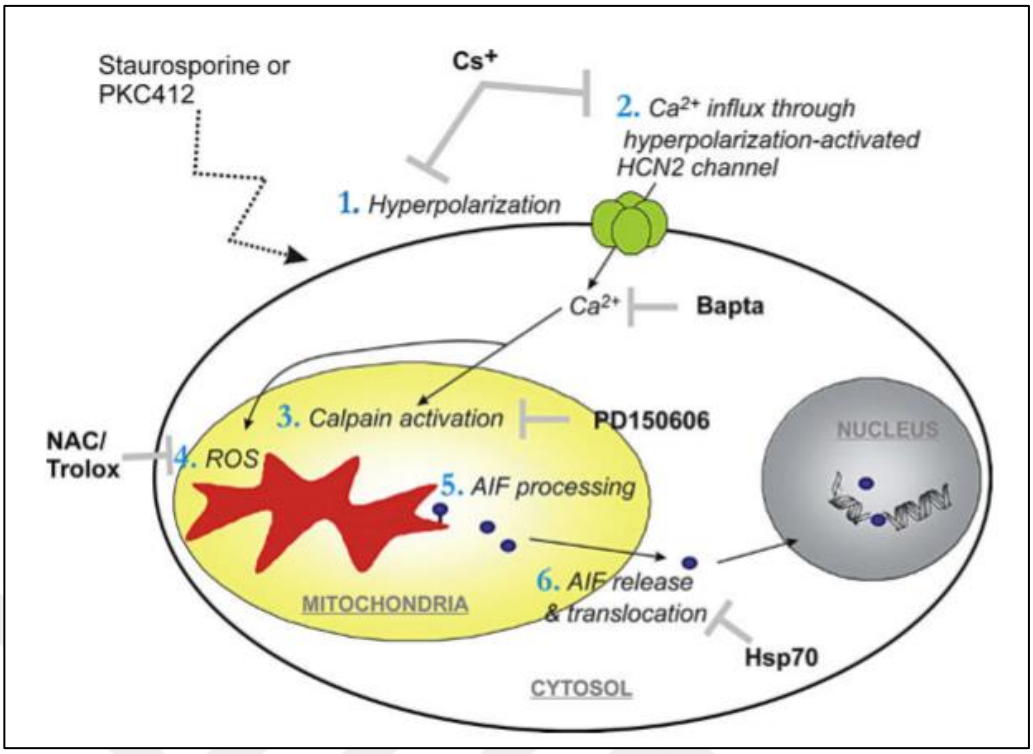


Figure 1.32 Hypothetical model of the mechanisms involved in AIF processing and release (Norberg, Orrenius, & Zhivotovsky, 2010)

However, both the increase and the suppression in the levels of reactive oxygen species were observed in the studies that were conducted with different cancer cells whose gene region expressing the AIF protein were deleted or silenced (Urbano et al., 2005). In a study of Hq model mice, the connection between AIF and mitochondrial ROS production could not be found (Chinta et al., 2009). Therefore, the effect of AIF on ROS levels is uncertain.

Upon treatment with certain apoptotic stimulus, AIF can be hydrolyzed via proteases from its membrane linkage and after this process 57-kDa soluble AIF fragment occurs. This fragment can be released to the cytosol from the inner mitochondrial space upon membrane permeabilization. The AIF protein contains two nuclear localization signals (NLS) and translocates to the nucleus when released into the cytosol. AIF contributes greatly to DNA fragmentation and chromatin condensation in the nucleus but the clear mechanism of its effect is not well known (Otera, Ohsakaya, Nagaura, Ishihara, & Mihara, 2005). Since the nuclear

translocation of AIF is not observed in non-cyclophilin (CypA) - / - neurons with hypoxia-ischemia, the protein is thought to require CypA to translocate into the nucleus and bind DNA (Zhu et al., 2007). As long as AIF can not translocate to the nucleus, it can not induce cell death. For this reason, the release of mitochondria is not sufficient to explain its function on cell death process (Cheung et al., 2006). It is also known that the AIF-mediated pathway is not required in all forms of apoptosis, and it changes according to stimulation and cell type.

It has been shown that AIF and endonuclease G proteins do not release from permeabilized mitochondria at the same time with other mitochodrial proteins like cytochrome c and Smac/DIABLO and requires extra signals for cytosolic release (Uren et al., 2005). In another study, it has been shown that recombinant calpain-I can hydrolyze and release AIF from the Bid-permeabilized mitochondrion (Figure 1.32) (Polster et al., 2005). Three different forms of AIF as 67, 62 and 57 kDa were found in mitochondrion of apoptotic cells. For this reason, the membrane bound has to be hydrolyzed for the release of AIF from mitochondria. It has been shown that calpain and kathepsin proteases play a role in AIF proteolysis. These proteases hydrolyze AIF from its Gly102/Leu103 position (Yuste et al., 2005).

It is now known that the AIF-mediated pathway is not effective in all cell death mechanisms. Therefore, the silencing or complete deletion of the gene region that expresses the AIF protein can not protect the cells from apoptotic cell death induced by many drugs. On the other hand, genetic inactivation of AIF in mice is embryonically lethal (Joza et al., 2001). According to current studies, AIF plays a critical role in the death of only certain cell types such as neurons and some tumor cells (Zhu et al., 2007). The type of apoptotic stimulation is another factor that determines whether AIF plays an active role in the cell death process. The major factors that stimulate AIF protein are those that directly affect intracellular Ca^{2+} homeostasis or cause early lysosomal permeabilization.

Another important feature of the role of AIF protein on cell death is its ability to time its own production and release in conjunction with other pro-apoptotic factors.

Mitochondria contain several specific pro-apoptotic proteins released from mitochondrial membranes during apoptotic signaling including Smac/DIABLO, cytochrome c and AIF. The Smac/DIABLO, cytochrome c and AIF proteins are of different sizes and are found differently in the intermitochondrial space. Smac/DIABLO is a 25 kDa protein in soluble form. While the 14 kDa cytochrome c binds to the cardiolipin in the intermitochondrial membrane with both electrostatic and hydrophobic interactions (Ott, Robertson, Gogvadze, Zhivotovsky, & Orrenius, 2005). 62 kDa AIF binds to intermitochondrial membrane via its transmembrane region. Therefore, it is thought that soluble proteins with small molecular weights are released before AIF by external mitochondrial membrane permeabilization, since AIF release requires an extra hydrolysis.

Similar to mammalian AIF, the yeast AIF homologue Ynr074cp regulates yeast apoptosis. It was shown by the fluorescent microscope and ^{35}S-labeled AIF1p precursor studies aiming to determine the localization of yeast AIF homologue Ynr074cp that the protein localizes in mitochondrial inner membrane or matrix (Wissing et al., 2004). As stated above, CypA is the protein that is vital for the translocation of mammalian AIF. Similarly, no apoptotic induction was observed in yeast under conditions where CypA homologue CPR1 was damaged even if AIF was overexpressed. This situation demonstrates the importance and necessity of CPR1 in AIF mediated apoptotic induction. Finally, it was shown that H_2O_2 and acetate induced yeast apoptosis in AIF dependent manner.

1.5.1.1.5 The mitochondrial serine protease (NMA111/YNL123W). HtrA (high-temperature requirement A) proteases were first described in Escherichia coli as serine proteases induced by heat shock and commonly found in almost all bacteria and eukaryotic genomes.

The apparent feature of the HtrA protein family is that the catalytic serine protease domain is in combination with one or two PDZ domains (Clausen, Southan, & Ehrmann, 2002). PDZ domains consist of small globular protein-protein interaction modules play a part in many signal pathways. As a result, HtrA proteases

play a role in many significant cell processes and dysfunction of mammalian HtrAs correlates with severe diseases such as Parkinson and Alzheimer's disease, myocardial ischemia and cancer.

Saccharomyces cerevisiae encodes an HtrA-like protein called Nma111p. This protein is ab nuclear mediator of apoptosis. Nma111p plays a role in lipid homeostasis and has chaperone activity (Padmanabhan et al., 2009). Nma111p is defined via dual-hybrid interaction with a yeast nuclear pore protein. In contrast to HtrA2/Omi which is localized to mitochondria in metazoa, it is localized in the nucleus (Walle, Lamkanfi, & Vandenabeele, 2008). Yeast cells lacking Nma111p are more resistant to elevated temperatures. These cells do not exhibit apoptotic characteristics such as chromatin condensation, phosphatidylserine externalization or ROS accumulation after being treated with H_2O_2, a known trigger of apoptosis in yeast. Conversely, overexpression of Nma111p triggers apoptotic cell death in yeast and this induction is dependent on the integrity of the serine protease domain. HtrA2/Omi induces apoptosis by binding and disrupting cellular IAPs (Inhibitor of apoptosis proteins) (Salvesen & Duckett, 2002). IAPs are characterized by the presence of BIR (baculovirus IAP repeat) domains (Huang et al., 2001). The yeast genome contains only one BIR protein called Bir1p. Bir1p was previously known for its role as a chromosomal passenger protein, but the latter appeared to be a substrate for Nma111p and a homologue of IAP in *S. cerevisiae*.

1.5.1.1.6 Caspases (MCA1/YOR197W). The discovery of cysteine proteases, called caspases, has led to significant improvements in understanding the molecular control mechanism of apoptotic cell death (Alnemri, 1997). Caspases can hydrolyze their substrates and related proenzymes from certain aspartic acid residues.

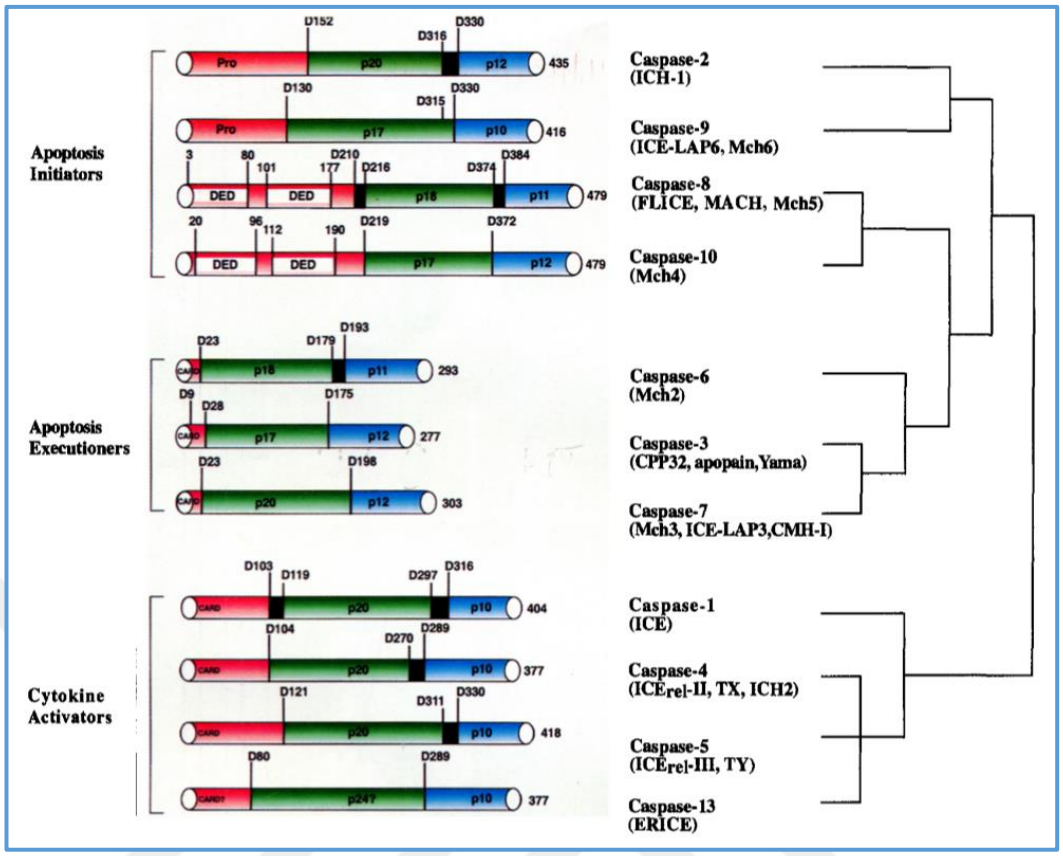


Figure 1.33 Classification of human caspases (Alnemri et al., 1997)

The caspase family in humans consists of 11 members and is separated to 3 subgroups according to phylogenetic analysis (Figure 1.33). The sequence similarity between these caspases ranges from 20 to 77% and X-ray crystallography and sequence analysis studies showed that caspases share a common structure (Wolf & Green, 1999). Caspases are synthesized as inactive zymogens and have molecular weights ranging from 30 to 55 kDa. Each zymogen has N-terminal pro-domain followed by a large subunit and small subunit at the C-terminus. Under normal conditions, these proteins are found as inactive, single polypeptide chains known as pro-caspases. Hydrolysis from specific aspartic acid residues that reveals large and small subunits and conformational rearrangement are required for their activation. As can be seen from Figure 1.33, while caspase-3 and -7 have a single hydrolysis site between two subunits, caspase-1, -2, -5, -6, -8 and -10 contain two hydrolysis sites. The physiological importance of the second hydrolyzed region is not clear and is thought to be used for regulatory purposes in some cell types. Activation occurs by the hydrolyze of the bond in this internal domain. On the other hand, there is no need

for proteolytic cleavage between subunits for pro-caspase-9 activity and the molecule can form the active holoenzyme complex with Apaf-1 and cytochrome c without any degradation (Rodriguez & Lazebnik, 1999; Stennicke et al., 1999).

In the large subunits of all caspases (17-21 kDa), the "QACXG" pentapeptide motif containing the active cysteine residue is conserved and in the small subunits (9-12 kDa) there are several conserved residues that determine the binding and specificity of the substrate. Active caspases are tetramers with two large and two small subunits. Subunits of caspases closely related to each other, such as caspase-3 and caspase-7, have been shown to form functional heterokomplexes (Fernandes-Alnemri, Litwack, & Alnemri, 1995). Based on functional assays, caspases are generally classified into 3 groups, including cytokine activators, apoptosis-inducing caspases and effector caspases.

Caspases are enzymes synthesized as zymogens with a general organization (Figure 1.34). The N-terminal domain in the caspases is followed by sequences that first encode larger subunits and then smaller subunits. In some procaspases, these subunits are separated from each other during the maturation of the enzyme via the hydrolysis of a small linkage from zymogen. All hydrolysis that take place in the maturation of caspases occur at the carboxyl end of the aspartate residues. In eukaryotic proteases, this specificity is seen only in caspases and cytotoxic T lymphocyte serine protease Granzim B.

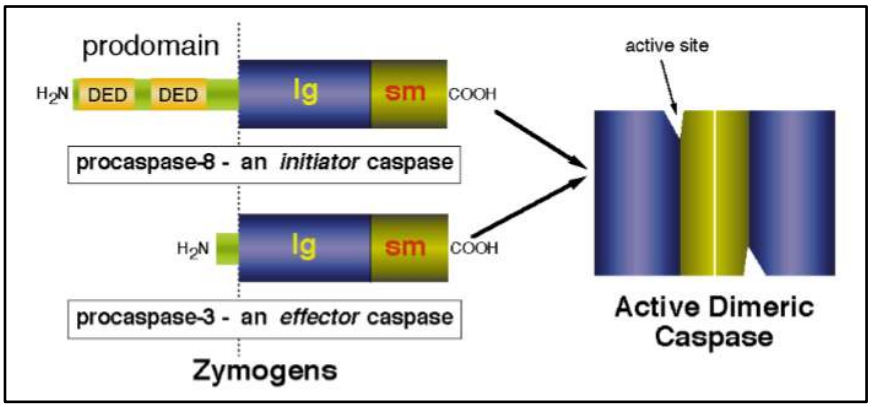


Figure 1.34 Diagram showing the structures of the zymogens for caspase 8, an initiator caspase, and caspase 3, an effector caspase. The amino-terminal prodomains are shown in green, large subunits are in blue, and small subunits are shown in green (Earnshaw, Martins, & Kaufmann, 1999)

When the sequences in the large and small subunits of eleven human caspases identified until today were compared, it was determined that 29 residues of at least 10 members of the family were identical. While 7 of these residues are responsible for the recognition of the substrate and catalysis, the remaining 22 residues are distributed throughout the protein. Since most of these residues are hydrophobic and usually do not localize on the surface, they are thought to be responsible for protecting the entire structure of the natural enzyme rather than catalytically active site.

The length of the caspase prodomains ranges from 23 amino acids for caspases 6 and 7 to 219 amino acids for caspase 10. Caspases with large prodomains are thought to induce apoptotic cell death and are called initiator caspases. The caspases with shorter prodomains are activated by the initiator caspases and are called effector/lethal caspases. Prodomains are much different than catalytic segments, but two related motifs are found in this region. It is thought that the "Death Effector Domain, DED" in caspase 8 and 10 interacts with adapter proteins such as MORT1/FADD and TRADD. "Caspase recruitment domain, CARD" is a domain found in caspases -1, -2, -4, and -9 and is significant both for the interactions of caspases with each other and with various regulatory and adapter proteins. The DED

and CARD motifs are similar to the death domains. All three regions are tightly packed six α-helix packages.

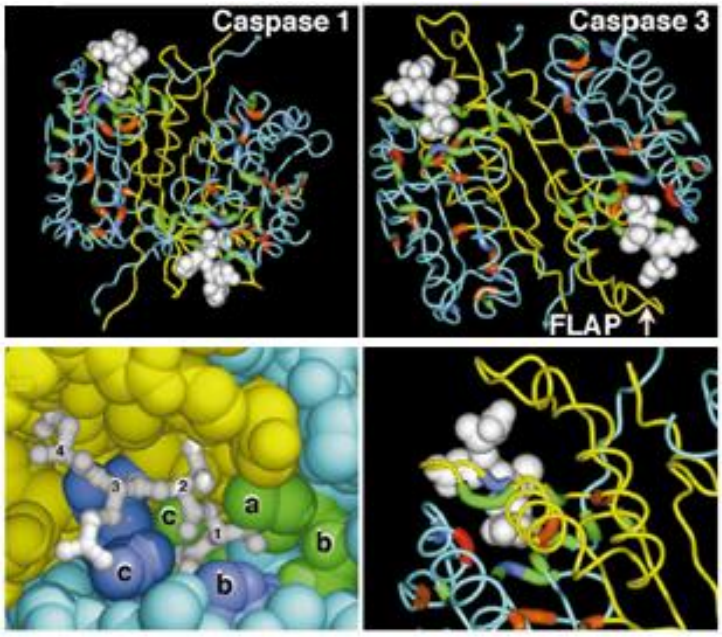


Figure 1.35 Three-dimensional structure of caspases. The YVAD (A) or DEVD (B) substrate is shown in the active sites in *white*. Space-filling representation of the substrate-binding pocket (*top view*) of caspase 3: a, Cys285 (*green*); b, His237 (*blue*), Gly238 (*green*); c, Gln283 (*green*), Arg341 (*blue*). α carbons of the P1, P2, P3, and P4 amino acids of the DEVD substrate are numbered 1–4, respectively, for orientation (C). The same substrate in the active site (rendered asan a-carbon tracing) but viewed from the orientation opposite (D) (Earnshaw, Martins, & Kaufmann, 1999)

Phylogenetic analyzes show that caspases are basically divided into two subfamilies. Caspase 1 superfamily (caspases -1, -4, -5 and -13) predominantly play a role in the control of inflammation, whereas caspase 3 superfamilies (caspases -3 and 6-10) are primarily specialized for apoptosis. Caspase -2 is structurally more like caspase 1 superfamily, but it is involved in some apoptosis types and can be grouped as a third caspase subfamily.

Each active caspase occurs with a self-assembly of two procaspase zymogens. The resulting tetrameric enzyme, defined as the homodimer of the heterodimers, contains two active sites at two opposite ends. The large and small subunits in each heterodimer interact with each other to form a nucleus that consists of ß-sheet folded with α-helix. This quaternary structure is specific to caspases among proteases.

Based on the distance between the N-terminus of the small subunit associated with the C-terminal of the large subunit, it is thought for both caspase -1 and caspase -3 that during the maturation of these precursors, p10 subunit of a zymogen complexes with p20 subunit of a second zymogen.

All caspases hydrolyze peptide bonds from the carboxyl side of an aspartate residue. This residue is called the P1 residue. Analysis of the active sites of caspase -1 and caspase -3 revealed that this hydrolysis is due to the fact that the Cys285 residue in caspase -1 is deprotonated from the sulfhydryl group by the imidazole ring in the His237 residue and consequently it has been understood that thiolate nucleophile attacking the carbonyl group of weak bond was formed to form a tetrahedral intermediate which is stabilized by hydrogen bonding to the amide nitrogen of Gly238 (Figure 1.35). This catalytic residue, Cys285, His237 and Gly238, is protected in 11 human caspases.

The aspartic acid residue must be present at the P1 position of the substrate. This residue is embedded in a deep pocket and is in the area called S1. This region is surrounded by residues Arg179, Gln283, Arg341 and Ser347 in caspase-1. This structure is completely preserved, except that Thr takes the place of Ser 347 in caspase 8. The two residues at the amino terminus of P1 aspartate (termed as P2 and P3) have limited activity in terms of substrate degradation. This is probably due to the fact that the side chains of the residues are away from the enzyme body. Conversely, it is believed that the sites of caspase-1 and caspase-3, which are bound by the P4 residue above the P1 aspartate, are responsible for different substrate specificities.

All caspases are found as hidden zymogens with very low intrinsic protease activity in normal cells (Muzio, Stockwell, Stennicke, Salvesen, & Dixit, 1998). An important difference between the caspases is the size of their prodomain. Initiating caspases such as caspase-8, -9 and -10 have partially larger prodomains than effector caspases such as caspase-3, -6 and -7.

Although the significance of a longer prodomain region of the initiator caspases had not been previously understood, it has been revealed by the subsequent studies that the initiator caspases are accumulated in the death complex or apoptosome by the interactions in their prodomain regions and thus require a longer prodomain region. In mammalian cells, it is thought that the accumulation of initiator caspases in DISC or apoptosomes, in other words, oligomerizations increase local enzyme concentration, which in turn promotes apoptosis by facilitating autoactivation.

No significant autoactivation is observed in short prodomain effector caspases such as caspases -3, -6 and -7. Such caspases require initiator caspases for their activation. Effector caspases are activated when hydrolyzed by the initiator caspases, and can hydrolyze cellular substrates or other pro-caspases. In this sense, effector caspases initiate the cascade of elevating apoptosis signals. For example, when adequate concentration conditions are provided, caspase -3 and -9 hydrolyze each other's proenzymes as well as other pro-caspases such as proaspase-6 and -7 effectively.

Another mechanism in caspase activation is mediated by aspartate-specific serine proteases, the most well-known example being Granzyme B in cytotoxic T lymphocytes and natural killer cells. Granzyme B is an important inducer of apoptotic cell death. Granzyme B activates most of the caspases, including pro-caspase-3 and pro-caspase-7 by hydrolysis from the critical Asp residue at the junction between the major and minor subunits (Barry et al., 2000). On the other hand, this region is also highly susceptible to proteolysis of many other serine proteases such as cathepsin G and subtilisin Carlsberg. Although the roles of prokaspase activation are not completely understood, it is known that other proteases such as proteasome, apoptotic serine proteinase p24, cathepsin D and calpain are involved in apoptotic cell death.

In plants, fungi, Dictyostelium and metazoa, paracaspases and metacaspases, which have similar homology to mammalian caspases but exhibit different substrate specificities have been identified. In *Saccharomyces cerevisiae*, a gene

(MCA1/YCA1) encoding metacaspase involved in cell death induction was detected via sequence homology search. Metacaspases are the homologs of caspases not in biochemistry but in sequence and function. On the other hand, caspases exhibit proteolytic activity from the C-terminus of aspartate while metacaspases from the C-terminus of to arginine or lysine.

1.6 Exogenous Factors that Trigger Apoptosis in Yeast

Numerous different external stimulis can induce yeast apoptosis. Such stimuli can be via heterologous expression of human pro-apoptotic proteins and in the form of chemical stresses.

1.6.1 Heterologous Expression of Human Proapoptotic Proteins

S. cerevisiae provides appealing alternative to bacterial and mammalian protein expression systems for the production of recombinant proteins. The main advantages of using yeast include an extensive toolbox of genetic modification strategies, production of authentic eukaryotic products, and low culture costs when compared with mammalian systems. Target recombinant proteins can be produced intracellularly or engineered to enter the yeast eukaryotic secretory pathway, resulting in secretion into the culture medium. This is essential if the target protein requires additional folding steps (e.g., disulfide bond formation) or modification (e.g., glycosylation). In addition heterologous expression of human proteins to yeast provides information related to the functions of the so-called protein in several biochemical pathways, one of which is the search of the anti- and pro-apoptotic roles of various mammalian proteins. Several membrane receptors, members of Bcl-2 family and α-synuclein are some of the human proteins whose importance in apoptotic cell death was highlighted with the use of heterologous expression in yeast. Bax is a pro-apoptotic Bcl-2-family protein in mammalians. Heterologous expression of the human key apoptotic inducer Bax causes rapid cell death in the yeast *S. cerevisiae* (Greenhalf, Stephan, & Chaudhuri, 1996) and *Candida albicans* (De Smet, Eberhardt, Reekmans, & Contreras, 1996). Manon and colleagues

demonstrated that Bax expression induces the release of cytochrome c from mitochondria (Manon, Chaudhuri, & Guérin, 1997). In addition, the generation of reactive oxygen species and other apoptotic features including DNA fragmentation and phosphatidylserine externalization were observed in Bax expressing yeast cells (Madeo et al., 1999). The cell-death inhibitor BI-1 (Bax inhibitor 1), which is conserved in mammals, plants, and fungi, can suppress apoptosis induced by Bax or hydrogen peroxide in yeast. Furthermore, heterologous expression of Bcl-2 or BclXL prevents the cytotoxic effect of Bax and at the same time causes enhanced resistance to H_2O_2 (Simizu, Umezawa, Takada, Arber, & Imoto, 1998) and acetic acid (Ludovico et al., 2002).

It has been shown that heterologous expression of α-synuclein (α-syn), an intracellular trigger of Parkinson's disease, has detrimental effects on yeast cells and induces apoptosis (Willingham, Outeiro, DeVit, Lindquist, & Muchowski, 2003). During chronological aging, α-syn decreases the life span and increases the frequency of apoptotic and necrotic events. This effect is abrogated in cells lacking mitochondrial DNA, revealing a strict requirement of functional mitochondria for α-syn toxicity.

The spinocerebellar ataxia type-3 (SCA3) is a disease caused by the expansion of poly-Q in the gene atxn3, which encodes a protein known as ataxin-3 (AT3) (Todi et al., 2010). The expression of AT3 in yeast is toxic and leads to accumulation of ROS, imbalance of the antioxidant defense system, loss in cell membrane integrity and necrotic cell death, without the induction of apoptosis.

Expression in yeast of a mutated form of DFNA5, a gene responsible for autosomal dominant hearing loss (HL), induces ROS accumulation leading to a caspase-independent cell death that relies on mitochondrial functions such as the mitochondrial fission protein Fis1p, the voltage-dependent anion channel Por1p, and the mitochondrial adenine nucleotide translocators Aac1p and Aac3p (Falcone & Mazzoni, 2016). More recently, it has been reported that ER and protein folding are also involved in RCD caused by DFNA5 both in yeast and in HEK293T cells.

1.6.2 Chemical Stress

According to the data obtained up to now, chemical stress factors that induce apoptotic cell death in yeast include a number of compounds especially acidified cell environment, various chemotherapeutics and some antifungal agents.

In the study conducted by in *S. cerevisiae* W303-1A, it was shown that acetic acid induced cell death associated with cycloheximide inhibitable chromatin condensation along the nuclear envelope, exposure of phosphatidylserine on the surface of the cytoplasmic membrane and the occurrence of DNA strand breaks (Ludovico, Sousa, Silva, Leão, & Côrte-Real, 2001). Almeida et al. showed that acetic acid induced apoptosis in *S. cerevisiae* BY4742 strain carried out by the involvement of TOR (target of rapamycin) pathway which mediates cell growth in response to nutrient availability. Arsenic is a toxic metalloid that was used as a therapeutic agent. This metalloid was used to treat the plague, malaria, and cancer (Miller, Schipper, Lee, Singer, & Waxman, 2002). Arsenic inhibits the growth of *S. cerevisiae* Y-1173 at moderate concentrations. It was also demonstrated that arsenic is able to trigger *S. cerevisiae* into an apoptotic process by showing characteristic markers including decrease in mitochondrial transmembrane potential and Yca1p activation (Du et al., 2007). Diamide is a sulfhydryl reagent which oxidizes sulfhydryl groups to the disulfide form. The POR1 (yeast voltage-dependent anion channel) mutant of *S. cerevisiae* W303-1B enhances apoptosis triggered by diamide together with the accumulation of ROS (Pereira, Camougrand, Manon, Sousa, & Côrte-Real, 2007). Another compound that induces apoptosis in yeast is ethanol. As known, ethanol is the most important fermentation product on earth. *S. cerevisiae* commonly used for fermentation dies when ethanol levels exceed a certain concentration. *S. cerevisiae* BY4743 strain treated with ethanol has demonstrated the evidence of apoptosis including chromatin condensation and fragmentation, DNA cleavage, and a requirement for de novo protein synthesis (Kitagaki, Araki, Funato, & Shimoi, 2007). In addition, it has been thought that among mitochondrial fission proteins, Fis1 mediates ethanol-induced apoptosis and ethanol-induced mitochondrial fragmentation in this strain.

According to their mode of action, antitumor drugs can be classified as alkylating agent, antimetabolites, plant alkaloids, antitumor antibiotics, monoclonal antibodies and hormone regulators (Figure 1.36).

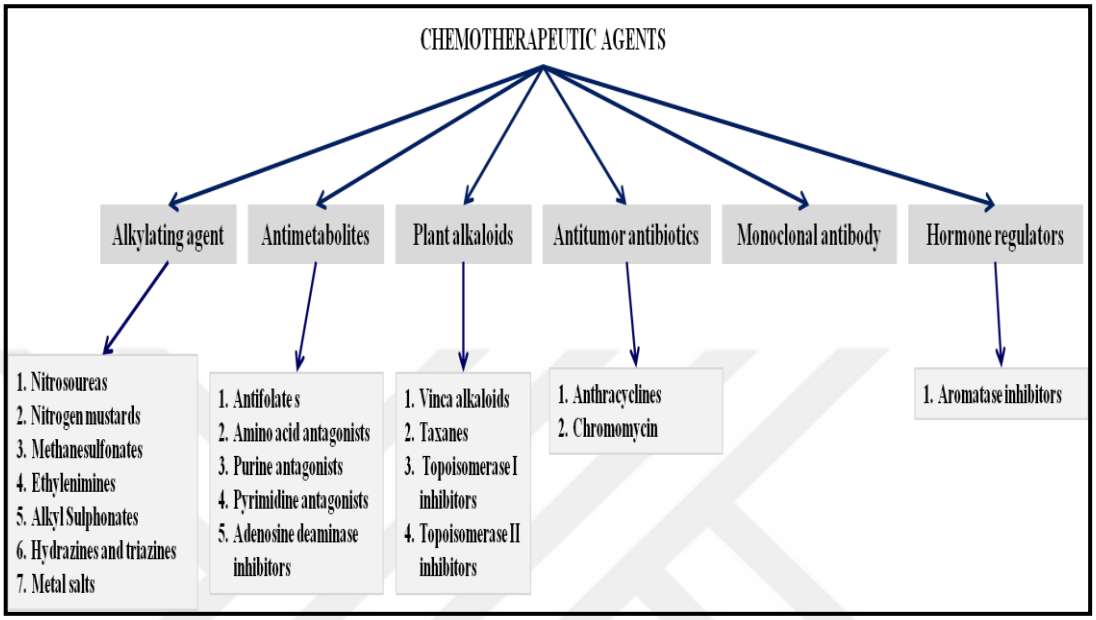


Figure 1.36 Classification of antitumor drugs according to their mode of action

Among these drugs, while topoisomerases inhibitors fredericamycin, camptothecin, etoposide and antimetabolites 5-fluorouracil have been known with their cytotoxic effects in yeast; microtubule-directed paclitaxel, DNA fragmenting bleomycin, histone deacetylase (HDAC) inhibitors valproate, and DNA intercalating doxorubicin represent the most well studied drugs inducing yeast apoptotic phenotypes.

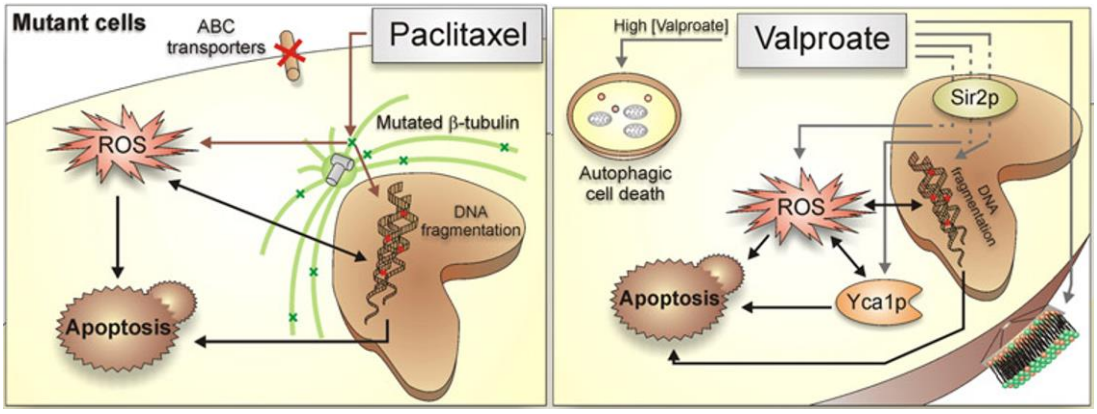


Figure 1.37 Action mechanisms of paclitaxel and valproate (Almeida et al., 2008)

Primary action mechanism of paclitaxel in cells is to cause a mitotic block by stabilizing microtubules, thereby decreasing the dynamic nature of these cytoskeletal structures (Jordan, 2002). Wild-type *S. cerevisiae* tubulin does not bind this agent. On the other hand, paclitaxel induced apoptosis in *S. cerevisiae* strain which mutated β-tubulin gene was introduced. Paclitaxel-treated mutant yeast cells showed DNA fragmentation and accumulated reactive oxygen species (Figure 1.37). Bleomycin is a radiomimetic anticancer drug that produces single- and double-stranded DNA breaks in mammalian tumor cells (Aouida, Mekid, Belhadj, Mir, & Tounekti, 2007). This agent induced apoptotic cell death characterized by sub-G1 region peak, chromatin condensation, and very rapid DNA fragmentation into oligonucleosomal-sized fragments in *S. cerevisiae* haploid strain YPH-1. Valproate belongs to the group of inhibitor of the class I HDACs (Gottlicher et al., 2001). It was found that valproate can trigger apoptosis in mammalian cells through caspase-dependent and -independent pathways (Kawagoe, Kawagoe, & Sano, 2002). The effects of valproate on the regulation of certain members of Bcl-2 family were also shown (Armeanu et al., 2005). Likewise, in yeast cells, valproate triggered a cell death process that is dependent on Yca1p (Mitsui, Nakagawa, Nakamura, Okamoto, & Tsurugi, 2005). At low valproate concentrations, yeast cells have undergone apoptotic cell death associated with DNA fragmentation, ROS accumulation, phosphatidylserine exposure and morphological alterations such as cell shrinkage. On the other hand, exposure to high concentrations of VPA induces cell death with morphological features similar to those of autophagic cell death (ACD), which is independent of Yca1p. Another antitumor drug that is known to induce apoptosis in yeast cells is

doxorubicin. The antibiotic doxorubicin originally isolated from *Streptomyces peucetius* was shown to induce apoptosis in *Candida utilis*, based merely upon morphological observations, with reported plasma membrane alterations and changes in mitochondrial shape and cristae organization (Keyhani & Keyhani, 2004).

Antifungal agent is a general term for drugs, chemicals, or other substances that either kill or slow the growth of fungal organisms. The common targets of antifungal drugs are the components of the fungal cell wall such as mannans, glucans and chitins; and a few of the enzymes of the ergosterol biosynthetic pathways which are unique to fungal cells. Among the enzymes of the ergosterol biosynthetic pathway, squalene epoxidase (ERG1), 14α-lanosterol demethylase or CYP51 (ERG11), Δ^{14}–reductase (ERG24) and Δ^{8}- Δ^{7}-isomerase (ERG2) have been the targets of most antifungal agents. Amphotericin B is one of the antifungal medications used especially for leishmaniasis. This antifungal arrests *Candida albicans* cells in G2/M phase and induced chromatin condensation, nuclear fragmentation, phosphatidylserine externalization and ROS accumulation (Phillips, Sudbery, & Ramsdale, 2003). Ciclopirox olamine is a synthetic antifungal agent for topical dermatologic treatment of superficial mycoses. In contrast to Amphotericin B, this antifungal induced apoptosis in *S. cerevisiae* BY4742 independent manner from ROS accumulation (Almeida et al., 2007). Ciclopirox olamine also leads to chromatin condensation, sub-G0/G1 population, arrest in G2/M and nuclear dysfunction. On the other hand, it does not require activation of metacaspase. Dermaseptin is another antifungal whose apoptotic phenotype has been well studied. It was found that this agent induces DNA fragmentation and ROS accumulation both *Candida albicans* IP886-65 and *S. cerevisiae* BY4742 strains. Similar to ciclopirox olamine, dermaseptin induced apoptosis also does not require metacaspase activation but it is dependent of the Aif1p and the proteosomal substrate (Stm1p).

Clotrimazole is another antifungal drug whose potential anticancer properties have been examined in mammalian cancer cells. In the scope of this thesis, the cytotoxic and apoptotic effects of the antifungal drug clotrimazole (CLT) on *S. cerevisiae* was investigated in detail.

1.7 Antifungal and Potential Chemotherapeutic Agent Clotrimazole

CLT that is one of the synthetic and lipophilic imidazole derivatives (Figure 1.38) is primarily used to treat infections caused by various fungi. It is known for a long time that *Candida spp., Vulvovaginal candidiasis, Trichophyton spp., Microsporum spp*. and *Malazzesia furfur* are the strains against which drug is most active (Sawyer, Brogden, Pinder, Speight, & Avery, 1975).

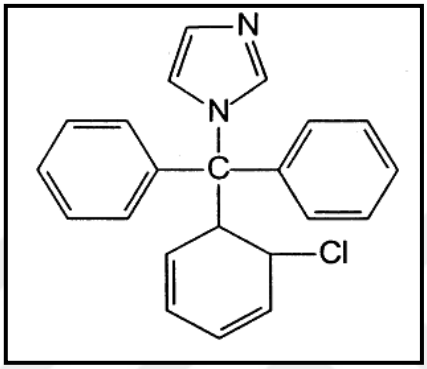


Figure 1.38 The structure of clotrimazole

As all azole-type antimycotic drugs, CLT inhibit the microsomal cytochrome P450 (CYP450) -dependent event 14-α-lanosterol demethylation, which is a vital step in ergosterol biosynthesis in fungi (Hitchcock, Dickinson, Brown, Evans, & Adams, 1990). The resultant depletion of ergosterol and its replacement with the aberrant sterol species, 14-α-methylsterol, perturb normal membrane permeability and fluidity.

Studies about CLT in the last 30 years have shown that as well as antifungal activity it can be used in the treatment of sickle cell anemia, joint inflammation, malaria (Tiffert, Ginsburg, Krugliak, Elford, & Lew, 2000) and tuberculosis (McLean et al., 2002), and may also have anticancer effects. In the first study conducted by Mukhtar et al. related to the antitumor effect of clotrimazole, it has been determined that local clotrimazole administration significantly reduces the formation of skin tumors by blocking the metabolic enzymes responsible for the conversion of precancerous polycyclic aromatic hydrocarbons to active carcinogenic metabolites (Mukhtar et al., 1984). Subsequent studies have also shown that

clotrimazole inhibits tumor cell growth in different cancer cells such as melanoma, human pancreatic cancer, human prostate cancer, lung and colon carcinomas (Penso & Beitner, 1998, 2002; Ito et al., 2002). In vitro studies carried out by Khalid have shown that CLT inhibits glioblastoma cell growth, arrests the cell cycle in G1 phase and induces apoptosis (Khalid, Tokunaga, Caputy, & Walters, 2005). In another study on how clotrimazole was affecting the cell cycle, U-87 MG glioblastoma cell line was used as a model and it was determined that the agent arrested the cell cycle in the late G1 phase (Liu, Li, & Raisch, 2010). In the same study, it was shown by the Annexin V test that clotrimazole induced apoptosis in the so-called cell line and also mitochondria-bound hexokinase II was translocated to the cytoplasm. In another study conducted by Meira et al., clotrimazole has been shown to exert cytotoxic effects on human breast cancer cell lines (Meira et al., 2005). Cancer cells are characterized by a high rate of glycolysis, which is the primary energy source. It is known that the glycolysis pathway is regulated by the reversible binding of glycolytic enzymes to the cytoskeleton as well as the allosteric regulators. In the study, it was determined that clotrimazole, which inhibited cell proliferation in a dose and incubation period dependent manner, distinguished 6-phosphofructo-1-kinase and aldolase among glycolytic enzymes enzymes from and this result was interpreted as apoptotic events may have begun in the cell and therefore the agent promised to use against breast cancer. In the research conducted to understand how clotrimazole affects glucose uptake, intracellular ATP content, basic glycolytic enzyme activities such as hexokinase, phosphofructokinase-1 and pyruvate kinase, MCF10A, MCF-7 and MDA-MB-23 cell lines that differ in their aggressiveness were used and it was found that the most aggressive cell line, MCF10A, was the line most affected by the drug (Furtado et al., 2012). In another study, it was shown that clotrimazole was cytotoxic to the human HA59T hepatoma cell line, induced apoptosis and also induced Ca^{2+} release from the endoplasmic reticulum dependent on phospholipase C (Horng et al., 2013).

In this thesis, the effects of this azole-type antimycotic and potential antitumor drug CLT on the growth and viability of wild type *S. cerevisiae* BY4742 and the apoptotic character of death were investigated by using methods for the

determination of viability and cell morphology that is characteristic for apoptosis, respectively.

1.8 The Methods for Determination of Cell viability and Metabolic Activity

Determination of cell viability in the understanding of cytotoxicity or genotoxicity of various chemicals, physical and environmental factors is one of the most commonly used methods. However, it is extremely important to determine cell viability in many industrial processes where microorganisms are used. Cell viability is generally defined as the percentage of viable cells in the whole population and can be determined by a variety of methods. These methods; Methods based on the ability of microorganisms to grow/proliferate in solid or liquid medium; determination of colony forming number, spotting test, determination of growth inhibition area diameters (IAD) and measurement of the optical density by culturing the sporesin liquid medium (Figure 1.39). The clear advantage of these methods is that they are easy and inexpensive to apply. However, it takes a long time to get the results with these techniques. Another important disadvantage is that these tests can only provide information about the percentage of growth inhibition, but not the number of viable cells or the number of proliferating cells.

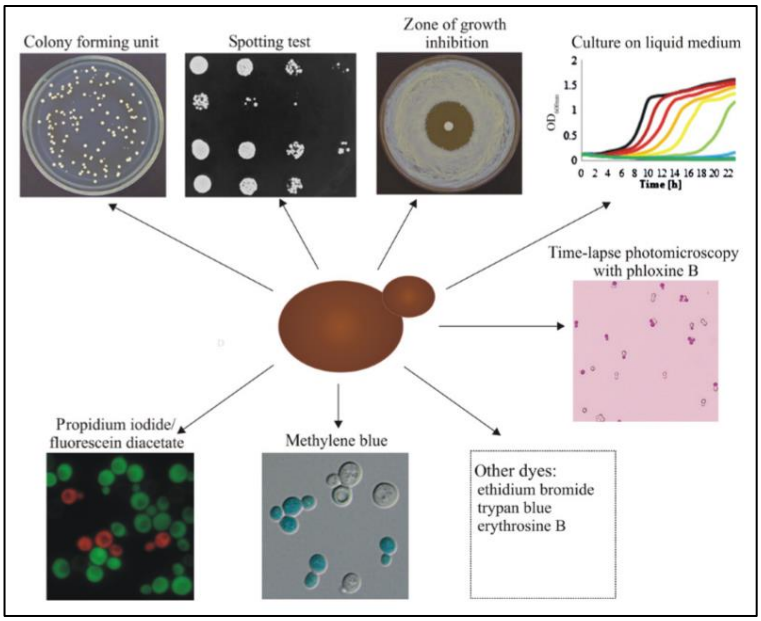


Figure 1.39 The methods used in determination of cell viability (Kwolek-Mirek & Zadrag-Tecza, 2014)

Another option in determining cell viability is dye-based methods in which various colorimetric and fluorimetric dyes are used. The mechanism of action of these dyes depends on the nature of the cell membrane. The cell membrane separates and protects the cell from the outer environment, thereby cell membrane damage causes cell to death. These dyes that are used to determine the cell viability are generally considered in two subgroups:

(1) Dyes dependent on changes in cell membrane integrity and functionality: Dyes that are in anionic form at physiological pH are excluded in living cells due to the negative charge of the cell membrane and can not penetrate into the cell. Such type of dyes can pass through damaged or dead cell membranes and stain the cell nucleus or cytoplasm. Therefore, the presence of dye in the interior of cell indicates that cell membranes are damaged or cell is dead. Some commonly used dyes in this group are fluorescent dyes attached to DNA such as propidium iodide and ethidium bromide and colorimetric ones as trypan blue and erythrocyte B.

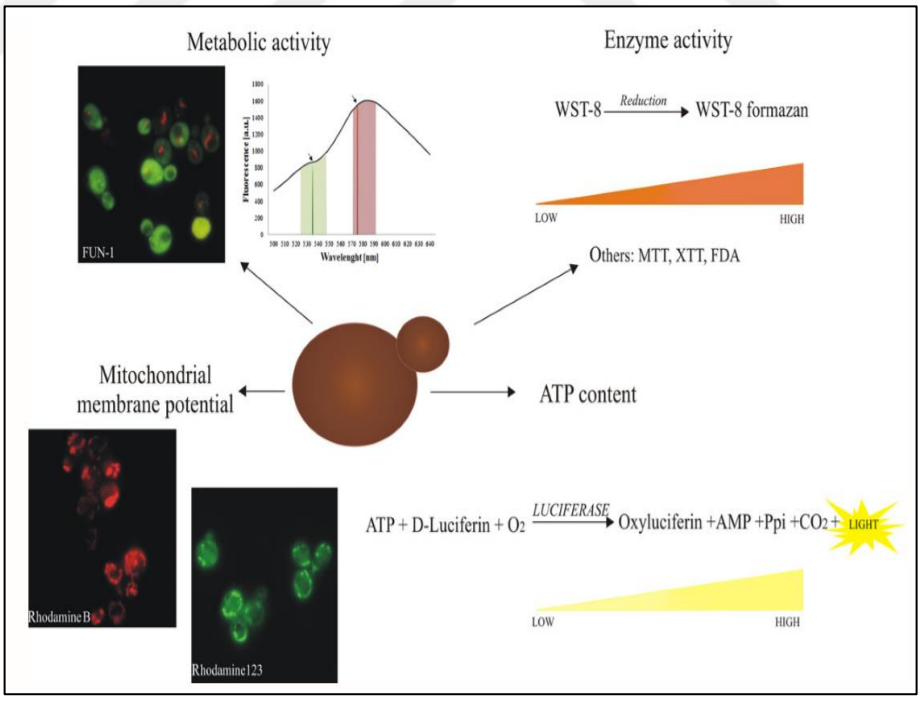


Figure 1.40 The methods used in determination of cell metabolic activity (Kwolek-Mirek & Zadrag-Tecza, 2014)

(2) Dyes that can penetrate both living and dead cells: Since living cells can exclude the dyes such as phloxine or can reduce the dyes such as methylene blue, there will be no colour when the cells are incubated with such type of dyes. On the other hand, the dead cells acquire the certain color that is specific for used dye since these cells do not have the ability to exclude or reduce the color. Viability tests based on the usage of this type of dyes offers very fast and reliable results. They also allow the measurement of living and dead cell percentages within the entire population.

All these methods used to determine cell viability only give information about the percentages of living and dead cells in the population. However, in many cases, the toxic effects of chemicals or physical factors do not directly cause cell death. Such factors can lead to a large number of morphological, intracellular or metabolic changes, and the cell can lose its ability to divide although it is still alive. This situation can be defined as the cell physiological capacity or cell metabolic activity. Methods for determining cellular metabolic activity are based on the investigation of physiological state of the cell in various aspects. These methods are divided into 3 subcategories: (1) Determination of cellular ATP content based on luciferin reaction; (2) determination of mitochondrial membrane potential with the dyes such as rhodamine 123 and rhodamine B; (3) determination of some enzyme activities (the intracellular enzyme activities for FUN-1, esterase activity for fluorescein diacetate, oxidoreductase activity for MTT, XTT and WST-8) (Figure 1.40).

1.9 The Methods for Determination of Apoptotic Cells

The biochemical and morphological changes that characterize apoptotic cell death include the loss of plasma membrane asymmetry, loss of mitochondrial membrane potential, and the condensing and eventual fragmentation of the cellular DNA. It is important to note that since there is no single parameter that defines programmed cell death, a combination of techniques is recommended for reliable detection of apoptosis (Figure 1.41).

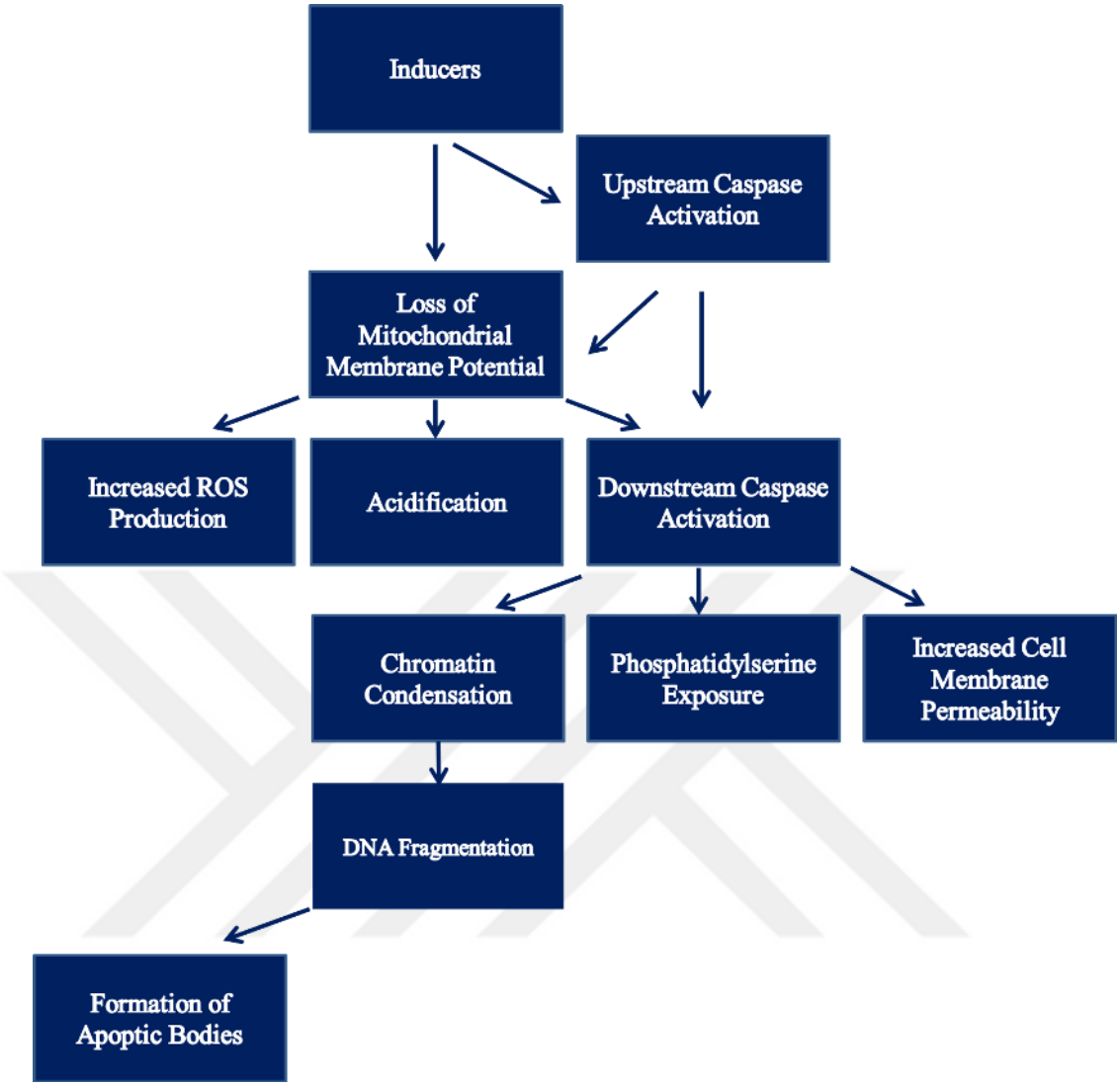


Figure 1.41 The biochemical and morphological changes that characterize apoptotic cell death according to the their formation priority

1.9.1 Phosphatidylserine Exposure

The Annexin-V binding assay is based on the relocation of phosphatidylserine to the outer cell membrane. Viable cells maintain an asymmetric distribution of different phospholipids between the inner and outer leaflets of the plasma membrane. Choline-containing phospholipids such as phosphatidylcholine and sphingomyelin are primarily located on the outer leaflet of viable cells and aminophospholipids such as phosphatidylethanolamine and phosphatidylserine (PS) are found at the cytoplasmic (inner) face of viable cells. The distribution of phospholipids in the plasma membrane changes during apoptosis. In particular, PS relocates from the

cytoplasmic face to the outer leaflet so called PS exposure. It must be stated that the extent of PS exposure can distinguish apoptotic cells from the non-apoptotic cells. Annexin-V is a 35-36 kDa calcium-dependent phospholipid binding protein with high affinity for PS (kDa ~ 5x10-10 M). When labeled with a fluorescent dye, Annexin-V can be used as a sensitive probe for PS exposure on the outer leaflet of the cell membrane (Figure 1.41).

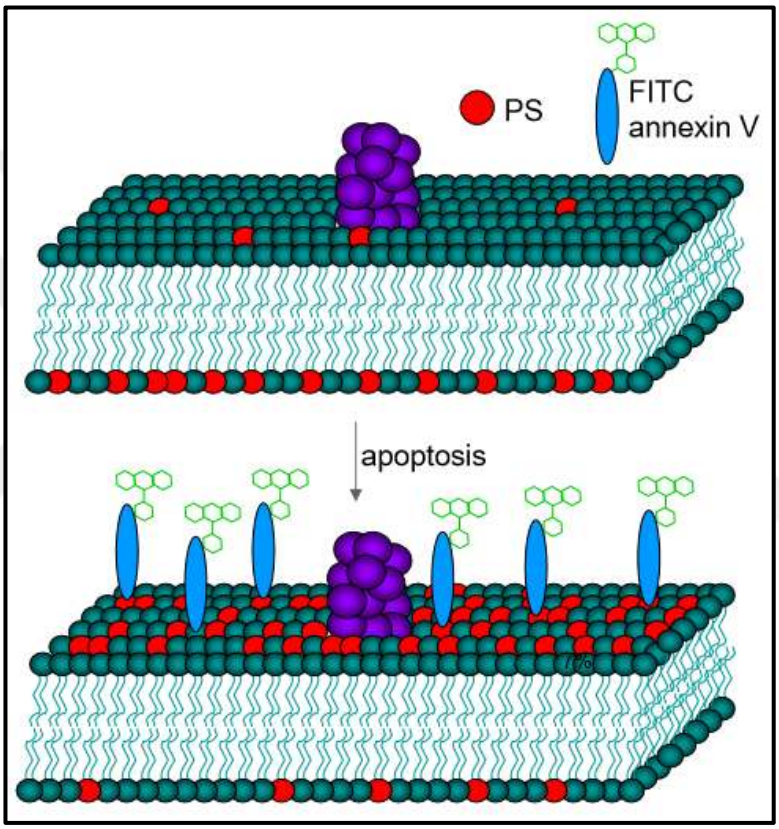


Figure 1.42 The Annexin-V binding on the relocation of phosphatidylserine to the outer cell membrane

The binding of Annexin-V conjugates such as Annexin-V FITC to cells permits differentiation of apoptotic cells (Annexin-V positive) from non-apoptotic cells (Annexin-V negative). Annexin-V binding is observed under two conditions. The first condition is observed in cells midway through the apoptosis pathway. Phosphatidylserine translocates to the outer leaflet of the cell membrane. The second condition is observed in very late apoptosis or when the cells become necrotic and membrane permeabilization occurs. This membrane permeabilization allows

Annexin-V to enter cells and bind to phosphatidylserine on the cytoplasmic face of the membrane. Since other causes besides apoptosis can result in necrosis, it is important to distinguish between necrotic and apoptotic cells. Membrane permeabilization also permits entry of other materials to the interior of the cell, including the fluorescent DNA-binding dye propidium iodide. Utilizing dual staining methodology, apoptotic populations can be distinguished from necrotic populations. For example, using the Annexin V-propidium iodide (PI) double staining regime, three populations of cells are distinguishable in twocolor flow cytometry.

1.9.2 Mitochondrial Membrane Depolarization

Loss of the mitochondrial inner transmembrane potential is often associated with the early stages of apoptosis and may be one of the central features of the process. Collapse of this potential is thought to coincide with the opening of the mitochondrial permeability transition pores, allowing passage of ions and small molecules. The resulting equilibration of ions leads in turn to the decoupling of the respiratory chain and subsequently to the release of cytochrome c into the cytosol. JC-1, JC-9, MitoTracker Red CMXRos and Rhodamine 123 are some of the dyes that used in terms of the determination of mitochondrial membrane depolarization.

1.9.3 DNA Fragmentation

Cell death by apoptosis is characterized by DNA fragmentation in 200-250 and/or 30-50 kilobases. Further internucleosomal DNA fragmentation in 180-200 base pairs may also occur. Such characteristics have been used to distinguish apoptotic cells from normal or necrotic cells. To detect apoptotic cells, whatever the pattern of DNA fragmentation, the TUNEL (Terminal deoxynucleotidyl transferase (TdT) mediated dUTP Nick End Labeling) method is commonly utilized (Figure 1.42). In the TUNEL assay, exogenous TdT is used to catalyze a template-independent addition of bromodeoxyuridine triphosphates (Br-dUTP) to the free 3'-hydroxyl ends of double or single stranded DNA fragments. After incorporation, the labeled BrDU can be identified by FITC conjugated anti-Bromodeoxyuridine (BrDU) antibodies and

analyzed using a flow cytometer or a fluorescence microscope. Due to the many free 3'-hydroxyl ends of fragmented DNA in apoptotic cells, a good signal is generated in affected cell populations. These cells can be visualized in tissue sections or quantified with flow cytometry. Compared to a single-step labeling FITC conjugated dUTP, this two step method provides a more sensitive, stronger signal.

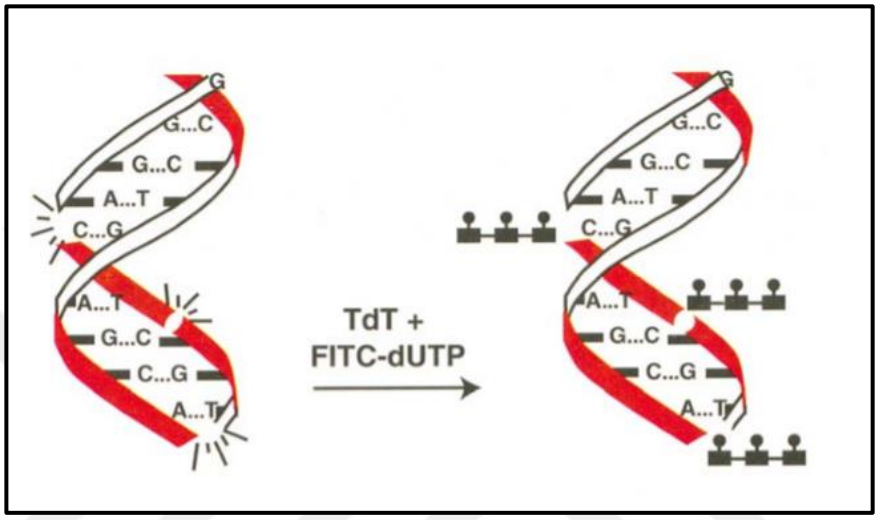


Figure 1.43 Terminal deoxynucleotidyl transferase (TdT) mediated dUTP Nick End Labeling

CHAPTER TWO
MATERIAL AND METHODS

2.1 Material

All chemicals used were of analytical grades or higher where appropriate.

2.2.1 Microorganism

A wild type yeast strain *Saccharomyces cerevisiae* BY4742 (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) was obtained from DSMZ - German Collection of Microorganisms and Cell Cultures. Isogenic yca1Δ (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yca1::kanMX4), aif1Δ (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 aif1::kanMX4), nuc1Δ (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 aif1::kanMX4) and nma1Δ (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 nma1::kanMX4) mutants of wild type BY4742 were obtained from Dharmacon - Yeast ORF library.

2.2 Methods

2.2.1 Microorganisms and Routine Maintenance Conditions

A wild type yeast strain *Saccharomyces cerevisiae* BY4742 (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and its isogenic yca1Δ (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yca1::kanMX4), aif1Δ (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 aif1::kanMX4), nuc1Δ (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 aif1::kanMX4) and nma1Δ mutants (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 nma1::kanMX4) were used. For routine maintenance, all the strains were grown on YPDA agar medium containing 10 g/L yeast extract, 20 g/ L peptone, 20 g/ L glucose and 20 g/ L bactoagar for 5 day at 30 ^{o}C (pH: 5.6).

2.2.2 Growth and Stress Conditions

Yeast extract-peptone-dextrose (YPD) broth was used as the growth medium for wild type and all mutant BY4742 strains. Geneticin (G418) antibiotic at a concentration of 200 mg/L was also added to the growth medium of mutant strains. YPD medium consist of 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose (pH: 5.6). 5 mL spore suspension whose optical density at 660 nm (OD_{660nm}) was 0.1 absorbance was inoculated to 100 mL broth medium in 250 mL erlenmeyer. The incubation was carried out on a rotary shaker at 180 rpm at 30 ^{o}C until mid-exponential phase of *S. cerevisiae.*

The cells were harvested at their mid-exponential phase and resuspended in the same media to obtain the initial absorbance as 0.240 at OD_{660nm}. CLT (50-125 μM) was added to the 20 mL subculture growth medium that containing $3x10^6$ cells per mL in 100-mL erlenmayer flask. Incubation was performed at 30 °C with agitation at 180 rpm. The samples were harvested for 15-90 min.

2.2.3 FUN-1 Viability Assay

FUN-1 staining that gives two colour fluorescent is a viability test for yeast and fungus and highly sensitive when compared with colony forming unit method. Normal cells with healthy metabolism convert the dye that gives green fluorescent into another derivative that gives red fluorescent (Millard, Roth, Thi, Yue, & Haugland, 1997). Dead or metabolically damaged cells cannot metabolize the dye and it will continue to give green fluorescent. Therefore, the red fluorescence/green fluorescence ratio is the indicator of cell viability (Figure 2.1)

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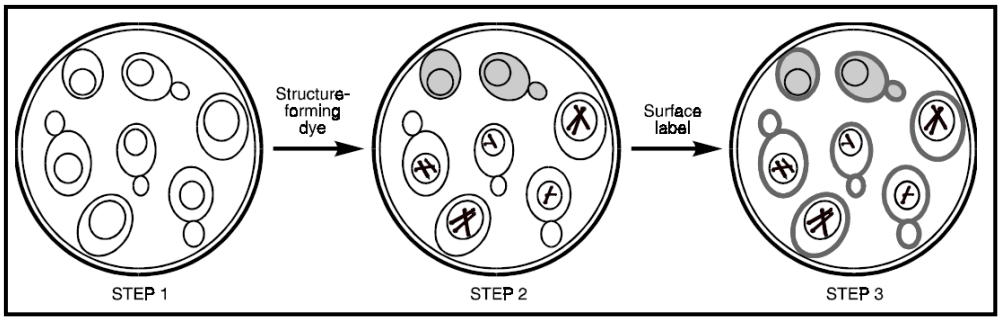

Figure 2.1 Labelling of yeast cells with FUN-1 stain

For viability assay, cells were harvested from the control and CLT-treated samples and centrifuged for 5 min at 10,000 g. The cells whose final count was $1x10^7$ cell.mL^{-1} were suspended in sterile 2% D-(+)-glucose solution (GH solution). 100 µL cell suspension in GH solution was mixed with 100 µl GH solution and 100 µl 4 µM FUN-1 stain and incubated at 30 ^{0}C with gentle shaking. The fluorescences were recorded in every 10 min at excitation wavelength of ~485 nm excitation/~530 nm emission (green) and ~485 nm excitation/~620 nm emission (red) for 150 min. The ratio of red fluorescence to green fluorescence was calculated to determine cell viability for both control and CLT-treated cells.

2.2.4 Annexin V-FITC and Propidium Iodide (PI) Double Staining

In viable and healthy cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. When cells undergo apoptosis, PS is translocated to the outer leaflet of the plasma membrane and exposed to the extracellular environment (Engeland et al., 1998). This is one of the earliest markers of apoptosis. The human vascular anticoagulant, Annexin V, is a 35–36 kDa Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS (Andree et al., 1990). Thus, Annexin V conjugated to green-fluorescent FITC can identify apoptotic cells by binding to PS exposed on the outer leaflet. On the other hand, only the membranes of dead and damaged cells are permeable to propidium iodide (PI) that stains these cells with red fluorescence.

Therefore, cells that are considered viable are both Annexin V and PI negative, while cells that are in early apoptosis are Annexin V positive and PI negative, and cells that are in late apoptosis or already dead are both Annexin V and PI positive.

Phosphatidylserine exposure was detected by Annexin V-FITC Apoptosis Kit from Biovision. For the efficient lysis of cell wall and spheroplast formation, $6x10^7$ cells were harvested, firstly washed with sorbitol buffer (pH: 5.8). Yeast cells were resuspended in buffer-3 and incubated with lyticase at the final concentration of 5 U/mL for 15 min at 28 °C. After efficient spheroplast formation, suspension washed twice with binding buffer and resuspended in binding buffer. 2 µL Annexin V-FITC and 2 µL PI were added to 38 µl cell suspension, incubated for 20 min at room temperature and analyzed under flow cytometry.

2.2.5 4,6-Diamidino-2-phenylindole (DAPI) Staining and Microscopy

4,6-Diamidino-2-phenylindole (DAPI) is a specific fluorescence dye that binds to DNA. This dye can penetrate from both viable and damaged cell membranes. On the other hand, according to the state of the cell membrane, permeability ratio may change. DAPI dye preferentially binds to double stranded DNA (dsDNA) especially to the regions that are rich in terms of Adenine (A) – Thymine (T) bases. The permeability of apoptotic cells to DAPI increases and therefore fluorescence intensity also increases. In apoptotic cells, the condensation of chromatin is easily observed after DAPI staining.

The standard protocol for DAPI nuclei staining was used, as described by Streiblova (1988). The cells treated with 110 µM CLT for 30 min and non-treated ones were collected through the centrifugation at 2000 rpm for 5 min. The cells were resuspended in 70% (vol/vol) ethanol for brief fixation and permeabilization, and then stained with 2 µg/mL DAPI solution with the incubation at 37 ^{0}C and for 15 min. Cell images were recorded at room temperature from a fluorescence microscope (model BX53; Olympus, Tokyo, Japan). A 100× objective lens was used.

2.2.6 Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) Staining

DNA fragmentation as one of the other central feature of apoptotic cells can be determined by TUNEL (Terminal deoxynucleotidyl transferase (TdT) mediated dUTP Nick End Labeling) method that is commonly utilized. In the TUNEL assay (Apo-BrDU) exogenous TdT is used to catalyze a template-independent addition of bromodeoxyuridine triphosphates (Br-dUTP) to the free 3'-hydroxyl ends of double or single stranded DNA fragments. After incorporation, the labeled BrDU can be identified by FITC conjugated anti-Bromodeoxyuridine (BrDU) antibodies and analyzed using a flow cytometer or a fluorescence microscope.

DNA strand breaks were monitored by TUNEL with the In Situ Cell Death Detection kit, Fluorescein (Roche Applied Science, Mannheim, Germany). Yeast cells were fixed with 3.7% (vol/vol) formaldehyde as described by Madeo et al. (1999), and the cell wall was digested with lyticase as described above for Annexin V-PI Staining. The cells were then applied to polylysine-coated slides. The cell slides were rinsed with phosphate-buffered saline (PBS), incubated in permeabilization solution (0.1%, vol/vol, Triton X-100 and 0.1%, wt/vol, sodium citrate) for 2 min on ice, rinsed twice with PBS, and incubated with 10 µl of TUNEL reaction mixture for 60 min at 37 °C. Finally, the slides were rinsed three times with PBS, and a coverslip was mounted. Microscope and image acquisitions were performed as described above for DAPI staining.

2.2.7 Determination of Mitochondrial Membrane Potential

Loss of the mitochondrial inner transmembrane potential is often associated with the early stages of apoptosis and is one of the other significant marker of apoptotic cells. JC-1, JC-9, MitoTracker Red CMXRos and Rhodamine 123 (Rho 123) are some of the dyes that used in terms of the determination of mitochondrial membrane depolarization. Rho 123 is a cationic fluorescent dye that is used to label respiring

mitochondria. There is an enhanced permeation of the dye in dysfunctional mitochondria and this result in increasing the fluorescence intensity (RLU).

The changes in mitochondrial membrane potential were determined with Rho123 staining, a cell-permeable cationic dye (Mathur, Hong, Kemp, Barrientos, & Erusalimsky, 2000). Rho123 solution was added into the cell suspension to obtain 2-μg/ml final concentration, and cells were further incubated at 37 °C for 30 min in the dark and washed twice with PBS. Finally, cells were analysed under microplate reader (Ex/Em = 485 nm/535 nm).

2.2.8 Yeast Caspase Activity

Detection of active metacaspase was performed using the 'CaspACE, FITC-VAD-fmk In Situ Marker' (Promega) and a protocol adapted from Madeo et al. (2002). Briefly, $5x10^6$ yeast cells were harvested, washed once in 1 ml PBS, and resuspended in 200 μl staining solution containing FITC-VAD-fmk. After incubation for 20 min at 30 °C, cells were washed in 1 ml PBS and resuspended in 200 μl PBS. Cells were subsequently incubated with 2 $\mu g.mL^{-1}$ of PI for 10 min at room temperature for double staining with PI. For flow cytometric analysis, stained cells were counted with excitation and emission settings of 488 nm and 525–550 nm.

2.2.9 Determination of ROS and RNS levels

2.2.9.1 Determination of the O_2^- level

O_2^- production was measured by chemiluminescence in the presence of the chemiluminogenic probe bis-N-methylacridinium nitrate (lucigenin) (Skatchkov et al., 1999). Lucigenin is a di-acridinium compound, which emits light on reaction with superoxide. The reaction involves an initial reduction of the lucigenin to a radical and this lucigenin radical can then react with superoxide in an addition reaction, leading to the decomposition of the lucigenin into two acridones, one of which is in an excited state, and decays to produce light. Lucigenin (5 μM) was

added to the plate that contained 250 µl cell suspensions from control and treated samples (OD_{660} = 0.100). The O_2^- level was determined after 1 h incubation at 37 °C in luminescence counter and the results were compared with non-treated controls.

2.2.9.2 Determination of the H_2O_2 Level

The H_2O_2 level was measured using a modification of the method described by Barja (1999). The assay was based on H_2O_2 and horseradish peroxidase (HRP) mediated oxidation of homovanillic acid (HVA) (nonfluorescent) for the production of biphenyl HVA dimer which is fluorescent in nature. Briefly, the cell supernatant was incubated at 30 °C with 1.5 mL phosphate buffer (pH 7.4) containing 0.1 mM EGTA, 3.0 mM $MgCl_2$, 145 mM KCl, 30 mM HEPES, 0.1 mM HVA, 2.5 mM pyruvate/malate and 850 U HRP. After 15 min, the reaction was stopped with 0.5 mL cold stop solution (0.1 M glycine, 25 mM EDTA-NaOH, pH 12.0) and then fluorescence was determined at excitation wavelength of 312 nm and emission wavelength of 420 nm. The H_2O_2 level was calculated using a standard curve of H_2O_2 and expressed in nmol/gww.

2.2.9.3 Determination of Total ROS Level

The total ROS levels were determined according to the method of Kirkland, Saavedra, & Franklin (2007). The cells were loaded with dye by replacing medium with fresh medium containing 10 µM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (C6827, Invitrogen, Grand Island, NY) for 1 h at 28°C. After loading, media were removed and replaced with fresh medium with or without CLT. Fluorescence measurements were made using microplate reader, using a 485/20 excitation, 528/20 emission.

2.2.9.4 Determination of HO•Level

HO• radical level was determined fluorometrically (Ruenroengklin, Yang, Lin, Chen, & Jiang, 2009). The assay was based on oxidative cleavage of 2-deoxyribose and the release of three-carbon aldehyde malondialdehyde (MDA) substrate that reacts on heating with thiobarbituric acid (TBA) to give a pink coloration. For the measurement of HO• radical levels, the cell supernatant was incubated at 37°C for 60 min in the presence of 10 mM deoxyribose. Color development was achieved by adding to the reaction mixture 0.14 mL of a 1% (w/v) aqueous TBA-TCA (pH 7.4) and heating at 100°C for 30 min. The fluorescent intensity of the product was measured at the excitation wavelength of 532 nm and the emission wavelength of 553 nm against the reagent blank solution.

2.2.9.5 Determination of NO• Level

Nitric oxide (NO•) was measured by using the Griess reaction (Giustarini, Rossi, Milzani, & Dalle-Donne, 2008). 100 µL of supernate was applied to a microtiter plate well, followed by 100 µL of Griess reagent (1 g/L sulfanilamide, 25 g/L phosphoric acid, and 0.1 g/L N-1-naphthylethylenediamine). After 30 min of colour development at room temperature, the absorbance was measured on a microplate reader at a wavelength of 540 nm.

2.2.10 Preparation of Crude Extract for Enzyme Activity Assays

The CLT-treated and control cells were harvested by centrifugation at 5000 rpm for 5 min and washed several times with 20 mM potassium phosphate buffer (pH 7.4) at 4 °C. The crude extracts were prepared by breaking the cells with glass beads using a Retsch Bead Mill Homogenizer for 14 min with 5 min time intervals at 4°C and centrifuged at 15000 rpm.

2.2.11 Enzyme Activity Assays

2.2.11.1 NADH Oxidase Activity Assay

The NADH oxidase activity was determined by spectrophotometry. The procedure was based on the disappearance of NADH at 340 nm (Anders, Hogg, & Jago, 1970). Briefly, sample was mixed with 250 µM NADH and the decrease in the A340 was recorded for 5-min in 30 seconds intervals. A millimolar extinction coefficient of 6.22 was used to calculate the NADH disappearance.

2.2.11.2 NADPH Oxidase Activity Assay

The NADPH oxidase activity was determined by spectrophotometry. The procedure was based on the disappearance of NADPH at 340 nm (Anders, Hogg, & Jago, 1970). Briefly, sample was mixed with 250 µM NADPH and the decrease in the A340 was recorded for 5-min in 30 seconds intervals. A millimolar extinction coefficient of 6.22 was used to calculate the volume activity of the enzyme.

2.2.11.3 SOD Activity Assay

A simple assay system for SOD was based on the inhibitory effect of the enzyme on the spontaneous autoxidation of 6-hydroxydopamine (6-OHDA). The autoxidation rate of 160 µM 6-OHDA (prepared in 1 mM KCl, pH 2.0, to avoid oxidation) in 0.1 M phosphate buffer (pH 7.4) was determined by observing the absorbance changes at 490 nm and 25 ^{0}C. One IU is the amount of SOD required to inhibit the initial rate of 6-OHDA autoxidation by 50% (Crosti, Servidei, Bajer, & Serra, 1987).

2.2.11.4 CAT Activity Assay

CAT activity was measured spectrophotometrically at 25 °C by following the absorbance decrease at 240 nm caused by the disappearance of 10.5 mM H_2O_2 (prepared in 50 mm phosphate buffer, pH 7.0) according to the Aebi method (1974). The molar extinction coefficient at 240 nm for H_2O_2 was 39.4 M^{-1} cm^{-1}. One unit of enzyme activity was defined as the amount of enzyme decomposing one µmole of H_2O_2 under standard assay conditions.

2.2.11.5 GR Activity Assay

The GR activity was assayed as reported by Massey and Williams, with slight modifications (Massey & Williams, 1965). The reaction rate at 340 nm was determined by using the NADPH extinction coefficient of 0.00622 μM^{-1}. One unit (U) was defined as the amount of enzyme that oxidised 1 nmol NADPH to NADP+ per minute at 25 ^{0}C.

2.2.11.6 GST Activity Assay

The GST activity was measured as reported by Habig et al., using chlorodinitrobenzene as a substrate (Habig et al., 1974). The reaction rate at 340 nm was determined by using the GSH-CDNB conjugate extinction coefficient of 0.0096 μM^{-1}. One unit (U) was defined as the amount of enzyme that conjugated 10.0 nmol of CDNB with reduced glutathione per minute at 25 ^{0}C.

2.2.11.7 GSHPx Activity Assay

The GSH-Px enzyme activity was measured as reported by Paglia and Valentine, with modifications (Paglia and Valentine 1967). The reaction rate at 340 nm was determined by using the NADPH extinction coefficient of 6.22 mM^{-1}. One unit (U) was defined as the amount of enzyme that oxidised by H_2O_2 of 1.0 µmol of reduced glutathione to oxidized glutathione per minute at pH 7.0 at 25 °C.

2.2.12 GSH and GSSG Levels

GSH and GSSG levels were measured as reported by Teare *et al.* (1993). This method is based on the reduction of GSSG to GSH in the presence of GR and NADPH and the formation of the coloured product, which is formed by the reaction of GSH with DTNB. The formation of the product is followed by measuring absorbance at 412 nm.

2.2.13 Total Protein Assay

Bradford method was used for the measurement of the total protein concentration in the samples (1976). Briefly, 100 µl sample was mixed with 900 µl Bradford reagent prepared by using Coomassie Brilliant Blue G-250 dye and after 2 min incubation, the absorbance in 595 nm was recorded against blank.

2.2.14 Membrane LPO Level Assay

Lipid peroxidation was measured by the formation of malondialdehyde (MDA) using the thiobarbutiric acid (TBA) reaction (Schmedes & Hølmer, 1989). Briefly, 500 µl cell suspension was incubated with 10% trichloroacetic acid (TCA) for 15 min at 90 ^{0}C. After 10 min. centrifugation, 1 mL supernatant was mixed with 1 mL TBA and again incubated for 15 min at 90 ^{0}C. The absorbance of MDA-TBA product in 532 nm was recorded against blank.

2.2.15 Protein Carbonyl Content

Protein carbonyl content was measured fluorometrically with using flourescein thiosemi carbazide. Fluoroscein-5Thiosemicarbazide (FTC), a fluorescent probe which covalently reacts with oxidized residues (i.e Cysteine, Lysine, Arginine, Histidine and Aspartic Acid) on proteins. The protein carbonyl content is determined by the generation of a stable fluorometric signal (Ex/Em 485/535 nm) and compared to the protein concentration determined.

Briefly, the protein concentration was adjusted to 1 $mg.mL^{-1}$ in all samples. 0.2 mM, 50 µL FTC was added to each 50 µL sample and was allowed to react with sample overnight in dark, at room temperature. After incubation, proteins were precipitated by the addition of 200 µL of 20% ice-cold TCA. The mixture was centrifuged for 10 min at 10000 x g at 4 ^{0}C. Pellet was washed 200 µl of 100% ice-cold isopropanol. Samples were centrifuged as above. Pellet was resuspended with 50 µl of 6 M guanidine and fluorescence was measured.

2.2.16 Statistical Analysis

Tukey's test, a multiple comparison test, was used to determine statistical significance. The values are the means of three separate experiments. Also comparison was made using Pearson's correlation.

CHAPTER THREE
RESULTS AND DISCUSSION

In the scope of this thesis, it was aimed to explore the effects of antifungal and potential antitumor drug CLT on eukaryotic model *S. cerevisiae* BY4742 cell growth and viability. The apoptotic character of the induced death was determined by Annexin V – PI assay. The dependency of CLT mediated apoptosis to DNA condensation – fragmentation and mitochondrial membrane potentials were also investigated. In addition by using isogenic knockout mutants, the roles of yca1, aif1, nuc1 and nma111 proteins in CLT induced apoptotic death were highlighted. To understand antioxidant and oxidant status of yeast cells, the variations in intracellular RNS and ROS levels together with NADH and NADPH oxidases besides antioxidant enzyme activities and MDA and protein carbonyl levels as the markers of the resulting damage were investigated in *S. cerevisiae* cells treated with CLT in the concentration range of 50-125 μM for between 15-90 min.

3.1 The effect of clotrimazole on growth and viability of wild type *S. cerevisiae* BY4742 cells

The growth and viability of wild type *S. cerevisiae* BY4742 cells treated with 10 μM CLT for different incubation times were determined and compared with their controls.

The growth inhibition effect of 10 μM CLT was investigated for 0 – 9 h by measuring optical density at 660 nm (OD_{660nm}). As can be seen from Figure 3.1, this concentration inhibited *S. cerevisiae* cell growth in a time dependent manner.

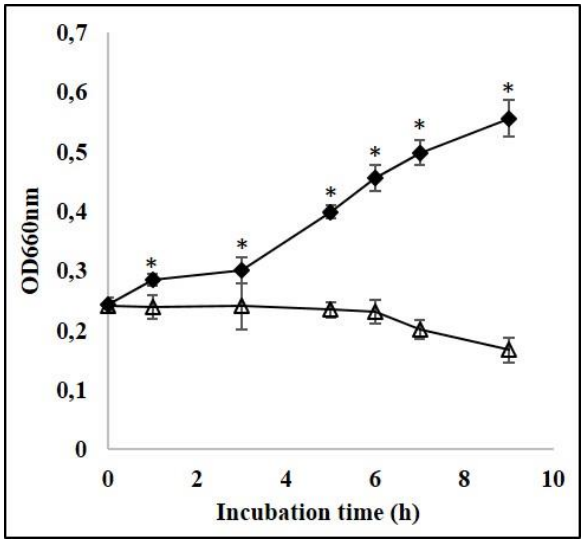

Figure 3.1 The growth inhibition of *S. cerevisiae* BY4742 cells treated with 10 µM clotrimazole (-Δ-) for 0 – 9 h compared to control (---■---)

The viability of wild type *S. cerevisiae* BY4742 cells treated with 10 µM CLT in comparison to control group was assessed by fluorometric FUN-1 stain that gives rise to red fluorescent cylindrical intravacuolar structures (CIVS) in metabolically active yeast while extremely bright, diffuse, green-yellow fluorescence in dead cells (Millard, Roth, Thi, Yue, & Haugland, 1997). As can be seen from Figure 3.2, 10 µM CLT decreased the cell viability of yeast by 24.82±0.81, 56.00±1.54 and 77.59±0.53% after 3, 6 and 9 h treatment, respectively.

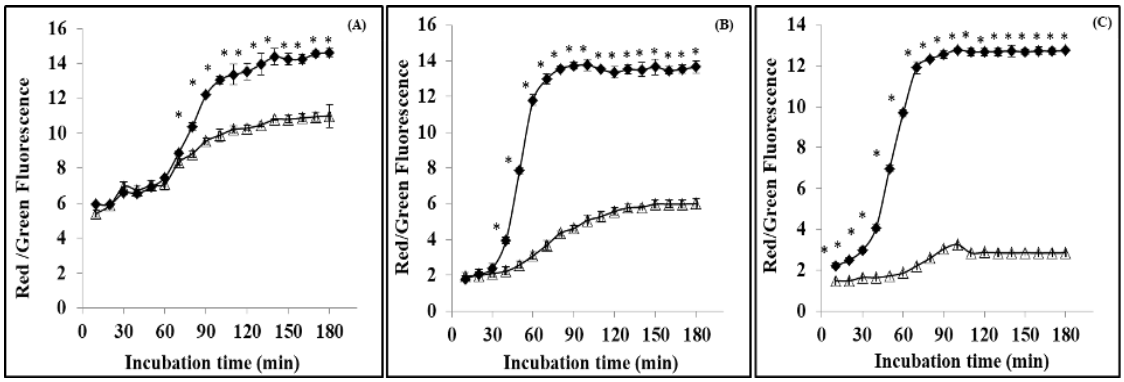

Figure 3.2 FUN-1 staining for the evaluation of *S. cerevisiae* BY4742 cell viability after treated with 10 µM clotrimazole (---Δ---) for 3 (A), 6 (B) and 9 h (C) compared to controls (---■---). The cells incubated with FUN-1 stain for 180 min

It has been shown that exposure to CLT triggers a significant reduction in various viable pathogenic fungal and non-pathogenic bacterial cell percentages. The growth

of Candida albicans, that is common member of the human gut flora, treated with 100 µM CLT for 24 h almost completely was blocked (Wächtler, Wilson, Haedicke, Dalle, & Hube, 2011). Minimal inhibitory concentrations (MICs) of CLT was in the range of 1 – 8 $\mu g.mL^{-1}$ for zoophilic yeast *Malassezia pachydermatis* isolates (Peano, Beccati, Chiavassa, & Pasquetti, 2012). *Trichophyton rubrum* that is a dermatophytic fungus was found very susceptible to CLT, which the MIC value was 0.03 $\mu g.mL^{-1}$ for this fungus (Fernández-Torres, Vázquez-Veiga, Llovo, Pereiro Jr, & Guarro, 2000). In another research, the MIC value of CLT against *Mycobacterium smegmatis*, a saprophytic, gram-positive bacterium, was determined less than 2 $\mu g.mL^{-1}$. In the same study, it was also showed that CLT was much more effective than fluconazole, imidazole and amphotericin B (>256 $\mu g.mL^{-1}$), tebuconazole (32 $\mu g.mL^{-1}$) and ketoconazole (16 $\mu g.mL^{-1}$) (Jackson, Lamb, Kelly, & Kelly, 2000). On the other hand, *Aspergillus niger*, *Penicillium chrysogenum*, and *Trichoderma viride* are the fungal species that CLT failed to substantially inhibit their growths at even 10 mg/mL (Clausen & Yang, 2005).

As stated previously, CLT is an azole antifungal drug that inhibits 14α-demethylase and its growth inhibition effect is assumed to be depletion of ergosterol and other sterols required for cell membrane production. In the study conducted by Abe et al., it was shown for an another azole antifungal fluconazole that deletion of ERG3 caused to 116-fold increase in IC_{50} value in comparison to its wild-type strain after 48 h treatment of the exponentially growing *S. cerevisiae* BY4742 cells (Abe, Usui, & Hiraki, 2009). Supportively, in the studies conducted with mammalian cells, cytotoxic effect of CLT was found to be time dependent and associated with the loss of cell membrane integrity (Meira et al., 2005).

3.2 Apoptotic cell death induction in wild type *S. cerevisiae* BY4742 cells depend on clotrimazole concentrations and incubation times

To explore the dominant cell death type induced by CLT, wild type *S. cerevisiae* BY4742 cells that were exposed to various concentration (0 - 120 μM) of the antifungal for different incubation times (0 – 240 min) were investigated after staining with FITC-coupled annexin V and the membrane impermeant fluorochrome PI.

In yeasts, as in mammalian cells, phosphatidylserine has an asymmetric distribution in the lipid bilayer of the cytoplasmic membrane (Cerbón & Calderón, 1991). The exposure of phosphatidylserine at the outer surface of the cytoplasmic membrane occurs at the early stages of apoptosis (Martin & Green, 1995) while membrane integrity is still retained. Therefore, with this technique, it is possible to distinguish viable, early, late and necrotic cells.

CLT induced cell death types in wild type *S. cerevisiae* were examined both for low and relatively higher concentrations of the drug (Figure 3.3 – 3.6). As can be seen clearly from the Figures, when cells exposed to low concentrations of CLT (3-10 μM) for 4 h, late apoptotic cell percentages were slightly increased while necrotic cells percentages were approximately constant with the increase in drug concentration. Similar trend was also observed for the cells treated with the same concentrations of CLT for 2 h. The changes in the percentages of cell death types were investigated for higher concentration of CLT by much more shortening the treatment period (5 – 45 μM for 90 min and 100 – 120 μM for 30 min). The best result was found for 110 μM CLT treatment which caused to cell death by 35.5±2.48% apoptotic and only 13.1±0.08% necrotic pathway within 30 min. On the other hand, higher concentrations of CLT induced predominantly necrotic cell death in *S. cerevisiae* cells.

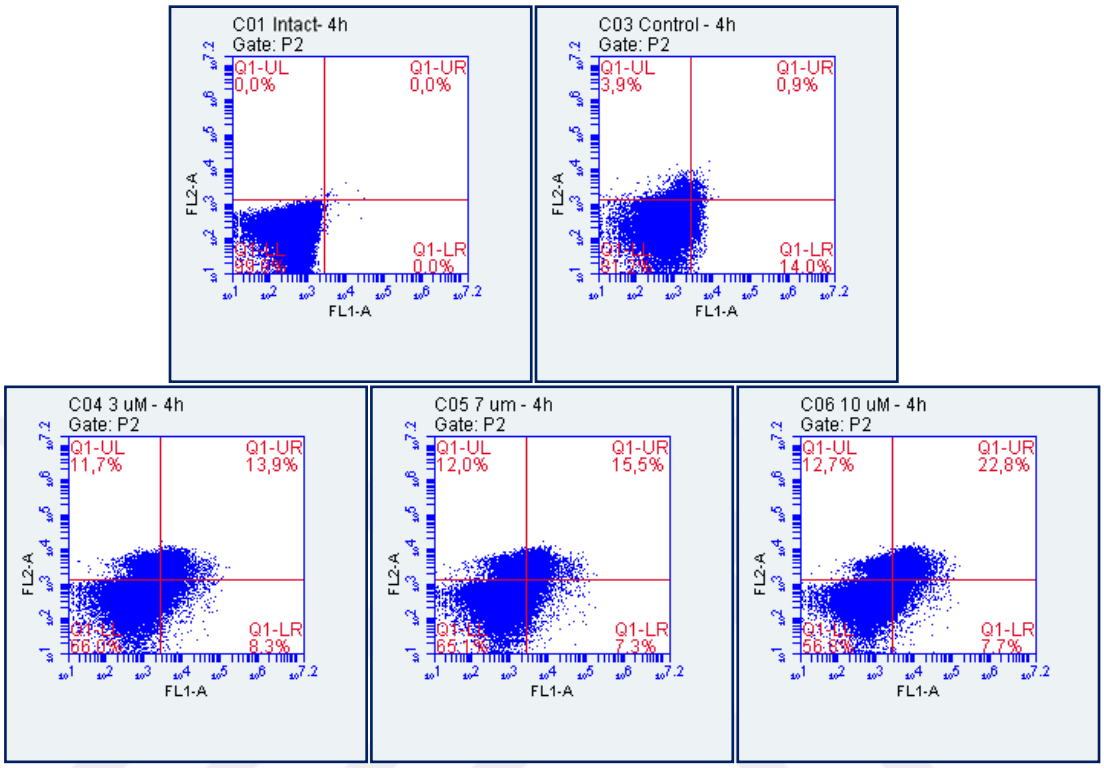

Figure 3.3 The percentages of live, early apoptotic, late apoptotic and necrotic *S. cerevisiae* BY4742 cells after treated with 3 - 10 µM CLT for 4 h

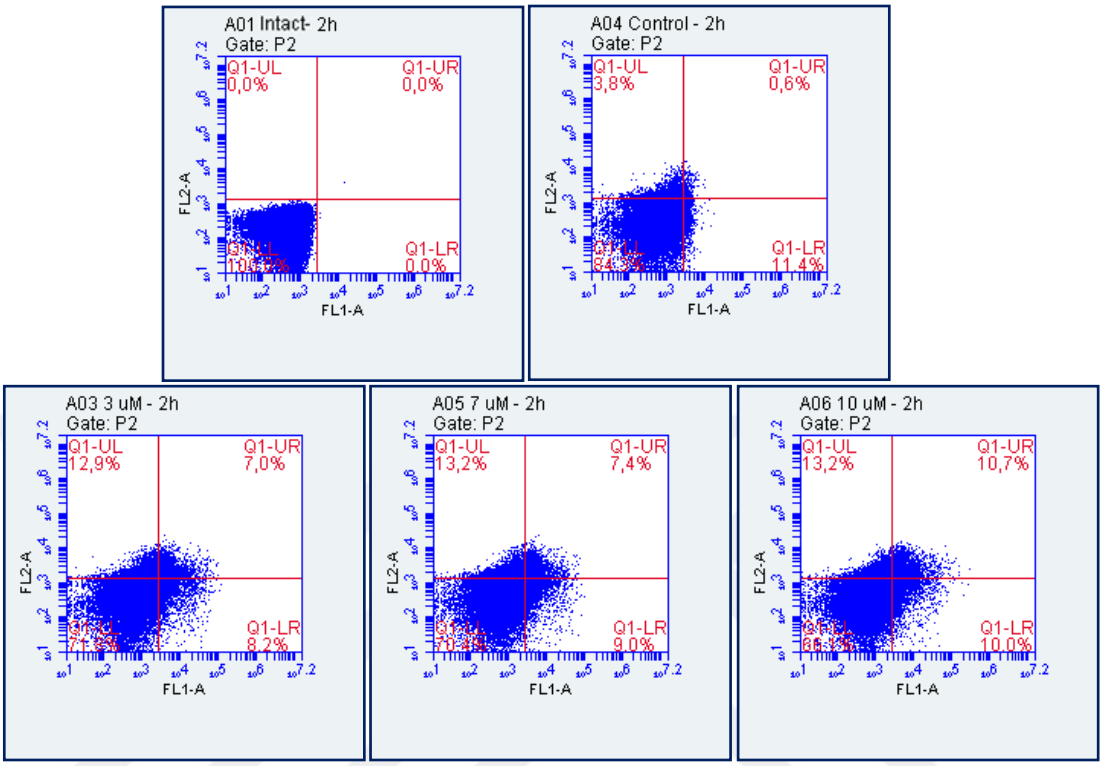

Figure 3.4 The percentages of live, early apoptotic, late apoptotic and necrotic *S. cerevisiae* BY4742 cells after treated with 3 - 10 µM CLT for 2 h

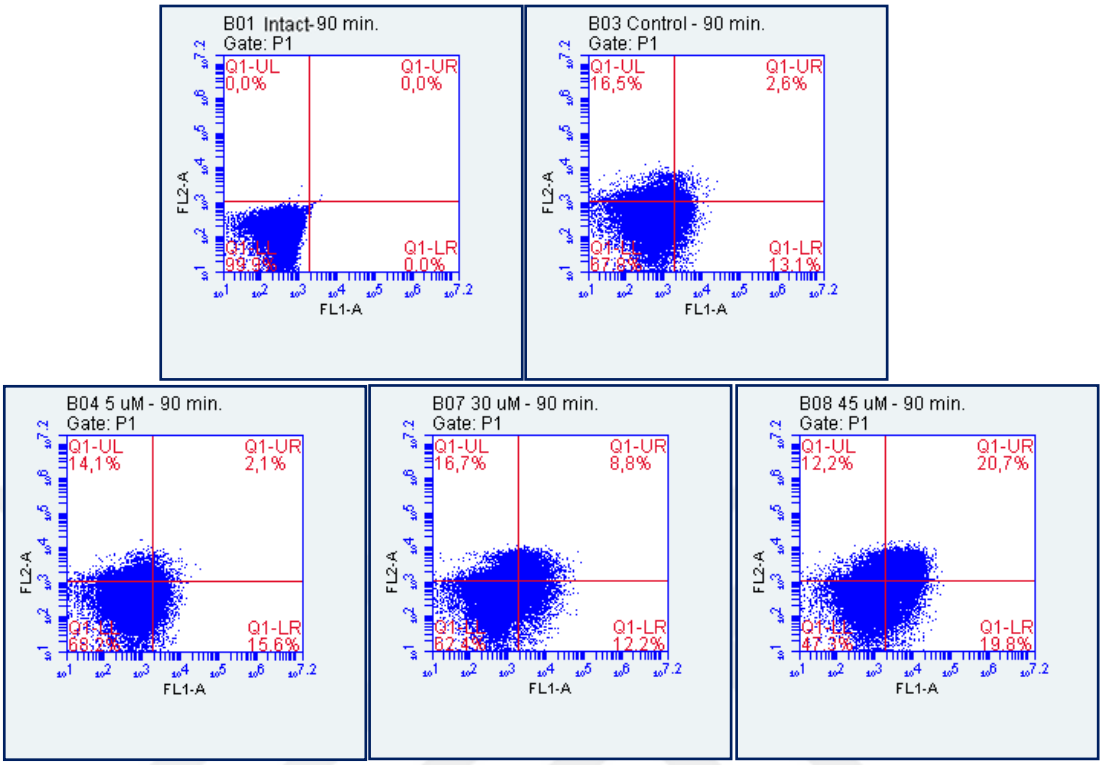

Figure 3.5 The percentages of live, early apoptotic, late apoptotic and necrotic *S. cerevisiae* BY4742 cells after treated with 5 - 45 µM CLT for 90 min

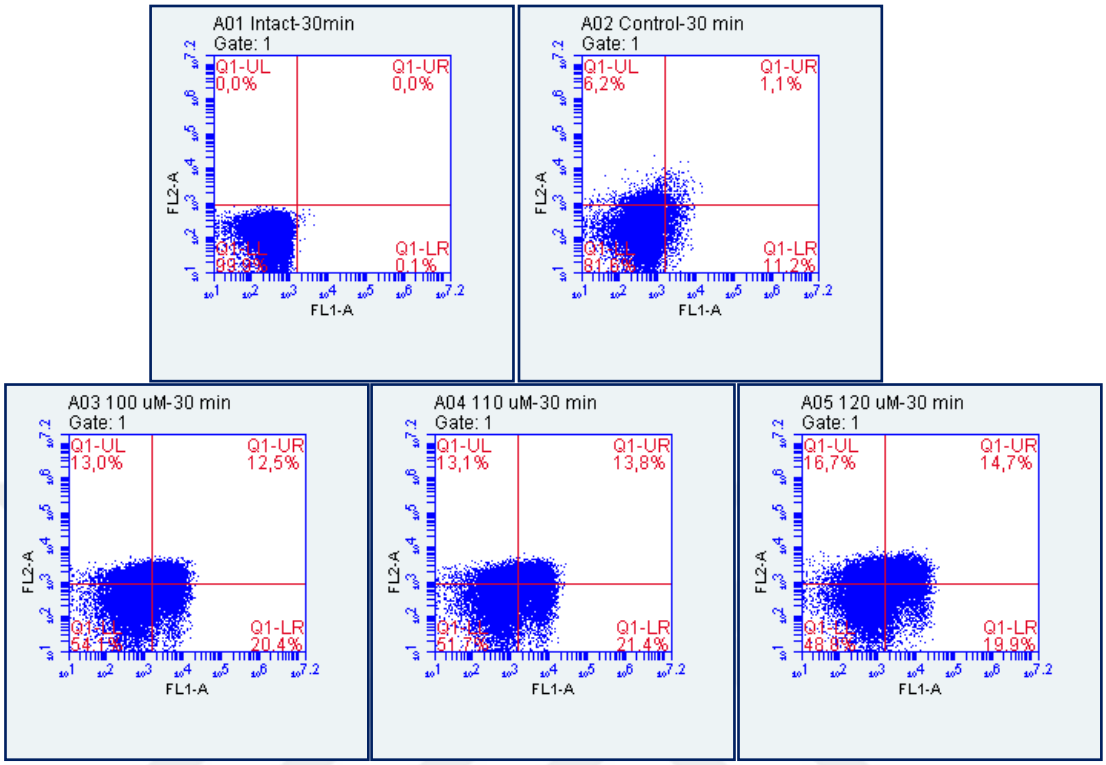

Figure 3.6 The percentages of live, early apoptotic, late apoptotic and necrotic *S. cerevisiae* BY4742 cells after treated with 100 - 120 µM CLT for 30 min

Besides early apoptotic event phosphatidylserine externalization, DNA condensation and fragmentation are the significant features of late apoptotic cell death and can be visualized by DAPI and TUNEL stainings, respectively. It was determined that wild type *S. cerevisiae* cells were DAPI – and TUNEL – positive for the condition of which 35.5±2.48% apoptosis induction was observed (Figure 3.7).

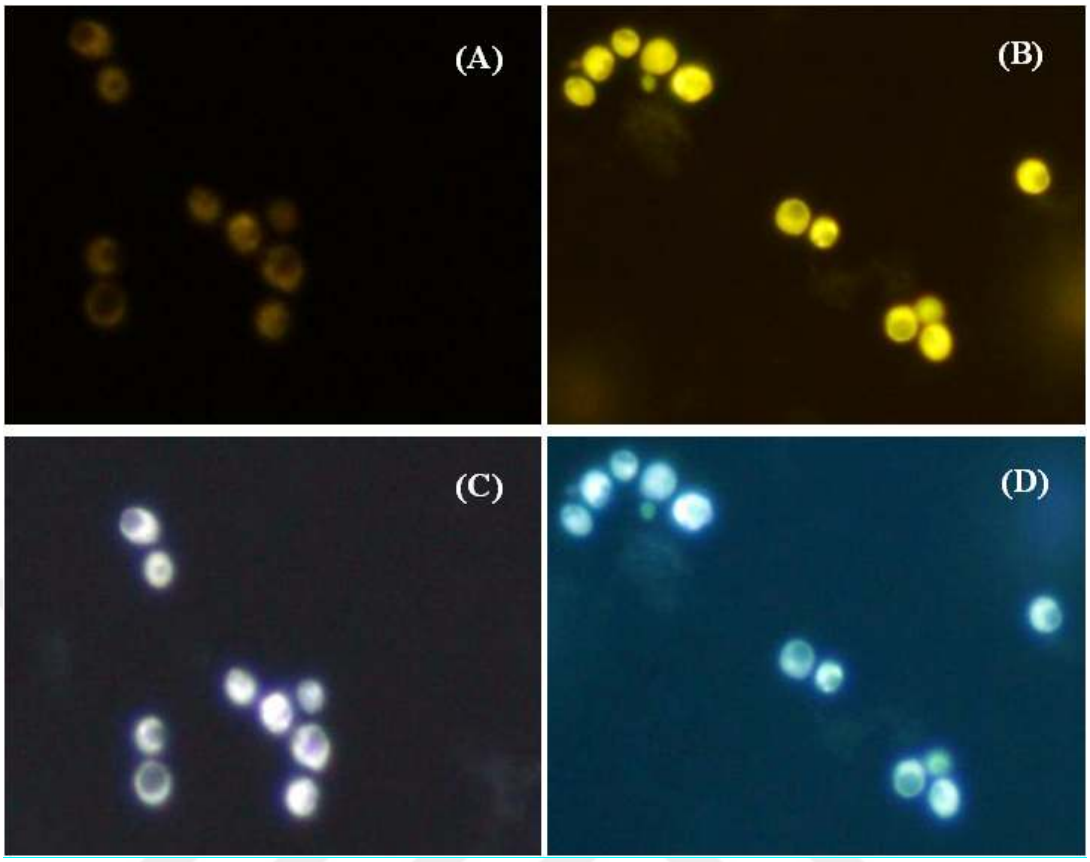

Figure 3.7 Clotrimazole-induced *S. cerevisiae* BY4742 cell death exhibits features of apoptosis. TUNEL staining of untreated control (A) and 110 µM clotrimazole treated cells for 30 min (B). DAPI staining of untreated control (C) and 110 µM clotrimazole treated cells for 30 min (D)

Cysteine-rich antifungal protein PAF (*Penicillium chrysogenum* antifungal protein), antifungal peptide HsAFP1, osmotin, melittin, psacotheacin, dermaseptin S3, lactoferrin, pradimicin A, amphotericin B and papiliocin are the compounds which show antifungal activity and induce apoptotic cell death in fungus and yeast. PAF which is effective against *Aspergillus nidulans*, *Aspergillus fumigatus*, *Aspergillus niger* and *Botrytis cinerea* is also able to induce apoptosis in these fungal strains (Leiter et al., 2005; Marx, Binder, Leiter, & Pocsi, 2008). The antifungal peptide HsAFP1 exerts strong antimicrobial activity against several fungi, including *C. albicans, C. krusei, Aspergillus flavus, B. cinerea and Fusarium culmorum* (Osborn et al., 1995). In the study conducted by Aerts et al., it was shown that exposure of *Candida albicans* to HsAFP1 induces apoptosis. Osmotin is a member of the pathogen related-5 family and has in vitro antifungal activity against a broad range of fungi, including *Fusarium oxysporum, Phytophtora infestans, Neurospora*

crassa and C. albicans (Narasimhan et al., 2003). This antifungal protein is known to cause permeabilization of the membrane and dissipation of the membrane potential. On the other hand, *S. cerevisiae* has resistance against osmotin that is mediated by Pirs (proteins with internal repeats), which are cell-wall-located stress proteins induced by heat and nitrogen limitation (Yun et al., 1997) and by Ssd1, a protein involved in cell wall biogenesis and composition, including sorting of Pirs (Ibeas et al., 2001). Pradimicin A and amphotericin B are the drugs which used in antifungal medications. These drugs induce apoptosis in *S. cerevisiae* and *C. albicans*, respectively by showing phosphatidylserine externalization, DNA condensation and fragmentation (Phillips, Sudbery, & Ramsdale, 2003).

There is no research dealing with the CLT induced death type in fungi. On the other hand, CLT is also known for its cytotoxic effect with apoptosis induction on various cancer cell lines as human breast carcinoma (Furtado, Marcondes, Sola-Penna, De Souza, & Zancan, 2015; Coelho et al., 2011), human glioblastoma cells (Liu & Raisch, 2010), human endometrial cancer (Mhawech & Fauceglia et al., 2011), human and B16-F10 mouse melanoma (Adinolfi et al., 2015; Penso & Beitner, 1998), lung carcinoma and colon adenocarcinoma (Penso & Beitner, 2002), mammary duct carcinoma (Meira et al., 2005), oral squmous cell carcinoma (Wang et al., 2014) and HA59T human hepatoma cells (Horng et al., 2013). Ito et al., showed apoptotic effect of CLT on acute lymphoblastic leukemia cells and stated that annexin V reactivity and cell membrane permeabilization were detectable after 24 h treatment with 10 μM CLT in contrast to our findings (Ito et al., 2002). Similarly, in the study conducted with glioblastoma cell line, a slight increase in apoptotic cells was observed after exposure to 40 $\mu mol.L^{-1}$ CLT for 24 h (Liu & Raisch, 2010). Horng et al. showed that 10 – 30 μM CLT significantly induced apoptosis in a concentration-dependent fashion in HA59T human hepatoma cells according to the flow cytometric Annexin V/PI staining. Human breast cancer cell lines MCF-7 and MDA-MB-231, showed DNA condensation upon CLT treatment (Sharma, Kumar, Ram, & Luthra-Guptasarma, 2017). In addition, it was previously stated that CLT showed its preferential effect on tumorigenic breast cell lines rather than normal breast cells most probably due to the more readily accumulation of CLT

in an aggressiveness-dependent pattern (Furtado, Marcondes, Sola-Penna, De Souza, & Zancan, 2012; Coelho et al., 2011). From this point of view, the apoptotic effects of CLT within short incubation times may be interpreted by the fact that the more sensitive exponentially growing *S. cerevisiae* BY4742 cells accumulate the agent more rapidly. In other words, it will not be wrong to say that the condition of the cell or tissue is one of the significant parameter that manipulates the effect of the CLT.

3.3 The variations in mitochondrial membrane potentials (Δψm) of wild type *S. cerevisiae* BY4742 cells depend on clotrimazole concentrations and incubation times

In addition to being the source of energy supporting life under aerobic conditions, mitochondria can also be the source of signals that initiate apoptosis in cells which exposed to receptor-independent stimuli (Gottlieb, 2000). It was demonstrated that these triggers invariably result in loss of the mitochondrial transmembrane potential (ΔΨm) and the release of pro-apoptotic proteins from mitochondria. In this thesis, ΔΨm changes in CLT (0 - 110 μM for 30 and 60 min) treated yeast cells were investigated by using the fluorescent probe Rho123. There is an enhanced permeation of the dye in dysfunctional mitochondria and this results in increasing the fluorescence intensity (RLU).

As can be seen from Figure 3.8, the RLU values increased 2.68±0.1 and 3.76±0.07-folds in 110 μM CLT treated cells compared to their controls within 30 and 60 min, respectively. According to the results, it can be certainly said that CLT caused to decrease in ΔΨm depend on drug concentration and incubation time, suggesting functional alterations in mitochondria. Supportingly, it was also observed that ROS levels measured with CMH2DCFDA induced 2.35±0.03 and 2.45±0.05-folds at 110 μM CLT compared to controls within 30 and 60 min, respectively. The increase in ROS levels can be seen as the indirect evidences of mitochondrial involvement to the CLT induced yeast apoptosis as in the case of amino acid starvation (Eisler, Fröhlich, & Heidenreich, 2004), valproic acid (Mitsui, Nakagawa, Nakamura, Okamoto, & Tsurugi, 2005), arsenic (Du et al., 2007), edelfosine (Zhang,

Gajate, & Mollınede, 2007) and jasplakinolide (Gourlay, Carpp, Timpson, Winder, & Ayscough, 2004). On the other hand, it must be stated that ROS production can occur in different cell locations under a death stimulus although mitochondria are the site of major ROS production in normal cells.

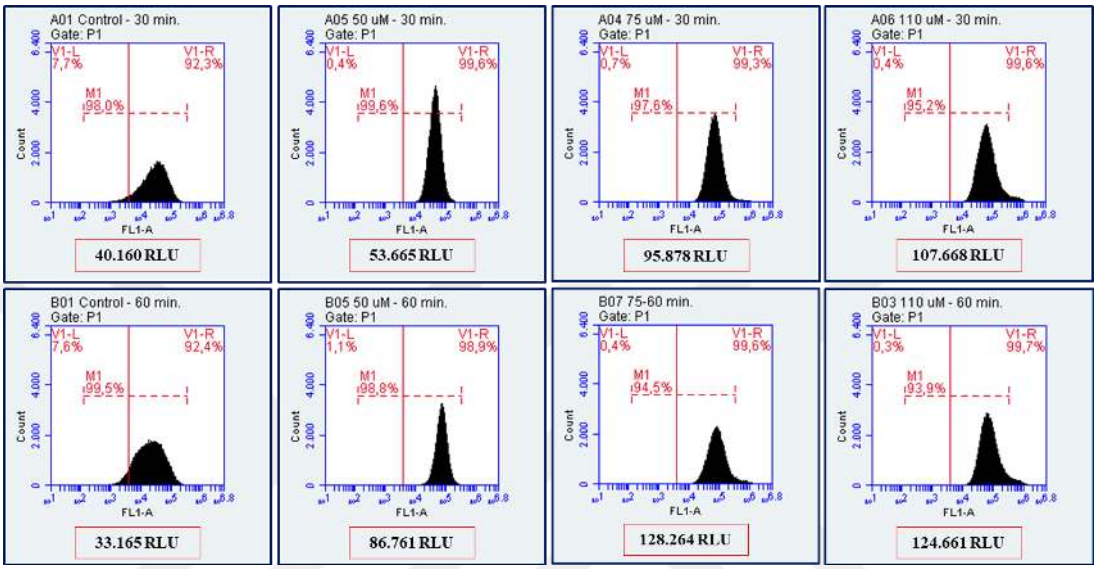

Figure 3.8 Measurement of mitochondrial membrane potentials using fluorescent rhodamine 123 of *S. cerevisiae* BY4742 cells after treated with 50, 75 and 110 µM clotrimazole for 30 and 60 min compared to untreated controls

3.4 The dependency of clotrimazole induced apoptotic cell death in wild type *S. cerevisiae* BY4742 cells on Yca1p activity

The caspases are members of a family, or structurally related group, known as cysteine proteases (Mazzoni & Falcone, 2008). The apoptotic events observed in *S. cerevisiae*, due to external stimuli as well as internal signals, can be dependent on the activity of the yeast metacaspase Yca1 which is a homologous of mammalian caspases (Du et al., 2007). It has been shown up to now that disruption of YCA1 reduces cell death and formation of an apoptotic phenotype in response to oxygen stress, aging (Herker et al., 2004) and arsenic treatment (Du et al., 2007). According to the study conducted by Amrita et al., cadmium exposure also led to YCA1 induction within 30 min in *S. cerevisiae* (Nargund, Avery, & Houghton, 2008).

To investigate whether Yca1p is involved in CLT-induced cell death, yeast caspase activities of untreated and 110 µM CLT treated wild type BY4742 cells for 30 min were measured by flow cytometrically. Cell populations were analyzed with FL1-A and FL2-A channels and the gating was done according to the untreated control group. As can be seen from Figure 3.9, yeast caspase activity was significantly induced up to 12.34±0.75-fold after the treatment with CLT compared to the untreated control.

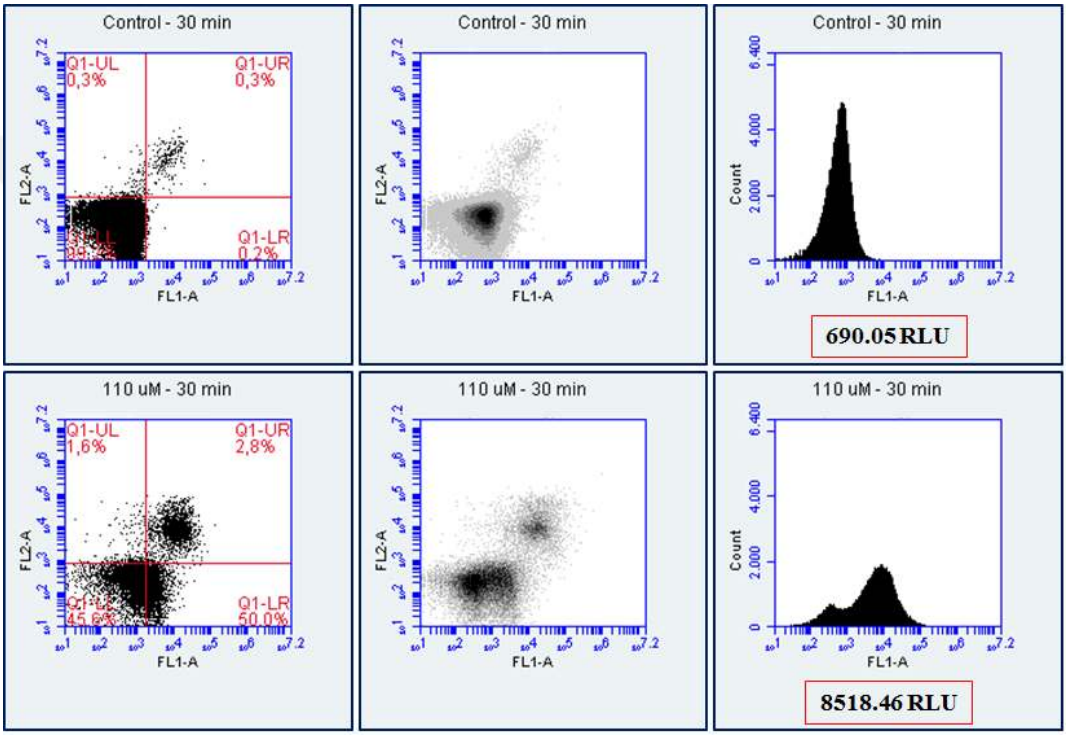

Figure 3.9 Flow cytometric detection of active metacaspase in *S. cerevisiae* BY4742 cells after treated with 110 µM clotrimazole for 30 min compared to untreated control

In addition, the YCA1-disrupted strain (Δyca1) that was also exposed to 110 µM CLT for 30 min were tested with Annexin V-PI and the results were compared with its isogenic wild type BY4742. In parallel with yeast caspase activity result, the Δyca1 deletion mutant displayed a considerable better survival compared with wild type. The YCA1-disrupted strain showed a 82.7% survival rate, much higher than that of the wild type (51.4%), after CLT treatment (Figure 3.10).

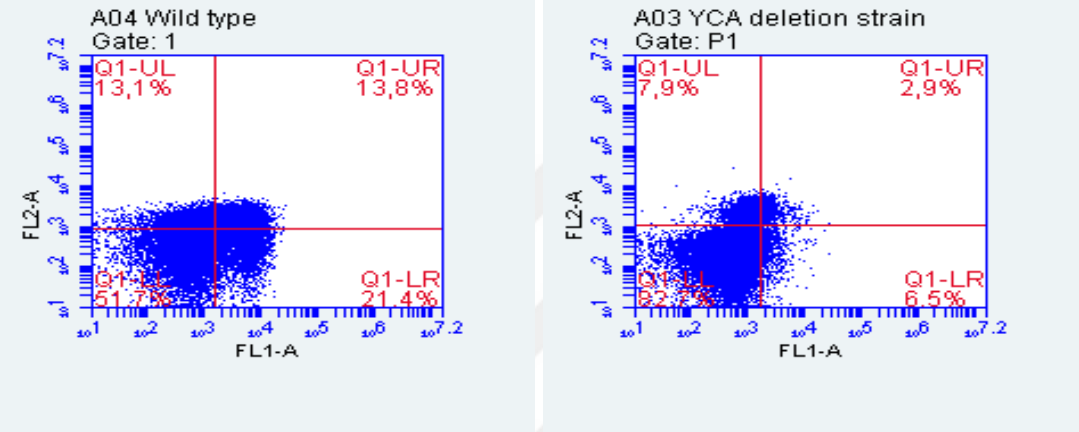

Figure 3.10 The live, early apoptotic, late apoptotic and necrotic cell percentages of YCA1-disrupted strain according to the Annexin V-FITC and PI double staining after treated with 110 µM CLT for 30 min compared to its isogenic wild type

In the study conducted by Iannelli et al., on the contrary, it was shown that CLT showed pro-survival and anti-apoptotic effects in rats with hepatic ischemia reperfusion injury (Iannelli et al., 2011). Ischemia-reperfusion injury represents a pathological condition that is characterized by tissue hypoxia, increased oxidative stress, inflammatory responses and cell death. According to the results of this study, as a result of pregnane X receptor (PXR) expression, an important component of the body's adaptive defense mechanism against toxic substances, in CLT treated rat liver, while expression of the activated form of caspase-3 decreased, the expression of the anti-apoptotic Bcl-xL protein increased significantly compared with controls. It may be said that PXR expression in response to CLT treatment has become more susceptible in hepatic tissues under this pathological condition. In another study conducted with liver cell line, it was shown that PXR overexpression in HepG2 cells

led to Bcl-2 induction upon CLT treatment and protects cells against Fas-induced apoptosis (Zucchini et al., 2005).

According to the other literatures, adiponectin, a protein involved in regulating glucose levels, increased caspase-3 activity approximately 6-fold over control in primary bovine capillary endothelial (BCE) cells (Bråkenhielm et al. 2004). Another outstanding result as 50-fold increase in caspase 3 activity was found for platinum-containing anti-cancer drug cisplatin treated renal cells (Cummings & Schnellmann, 2002).

3.5 The dependency of clotrimazole induced apoptotic cell death in wild type *S. cerevisiae* BY4742 cells on Aifp, Nuc1p and Nma111p proteins

Besides yca1p activity, it was also investigated whether CLT induced apoptotic cell death in *S. cerevisiae* BY4742 cells is dependent on Aifp, Nuc1p and Nma111p proteins. According to the literatures, it is generally thought that all these proteins induce apoptotic cell death in an independent manner from caspases, in the case of yeast, from metacaspase. In order to highlight, the contributions of these proteins to CLT-induced apoptosis, AIF1 (Δaif1), NUC1 (Δnuc1) and NMA111 (Δnma111) distrupted strains were used. These knockout mutants were also exposed to 110 μM CLT for 30 min, apoptotic cell percentages were determined with Annexin V-PI and the results were compared their isogenic wild type BY4742.

As can be seen from Figure 3.11., while the percentage of living cells was 51.7% in wild type yeast, this value was increased to 63.6, 59.9 and 62.4% in Δaif1, Δnuc1 and Δnma111 mutant strains, respectively. According to the results, necrotic cell percentages increased especially in Δaif1 and Δnma111 mutants, up to approximately 2-folds of wild type. AIF is known as a NADH oxidase with a local redox function that is essential for optimal oxidative phosphorylation and for an efficient antioxidant defense in mammalian cells (Modjtahedi, Giordanetto, Madeo, & Kroemer, 2006). But it has been stated for yeast cells that AIF synergizes with oxidative stress to induce cell killing (Wissing et al., 2004). Therefore, it is not clear whether it has

protective function or not against necrotic cell death. In the case of Δnuc1 mutant, slight decreases in early apoptotic and necrotic cell death percentages were observed. According to these results, it can be said that these three proteins are not as effective as yca1p in CLT-induced apoptosis in yeast.

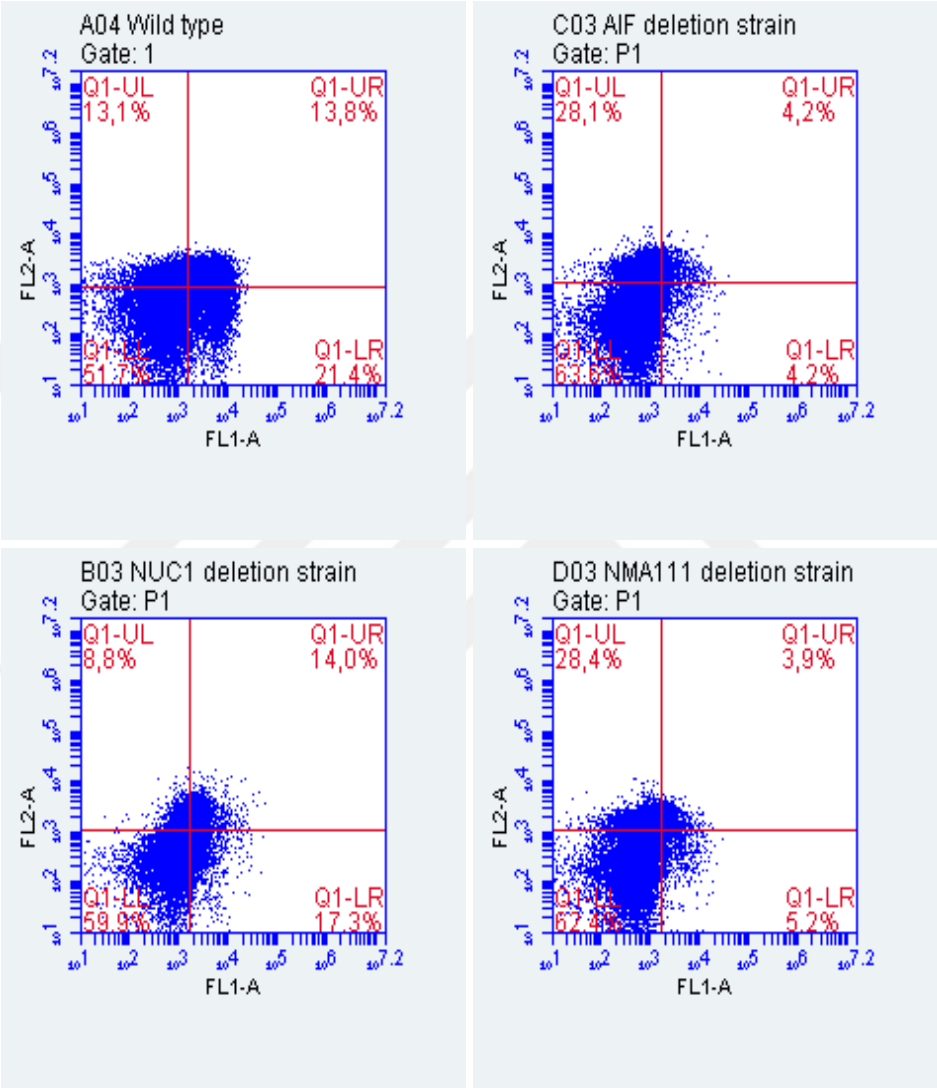

Figure 3.11 The live, early apoptotic, late apoptotic and necrotic cell percentages of AIF1, NUC1 and NMA111-disrupted strains according to the Annexin V-FITC and PI double staining after treated with 110 μM CLT for 30 min compared to their isogenic wild type

There are certain stimulus that induced apoptotic mechanism in yeast depends on Aif1p, Nma111p and Nuc1p. It was shown that while H_2O_2 induced yeast death is sensitized by the overexpression of Aif1p, disruption of AIF1 largely inhibits death killing. Besides H_2O_2, AIF1 deletion mutant exhibits improved survival following

acetate treatment and during aging (Wissing et al., 2004). Amphibian-derived peptides from the dermaseptin family can induce Aif1p-dependent but Yca1p-independent yeast apoptosis (Morton, Dos Santos, & Coote, 2007). While NMA111 deletion reduces apoptotic markers, its overexpression promotes cell death upon hyperthermia or H_2O_2 treatment (Fahrenkrog, Sauder, & Aebi, 2004). Toxicity of intracellular trigger of Parkinson's disease, α-synuclein depends on chronological aging, functional mitochondria and the presence of Nuc1p (Buttner et al., 2013).

3.6 Variation in Intracellular RNS and ROS levels in CLT treated *S. cerevisiae*

3.6.1 NO• levels

NO• is a free radical endogenously produced in biological tissues that exerts a large number of critical regulatory functions and also plays an important role in the pathogenesis of cellular injury. NO• reacts very fast with $O_2•^-$ to form peroxynitrite (Squadrito & Pryor, 1998). In this regard, NO• functions as a potent antioxidant (Chiueh, 1999) with the capability to scavenge $O_2•^-$ produced in the cytosol or mitochondria. According to the obtained results, NO• levels were generally over the control values in *S. cerevisae* exposed to CLT (Figure 3.12). The initial NO• levels were about 1.35-fold of control except in cells treated with 125 μM CLT and then they showed decreasing tendencies depending on time for all investigated concentrations. The levels dropped under control values at 125 μM from 60^{th} min ($p>0.05$). In the earlier studies of Jiang et al., it was stated that hydroxyurea-derived NO• may play a subsidiary role in the anticancer activity of this antineoplastic (Jiang et al., 1997). According to the Ferreira et al., RNS level in *Cryptococcus gatti* was induced 1.52-fold compared to its control within 1 h at even 0.27 μM of the other antifungal drug amphotericin B (Ferreira et al., 2013).

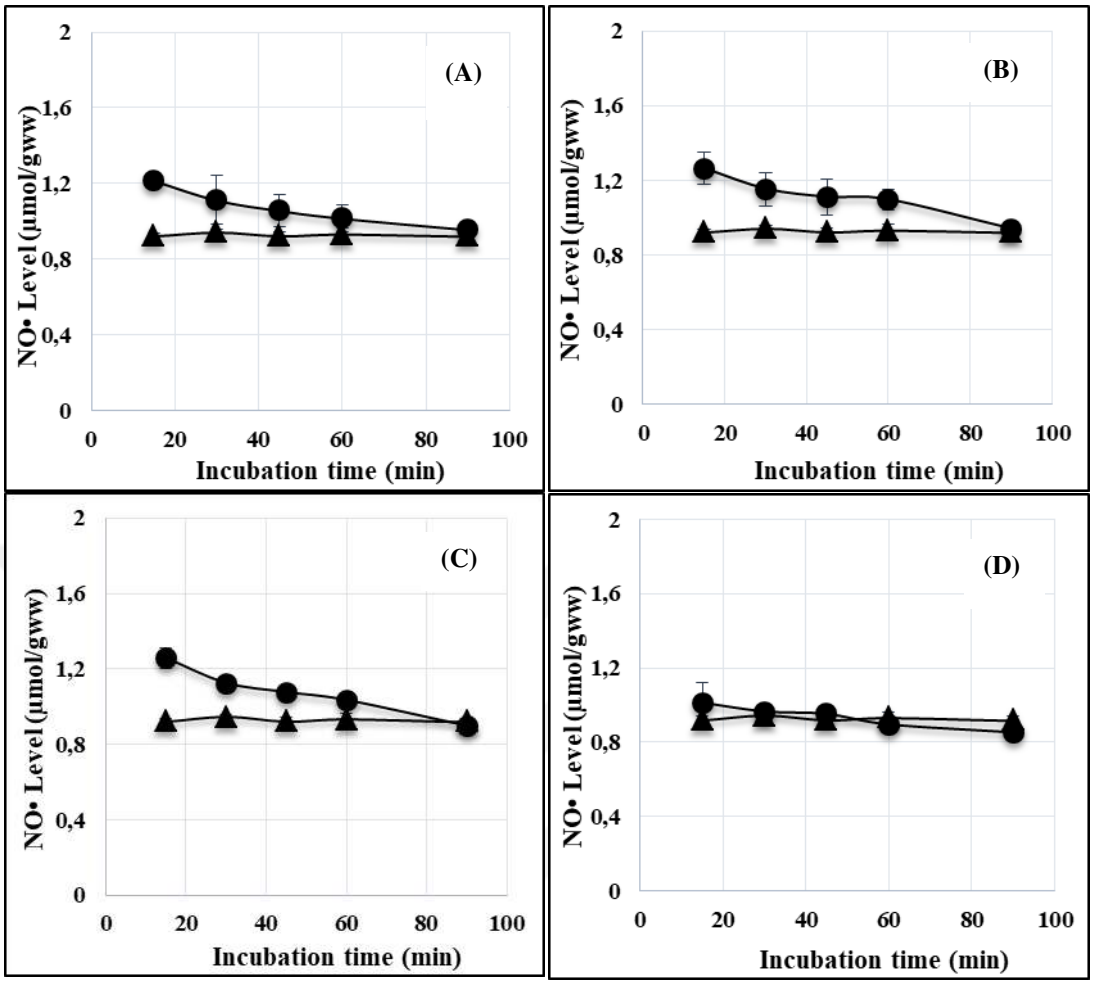

Figure 3.12 Variation in NO• levels depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

3.6.2 $O_2^{\bullet-}$ level

$O_2^{\bullet-}$, that is mainly produced by the conversion of enediol radical anion, undergoes dismutation to H_2O_2 or interacts with iron ions in iron-sulphur proteins and NO• to form reactive peroxynitrite (ONOO) (Bozinovski et al., 2016). According to the obtained findings, $O_2^{\bullet-}$ levels in *S. cerevisiae* exposed to CLT were increased with the increasing concentrations of the antifungal and showed decreasing tendency from the beginning of the incubation but never decreased under control values (Figure 3.13). The highest $O_2^{\bullet-}$ level was observed as 29.65 RLU that was 1.95-fold of its control at 125 µM for 15 min treatment. According to the literature view, there is no study dealing with the effect of CLT treatment on the production of $O_2^{\bullet-}$. Camera et al., determined *in vitro* $O_2^{\bullet-}$ scavenging activity of another ergosterol inhibitors terbinafine at its therapeutic concentrations (Camera, Cannistraci, Briganti, Colombo, & Picardo, 1999). The mycotoxins patulin and citrinin did not induce $O_2^{\bullet-}$ levels in *Schizosaccharomyces pombe* when they administered together (Papp, Máté, Mike, Gazdag, & Pesti, 2016). On the other hand, in the study conducted by Abrashev et al. (2011), it was observed that $O_2^{\bullet-}$ level increased steadily in paraquat concentration range of 0.1 - 3 mM and reached to 3.5-fold of its control within 1 h (Abrashev et al., 2011). The environmental contaminant methoxychlor also significantly increased $O_2^{\bullet-}$ level in rat testis in a dose-dependent manner and after 200 mg/kg methoxychlor treatment for 7 day, the level increased about 1.77-folds of its control (Latchoumycandane, Chitra, & Mathur, 2002) . It was found that anthracycline antibiotic doxorubicin that is especially used in hematological malignancies increased $O_2^{\bullet-}$ levels up to 4-fold of control at 250 nM and higher concentrations in human cardiomyocyte cell line (Yuan et al., 2016). Wenzel et al. determined mitochondrial $O_2^{\bullet-}$ generation of another antineoplastic compound camptothecin and seemed this radical formation as the cause of apoptosis triggering by the drug in human preneoplastic colonocytes (Wenzel, Kuntz, & Daniel, 2003). In the same study, it was also stated that although a decline was observed in endogenous free NO• concentration, when the NO• donor sodium nitroprusside was applied, a sustained increases in its levels efficiently prevented the camptothecin-induced generation of mitochondrial $O_2^{\bullet-}$ and apoptosis. In this study, positive

correlations in between NO• and O_2•$^-$levels for all investigated CLT concentrations were also observed ($r_{50\mu M}$=0.995; $r_{75\mu M}$ =0.984; $r_{110\mu M}$ =0.932; $r_{125\mu M}$= 0.772), which may reflect the reactions of these radical to form particularly peroxynitrite (ONOO). On the other hand, these reactions could not inhibit CLT-induced *S. cerevisiae* apoptosis determined by flow cytometric annexin-V/PI analysis most probably due to inadequate levels of NO•.

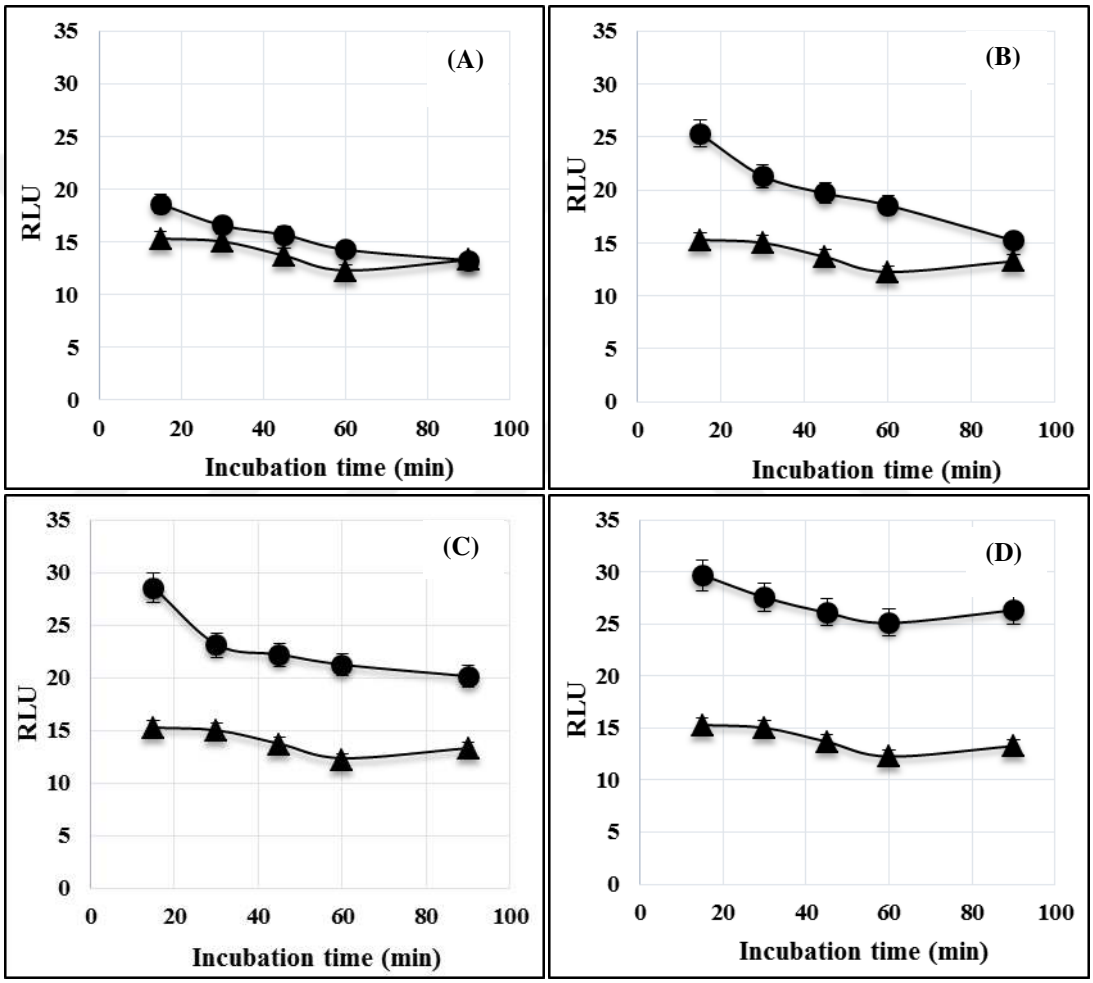

Figure 3.13 Variation in O_2•$^-$ levels depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

3.6.3 H_2O_2 level

H_2O_2 is one of the freely diffusible and relatively long-live non-radical ROS. On the other hand, it is the precursor of the HO• radical by Fenton reactions (Halliwell, 1991, 2006). As can be seen from Figure 3.14, intracellular H_2O_2 levels in *S. cerevisiae* that increased with increasing CLT concentrations were significantly over the control and generally increased slightly throughout incubation period. The observed highest H_2O_2 level was determined as 25.58 nmol.gww^{-1} after 60 min treatment with 125 µM CLT that was 2.85-fold higher than its control. As in the case of O_2•$^-$, there is no data about the effect of CLT on the production of H_2O_2. On the other hand, another exogenous factor menadione was found to induce H_2O_2 levels in a similar manner with CLT as 2.2-, 1.2-, 2.4-folds compared to their controls in *Phanerochaete chrysosporium*, *Schizosaccharomyces pombe* and *Penicillium chrysogenum*, respectively (Emri, Pócsi, & Szentirmai, 1999; Tongul & Tarhan, 2014).

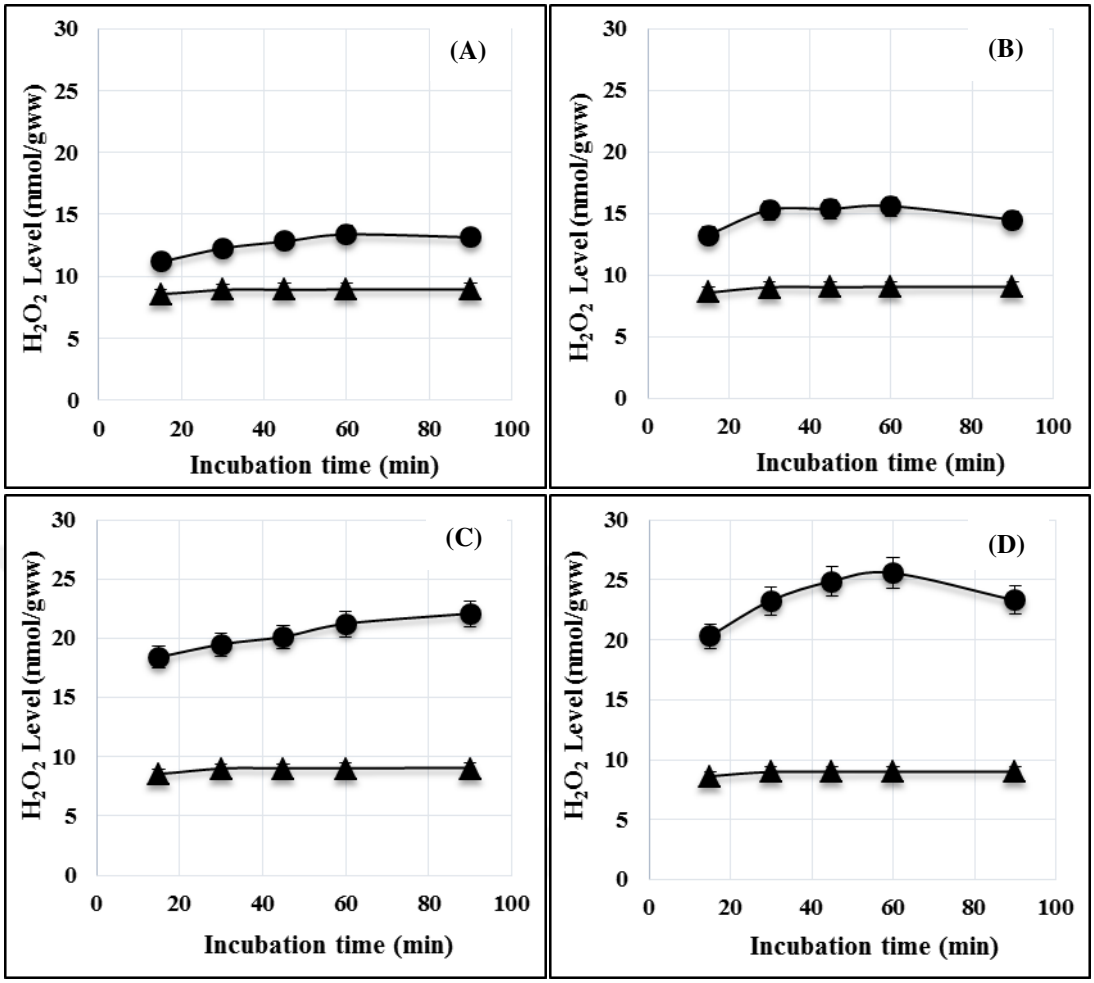

Figure 3.14 Variation in H_2O_2 levels depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

3.6.4 HO· level

In living or ganisms, HO· radical is one of the major reactive oxygen species together with $O_2•^-$ radical that are being continuously formed in a process of reduction of oxygen to water. In the Haber-Weiss reaction HO· radicals are generated in the presence of H_2O_2 and iron ions. As seen from Figure 3.15, HO· levels in all CLT treated *S. cerevisae* cells were generally over the controls and increased throughout the incubation period. The highest levels were similar at 110 and 125 µM and determined approximately as 5.29 µmol.gww^{-1} that was 1.3-fold higher than control in the end of incubation period. In the study conducted by Cho and Lee, it

was demonstrated that treatment with 9 μM antimicrobial peptide arenicin-1 for 2 h induced significant increases in especially cytotoxic HO• levels besides total ROS and H_2O_2 in *C. albicans* (Cho & Lee, 2011). Sampson et al. investigated the whether HO• radicals were induced during the observed rapid polymyxin killing of *Acinetobacter baumannii* and found that 5 ppm kanamycin and 2 ppm colistin treatments for 30 min caused approximately 2.50- and 2.00-fold increases in the radical levels (Sampson et al., 2012). The researchers also stated that reactive oxygen species initiate an exponential increase in hydroxyl radical production through an intracellular Fenton reaction in the killing mechanism of polymyxins. ·OH radical can also be maintained with the conversion of $O_2•^-$ by Haber-Weiss and other cellular reactions. In accordance with this knowledge, negative correlations were observed in between HO• and $O_2•^-$ levels in *S. cerevisiae* cells treated with CLT ($r_{50\mu M}$=-0.899; $r_{75\mu M}$ =-0.941; $r_{110\mu M}$ =-0.835; $r_{125\mu M}$= -0.159).

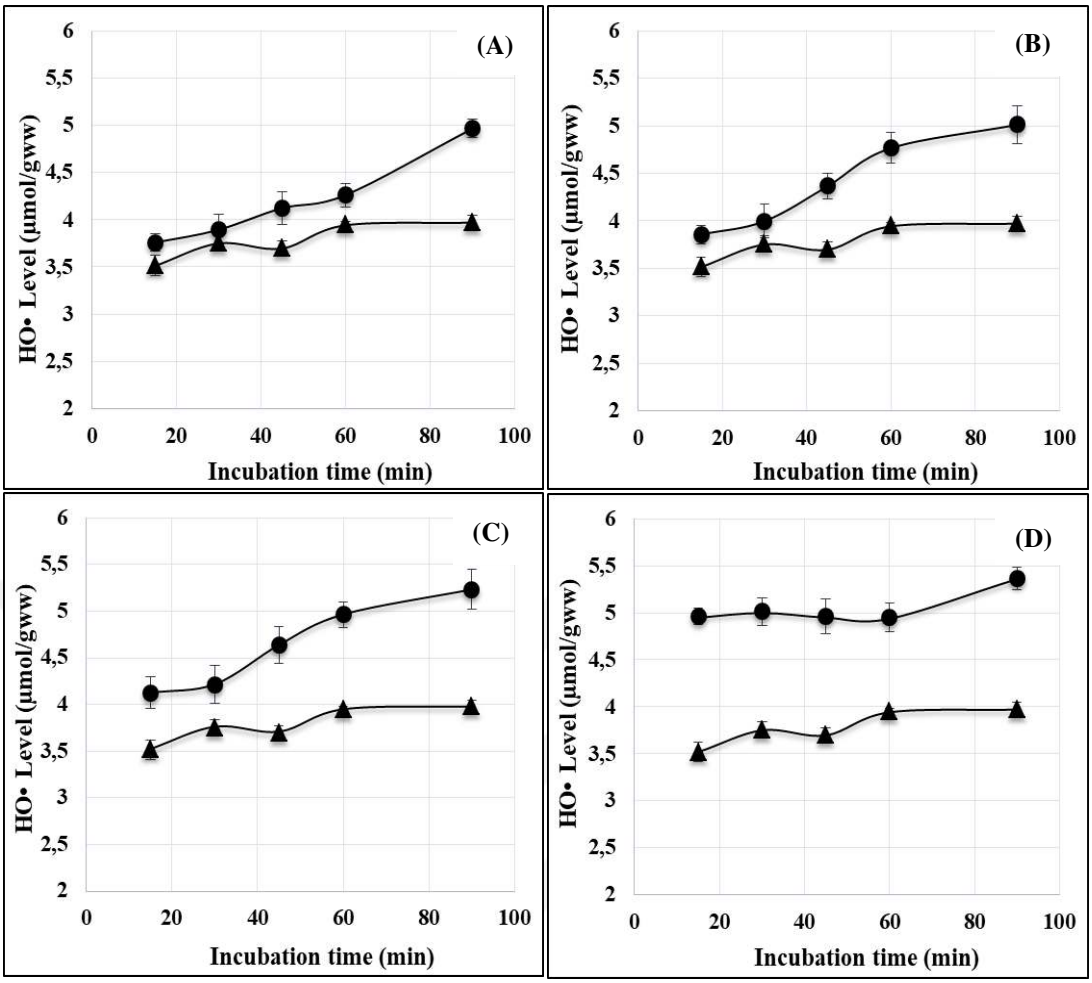

Figure 3.15 Variation in HO• levels depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

3.6.5 Total ROS level

Total ROS levels were measured with CMH2DCFDA which is mainly specific for hydrogen peroxide and other hydroperoxides. The total ROS levels in *S. cerevisiae* for all investigated CLT concentrations were over the control throughout the incubation period ($p<0.05$) (Figure 3.16). The increases in total ROS levels slightly increased depending on CLT concentrations and the highest value was found approximately as 2.53 RLU for all CLT concentrations except 50 µM in the end of the investigated incubation period. The increases in total ROS levels except 125 µM can be explained by the formation of other hydroperoxides besides H_2O_2 because of its relatively constant levels. In the study conducted with another antifungal amphotericin B, the total ROS level in *Candida albicans* after the treatment with 10 µM amphotericin B for 200 min was found as 2.00-fold of its control (Philips, Sudbery, & Ramsdale, 2003). It was shown that miconazole and to a lesser extent fluconazole treatments dose-dependently increased ROS production in *Candida albicans* and miconazole at 12.5 µg/mL caused 4-fold increase after 2 h treatment (Kobayashi et al., 2002). Total ROS level in *Cryptococcus gattii* exposed to 104 µM fluconazole increased 2.5-fold compared to related control after 1 h at which 2.26-fold increase was observed in 110 µM CLT treated *S. cerevisiae* (Ferreira et al., 2013). On the other hand, only 1.39-fold increase was observed in *Schizosaccharomyces pombe* cells treated with 250 µM patulin and citrinin micotoxins at the same time for 1 h (Papp, Máté, Mike, Gazdag, & Pesti, 2016).

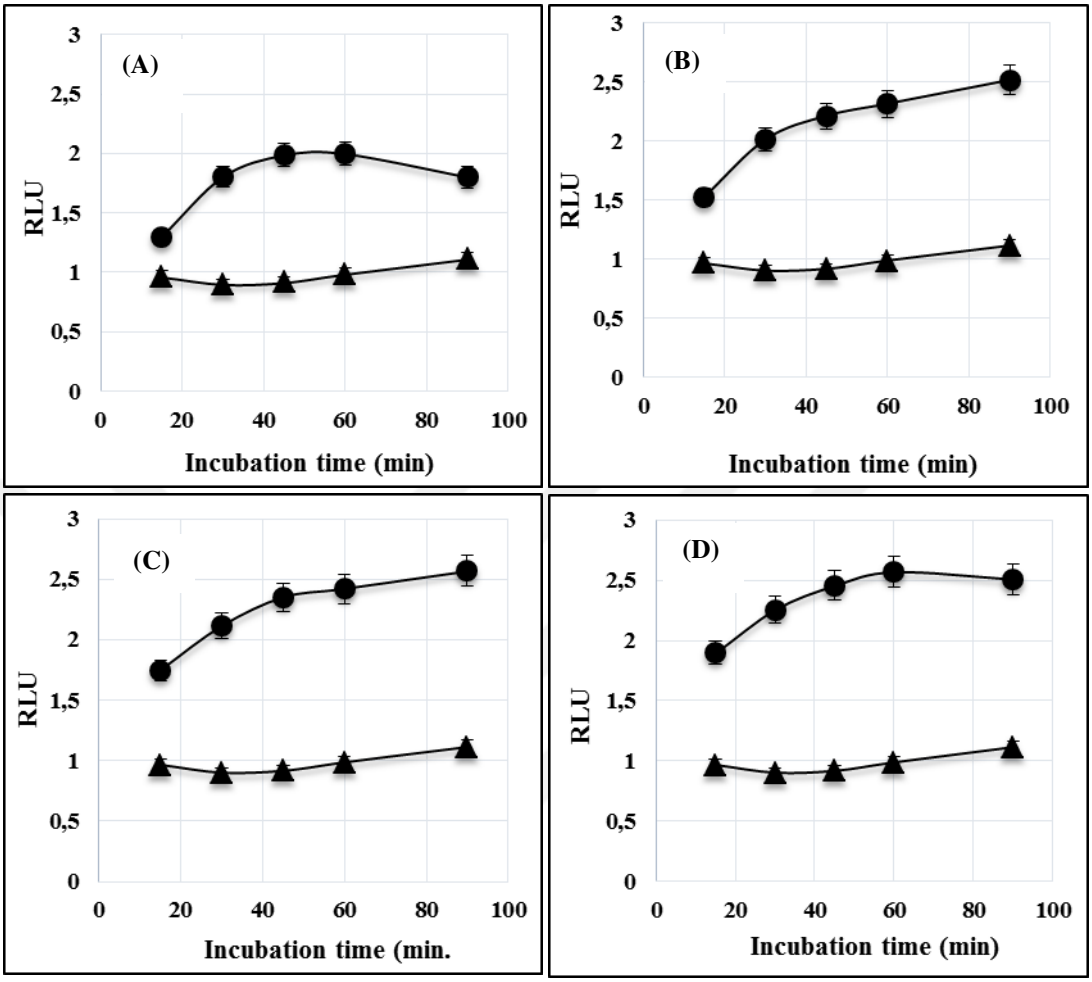

Figure 3.16 Variation in Total ROS levels depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

3.7 Variations in NADH and NADPH oxidase activity levels in CLT treated *S. cerevisiae*

NADH and NADPH oxidases are the enzymes that catalyze the production of $O_2^{\bullet -}$ from oxygen and NADH/NADPH and so known as the $O_2^{\bullet -}$ producer enzymes as endogenous sources of ROS. As can be seen from Figure 3.17, NADH oxidase activities in CLT treated *S. cerevisiae* were over their controls except 50 μM in all investigated incubation times ($p<0.05$). On the other hand, the activities were below the control and showed similar values in cells treated with 50 μM CLT throughout all incubation period. The activities showed similar tendencies at 75 and 110 μM for the investigated incubation period and reached their maxima in 30^{th} min as 122.12 and 142.72 U/mg, respectively. On the other hand, although the initial NADH oxidase activity was 3.08-fold of its control at 125 μM, it showed decreasing tendency from the beginning of incubation to 60^{th} min. According to the results, NADPH oxidase activities showed also similar tendencies with NADH oxidase in all investigated CLT concentrations and incubation times (Figure 3.18). The observed highest activity was 45.87 U/mg at 110 μM CLT in 30^{th} min. The positive correlations were found between $O_2^{\bullet -}$levels and NAD(P)H oxidase activities in 125 μM treatments (for NADH oxidase-$O_2^{\bullet -}$: $r_{125\mu M}= 0.89$; for NADPH oxidase-$O_2^{\bullet -}$: $r_{125\mu M}= 0.68$).

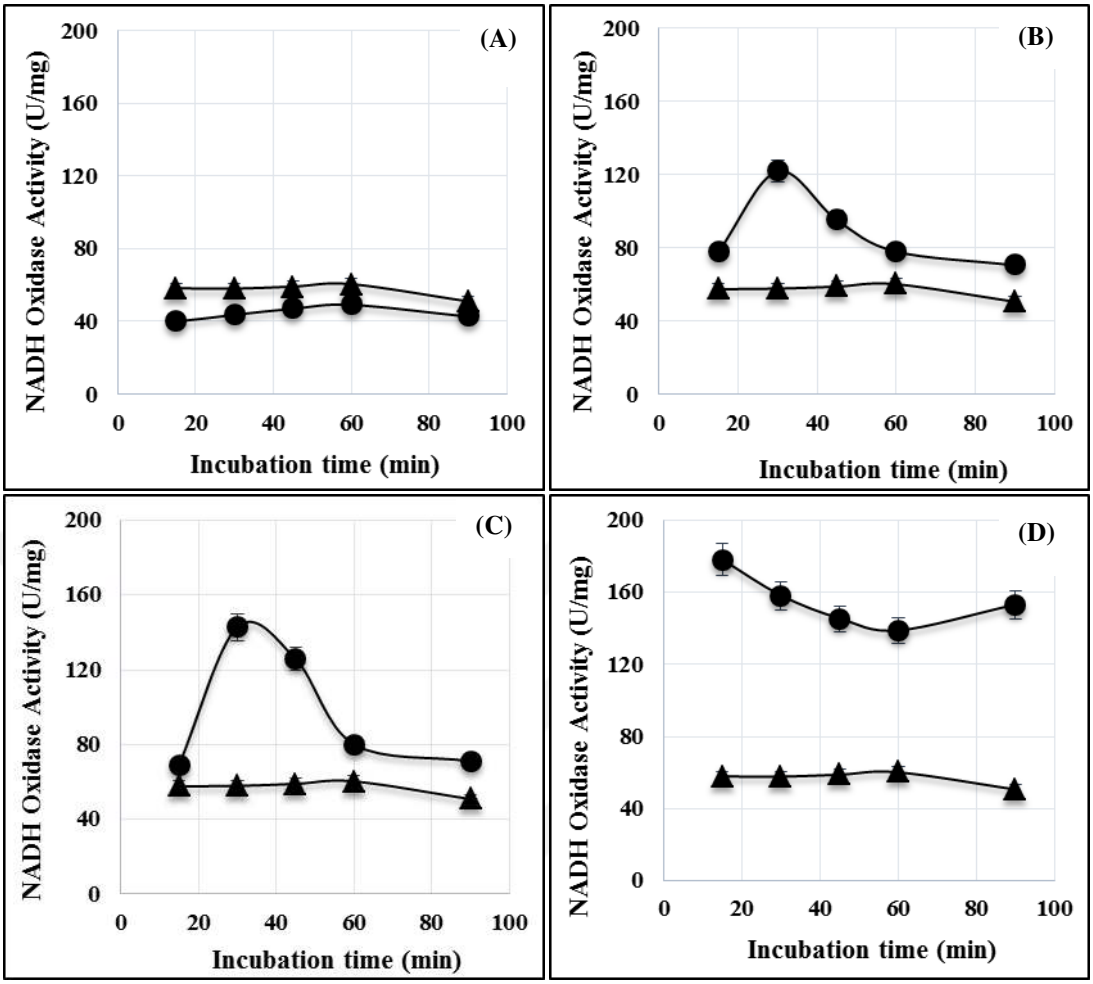

Figure 3.17 Variation in NADH oxidase activities depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

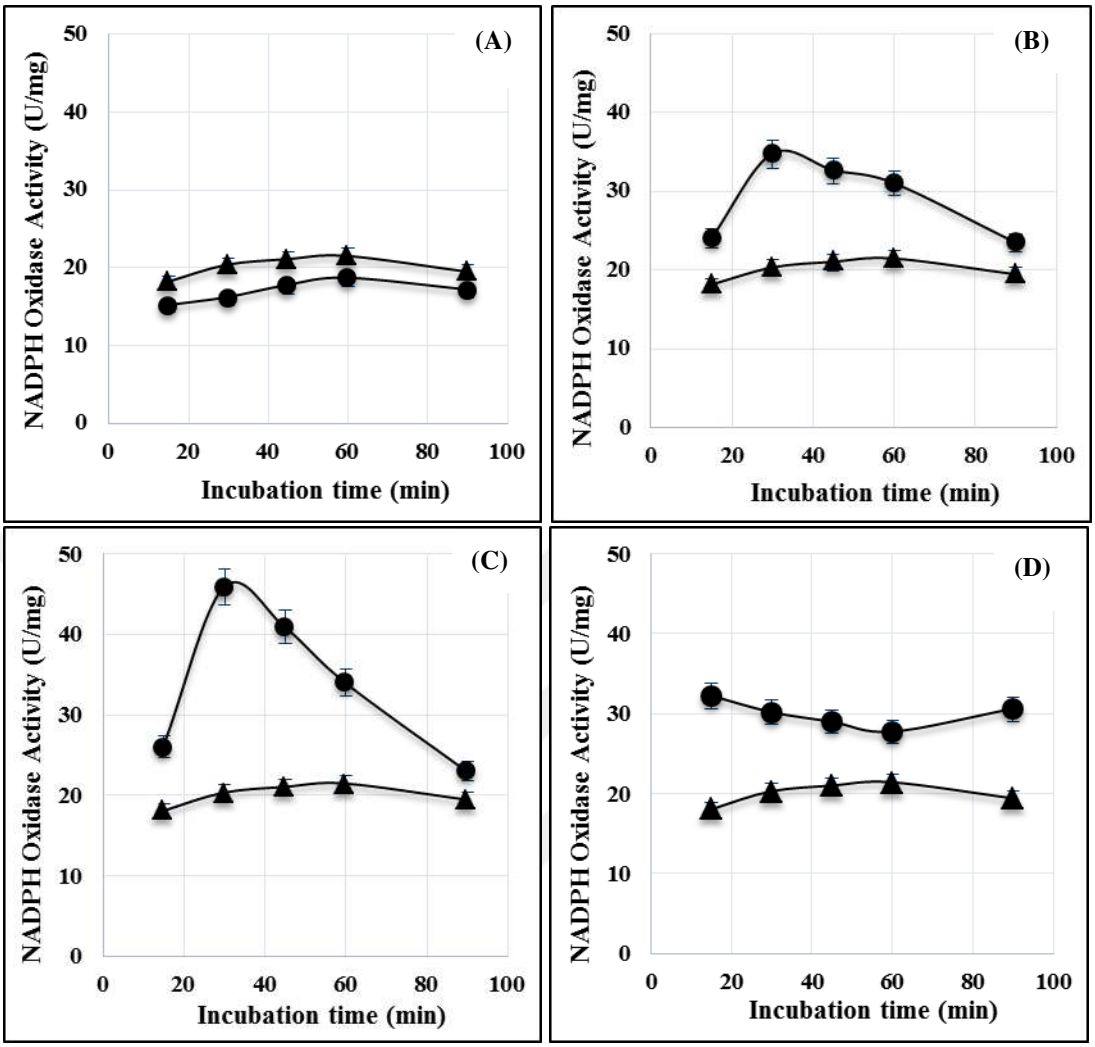

Figure 3.18 Variation in NADPH oxidase activities depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

In contrast to the findings of this thesis, it was shown that another potential antitumor substance bullatacin that was isolated from plants of the Annonaceae has an ability to inhibit NADH oxidase activity of plasma membrane vesicles isolated from HeLa and HL-60 cells, even at much lower concentrations as 5-10 nM (Morre, de Cabo, Farley, Oberlies, & McLaughlin, 1994). In the study conducted by Mizutani et al., doxorubicin induced apoptosis in HP100 cells, derived from human leukemia HL-60, was investigated. It was determined that doxorubicin causes DNA damage by indirect H_2O_2 generation through NAD(P)H oxidase activation and NAD(P)H

oxidase inhibitors prevented doxorubicin induced DNA ladder formation (Mizutani, Tada-Oikawa, Hiraku, Kojima, & Kawanishi, 2005).

3.8 Variations in antioxidant enzyme activity levels in CLT treated *S. cerevisiae*

3.8.1 SOD activity

SOD converts superoxide anion radicals produced in the body to hydrogen peroxide, thereby reducing the likelihood of superoxide anion interacting with nitric oxide to form reactive peroxynitrite. As seen Figure 3.19, SOD activities were significantly over the control values in all of the investigated CLT treatments ($p<0.05$). The activities at 50 and 75 µM increased with the increase of time. While SOD activities induced up to 45th min in 110 µM treated cells, the continuous decreases were observed from the beginning of incubation at 125 µM. The highest activity was found as 20.36 U/mg that was 1.59-fold of its control at 110 µM in 30 min. On the other hand, the activity was 20.34 U/mg at the beginning of the incubation in 125 µM CLT treated *S. cerevisiae*. The strongly negative correlations between $O_2\bullet^-$ level and SOD activity were found at 50 µM CLT ($r_{50\mu M}$=-0.96) and up to 45th min at 75 and 110 µM ($r_{75\mu M}$=-0.99; $r_{110\mu M}$ =-0.88), while the positive correlation was observed at 125 µM ($r_{125\mu M}$ =0.95). It can be said that especially decreased SOD activities at 110 and 125 µM could not significiantly decrease $O_2\bullet^-$ levels. In addition, there were positive correlation between H_2O_2 level and SOD activity at 50 and 75 µM CLT ($r_{50\mu M}$ =0.87; $r_{75\mu M}$ =0.77).

From other triazole antifungals, fluconazole did not affect SOD activity at 104 µM and 0.7 µM itraconazole increased the enzyme activity 1.12 fold compared to the control in C. gattii. In the study of Khan et al., SOD activities in *C. albicans* treated with 133 µM thymol and 67 µM carvacrol reached 2.04- and 2.12-fold higher values compared to the their controls, respectively. Oberley showed that overexpression of MnSOD via plasmid and adenovirus transfection in various cancer cell types leads to tumor suppression through noncytotoxic mechanism of cell-cycle perturbations (Oberley, 2001). The researcher have also stated that MnSOD overexpression causes

the anticancer drug 1,3- bis(2-chloroethyl)-1-nitrosourea (BCNU) to have increased cytotoxicity due to the fact that BCNU inhibits removal of peroxide which increased in response to MnSOD overexpression. On the contrary, according to the Kuninaka et al., highly expressed MnSOD confers resistance in colon cancer types (Kuninaka, Ichinose, Koja, & Toh, 2000). From this point of view, although SOD activity induction increased with the increase in CLT concentration, it could not inhibit CLT-based apoptosis in *S. cerevisiae*. This situation is most probably because of the difference in cell type of yeast and independency of CLT induced apoptosis from SOD protection.

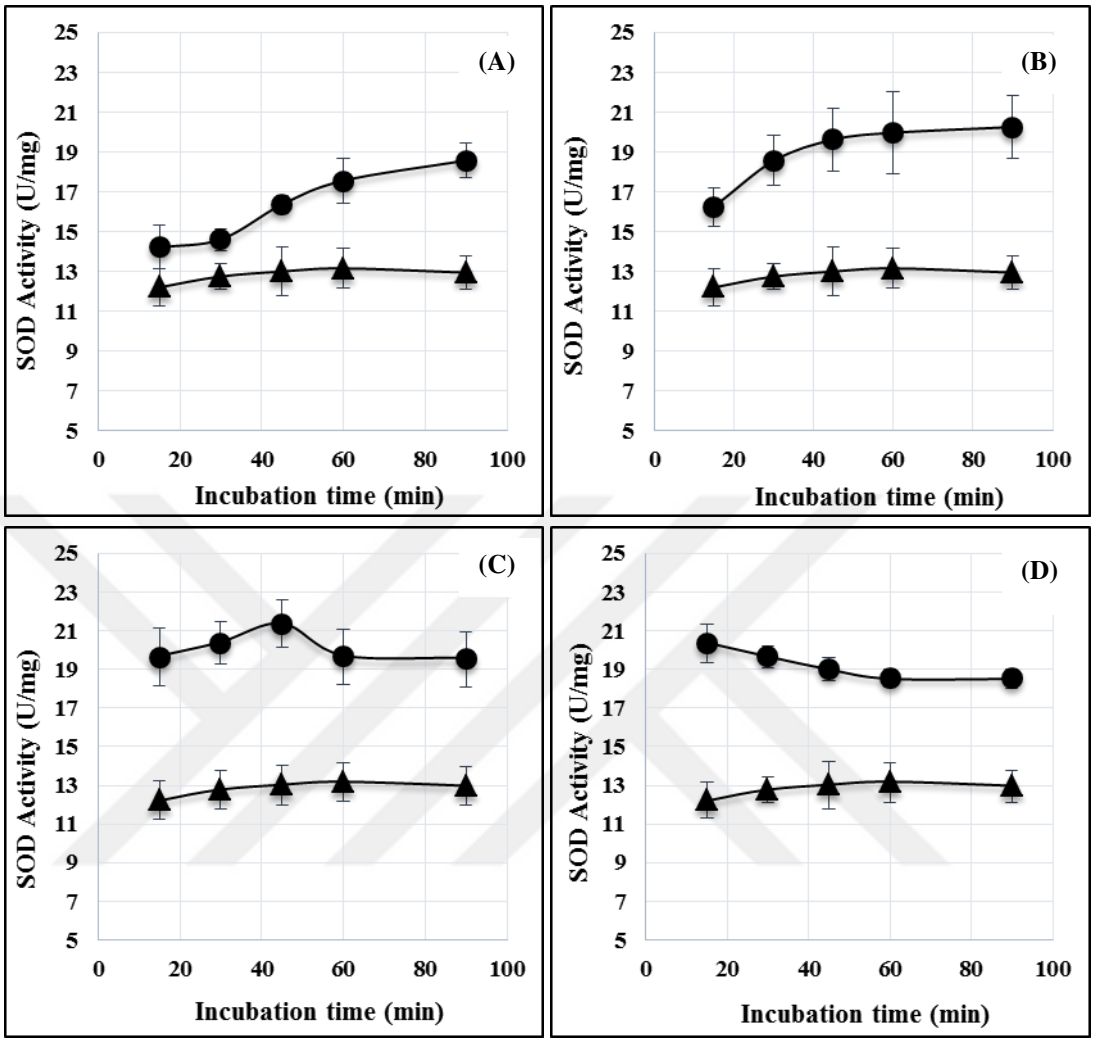

Figure 3.19 Variation in SOD activities depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

3.8.2 CAT activity

CAT which is mainly located in peroxisomes decomposes hydrogen peroxide to water and oxygen (Mandell, 1975). It must be stated that CAT activity in *S. cerevisiae* significantly varies depending on the growth phase of this microorganism. According to obtained findings, the activities increased approximately 10-fold in the yeast cells in their stationary phase compared to cells used in this thesis that is in their exponential phase. CAT activities in all CLT treated *S. cerevisiae* were generally significantly over the control values from the beginning of incubation and increased with increasing concentrations (Figure 3.20). While CAT activities continuously slightly increased at the concentration range of 50-110 µM throughout the investigated incubation period, they showed similar values at 125 µM. The observed highest activity was 0.92 U/mg that was 1.21-fold of control at 110 µM in the end of incubation period. It can be certainly said that this activity was maintained at 125 µM but without any additional induction throughout all incubation period. There were positive correlations between CAT activities and H_2O_2 levels in all treatments ($r_{50\mu M}$=0.93; $r_{75\mu M}$=0.24; $r_{110\mu M}$=0.92; $r_{125\mu M}$=0.66). Therefore, it can be said that induced CAT activities could not be sufficient to decrease the H_2O_2 but may play role in keeping in a certain level.

According to the literature, similar with SOD, catalase has also inhibition effect upon apoptosis. In the study conducted by Bai and Cederbaum, the effect of catalase on HepG2 cell apoptosis induced by DNA-damaging agents was investigated (Bai & Cederbaum, 2003). For this aim, HepG2 cells were infected with adenovirus containing the cDNA of catalase and the activity was increased 7–10-fold compared with control cells. After treatment with Vp16 or mitomycin C, control cells underwent apoptosis in a p53-dependent manner; however, overexpression of catalase inhibited this apoptosis. Among the antineoplastic drugs, while dacarbazine (8 µg.ml $^{-1}$), cyclophosphamide (50 µg.mL^{-1}) and cytarabine (1 µg.mL^{-1}) are not effective against catalase activity, methotrexate (2 µg.mL^{-1}) increased catalase activity about 2.61-fold of related control after 48 h treatment (Linares et al., 2006). In the study conducted by Gonzalez-Parraga, CAT activity in *C. albicans* treated

with 500 µM H_2O_2 for 1 h was 1.5-fold higher than its control (González-Párraga, Hernández, & Argüelles, 2003). Finally, 250 µM ketoconazole treatment increased CAT activity up to 2.79-fold higher than its control within 1 h in *S. cerevisiae* (Tongul & Tarhan, 2016).

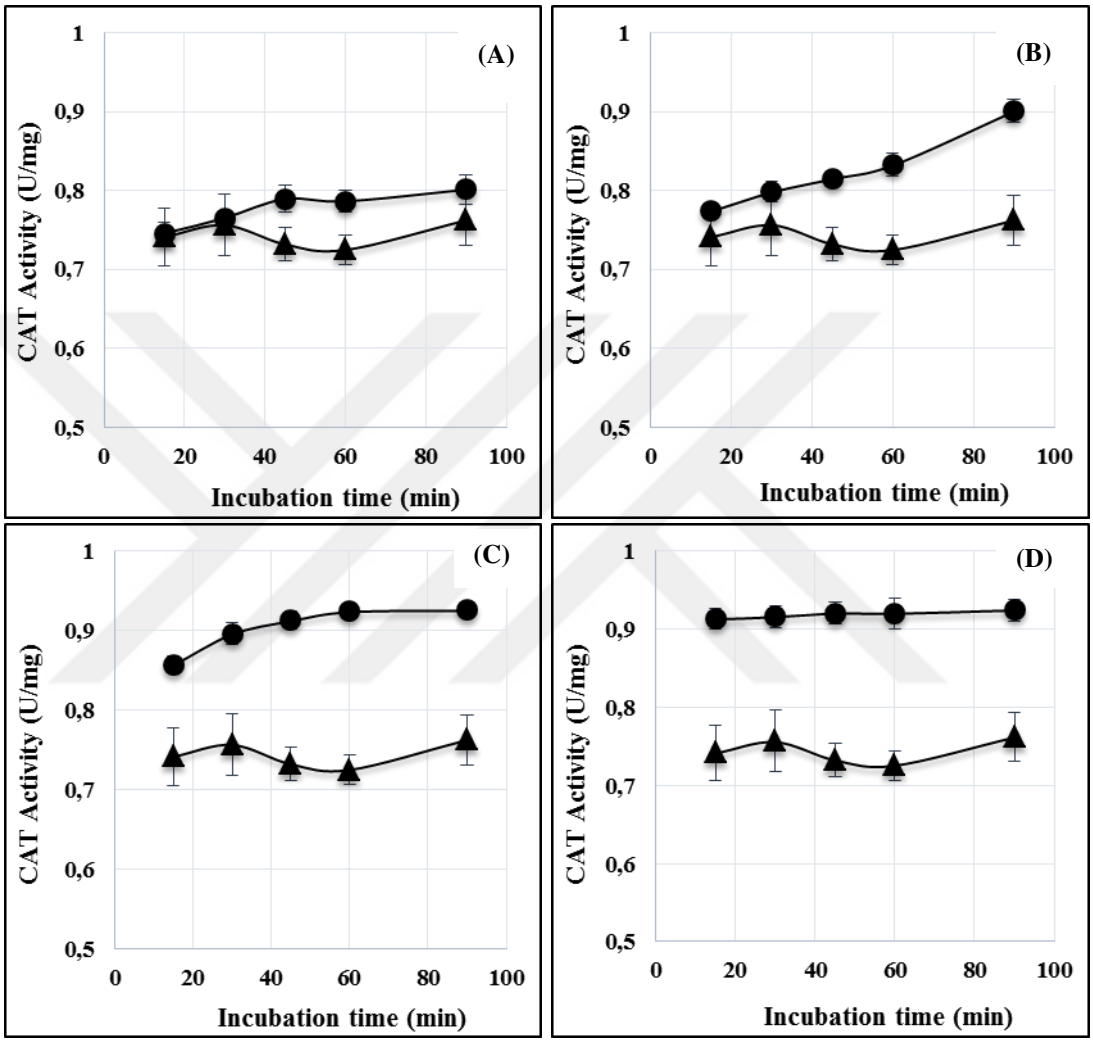

Figure 3.20 Variation in CAT activities depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

3.9 Variations in GSH related enzyme activities in CLT treated *S. cerevisiae*

3.9.1 GR Activity

GR is an essential enzyme that recycles oxidised glutathione back to the its reduced form and therefore maintain the reducing environment of the cell. This enzyme is highly conserved across nature and a high degree of similarity has been shown between its three-dimensional structures from *E. coli*, *S. cerevisiae* and *Homo sapiens* (Yu & Zhou, 2007; Karplus & Schulz, 1987; Mittl & Schulz, 1994). GR has been shown to be modulated under different type of stresses such as heavy metal ions, cadmium and zinc; nickel and cadmium (Schickler & Caspi, 1999). It was observed that CLT treatment resulted in the inductions of GR activities in all concentrations of the drug and they were generally significantly higher than control values ($p<0.05$) (Figure 3.21). While GR activities reached their maximums in 30^{th} min of the treatment period for both 50 and 110 µM, they continuously increased at 75 µM throughout the incubation period. On the other hand, the highest GR activity was observed in 60^{th} min at 125 µM CLT treatment as 252 U/mg, 4.38-fold of control and then sharply decreased at the end of incubation period. It should be stated that although there was positive correlations between GR activities and GSH levels in all treatments ($r_{50\mu M}=0.332$; $r_{75\mu M}=0.961$; $r_{110\mu M}=0.535$; $r_{125\mu M}=0.447$), the increase in GR activities could not maintain GSH:GSSG ratios especially at 110 and 125 µM.

According to all these results, it can be said that CLT shows its cytotoxicity in *S. cerevisiae* independently from GR. Besides potential antitumor drug CLT, the known chemotherapeutics including melphalan, busulfan, chlorambucil, daunorubicin and doxorubicin did not affect pure GR activity. On the other hand, 100 µM carmustine almost completely inhibited GR activity within 30 min (Witte, Anestål, Jerremalm, Ehrsson, & Arnér et al., 2005). In addition, it was found by Tunç et al. that antimony (III) complexes inhibited GR activity in the concentration range of 4.87–5.87 µM (Tunç, Koç, Açık, Karacan, & Karacan, 2015).

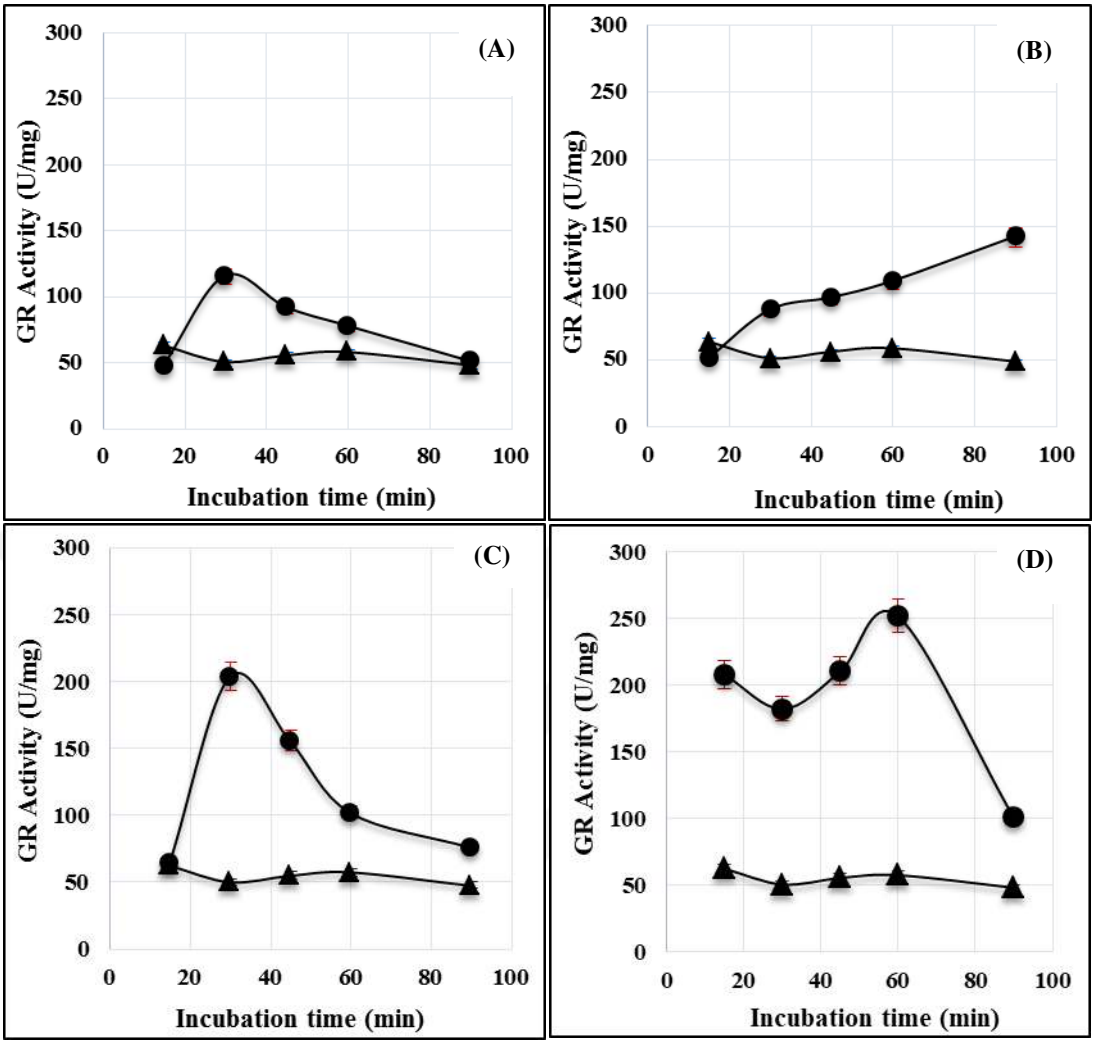

Figure 3.21 Variation in GR activities depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

3.9.2 GST Activity

GSTs are xenobiotic metabolizing enzymes that protect cells from toxic drugs and environmental electrophiles. These multiple isozymes are responsible from catalysing the conjugation of electrophilic xenobiotics with the tripeptide GSH to form polar, non-toxic peptide conjugates. In addition, GST has peroxidase activity, and can reduce the hydroxyl-peroxide toxicity (Marrs, 1996). As in the case of GR, GST activities were also significantly induced all applied CLT concentrations ($p<0.05$) (Figure 3.22). It can be said that GST activities increased with the increases in CLT concentrations except 110 µM. Similar with GR, GST activities reached their highest levels at 125 µM in 30^{th} min as 6.81-fold of its control. It should be noted that strong negative correlation ($r_{110\mu M}$=-0.932) was determined at 110 µM CLT treatment that was also identified as the condition which highest apoptotic induction was observed.

GSTs as xenobiotic metabolizing enzymes are one of the important factors that lead to resistance to antineoplastic drugs. Recently, Li and colleagues stated that one of cisplatin resistance mechanisms is associated with overexpression of GSTs, which would accelerate the deactivation of cisplatin and decrease its antitumor efficiency (Li et al., 2017). Besides cisplatin, GSTs play significant roles against vast variety of exogenous stimuli such as DDT, abamectin (Hayes & Wolf, 1988), acrolein (Sthijns et al., 2017), arsenate (Bianucci et al., 2017), various pesticides, benzene oxide (Zarth, Murphy, & Hecht, 2015) and different insectisides. In the case of CLT, it can be interpreted that although GST activity inductions were observed in all studied drug concentrations, these inductions were not sufficient to prevent CLT induced cell death and damage in *S. cerevisiae* BY4742 cells.

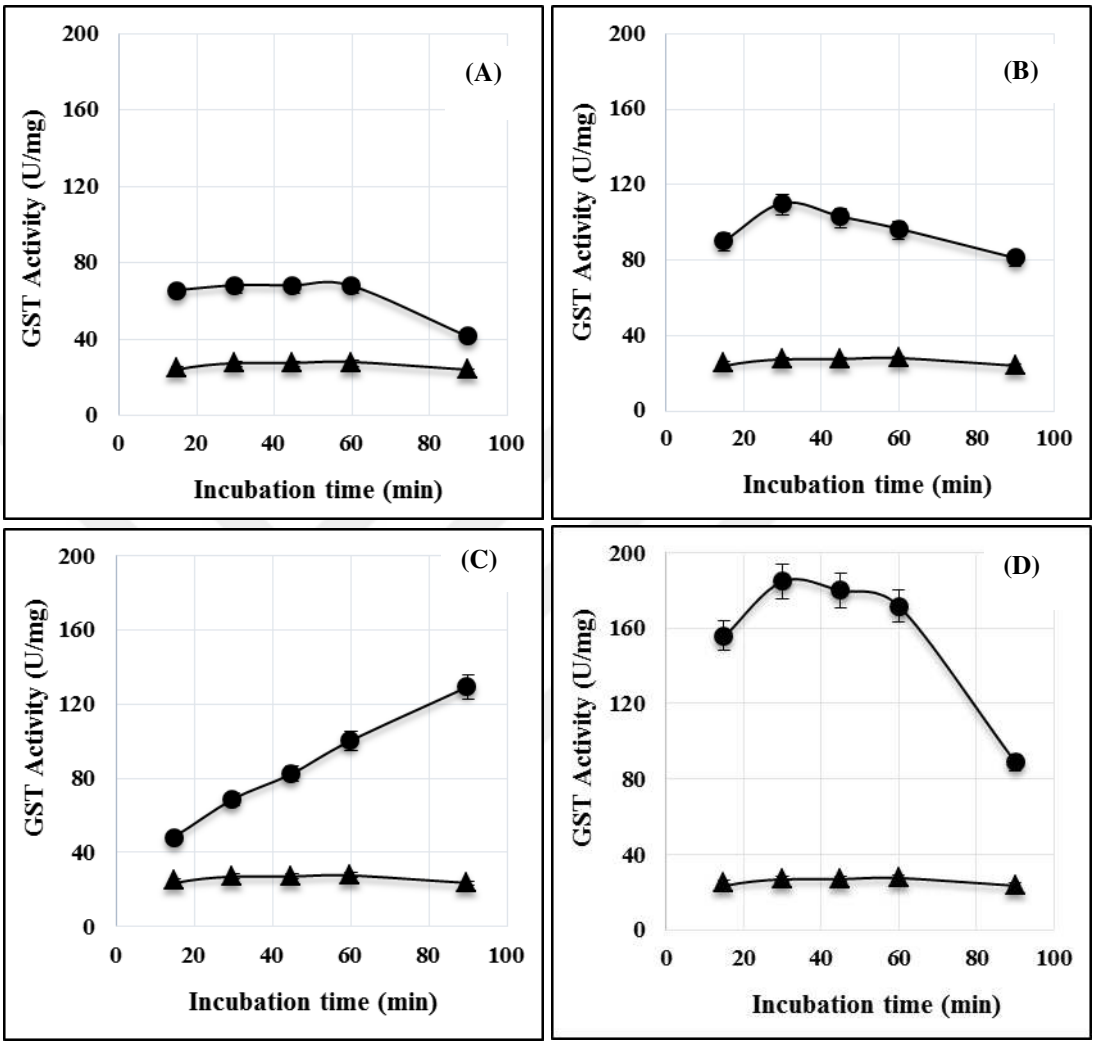

Figure 3.22 Variation in GST activities depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

3.9.3 GSHPx Activity

GSH-Px is a powerful scavenger of H_2O_2 and lipid hydroperoxides, with the help of GSH and suggested to protect the cell against low levels of oxidative stress (Mates, 2000). According to the results, GSHPx activities were higher than control values in CLT-treated *S. cerevisiae* cells except 50 µM. In general, GSHPx activities increased with the increase in CLT concentration throughout incubation period. The highest GSHPx activity was determined at 125 µM in 30^{th} min as 2.56-fold of its control (Figure 3.23). On the other hand, sharp decrease was observed towards the end of the incubation period, likewise, GR and GST activities. While positive correlation was found between GSHPx activities and H_2O_2 levels at 75 µM and 125 µM CLT ($r_{75\mu M}$=0.517, $r_{125\mu M}$=0.339), negative correlation was observed at 50 µM and 110 µM CLT ($r_{50\mu M}$=-0.165, $r_{110\mu M}$=-0.493). When these results are considered together with the positive correlations between CAT activities and H_2O_2 levels, it is clear that neither CAT nor GSHPx activity inductions were adequate to scavenge H_2O_2.

Acetaldehyde, the primary ethanol metabolite, has been implicated in the pathogenesis of alcoholic liver disease. On the other hand, it was determined that acetaldehyde at the concentration range of 30 – 300 µM did not affect GSHPx activity after 30 min treatment in liver (Pivetta et al., 2006). Doroshow showed that overexpression of the cDNA for the major cytoplasmic GSHPx isoenzyme, GSHPx-1, in human MCF-7 breast cancer cells has been shown to significantly increase the tolerance of these cells to oxidative stress produced by hydrogen peroxide or by the redox cycling of the quinone-containing anticancer agent doxorubicin (Doroshow, 1995). Missall examined the significance of two GPx, GPx1, and GPx2 in *Cryptococcus neoformans* for oxidative stress resistance. It was observed that the gpx1Δ mutant was hypersensitive to t-butylhydroperoxide, and gpx2Δ mutant was hypersensitive to cumene hydroperoxide (Missall, Cherry-Harris, & Lodge, 2005). Salicylic acid is another compound cause to reductions in oxidative stress through the elevation in GSHPx activity of colon tissue (Drew et al., 2005).

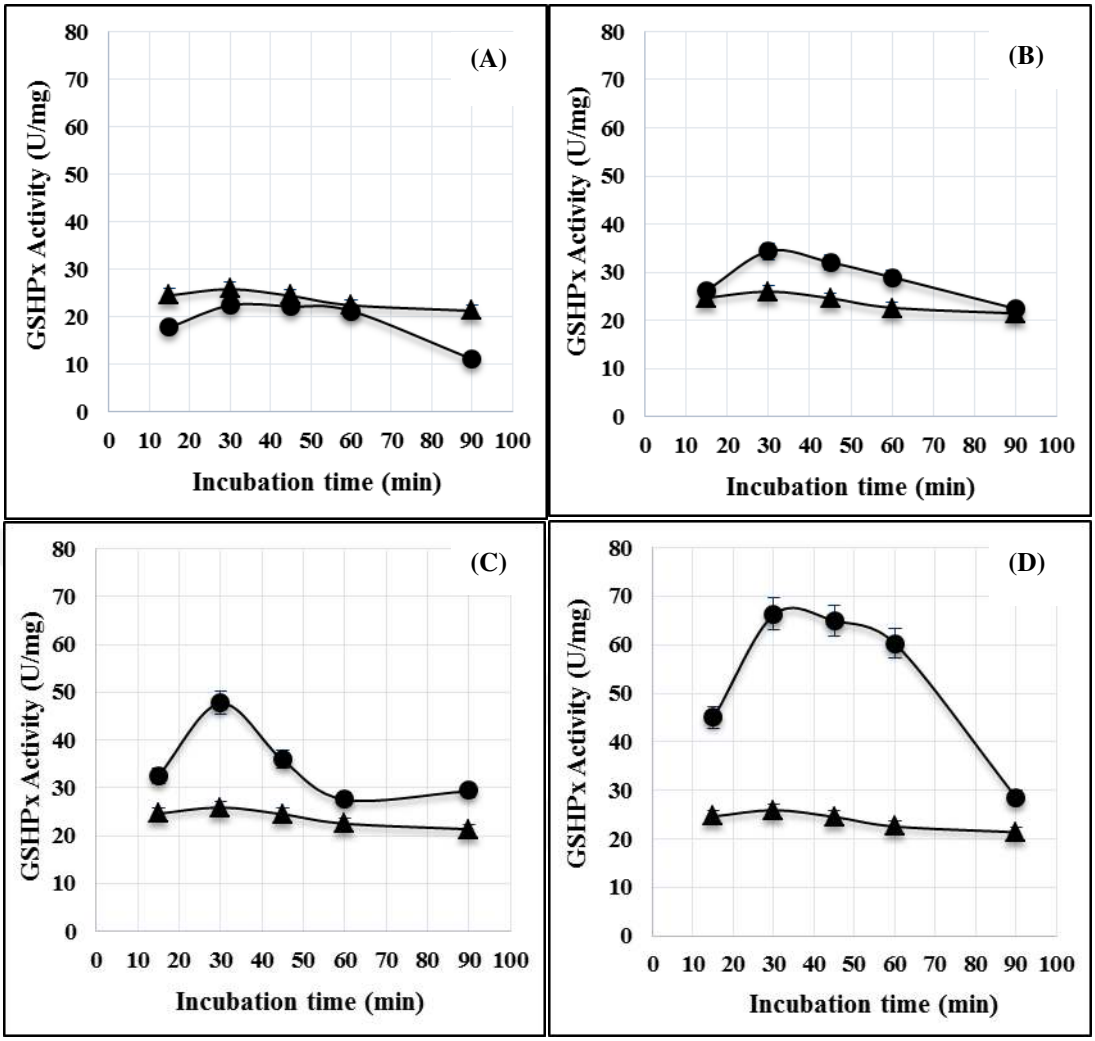

Figure 3.23 Variation in GSHPx activities depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

3.10 Variations in GSH – GSSG Levels and GSH/GSSG Ratios in CLT Treated *S. cerevisiae*

GSH is the predominant non-protein thiol present in nearly all microbial cells (Copley & Dhillon, 2002), especially in yeasts. GSH/GR system is obligatory for maintaining the redox balance and defending the oxidative stress in yeast cells.

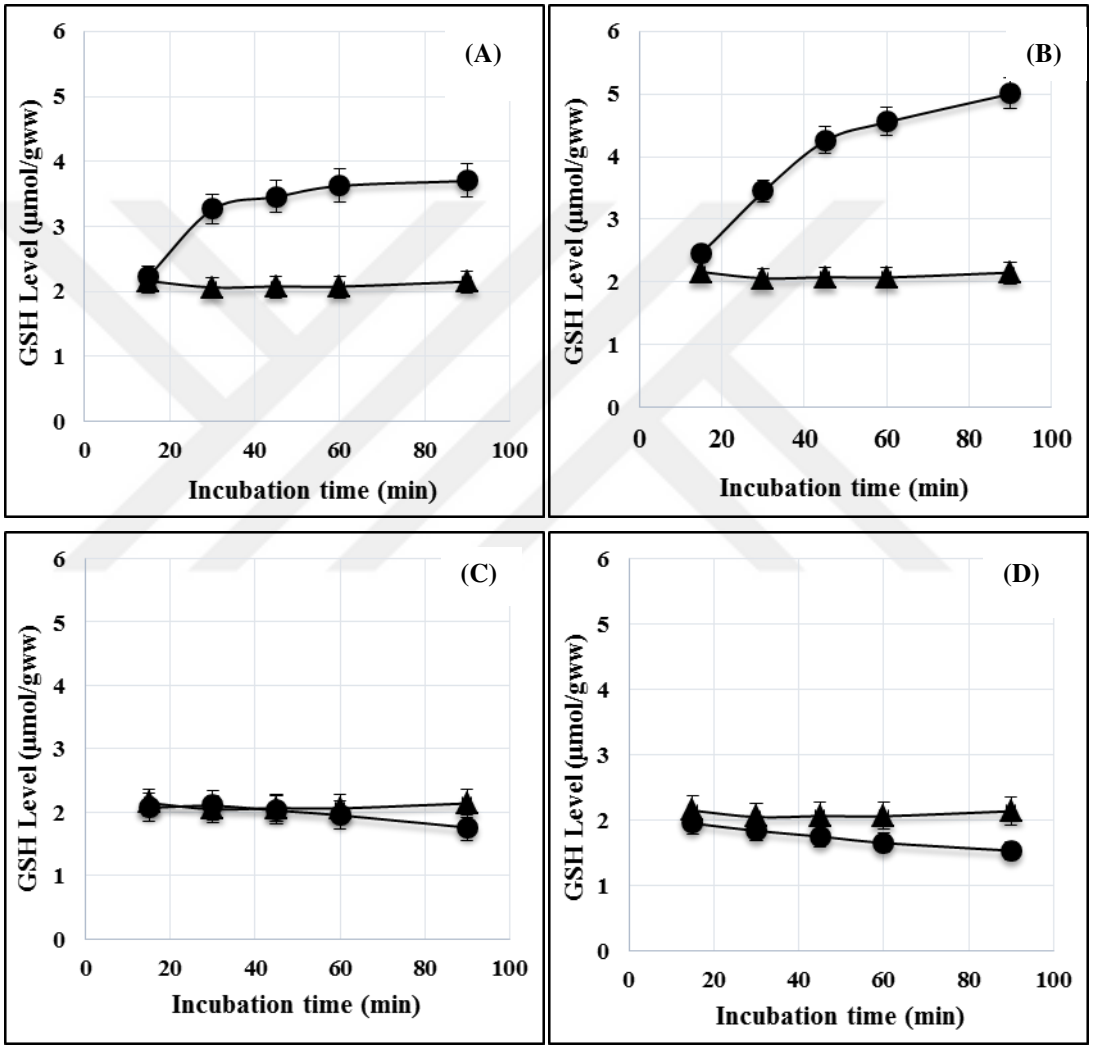

Figure 3.24 Variation in GSH levels depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

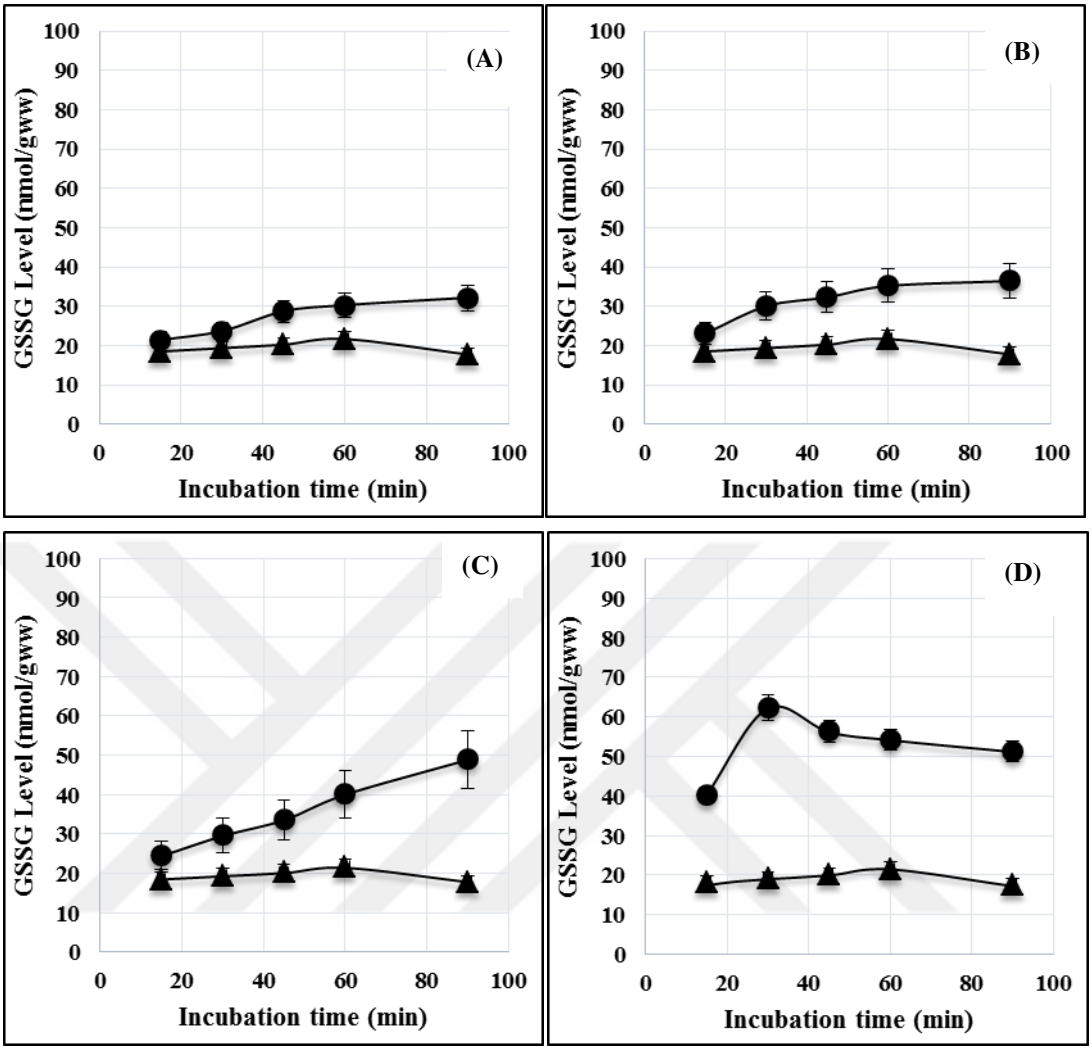

Figure 3.25 Variation in GSSG levels depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

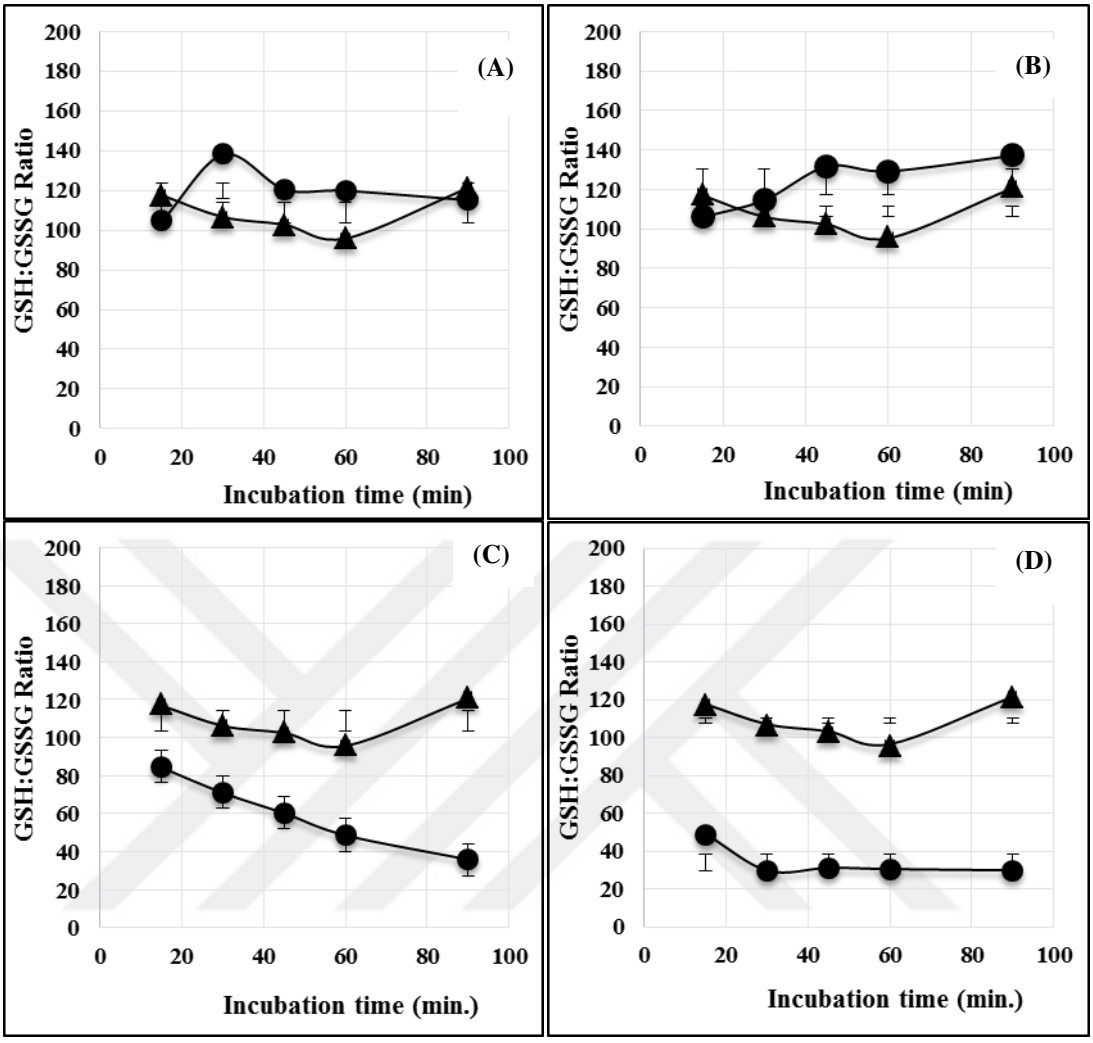

Figure 3.26 Variation in GSH:GSSG ratios depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

According to the obtained results, GSH levels in *S. cerevisiae* were induced in 50 and 75 µM CLT treatments (Figure 3.24). On the other hand, when higher concentrations of CLT were applied, GSH levels dropped under control values in 90th min for 110 µM and from the beginning of incubation for 125 µM CLT. Although there was no correlation between the decreases in GSH levels and CLT concentration, GSSG levels increased in parallel with the increase in CLT concentrations. GSSG levels reached their maximum at 125 µM CLT in 30th min as 3.24-fold of control (Figure 3.25). As can be seen from Figure 3.26., while

GSH:GSSG ratios could be kept higher than control values at 50 and 75 μM, they decreased under control with time at 110 and 125 μM CLT treatments. The highest decrease in GSH:GSSG ratios was observed at 125 μM in the end of incubation period as 4.06-fold under control.

There is no other study that dealing with the effects of CLT on GSH metabolism in microorganisms. On the other hand, it was stated that CLT did not affect the kinetics of GSH degradation in normal human erythrocytes (Lisovskaya, Schherbachenko, Volkova, & Ataullakhanov, 2009). Ebselen is a synthetic organoselenium drug molecule with anti-inflammatory, anti-oxidant and cytoprotective activity. However, it exhibits potent antifungal activity against *Candida* and *Cryptococcus spp* by regulating GSH levels (Thangamani et al., 2017). 20 μg/ml ebselen caused to 40% depletion of GSH in the so-called fungi. In the case of *S. cerevisiae* BY4742 cells, although all inductions that were observed in antioxidant enzyme activities, this primary redox balance defence could not be maintained in CLT treated yeast.

3.11 LPO Levels in CLT Treated *S. cerevisiae*

Lipid peroxidation is a complex process known to occur in all living organisms. MDA, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of the peroxidation reaction (Valverde, Trejo, & Rojas, 2001). As shown in Figure 3.27, the membrane LPO levels in all CLT treated *S. cerevisiae* were significantly over the controls in all incubation periods ($p<0.05$). The observed highest membrane LPO level was 7.43 nmol MDA/gww that was 4.57-fold of its control, at 125 μM after 90 min treatment. In contrast to these results, Usul et al. stated protective effect of clotrimazole on spinal cord lipid peroxidation (Usul et al., 2006). On the other hand, it was shown in the study conducted with other two antifungal agents that while LPO levels in fluconazole treated *Cryptococcus gattii* cells did not change at 104 μM for 1 h, 0.7 μM itraconazole caused 3-fold increases (Ferreira et al., 2013). In the study conducted by Kharasch and Novak, it was found that while new anthracenedione antineoplastic agents ametantrone and mitoxantrone produced a concentration-dependent inhibition of hepatic microsomal lipid peroxidation, adriamycin stimulated lipid peroxidation over two fold under similar conditions (Kharasch & Novak, 1982).

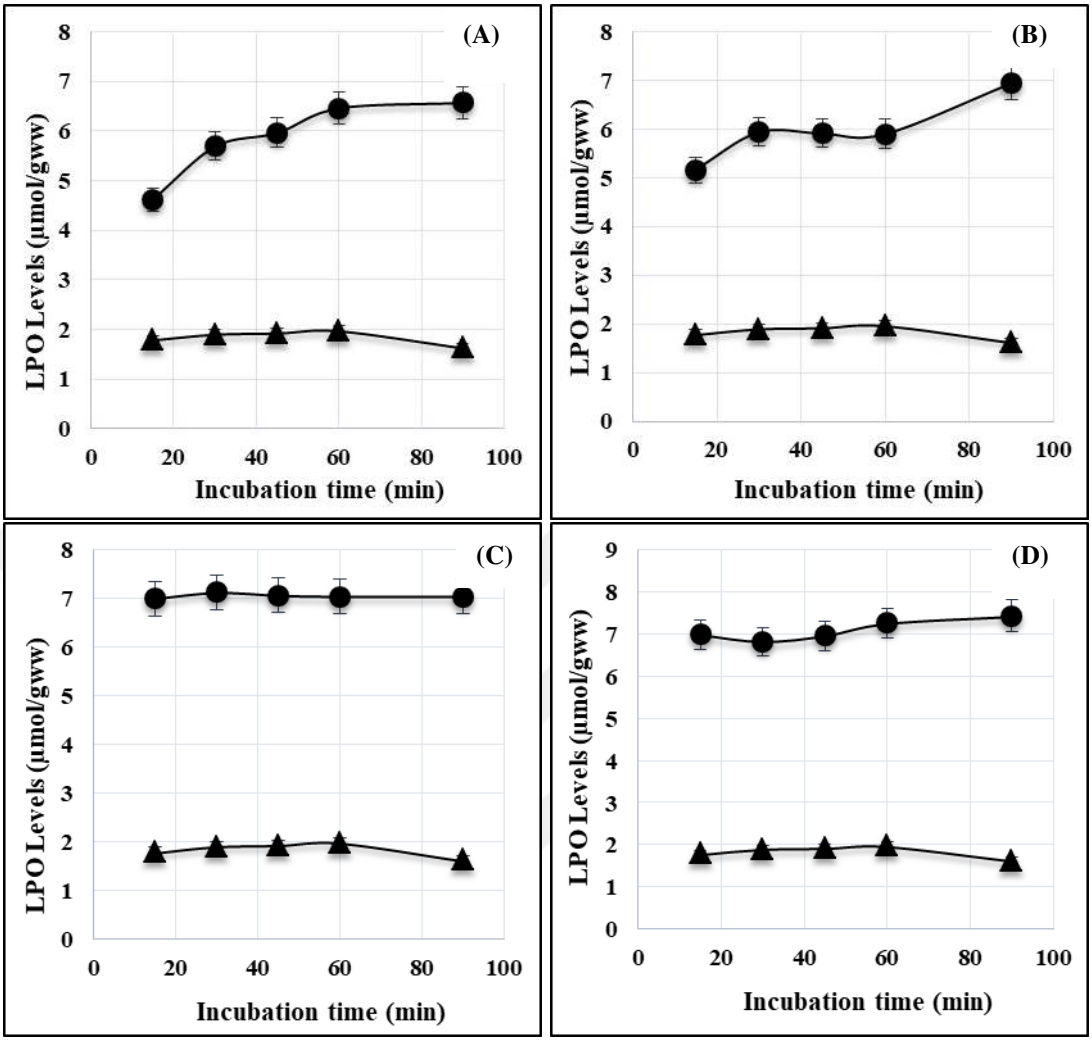

Figure 3.27 Variation in LPO levels depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

3.12 Protein Carbonyl Levels in CLT Treated *S. cerevisiae*

The determination of protein carbonyl levels is another significant marker of oxidative stress condition. The usage of protein carbonyl groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins.

Propiconazole, a widely used fungicide, is hepatotoxic and hepatotumorigenic in mice. The mode of propiconazole-induced toxicity in mouse liver primarily involves oxidative damage to cellular proteins and formation of protein carbonyl groups (Bruno, Moore, Nesnow, & Ge, 2009). 8 mM $Cu(NO_3)_2$ caused to increase in protein carbonyl levels by approximately eight-fold after 15 min in yeast cells (Shanmuganathan, Avery, Willetts, & Houghton, 2004). In the study conducted by Khan et al., it was shown that upon exposure to H_2O_2, the accumulation of protein carbonyls in *S. cerevisiae*, is much greater in a Δyca1 strain lacking the YCA1 gene than in the wild type (Khan, Chock, & Stadtman, 2005). In addition, apoptosis was abrogated in the Δyca1 strain, whereas wild type underwent apoptosis as measured by externalization of phosphatidylserine and the display of TUNEL-positive nuclei. In the study which compared biochemical changes in wild-type yeast and yeast strains deficient in various elements of the antioxidant defense during prolonged incubation of stationary cultures of *S. cerevisiae*, the most pronounced increase in protein carbonyl levels was found for the glutathione-deficient yeast strain (Jakubowski, Biliński, Bartosz, 2000).

As can be seen Figure 3.28, protein carbonyl levels were generally significantly higher than control values ($p < 0.05$) and increased with increase concentrations of CLT. In a similar manner with LPO results, there was no significant difference between the samples treated with 110 and 125 μM CLT. The highest protein carbonyl levels were similarly observed at 110 and 125 μM CLT in the beginning of incubation period as 164.07 and 166.67 RFU/mg protein, approximately 1.68-fold of control, respectively.

Collectively, it can be certainly stated that although inductions were observed in antioxidant defence system in *S. cerevisiae* BY4742, in the range of studied concentrations CLT leads to oxidative stress, oxidative damage and consequently death of yeast cells.

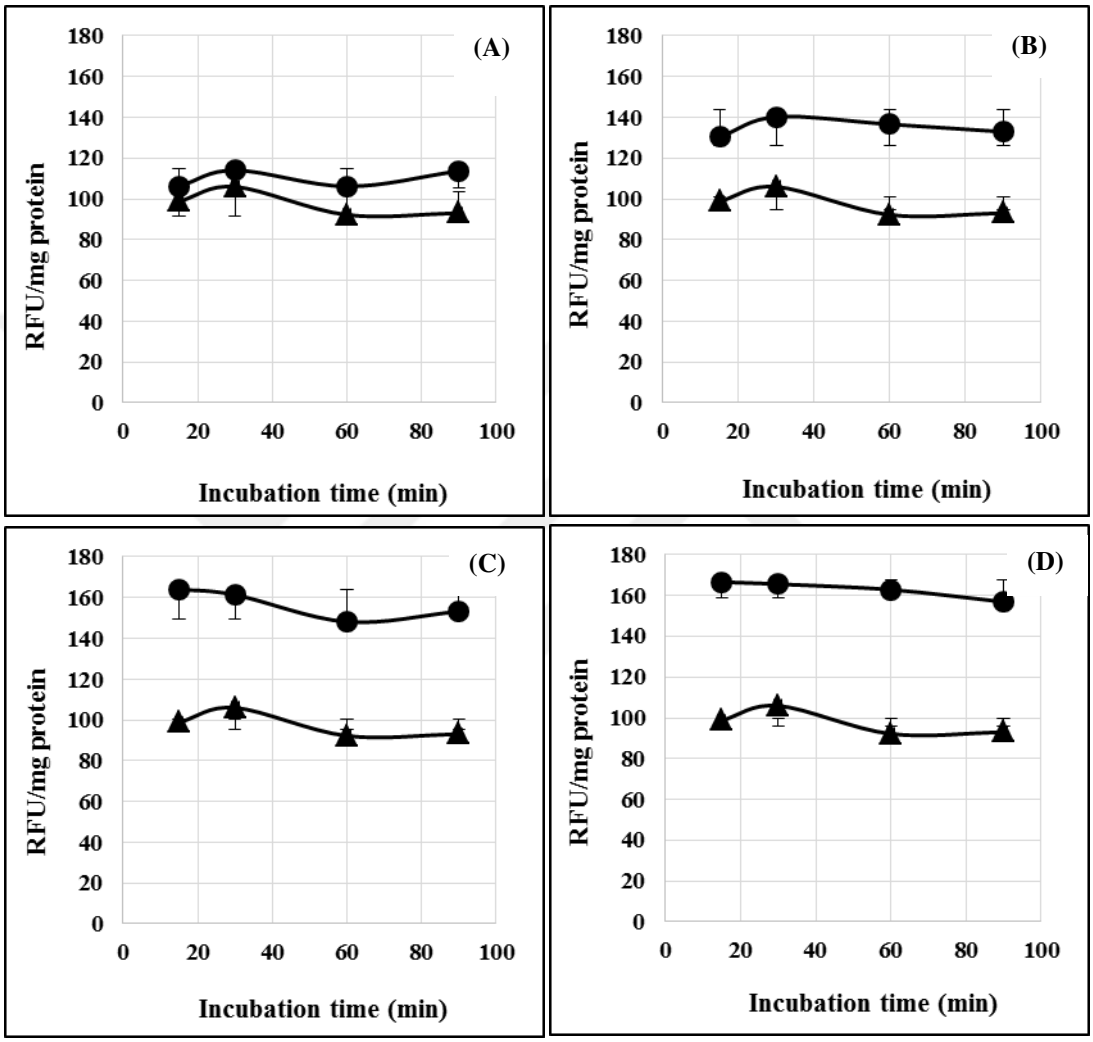

Figure 3.28 Variation in GSH:GSSG ratios depending on the incubation period and concentration of clotrimazole; control (–▲–) and clotrimazole treatments (–●–); 50 µM (A), 75 µM (B), 110 µM (C), 125 µM (D) for 90 min

CHAPTER FOUR
CONCLUSION

Clotrimazole (CLT, 3 1-(α-2-chlorotrityl)imidazole) is an azole derivative that is effective against a wide range of fungal pathogens. The fungistatic mechanism of CLT is associated with inhibition of sterol 14 α-demethylase and microsomal P-450-dependent enzymes. On the other hand, besides its antifungal action, CLT has diverse biological activities including growth inhibition of certain pathogens such as *Plasmodium falciparum*, induction of apoptosis in lung carcinoma, colon adenocarcinoma and leukemic lymphoblast cells and inhibition of angiogenesis and also irreversible inhibition of transient receptor potential (TRP) which is significant in the case of oxidative stress.

S. cerevisiae has been gradually became a preferred and popular research tool over years due to the fact that this organism can be easily handle and also genetically modified. In addition, high degree of conservation of basic cellular processes between yeast and higher organisms makes yeast cell a suitable model organism to investigate human diseases.

CLT-induced cell death in *S. cerevisiae* was firstly characterized in the content of this thesis. It was shown that apoptotic cell death percentage increased when cells exposed higher concentrations of CLT for short-term incubation and the highest apoptosis induction was detected in 110 μM CLT treatment for 30 min. This was most probably that exponentially growing *S. cerevisiae* BY4742 cells accumulated the agent more rapidly and within short incubation times. We further investigated the dependency of CLT induced apoptotic death to mitochondrial proteins including yca1p, aif1p, nuc1p and nma111p. When results were evaluated, it was seen that CLT induced apoptosis in *S. cerevisiae* is predominantly depend on loss of mitochondrial membrane potential, release of pro-apoptotic factors from mitochondria to cytosol and activation of yeast caspase, supportively yeast caspase activity increased 12.34-fold after drug treatment.

The effects of potential antineoplastic agent CLT on the oxidant-antioxidant system of *S. cerevisiae* were investigated at large range of CLT concentrations and treatment period which also include the condition that highest apoptosis induction was observed.

According to the results, it can be concluded that

- the levels of reactive species increased but generally stayed in a certain level, especially after 110 µM CLT treatment,
- antioxidant enzyme activities were generally induced significantly above control, but sharp decreases were observed in the end of incubation period, especially at 125 µM CLT,
- GSH:GSSG ratios significantly decreased relative to control values especially in higher concentrations of CLT,
- MDA and protein carbonyl levels which were significantly higher than control values were similar between 110 µM and 125 µM CLT treatments.

When all these results evaluated, it can be certainly said that CLT showed its destructive effect on *S. cerevisiae* cells at 110 µM and higher concentrations but for short treatment periods, the major death type is apoptosis that is tightly dependent on yca1p activity.

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