DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

CLARIFICATION OF APOPTOSIS PATHWAY IN *SACCHAROMYCES CEREVISIAE* DEPENDING ON KETOCONAZOLE INDUCED CONDITIONS

by

Burcu TONGUL

November, 2017

İZMİR

CLARIFICATION OF APOPTOSIS PATHWAY IN *SACCHAROMYCES CEREVISIAE* DEPENDING ON KETOCONAZOLE INDUCED CONDITIONS

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by
Burcu TONGUL

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Ph.D. THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"CLARIFICATION OF APOPTOSIS PATHWAY IN SACCHAROMYCES CEREVISIAE DEPENDING ON KETOCONAZOLE INDUCED CONDITIONS"** completed by **BURCU TONGUL** 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.

Prof. Dr. Leman TARHAN

Supervisor

Prof. Dr. Ayşegül PALA

Thesis Committee Member

Prof. Dr. Levent Cavas

Thesis Committee Member

Prof. Dr. Figen Zihnioğlu

Examining Committee Member

Prof. Dr. Tülin Aydemir

Examining Committee Member

Prof. Dr. Kadriye ERTEKİN
Director
Graduate School of Natural and Applied Sciences

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ABSTRACT

In the scope of this thesis, it was aimed to clarify the effect of ketoconazole which is an antifungal and promising anticancer drug, on the viability and induced cell death types in *Saccharomyces cerevisiae* BY4742. Cell viability, phosphatidylserine externalisation, chromatin condensation, DNA fragmentation, mitochondrial membrane potentials were examined in *S. cerevisiae* to decide the cell death types triggered by ketoconazole in time- and concentration- dependent manner. The apoptotic pathways were investigated through the mutant *S. cerevisiae* BY4742 cells in which the known most efficient apoptotic genes deleted. Furthermore, alterations in intracellular reactive oxygen species, activities of radical producing enzymes and the response of enzymatic and non-enzymatic antioxidant systems and lipid peroxidation and protein carbonyl levels were investigated in a time and concentration dependent manner. The predominant cell death was found as necrosis at relatively higher ketoconazole concentrations and longer treatment time. In addition, there wasn't any cell death with the treatment of lower concentrations of ketoconazole. On the other hand, after optimising apoptotic conditions, apoptotic cell death occurred in a large amount of cell population. It was found that ketoconazole induced more than one apoptotic pathway in *S. cerevisiae* and all the investigated proteins participated in this process and the lowest efficient protein was determined as yeast caspase. After the treatment of ketoconazole, although the antioxidant system of *S. cerevisiae* BY4742 was induced strongly, hydrogen peroxide, hydroxyl radical and total ROS levels were over the control values in all treatments. However, the levels of superoxide anion radical and nitric oxide decreased to below the control level at the end of the investigated incubation period. Additionally, due to the oxidative stress resulting from the imbalance between the ketoconazole-induced ROS formation and the antioxidant defence system, membrane lipid peroxidation and

protein carbonyl levels increased depending on ketoconazole concentration and incubation time.

Keywords: Ketoconazole**,** *Saccharomyces cerevisiae*, apoptosis, reactive oxygen species, antioxidant response system, lipid peroxidation, protein oxidation

SACCHAROMYCES CEREVISIAE DA KETOKONAZOL İNDÜKLÜ KOŞULLARA BAĞIMLI APOPTOZİS YOLAĞININ AYDINLATILMASI

ÖZ

Tez çalışması kapsamında, antifungal ve umut verici bir antikanser ilaç olan ketokonazolün *Saccharomyces cerevisiae* BY4742'deki sitotoksik ve indüklenen hücre ölümü tipleri üzerindeki etkisini belirlemek amaçlanmıştır. Ketokonazol tarafından indüklenen hücre ölüm türüne zaman ve konsantrasyona bağımlı *S. cerevisiae*'de hücre canlılığı, fosfatidilserin eksternelizasyonu, kromatin yoğunlaşması, DNA fragmentasyonu, mitokondriyal membran potansiyelleri incelenerek karar verilmiştir. İndüklenen apoptotik yolizleri, bilinen en etkin apoptotik genlerin silindiği mutant S. cerevisiae BY4742 hücreleri ile belirlenmiştir. Ayrıca, hücre içi reaktif oksijen türlerinde meydana gelen değişiklikler, radikal üreten enzimlerin aktiviteleri ve enzimatik ve enzimatik olmayan antioksidan sistemlerin yanı sıra lipid peroksidasyonu ve protein karbonil düzeyleri, zamana ve konsantrasyona bağımlı araştırılmıştır. Nispeten yüksek konsantrasyon ve uzun inkübasyon süresinde baskın hücre ölüm türü nekroz olarak belirlenmiştir. Ayrıca düşük konsantrasyonlarda hücre ölümü gözlenmemiştir. Bununla birlikte apoptotik koşulların optimizasyonu ile çok büyük oranda apoptotik hücre ölümü gözlenmiştir. Ketokonazol indüklü apoptoziste incelenen tüm proteinlerin etkisinin olduğu belirlenmiş olup en az etki maya kaspazdan kaynaklanmaktadır. Ketokonazol uygulamasına *S. cerevisiae*'nin antioksidan savunma sistemi güçlü bir şekilde cevap vermesine rağmen, hidrojen peroksit, hidroksil radikali ve total ROS düzeyleri tüm uygulamalarda kontrolün üstündedir. Bununla birlikte süperoksit anyon radikal ve nitrik oksit düzeyleri inkübasyon periyodunun sonlarında kontrolün altına düşmüştür. Ayrıca ketokonazol ile indüklenen ROS oluşumu ve antioksidan savunma sistemi arasındaki dengesizlikten kaynaklanan oksidatif stres nedeniyle, lipit peroksidasyonu ve protein karbonil seviyeleri, ketokonazol konsantrasyonuna ve inkübasyon süresine bağımlı artmıştır.

Anahtar Kelimeler: Ketokonazol, *Saccharomyces cerevisiae*, apoptozis, reaktif oksijen türleri, antioksidan savunma sistemi, membran lipit hasarı, protein hasarı

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CHAPTER ONE
INTRODUCTION

It has been proven that there are eight million and seven hundred thousand living things in the world (Yarza et al., 2008). On the other hand, it is thought that the discovery of all living species can be explored over a thousand years. Cellular structure is known as the most basic common feature of living things from the unicellular to multicellular organisms (Alberts et al., 2013). Understanding the structure, function and organisation of the cells provides opportunity to the researchers to be able to understand the life and develop new ideas about diseases and ageing.

1.1 Life in Cell

Cell is the smallest structural and functional building block of all organisms or populations (Arias, 2006). It has complex structure containing protein, nucleic acid and polysaccharides. According to cell types, some of them also contain functionally specialized organelles. The cell fulfils the functions related with being alive such as growth, excretion, movement, fertility, and communication at microscopic level (Kües, 2000). Due to this situation, the organism can perform these functions macroscopically.

Cells are classified into two main classes and are defined if they contain a nucleus or not (Woese, et al., 1990). While prokaryotic cells do not contain a nuclear membrane, eukaryotic cells have gathered all their genetic material into the nucleus and separated them from cytoplasm. Eukaryotic cells are bigger and more complex than prokaryotic cells. Beside presence of their nucleus, their genomic structures are more complicated and have cytoskeleton and cytoplasmic organelles.

1.1.1 Prokaryotic Cells

Prokaryotes, including several species of bacteria, can be divided into three groups: archaebacterial, eubacteria and cyanobacteria (Woese, et al., 1990). Archaebacteria can live in extraordinary environment conditions involving hot sulphurous areas, brines, extremely low pH values up to 2 and high temperatures reaching to 80 °C (Wharton, 2007). Eubacteria can survive in more moderate environments such as soil, water and air. Cyanobacteria live in water and have the ability to produce their own food by photosynthesis (Cord-Ruwisch et al., 1987). Although they do not have chloroplasts, they are capable of photosynthesis due to phycobiliproteins and are the most complex prokaryotes.

Most prokaryotic cells are rod-shaped, spherical or spiral, ranging in diameter from 1 to 10 μm (Kanhere & Bansal, 2005). The DNA content contains 0.6-5 million base pairs to encode about 5000 different proteins. A typical prokaryotic cell structure can be defined in *Escherichia coli*, which is commonly found in human intestines (Copper, 2000). The cell is rod-shaped, about 1 μm in diameter and about 2 μm in length. In *E. coli*, there is a cell wall composed of polysaccharides and peptides as in other prokaryotes (Bassler & Losick, 2006). There is a lipid bilayer cell membrane composed of phospholipids and proteins under the cell wall. Extracellular components can enter from the cell wall, but the cell membrane provides selective permeability. Unlike eukaryotic nuclei, DNA of *E.coli* is like a circular molecule with no nucleus membrane. There are about 30,000 ribosomes in the cytoplasm and this creates a granular appearance (Lodish et al., 2000). Because of their small size, simple diffusion is sufficient to distribute nutrients throughout the cytoplasm.

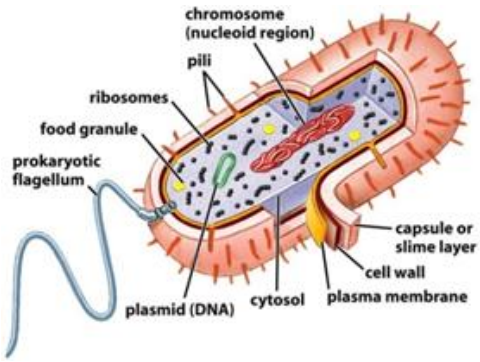


Figure 1.1 Structure of prokaryotic cells (Audesirk et al., 2008)

Some of the prokaryotes use CO_2 as a carbon source while others use organic molecules from dead plants and animals (Madigan et al., 1997). There are also species that is fed with inorganic compounds on rocks. One bacterium, *Thiobacillus concretivorans*, consumes metal-melting sulfuric acid (Kuenen & Robertson, 1992). The needs of prokaryotes to oxygen also vary from species to species. While some cannot live without oxygen, some are poisoned with oxygen (Halliwell, 1999). On the other hand, some of them can live with or without O_2 according to their adaptation to environment. Nitrogen-fixing prokaryotes can convert atmospheric nitrogen to ammonia. Plants and other organisms then use this ammonia to synthesize amino acids and nucleotides. Nitrifying bacteria living in the soil can transform ammonia to nitrate and nitrites, which can be also absorbed by plants. Denitrifying bacteria metabolizes these nitrates to N_2, allowing nitrogen atoms to pass from the soil to the air.

1.1.2 Eukaryotic Cells

All eukaryotic cells, similar with prokaryotic cells, are surrounded by plasma membrane and contain ribosomes (Lodish et al., 2000). However, eukaryotic cells are much more complex and contain a nucleus, various cytoplasmic organs and a cytoskeleton. The largest and most prominent organelles of eukaryotic cells are those with a diameter of about 5 μm. The nucleus contains the genetic information of the cell, arranged linearly in place of the circular DNA molecules in eukaryotes. Core DNA replication and RNA synthesis; the conversion of RNA into proteins occurs on the ribosomes in the cytoplasm.

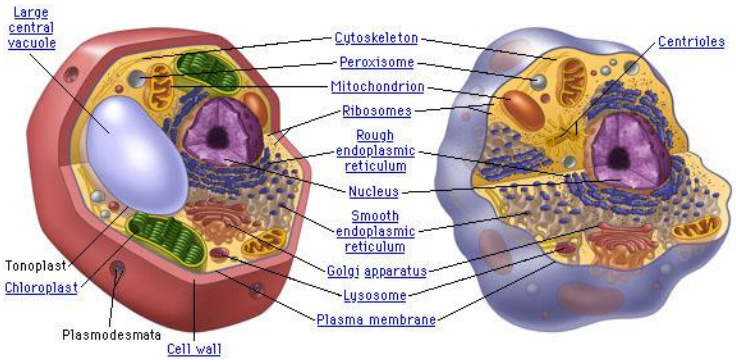


Figure 1.2 Structures of plant and animal cells (Audesirk et al., 2008)

Eukaryotic cells, in addition to their nuclei, also have organelles surrounded by various membranes in their cytoplasm (Lodish et al., 2000). These organelles are the areas where different metabolic activities take place. Eukaryotic cells usually have at least a thousand times more cell volume than prokaryotic cells. The division of labour provided by the cytoplasmic organelles allows eukaryotic cells to function effectively. Two of these organelles are mitochondria and chloroplasts, which play a critical role in energy metabolism. Mitochondria is presents in almost all eukaryotic cells undergo oxidative metabolism and are therefore responsible for producing a large proportion of the ATP produced by the breakdown of organic molecules. Chloroplasts are the areas where photosynthesis is occurred and are found only in plants and green algae cells. Lysosomes and peroxisomes have special metabolic functions for the digestion of macromolecules and various oxidative reactions, respectively. In addition, most plant cells contain vacuoles which have the task of digesting and storing nutrients and waste products.

Due to the complexity and size of eukaryotic cells, transporting proteins to the right places in the cell is a very difficult task (National Research Council, 1989). The endoplasmic reticulum and Golgi apparatus are specialized for the release of proteins, the separation and transport of proteins to the plasma membrane and lysosomes. Endoplasmic reticulum is a network system that provides a wide range of intracellular communication from nuclear membrane to cytoplasm. It functions both in the processing and transport of proteins as well as in the synthesis of lipids. Proteins are transported in small vesicles to the Golgi apparatus and processed to be transferred to the final destination with a series of modifications. Golgi apparatus also allows the synthesis of some of the polysaccharides that form the cell wall.

Eukaryotic cells have a network of protein filaments throughout the cytoplasm (Alberts et al., 2002). The cytoskeleton regulates the cytoplasm and forms the shape of cell. It is also responsible for the intracellular movements of the organelles and the movement of the whole cell.

Eukaryotic cells can be unicellular or multicellular while prokaryotes are only unicellular (Michod, 2007). Unicellular cells have the ability of self-replication and create their own population quickly (Sipper, 1998). One of the simplest eukaryotes is yeasts. Yeast is smaller and simpler compared to cells of animals and plants but more complex and larger than bacteria (Botstein et al., 1997). On the other hand, unicellular cells also have almost all of the metabolic activities possessed by multicellular eukaryotes. Although it is similar to prokaryotes in terms of rapid production and easy control, the eukaryotic nature gives them a functional advantage. The high similarity between the higher organisms and the yeast in terms of basic cellular processes and the easy genetic manipulations have made unicellular eukaryotes, especially yeast, valuable for investigation of molecular and cellular pathways underlying mammalian diseases (Hartwell, 2004).

In multicellular organisms, cells with different functions come together to form tissues. The function of tissue is dependent on the types and coordination of the cells in it (Bryant & Mostov, 2008). For example, some cells in the endothelial tissue in the human gastrointestinal tract provide absorption of nutrients, while some cells secrete lubricious mucus. In addition to the functions of the cells in a tissue, transcriptional programs are also different and the cells are divided at different rates. Proper planning of these rates is necessary for tissue function and repair. Localization of cells in tissue is also very important. In addition, signals from neighbouring cells or extracellular matrix significantly affect the regulation of cells in a tissue.

Plant cells contain three main internal tissue systems, including the ground tissue and the dermal tissue (Evert, 2006). Cells found in animals are more different and diverse than plant cells. For example, the human body contains more than 200 different cell types known as the cells of five major types of tissue (epithelial tissue, connective tissue, blood, nerve tissue and muscle).

All of these cell types are subject to aging processes so they will die in order to protect their surrounding cells and younger generations (Cooper, 2000). About a

century ago, animals with high metabolic rates were found to have shorter life span. According to this information, it has been revealed that the aging process of the organism is not only dependent on exogenous factors but also on endogenous factors. In the mid-1950s, Denham Harman defined the "free radical theory" of aging and claimed that oxygen radicals are produced endogenously in cells and cause cumulative damage (Harman, 2002). Most of the energy needed by the cell is produced by mitochondria and consumes cellular oxygen in this mechanism. For this reason, the free radical theory of aging is thought to be similar with the hypothesis of rate-of-living. That is, the higher the metabolic rate of an organism, the higher the production of reactive oxygen species (ROS) and therefore the shorter the life span. However, this important correlation between metabolic rate and life span is not seen in some species (Speakman, 2005). This is especially valid for birds and primates. The studies have shown that mitochondria produce less ROS compared to other species at a given metabolic rate in these species. This suggests that long life span is correlated with ROS production rather than the rate of metabolism. The various reactive species can either be generated exogenously or produced intracellularly from several different sources.

ROS can be produced from many different sources exogenously or endogenously (Finkel & Holbrook, 2000). Intracellular sources of ROS can be mainly specified as mitochondria, peroxisomes, NAD(P)H oxidases, lipoxygenase and cytochrome p450 enzyme systems (Holmström & Finkel, 2014). Potential sources of ROS production are phagocytic cells such as polymorphonuclear leukocytes (neutrophils), monocytes and macrophages (Klebanoff, 1980). These cells use the NADPH oxidase enzymatic system to directly produce the $O_2^{\bullet -}$ radical as part of defence systems against the invading microorganisms. In physiological conditions, the $O_2^{\bullet -}$ radical (and related species) produced by NADPH oxidase must be directed towards the microorganisms encapsulated by phagocytic phagolysosomes. Additionally, H_2O_2 can be converted to HClO with myeloperoxidase (Kettle, & Winterbourn, 2001; Winterbourn, et al., 2006). HClO can react with the $O_2^{\bullet -}$ radical to produce H_2O_2. These produced ROS enable bacteria to be killed effectively, so they are very important for the immune system. Furthermore, there may be toxic metabolite production or e^- escapes in the

P450 catalytic cycle, resulting in the $O_2^{\bullet -}$ radical and H_2O_2 (Guptaet al., 1997). Additionally, the tissues under chronic inflammatory conditions are directly affected by $O_2^{\bullet -}$ radical and its metabolites at high levels. Such chronic inflammatory conditions are characteristic of many diseases such as rheumatoid arthritis, lupus erythematosus and psoriatic arthritis. Various mammalian cell types express NADH- and/or NADPH-dependent oxidases on the membrane surfaces (Afanas'ev, 1991). Such oxidase complexes function similarly to phagocytic cell NADPH oxidases and protect the cell by forming an "antiseptic superoxide pool" against bacterial/fungal infections. Exogenous sources of ROS for cells are ultraviolet light, ionizing radiation, chemotherapeutics, inflammatory cytokines and environmental toxins (Babizhayev, 2016). The cumulative damage of cells caused by endogenously or exogenously produced ROS causes the loss of cell functions. At this point, the cells must die under control to protect whole organism's health (Duke et al., 1196). There are many cell death types such as apoptosis, necrosis, autophagy, and cornification. Through the controlled cell death types, the dead cells are removed from the specific immune system cells without causing any inflammation and damaging the surrounding cells. The most important, effective and investigated cell death type among cell deaths is apoptosis, which involves highly complex energy-dependent control mechanisms (Elmore, 2007). On the other hand, if the imbalance occurs between cell death and growth, lots of disease including Alzheimer's disease, Parkinson's disease, chronic lung disease, diabetes and cancer arise (Dong et al., 2009).

To determine the causes of diseases and treatment methods, model organisms are commonly used in researches (Engel, 1989). Model organisms are a group of research organisms used to understand human biology, diseases and treatment methods. These are usually yeast, fruit nymphs, worms, zebrafish and mice (Hariharan & Haber, 2003). Many aspects of the biology of these organisms are similar, including metabolisms, pathways, detoxification mechanisms, to those of humans and have much knowledge about the genetic structure. For this reason, conducting research with these model organisms helps scientists learn more about human health. The appearance of a human disease and its symptoms can last for

decades. In contrast, in a model organism, a disease can be quickly developed. In this way, researchers learn with more information about the disease.

Yeast cells are widely used as model organisms (Guarente & Kenyon, 2000). Among these studies, cell proliferation and death studies were carried out (Madeo et al., 2002). Since a great deal of cancer drugs interfere with the cell cycle, many researchers who have conducted cancer researches have provided crucial information using yeast. It also allowed us to understand how the genes behave in the disease mechanisms. Yeast is a common term for single-cell ascomycetous and basidiomycetous fungi (Flegel, 1981). Most yeast (such as *Saccharomyces spp*.) buds as a small "girl cell" from the "mother cell" and grows gradually. Some (such as *Schizosaccharomyces spp*.) multiplies by fission and form a septum, dividing the cell into two equal-sized cells. Most yeast can also multiply with meiosis. Meiosis (sporulation) results in the formation of haploid spores (ascuspores, basidiospores, respectively) from a diploid cell exhibiting different mating types. The haploids of *S. cerevisiae* secrete specific mating pheromones (a or α factor) that cause the mating process and bind to the receptors of the other mating pathway cells (MTa or MTα, respectively), bringing the diploid cell.

S. cerevisiae is the best researched yeast and probably the best known eukaryotic organism (Galao et al., 2007). In 1996, the genome was fully clarified and it is widely preferred model organism in many areas of cell biology (Botstein et al., 1997). Although it is similar to bacteria in terms of rapid production and easy control, the eukaryotic nature of *S. cerevisiae* cells gives them a functional advantage. The high similarity between the higher organisms and the yeast in terms of cellular processes and easy genetic manipulations in yeast have made *S.cerevisiae* the most advantageous model organism that has been used successfully to study the molecular and cellular pathways underlying mammalian diseases (Hartwell, 2004).

In addition to other cellular processes, the apoptotic mechanism of the human apoptosis has provided high degree of resemblance to apoptotic processes of which deregulation resulting in neoplasia, neurodegenerative diseases, and an appropriate

model organism that can provide answers to the pending questions in the aging process (Kazemzadeh et al., 2012). In recent years, *S. cerevisiae* have been used as eukaryotic model organism in the studies concerning the clarification of apoptosis mechanisms induced by various environmental stresses because of having similarity with the mammalian cells, especially in terms of intrinsic pathway (Madeo et al., 1999; Ludovico, 2001; Apel and Hirt, 2004; Eisenberg et al., 2007; Carmona-Gutierrez, 2010). With the increasing interest in researches conducted with yeast apoptosis, many genes, which express the homolog proteins with mammalian cells, were found. Some of these are metacaspase (Yca1/Mca1) (Madeo et al., 2002), homologous of htra2 / OMI; Nma111 (Fahrenkrog et al., 2004), the two different homologous of AIF/AMID; Aif1 and Ndi1 (Wissing et al., 2004) and the homolog of proapoptotic Endo G; Nuc1 (Li et al., 2001; Wissing et al., 2004).

As mentioned above, the most important inducer of aging, neurodegenerative disease and cancer is oxidative stress caused by the imbalance between intracellular ROS production, antioxidant and repair mechanisms. As a result of this imbalance, cell might die via programmed cell death or necrosis or the cells become immortalize and cancer occurs.

1.2 Reactive Oxygen Species and Oxidative Stress

Eukaryotic aerobic organisms cannot exist in an anaerobic condition, at the same time; the nature of oxygen poses a great danger to their existence (Davies, 1995). This negative effect of oxygen is directly related to the fact that every oxygen atom has an unpaired electron and O_2 has two unpaired electrons in their outer orbitals (Boveris, 1998). 95% of the intracellular O_2 gives the 4 e-reduction reaction catalysed by the cytochrome oxidase (cytochrome c: oxygen oxidoreductase) enzyme of complex IV to produce water.

$$O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$$

The cytochrome oxidase is terminal e-receptor in mitochondrial electron transport system (ETS) and should give up the reducing equivalents to continue e-transport; if the electrons stop flowing through the electron transport chain, the proton motive force (PMF) is disturbed and ATP production cannot continue. Therefore, the main role of O_2 for all aerobic organisms in the electron transport system is to act as a ground for discharge electrons. Although the mitochondrial electron transport chain is effective system, it is possible that each electron carrier can undergo side reactions with O_2 due to the nature of the single electron oxidation and reduction reactions (Cadenas & Davies, 2000). The tetravalent reduction of oxygen to produce water by the mitochondrial electron transport chain (ETS) is considered to be a relatively safe mechanism (Galli et al., 2005). In addition to this, the monovalent reduction of O_2 causes the production of reactive oxygen species.

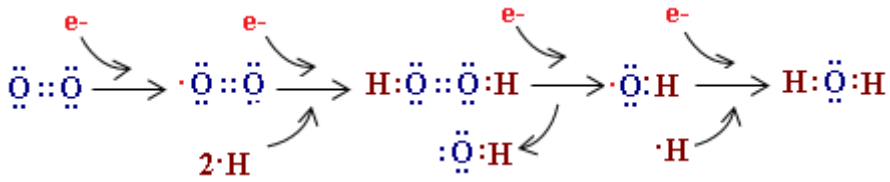


Figure 1.3 Monovalent pathway for oxygen reduction

The reductive property of the cellular environment provides oxygen with a monovalent reduction (Ziech et al., 2010). In this case, the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the excessively reactive hydroxyl radical ($HO^{\bullet}$) are common products of life in an aerobic environment, these intermediate components appear to be responsible for oxygen toxicity. In addition, singlet oxygen ($^1O_2$) is another reactive species originating from O_2, but redox reactions are not responsible for the production of this species (Turrens, 2003). Aerobic organisms produce antioxidant compounds that dissolve in water or lipid to survive in a highly toxic oxygen environment. Furthermore, a number of antioxidant enzymes whose role is to scavenge and inactivate the reactive oxygen intermediates are synthesized by all known aerobic organisms.

ROS can be produced by endogenous and exogenous sources. Potential endogenous sources include mitochondria, cytochrome P450 metabolism,

peroxisomes, and inflammatory cell activation (Inoue et al., 2003). Oxygen based toxic metabolites, known as reactive oxygen species (ROS), can be radicals ($O_2^{\bullet -}$, $HO^{\bullet}$, peroxyl ($RO_2^{\bullet}$), and nitric oxide $NO^{\bullet}$) and also non-radical (hypochlorous acid (HOCl), $^1O_2$, peroxynitrite (ONOO-), ozone (O_3), and H_2O_2 (Eberhardt, 2000). Although being very important, antioxidant enzymes and compounds are not entirely effective in preventing ROS-mediated cellular damages. A series of damage-repair enzymes for protein, lipid and DNA are also synthesized to cope with oxidative stress-induced damage caused by the imbalance of the ROS and antioxidant system (Davies, 2000). Furthermore, since oxidative stress exposure levels can change from time to time, organisms can adapt to the stressful environments by leading to the synthesis or induction of antioxidant and damage-repair enzymes (Parsons, 2005). Despite the stated antioxidant, repair and adaptation mechanisms, oxidative damage is an inevitable consequence of oxygenated life.

Table 1.1 Half Life of Reactive Oxygen Species

Name of ROS	**Symbol**	**Half Life**
Singlet Oxygen	$^1O_2$	10^{-6} second
Superoxide Anion Radical	$O_2^{\bullet -}$	10^{-6} second
Hydrogen Peroxide	H_2O_2	Minutes
Hydroxyl Radical	$HO^{\bullet}$	10^{-9} second
Hydroperoxyl Radical	$HOO^{\bullet}$	Second
Lipid Peroxide Radical	$ROO^{\bullet}$	Second
Nitric Oxide	$NO^{\bullet}$	Second
Peroxy Nitrite	$ONOO^{\bullet -2}$	10^{-3}
Hypochlorous acid	HClO	<1 minute
Ozone	O_3	<15 min

Oxidative stress can be defined as the damage state that occurs in biomolecules with increasing intracellular oxidation level and the imbalance between antioxidant system and ROS production (Halliwell, 2006). The size of the oxidative stress exposed to cells, tissues, or organisms can be understood as a consequence of the

oxidative damage of biologically important biomolecules, such as proteins, lipids, carbohydrates and nucleic acids (DNA and RNA). It has been associated with chronic inflammatory diseases such as rheumatoid arthritis, lupus erythematosus and psoriatic arthritis, acute inflammatory problems, photo oxidative stress such as cataracts, some familial amyotrophic lateral sclerosis forms, several glutathione peroxidase-associated adolescent seizures, central nervous system disorders such as Parkinson and Alzheimer's, dementia disease and syndrome (Rani et al., 2016). These oxidation-dependent diseases may also be initiated or worsened by numerous environmental pro-oxidants, pro-oxidant drugs and foods.

Radicals are components that have high reactivity and a very short half-life due to their unpaired electrons in their last orbital (Reczek & Chandel, 2015). For this reason, oxidative damage is occurred by these molecules, reacting with other molecules, especially vital intracellular biomolecules such as lipid, protein and DNA.

1.2.1 The Formation of ROS and Their Intracellular Effects

1.2.1.1 Singlet Oxygen ($^1O_2$)

$^1O_2$ is not only produced by redox reactions, but also by the absorption of electromagnetic energy that temporarily reverses the spin of one in two unpaired electrons of oxygen; so that both spins reach anti-parallel orientation (Foote & Denny 1968). O_2 cannot accept two e^- directly (bivalent reduction) because the addition of one pair of parallel-electrons is restricted to the spin-parallel state of e-. However, there are anti-parallel e^- spins in $^1O_2$, and since there is no spin restriction, it is a very suitable two-electron oxidant for many biomolecules (Wijk et al., 2008). However, in order to produce $^1O_2$, a large amount of electromagnetic energy must be absorbed by O_2, which is largely limited to photochemical events. The interest in $^1O_2$ is therefore largely focused on environmental chemistry, plant biology, skin and oxidative reactions in the eye (Davies, 1995).

1.2.1.2 Superoxide anion radical ($O_2^{\bullet -}$)

$O_2^{\bullet -}$ production is mainly occurred in mitochondria responsible for ATP synthesis in cells (Cadenas & Sies, 1998). During energy production, electron leak is formed in mitochondria and the $O_2^{\bullet -}$ radical is generated when these electrons are transferred to O_2. In the electron transport chain, a ubiquinone that can be found as quinone (fully oxidized), semiquinone (e^--reduced) and quinol (completely reduced with two e^-) tends to give electrons directly to O_2 rather than to the other carrier in the chain. In addition, the iron-sulphur units in the respiratory chain also give rise to side reactions with oxygen that produce the $O_2^{\bullet -}$ radical (Bresgen et al., 2015). On the other hand, the $O_2^{\bullet -}$ radical participates in the dismutation reaction to oxidize itself to O_2 and reduce to H_2O_2 (Halliwell, & Gutteridge, 1999).

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

This reaction is faster at the acidic pH than at the alkaline pH. $O_2^{\bullet -}$ radical has functions as both reducing and oxidizing agent in the cell (Robertson, 2010). The interaction of $O_2^{\bullet -}$ radical with iron ions is of particular importance for organisms. $O_2^{\bullet -}$ can reduce Fe (III), as well as oxidize Fe (II). While it can cause the reduction of cytochrome c and plastocyanin which are involved in the reactions of ETS and photosynthesis, respectively, it causes the oxidation of ascorbic acid (AH_2) (Takahashi & Asada, 1983).

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

$$Cyt\text{-}c\text{-}Fe(III)_{red} + O_2^{\bullet -} \longrightarrow O_2 + Cyt\text{-}c\text{-}Fe(II)_{ox}$$

$$Plastocyanin\text{-}Cu(II)_{red} + O_2^{\bullet -} \longrightarrow O_2 + Plastocyanin\text{-}Cu(I)_{ox}$$

$$AH_2 + O_2^{\bullet -} \longrightarrow A^{\bullet -} + H_2O_2$$

$O_2^{\bullet -}$ radical does not have ability to oxidize NADH or NADPH in measurable levels (Zorov et al., 2014). However, it may interact with the active site of lactate dehydrogenase, which contains NADH and at the end produce NAD• radical (Chan & Bielski, 1974). Additionally, NAD(P)H oxidase enzyme has a vital role as one of

the important sources of $O_2^{\bullet -}$ radicals, especially in vascular, endothelial and phagocytic cells. NAD(P)H oxidase allows e^- to pass through the membrane, and these electrons bring the $O_2^{\bullet -}$ radical when O_2 is present (Selemidis et al., 2008). Various mammalian cell types express NADH- and/or NADPH-dependent oxidases on the membrane surfaces. Such oxidase complexes function similarly to phagocytic cell NADPH oxidases and protect the cell by forming an "antiseptic superoxide pool" against bacterial/fungal infections (Afanas'ev, 1991).

$$\text{Enzyme-NADH} + O_2^{\bullet -} + H_2O_2 \longrightarrow \text{Enzyme-NAD}^{\bullet} + H_2O_2$$

$$2O_2 + \text{NADPH} \xrightarrow{\text{NADPH Oxidase}} O_2^{\bullet -} + \text{NADP}^+ + H^+$$

Furthermore, there may be toxic metabolite production or e- escapes in the P450 catalytic cycle, resulting in the $O_2^{\bullet -}$ radical and H_2O_2 (Gupta et al., 1997). Additionally, tissues under chronic inflammatory conditions are directly affected by $O_2^{\bullet -}$ radical and its metabolites at high levels. Such chronic inflammatory conditions are characteristic of many diseases such as rheumatoid arthritis, lupus erythematosus and psoriatic arthritis.

The reactivity of $O_2^{\bullet -}$ radical is very low at physiological conditions (pH 7.4) so the direct biological damage that can be caused by the $O_2^{\bullet -}$ radical is very limited and requires interaction with the other components such as iron ions that are located in iron-sulphur proteins or NO• radicals (Grune, 2005).

$O_2^{\bullet -}$ accelerates the Fenton reaction, one of the reactions responsible for the HO• radical generation by forming Fe(II)ox. Additionally, $O_2^{\bullet -}$ also directly cause HO• radical formation via Haber-Weiss reaction (Kehrer, 2000);

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

$$Fe(III)_{ox} + H_2O_2 \longrightarrow Fe(III)_{red} + HO^{\bullet} + HO^- \qquad \text{Fenton Reaction}$$

$$O_2^{\bullet -} + H_2O_2 + H^+ \longrightarrow O_2 + H_2O + HO^{\bullet} \qquad \text{Haber-Weiss Reaction}$$

$O_2^{\bullet-}$ is not reactive against lipids, carbohydrates or nucleic acids, but interacts with some proteins and causes damage (Bergamini et al., 2004). The data obtained so far have shown that $O_2^{\bullet-}$ causes damage to the prosthetic groups of proteins containing transitional metals such as haem or Fe/S. These transitional metal-mediated reactions usually result in the loss of the active side or proximal amino acids and protein/enzyme function directly in the metal catalyst. A good example of these reactions is the loss of the aconitase activity of Fe/S centres after exposure of the bacteria to the $O_2^{\bullet-}$ radical (Bulteau et al., 2003). One of the most important reactions of $O_2^{\bullet-}$ radical is a reaction with another $O_2^{\bullet-}$ radical which is called dismutation reaction.

1.2.1.3 Hydroxyl radical ($HO^{\bullet}$)

$HO^{\bullet}$ radical is one of the most toxic radicals having a half-life of about 10^{-9} seconds. Due to its relatively short half-life, this radical can interact with any molecule in the environment in which it is formed (Pastor et al., 2000). The $HO^{\bullet}$ radical can be produced in biological systems by many reactions.

One of these is, as mentioned above, the Fenton reaction, catalysed by transition metals. In fact, Fe ions in organisms are not free but under stress conditions, $O_2^{\bullet-}$ radical breaks Fe from Fe-containing molecules and makes it free (Valko et al., 2007).

In the sun-exposed skin, $HO^{\bullet}$ is produced by the homolytic fission of the O-O bond in the UV-exposed H_2O_2 (Schallreuter et al., 1991). In addition, $HO^{\bullet}$ can also be produced during the decomposition of O_3 and peroxynitric acid and also ethanol metabolism. Other $HO^{\bullet}$ sources include ionizing radiation, reaction between hypochlorous acid and $O_2^{\bullet-}$ radical, ultrasonography and lithotripsy (Graves, 2012).

1.2.1.4 Hydrogen peroxide

H_2O_2 is one of the non-radical reactive species, it can easily diffuse into the cell with high concentrations and causes the oxidation of cellular biomolecules, including

in electron transport systems which is responsible for energy formation (Rahal, 2014). Many enzymes found in vivo, such as xanthine, urate and D-amino acid oxidases, can produce H_2O_2 (Allegra et al., 2003). In addition, it is formed by dismutation of $O_2^{\bullet -}$ in many biological systems (Henle& Linn, 1997) . H_2O_2 is a weak oxidant and reducing agent. It is known that H_2O_2 has the ability to directly affect enzymes by causing the oxidation of basic thiol groups in the active sites of the enzymes (Birben et al., 2012).

If DNA, lipids and proteins are incubated with H_2O_2, oxidation may not appear even at milimolar levels, but H_2O_2 can be easily diffused into membranes and despite being a weak ROS compared to other species, it can react with Cu and Fe ions in the cells to produce hydroxyl radical with Fenton and Haber-Weiss reactions (Imlay et al., 1988). In this case, H_2O_2 indirectly damages biomolecules (Spencer et al., 1995).

Proteins containing Fe or Cu prosthetic groups can also undergo reactions with H_2O_2 that result in metal oxidation. For example, hemoglobin can react with H_2O_2 to produce a ferryl-hemoglobin (Fe^{4+}) (Winterbourn, 1985). Such high oxidation states give similar damage with $HO^{\bullet}$ radical, which causes significant oxidative damage. The main difference is that $HO^{\bullet}$ is a free radical that reacts almost at diffusion controlled rates. On the contrary, components such as ferryl-hemoglobin have less mobility, limited reproducibility, and the presence of the metal on the protein and the potential presence of the site determine the availability of the radical (Goldman, 1998).

1.2.1.5 Peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals

Peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals are other oxygen-derived radicals. (Riley, 1994). The $RO^{\bullet}$ radical formed in biological systems often undergoes a rapid molecular change to transform into other radical species. The simplest peroxyl radical is hydroperoxyl ($HOO^{\bullet}$) which is the protonated form of $O_2^{\bullet -}$ radical. These radicals are mainly formed when lipid peroxidation chain reactions begins. $ROO^{\bullet}$

radical oxidize ascorbate and NADH, leading to the formation of $O_2^{\bullet -}$ in the presence of O_2:

$$ROO^{\bullet} + NADH \longrightarrow RO_2H + NAD^{\bullet}$$
$$NAD^{\bullet} + O_2 \longrightarrow NAD^{+} + O_2^{\bullet -}$$

$ROO^{\bullet}$ and $RO^{\bullet}$ radicals can break H^+ from other molecules, an important reaction to lipid peroxidation (Buettner, 1993). Some $ROO^{\bullet}$ species may form the radical $O_2^{\bullet -}$. In addition, when $HO^{\bullet}$ radical reacts with glucose, 6 different $ROO^{\bullet}$ can be formed. However the aromatic alkoxyl and peroxyl radicals are less active because e^- can delocalize to the benzene ring (Sisein, 2014).

1.2.1.6 $NO^{\bullet}$ Radical

$NO^{\bullet}$ radical can easily diffuse into the cell (Beckman & Koppenol, 1996). One electron reduction of $NO^{\bullet}$ causes the formation of the nitroxyl anion ($ONNO^{\bullet -}$). $ONNO^{\bullet -}$ is a reactive and short-lived species and gives reaction with $NO^{\bullet}$ to form nitrogen oxides, N_2O and $HO^{\bullet}$ radical.

$$NO^{\bullet} + NO^{\bullet} \longrightarrow ONNO^{\bullet -}$$
$$ONNO^{\bullet -} + NO^{\bullet} \longrightarrow N_2O + NO_2^{\bullet -}$$
$$ONNO^{\bullet -} + H^{+} \longrightarrow N_2O + HO^{\bullet}$$

Nitric oxide synthases (NOS) which metabolize arginine to citrulline, leading to the formation of $NO^{\bullet}$ (Ghafourifar, & Cadenas, 2005). Cells, responsible for the immune system during inflammatory processes, produce $O_2^{\bullet -}$ and $NO^{\bullet}$. Under these conditions, $O_2^{\bullet -}$ and $NO^{\bullet}$ can react to produce peroxynitrite (ONOO-), which has relatively high reactivity and causes DNA fragmentation and lipid peroxidation (Carr, McCall, & Frei, 2000).

1.2.1.7 Hypochlorous acid (HClO)

Hypochlorous acid (HClO) is one of the non-radicalic ROS. When an inflammation occurs, myeloperoxidase produces HClO (Weiss, 1989). H_2O_2 can be converted to HClO with myeloperoxidase (Kettle, & Winterbourn, 2001; Winterbourn et al., 2006). This HClO can react with the $O_2^{\bullet-}$ radical to produce H_2O_2. These produced ROS enable bacteria to be killed effectively, so they are very important for the immune system. Although the importance of phagocytosis in the bactericidal effect is unclear, HClO can damage biomolecules both directly and through chlorine at this reaction. In addition, HClO can oxidize the thiols, mercury, NAD(P)H, and can lead to the chlorination of DNA bases, especially pyrimidines and tyrosine residues in proteins.

1.3 Antioxidant Defence Systems

Only aerobic organisms can survive in the presence of oxygen because these organisms have developed a number of defence mechanisms to protect themselves from the toxicity of oxygen, ROS and other reactive metabolites (Cadenas, 1997). Antioxidant defence systems include enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidases, which catalytically degrade ROS and other free radicals; (Scandalios, 2005), non-enzymatic antioxidants such as metallothionin, hemopexin and low molecular weight ROS scavenger molecules such as heat shock proteins, glutathione, α-tocopherol and ascorbic acid.

1.3.1 Enzymatic Antioxidant System

The enzymatic antioxidant system has vital role in all aerobic cells. The exposure time of the cells to ROS is greatly reduced due to their ability of removing ROS catalytically.

1.3.1.1 Superoxide dismutase (SOD) (EC1.15.1.1.)

SOD is a metalloenzyme, which catalyses the degradation of $O_2^{\bullet -}$ radical to O_2 and H_2O_2, less reactive species than $O_2^{\bullet -}$ radicals (Dat et al., 2000).

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

SOD has several isoforms such as Cu/ZnSOD, MnSOD and FeSOD. Cu/ZnSOD is found in almost all eukaryotic cells. It is known that Cu/ZnSOD is present in cytosol, lysosomes, peroxisomes, nuclei and mitochondrial membranes (Halliwell, & Gutteridge, 1999).

MnSOD catalyses the same reaction with Cu/ZnSOD, despite their structural differences (Perry et al., 2010). MnSOD is common in bacteria, plants and animals. MnSOD is located in mitochondria in most animal tissues and yeasts. The relative activity of MnSOD and Cu/ZnSOD depends on the tissue and species. The apparent variable is the number of existing mitochondria, for example mammalian erythrocytes, which do not contain mitochondria, does not contain MnSOD.

FeSOD usually contains two subunits, one of which may contain one iron and the other of which contains two irons (Noodleman & Han, 2006). FeSOD has lower activity at higher pH compared with pH 7.0. The rate constant for the $O_2^{\bullet -}$ radical is very low compared to other SOD species. Some bacterial species and plant tissues contain FeSOD, but not found in animals. The intracellular localizations of SOD species with different prosthetic groups also differ.

Yeast cells contain two genes encoding the SOD enzyme; SOD1 encodes Cu/ZnSOD in cytoplasm and SOD2 encodes MnSOD in mitochondria, as well as many eukaryotes (Gralla and Kosman, 1992).

It has been shown that Cu/ZnSOD and some part of its metalochaperone are localized together in the mitochondrial intermembrane space and this enzyme helps

to protect mitochondria against oxidative damage. Mutation of the SOD1 gene resulted in increased carbonylation of mitochondrial proteins and expression of Cu/ZnSOD prolonged the viability of yeast cells in the stationary phase (Sturtz et al., 2001).

Treatment of paraquat and O_2 increases the level of intracellular Cu/ZnSOD in non-fermentable carbon sources (Lee & Hassan, 1985; Gralla & Kosman, 1992) and also the activity is increased in copper ion exposure (Lee and Hassan, 1985; Greco et al, 1990). Transcription of the SOD2 gene encoding mitochondrial MnSOD is also induced by growth in oxygenated media or with non-fermentative carbon sources and by treating cells with superoxide-producing agents such as paraquat. In addition, SOD in some strains is more induced than paraquat when they are in post-diauxic growth phase in the starvation or in glucose environments (Lowry and Zitomer, 1984; Westerbeek-Marres et al., 1988; Chang et al., 1991; Galiazzo and Labbe-Bois, 1993). MnSOD provides a great defence against $O_2^{\bullet -}$ toxicity and one of its important roles is to protect mitochondrial Fe-S enzymes such as aconitase from damage caused by superoxide (Longo et al., 1999). With the deletion of both SOD genes, *S. cerevisiae* mutants could not synthesize methionine and lysine and also sporulation deterioration and mutation rate increased (Liu et al., 1992). Strains in which the SOD1 and SOD2 genes are deleted are hypersensitive to O_2. When cells were incubated in an unfermentable carbon source and at low oxygen ratios, both single gene mutant strains and double gene mutant strains grew, but at high oxygen ratios, the growth of single gene mutants decreased and the double gene mutant strains did not grow (Longo et al., 1996).

1.3.1.2 Catalase (CAT) (EC 1.11.1.6.)

Catalase is a water-soluble enzyme and catalyses the reaction of H_2O_2 to H_2O and O_2 (Qi et al., 2007). Most aerobic cells contain catalase enzyme (Deisseroth & Dounce, 1970). Very few aerobic cells such as *Bacillus popilliae,* mycoplasma pneumoniae, and euglena do not contain catalase. Catalase is found at high amounts in blood, bone marrow, liver, kidney and mucous membranes. The catalase in the

erythrocytes helps to protect them against H_2O_2. Brain, heart and skeletal muscle contain lower catalase levels than other tissues (Kang et al., 1996). Mitochondria, chloroplasts and endoplasmic reticulum contain catalase at low amounts.

In the cases where the formation rate of H_2O_2 is low, the catalase reduces H_2O_2 to H_2O by peroxidative reaction. At high H_2O_2 formation rate, catalase catalytically oxidizes H_2O_2 to O_2.

$$H_2O_2 + AH_2 \longrightarrow 2H_2O + A$$
$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2$$

S. cerevisiae contains two isoform of haemprotein catalase. CTA1 gene encodes Catalase A, located in peroxisomes and CTT1 encodes cytosolic catalase T (Cohen et al., 1985, Spevak et al., 1986; Ruis and Hamilton, 1992). These two catalase isoforms do not have the ability to react with larger hydroperoxides such as tert-butyl hydroperoxide (Ruis and Hamilton, 1992).

1.3.1.3 Glutathione peroxidase (GSHPx) (EC 1.11.1.19.)

Glutathione peroxidase (GSHPx) reduces H_2O_2 to H_2O by oxidation of reduced glutathione (GSH) (Halliwell, & Gutteridge, 1999). GSH is the substrate of GSHPx, a low molecular weight, thiol containing tripeptide. It is usually found at milimolar levels in animals, plants and many aerobic bacteria, but rarely in anaerobic bacteria.

$$H_2O_2 + 2GSH \longrightarrow GSSG + H_2O$$

Glutathione peroxidase is not found in advanced plants. Its presence is reported only for a few algae and fungi, including *S. pompe, S. cereviaise, P. chrysosporium* (Morel, 2009; Tanaka, 2005; Yamada, 1999). Glutathione peroxidases cannot act on fatty acid peroxides, esterified lipid molecules in lipoproteins or membranes. These peroxides must first be released by the action of lipase enzymes.

1.3.1.4 Glutathione Reductase (GR) (EC 1.8.1.7)

GSSG should be regenerated to GSH because in healthy cells the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) must be high in order to protect intracellular redox balance (Pastore, et al., 2003). Glutathione reductase enzyme is responsible for this mechanism.

1.3.1.5 Glutathione S-Transferases (GSTs)(EC 2.5.1.18)

Glutathione S-transferases are widely found in microorganism, plants and mammalian. Most of the GSTs catalyses the conjugation reaction of GSH with the compounds which has electrophilic centre by forming a thioether bond between the sulphur atoms in GSH and substrate (Chasseaud, 1979; Mannervik, 1985). Beside conjugation reaction, GSTs catalyse the reduction of organic hydroperoxides and isomerisation of unsaturated components (Ketterer & Mulder, 1990). Especially, the liver is rich in these enzymes and the produced glutathione conjugates are usually thrown into the bile using ATP-dependent glutathione S-conjugate (Halliwell, & Gutteridge, 1999). Compounds metabolized by GST in animals include chloroform, organic nitrates, bromobenzene, aflatoxin, dichlorodiphenyl trichloroethane, naphthalene and paracetamol. Excessive amounts of such xenobiotics may reduce liver GSH concentrations, thereby weakening the antioxidant defence capacity of the liver. Additionally, GSTs metabolize aldehydes produced during lipid peroxidation to 4-hydroxynonal.

All eukaryotes have multiple cytosolic and membrane-bound GST isoenzymes with different substrate specificities (Aliya et al., 2003). Many GSTs are also used as cellular carrier proteins for hemol, bilirubin, bile pigments and steroids that cannot enzymatically bind to proteins, as well as their catalytic functions.

1.3.1.6 Glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49.)

Glucose-6-phosphate dehydrogenase is the rate-limiting enzyme of pentose phosphate pathway (PPP) (Fujita et al., 2007) and also responsible for formation of NADPH, which is predominantly used to regenerate GSH.

It was shown that deletion of the gene responsible for G6PDH made the cells more sensitive and unhealthy compared with wild type and the reason was shown as the depletion of GSH levels in cells. Similar results were shown in another study related with mouse embryonic stem cells (Pandolfi et al., 1995). Oxidative stress causes gene expression of G6PD by increasing the $O_2\bullet^-$ radical or decreasing the amount of GSH. Since it is known to be effective in the regeneration of GSH, G6PD is known as an antioxidant enzyme.

1.3.2 Non-enzymatic Antioxidants

1.3.2.1 Reduced Glutathione (GSH) and Thioredoxin

Glutathione is one of the most abundant intracellular antioxidants in the antioxidant system and contains thiol groups (Masella et al., 2005). In addition to being a cofactor of GSHPx, GSH is involved in many metabolic processes, including ascorbic acid metabolism, protection of intercellular communication, prevention of oxidation and cross-linking of protein-SH groups (Foyer & Halliwell, 1976). It is also involved in intracellular copper transport; GSH can interact with copper ions to reduce the ability to produce free radicals, or at least transfer the radicals out of the cell.

Additionally, GSH is a cofactor for many enzymes involved in different metabolic pathways, including enzymes involved in the synthesis of glyoxylases and leukotrienes (Beutler, 1989). It also plays important role in protein folding containing disulfide bonds such as insulin. Protein sulfhydryl in DNA repair and expression

system are stabilized by GSH with the protection of the redox balance of these proteins (Ji et al., 1999).

In vitro, GSH can react with HO•, HOCl, ONOO-, RO•, RO2• and carbon-centered radicals. The reaction with free radicals will produce glutathione radicals (GS•). If the GS• radicals do not scavenge each other, they may produce the $O_2^{\bullet -}$ radical (Karoui et al., 1996) and for the formation of non-radical components, GS• may dimerize and produce GSSG.

GSSG accumulation in the cell is toxic and disrupts the intracellular redox balance. In addition, the GSH/GSSG ratio is an important criterion for oxidative stress (Hwang et al., 1992). Optimal GSH:GSSG ratio in cells is one of the important parameters affecting cell viability and health. The decrease in this ration makes the cells unresistant to oxidative stress by altering intracellular redox potential. The ratio of GSH to GSSG is an indicator of cellular health, and reduced GSH constitutes up to 98% of cellular glutathione under normal conditions. GSH:GSSG ratios are low in many pathological conditions such as cancer, neurodegenerative diseases, HIV and aging.

GSSG can react with sulfhydryl groups of proteins, resulting in the formation of protein-glutathione disulphide; as a result, many enzymes can be damaged by high concentrations of GSSG.

$$\text{GSSG} + \text{Protein-SH} \rightleftharpoons \text{Protein-SSG} + \text{GSH}$$

GSH is synthesized in two steps, catalysed by glutamyl cysteine synthase encoded by the yeast GSH1 gene and glutathione synthetase encoded by the GSH2 gene (Ohtake & Yabuuchi, 1991; Grant et al., 1997a). When GSH is used in the cell, it is usually oxidized to the form of disulfide (GSSG). GSSG is then regenerated in the presence of NADPH by glutathione reductase, which is further encoded by the GLR1 gene (Collinson & Dawes, 1995). gsh1 mutants showed hypersensitivity to oxidants, including H_2O_2, tert-butyl hydroperoxide, along with the need for reductants for

growth (Wu & Moye-Rowley, 1994; Izawa et al., 1995). This situation became complicated by the fact that gsh1 mutants are phenotypically composed of petit cells and the sensitivity of these petit cells to oxidants is increased (Collinson & Dawes, 1992).However, gsh1 mutants have been found to be more sensitive to oxidants than an isogenic petite. Interestingly, gsh1 mutants require glutathione to be 100 times higher compared to non-mutant cells.

The thioredoxin system contains two reduced -SH groups, which can be converted to disulfide units and give redox reactions with many proteins (Nakamura, & Yodoi, 1997):

$$\text{Thioredoxin-(SH)}_2 + \text{Protein-S}_2 \longrightarrow \text{Thioredoxin-S}_2 + \text{Protein-(SH)}_2$$

Regeneration of the disulphide dithiol form is catalysed by thioredoxin reductase (TR) and the electron source is NADPH, as being in GSH regeneration.

1.3.2.2 Ubiquinol

Coenzyme Q or ubiquinone is well known with its role in the mitochondrial respiratory chain, but it is also known that the reduced form of ubiquinol has an important role as a potent antioxidant that can inhibit lipid peroxidation (Beyer, 1992; Ernster & Forsmark-Andree, 1993; Ernster & Dallner, 1995). It is found in many intracellular membranes, including microsomes, lysosomes, peroxisomes and plasma membranes, and this location is compatible with its antioxidant role (Takada et al., 1982, Kalen et al., 1987). Different organisms contain ubiquinones with different isoprenoid side chain lengths; Six units of ubiquinone-6 in *S. cerevisiae*, ubiquinone-8 in *E. coli*, and ubiquinone-10 in humans and *Schizosaccharomyces pombe* (Suzuki et al., 1997, Takada, 1984). Tzagoloff et al. (1990), Have identified eight effective complementary groups (coq1-coq8) in the synthesis of coenzyme Q in the yeast. Mutations in the COQ gene lead to the petit phenotype, which leads to faults in the respiratory chain. When treated with polyunsaturated fatty acids, the Coq3 mutant is highly sensitive compared to wild type strains. This sensitivity has

been concluded due to the important role of ubiquinol in protecting the cells against lipid autoxidation products, as it is reduced by the addition of synthetic antioxidant butylated hydroxytoluene or α-tocopherol (Do et al., 1996).

1.3.2.3 L-ascorbate and D-erythroascorbic acid

L-Ascorbic acid (vitamin C) is found in many organisms and has the ability to scavenge many free radicals such as lipid peroxyl, $O_2^{\bullet -}$ and $HO^{\bullet}$ radicals (Bendich et al., 1986). It is known that vitamin C plays an important role in the regeneration of vitamin E after its reaction with lipid radicals. On the other hand, it can reduce ferric ions to ferro ions and act as a pro-oxidant in the presence of H_2O_2 to stimulate $HO^{\bullet}$ formation through the Fenton reaction (Halliwell, 1994). Since the 1980s, cell proteins, carbohydrates and nucleic acids have been shown to be highly susceptible to autoxidation. Components such as vitamin C (ascorbic acid) and uric acid are known to provide important protection against the effects of protein, carbohydrate and nucleic acid oxidation in cells (Machlin & Bendich, 1987). It is known that *S. cerevisiae* does not produce significant amounts of L-ascorbic acid. D-erythroascorbic acid is produced 10 times more than L-ascorbic acid. This suggests that ascorbic acid has little or no role in yeast metabolism (Leung & Loewus, 1985).

1.3.2.4 Vitamin E

Vitamin E is a fat-soluble antioxidant and has eight isoforms. α-tocopherol is the active form of Vitamin E in particular and is known as the most important membrane-bound antioxidant (Burton, & Ingold, 1989).Vitamin E often protects the cells from lipid peroxidation (Pryor, 2000). α-tocopherol and ascorbate work together 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. The role of vitamin C in this process is regeneration of α-tocopherol (Kojo, 2004). The autoxidation of biological molecules can form very important *in vivo* ROS source. There is great interest in particularly the autoxidation of membrane lipids, and this approach has provided significant advances in food chemistry, including the

development of effective antioxidant compounds and packaging processes that ensure long-term preservation of foods. Increased interest in lipid autoxidation has also led to understanding of the importance of physiological antioxidant vitamin E, which is soluble in lipids and can affect all cell membranes.

1.3.2.5 Carotenoids

Carotenoids are a group of coloured pigments that are produced commonly in plants (Tanaka et al., 2008). They are also found in some animals and bacteria. Nutritional carotenoids are found in human tissues and in some other mammals, but many animals cannot absorb them. In humans, carotenoids are the most abundant antioxidant in fatty tissues and the liver (Fang et al., 2002). In plants, carotenoids act as antioxidants and help 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 peroxidation in lipid systems at low O_2 concentration, but not at high O_2 concentrations. However, studies with Low Density Lipoprotein (LDL) have not shown that β-carotene protects LDL against peroxidation, regardless of O_2 concentration (McCall & Frei 1999).

1.3.2.6 Transferrin

Transferrin binds free metal ions without participating in free radical reactions (Gutteridge et al., 1994). Transition metals are required for numerous biological processes such as DNA, RNA or protein synthesis due to the fact that being cofactor of many enzymes. When the levels of these metals increased, the metabolic processes will deteriorate (Silva, 2007). However, excessive accumulation of these metals in the tissues is cytotoxic for the cells (Han et al., 2006). At this point, the antioxidant properties of transferrin are prominent, and they prevents from entering radical-producing reactions such as Fenton and Haber Weiss by binding metals that accumulate in the cell.

1.3.2.7 Heat Shock Proteins

All organisms have an optimum temperature that they can grow better (Brock et al., 1972). For cells exposed to higher temperatures than the optimum, damage can be occurred in proteins, such as the aggregation or denaturation of proteins, changes in permeability and cell membrane damage leading to ion leakage, ribosome break, and DNA chain break. It has been found that in oxotrophic or temperature-sensitive mutants, cell membranes are the most affected cellular target of heat shock (Walton, 1976). It is also known that incubation of *S. cereviaise* at maximum growth temperature causes the formation of petites and respiratory failure (Sherman, 1959). During heat damage, changes in membrane protein composition, activation of lipid saturation is observed by increasing the permeability of the membrane.

A major change in cells exposed to heat shock is the increased expression of a number of heat shock genes encoding a limited number of proteins, despite the synthesis of lots of cellular proteins are stopped. Heat shock proteins are proteins that act as molecular protectors that regulate the function of other proteins by regulating their functions, transport and folding states (Diller, 2006, Lenaerts et al., 2007).

1.4 Oxidative Damages in Biomolecules

1.4.1 Oxidative Damage in Lipids

The increase in end-product concentration of lipid peroxidation is a clear proof that ROS participates in the process of disease formation (Negre, et al., 2008). Increased oxidative damage is seen in most of human diseases although it plays a significant, pathological role only in some cases. For example, peroxidation appears to be important in atherosclerosis and worsening of initial tissue damage caused by ischemic or traumatic brain injury. Oxidative stress can damage many biological molecules; proteins and DNA are usually the most important targets of extensive damage compared to lipids and lipid peroxidation is often seen in the injury process.

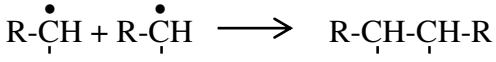
1.4.1.1 Lipid Peroxidation

Lipid peroxidation is described as an oxidative degradation of polyunsaturated lipids. Polyunsaturated fatty acids (PUFAs) contain two or more double bonds between carbon-carbon elements (Tappel et al., 1999). Some proteins may be loosely attached to the surface of the membranes, while many are bound tightly or partially embedded in the membrane. Because of the position of the proteins in the membranes, lipid peroxidation can also damage membrane proteins (Catalá, 2009).

Lipid peroxidation is a chain reaction and has three steps, as in all chain reactions; initiation, propagation and termination (Porter et al., 1995). Initiation of lipid peroxidation occurs by abstracting a hydrogen atom from a methylene group. Fatty acids with no or a single double bond are resistant to such attack compared to PUFAs. Because the double bond presence reduces the C-H bonds in the adjacent carbon atom to the double bond (Gutteridge, 1995). This causes the sensitivity of the polyunsaturated fatty acid side chains of the membrane lipids to peroxidation. C-radical is formed by removal of a hydrogen atom from a fatty acid and by molecular arrangement; then this radical interacts with O_2 to produce the peroxyl radical which has the ability to spontaneously break off the H-atom from other fatty acids; in this case the chain reaction begins. If the chain-breaking antioxidants do not interact with the fatty acid chain reaction, the peroxidation may last until the end of the substrate. $HO^{\bullet}$ radical can initiate peroxidation and produce a C-cantered radical. The $HO^{\bullet}$ radical outside of membrane can also attack extrinsic proteins or "head groups of phospholipids". On the other hand, the $O2^{\bullet -}$ radical is not reactive enough to abstract H- from lipids and the charge of this radical prevents from entering the hydrophobic interior of the cell membrane.

Hydroperoxyl ($HO_2^{\bullet -}$), the protonated and uncharged form of $O_2^{\bullet -}$ radical, is more reactive and has ability to oxidize fatty acids such as linoleic acid and arachidonic acid (Frankel, 2014). In addition to $RO^{\bullet}$, $RO_2^{\bullet}$, $HO^{\bullet}$, $HO_2^{\bullet}$ and several iron-oxygen complexes have the ability to abstract H- and initiate peroxidation.

After the hydrogen atom is abstracted from -CH2- groups, the unpaired electron remains on the carbon. The carbon radical is generally stabilized by a molecular arrangement to form a conjugated diene. Carbon radicals (C•) can participate into many reactions, for example, when two carbon radicals interact, they can crosslink the fatty acid side chains (Halliwell, 1991).



On the other hand, C• radicals can react with O_2 in aerobic conditions. This reaction results in peroxyl radical formation (Gardner, 1989). Peroxyl radicals can also abstract H- from another lipid molecule. This is the propagation of lipid peroxidation. The peroxyl radical combines with the hydrogen atom and forms LOOH. LOOH may react with Fe^{+2} and form an alkyl radical to form an alkoxyl radical (RO•) for propagation of lipid peroxidation, and alkoxyl may remove H from PUFAs. Additionally, an alternative product of the peroxyl radical is cyclic peroxides.

In the termination step, the chain reaction is terminated by the reaction and scavenging of peroxyl with other radicals or an antioxidant (Porter et al., 1995). Lipid peroxidation forms a variety of oxidation products and the mains are LOOH. Malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE) as aldehydes formed during lipid peroxidation were extensively studied by Esterbauer (1993). While MDA appears to be the most mutagenic product of lipid peroxidation; 4-HNE is the most toxic one.

MDA has been widely used for many years as a biomarker for lipid peroxidation of fatty acids due to reaction with thiobarbituric acid (TBA) (Del Rio et al., 2005). The TBA test is based on the reactivity of the TBA to 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 (Corongiu & Milia, 1983). In recent years, attempts have been made to produce GC-MS/MS, LC-MS/MS and various derivation-based strategies to determine free and total MDA

levels (Giera et al., 2012). MDA is one of the most common and reliable indicator for oxidative stress in clinical researches, its reactivity and toxicity makes this molecule to be of interest in biomedical research communities.

4-HNE was first discovered in the 60's (Schauenstein, 1967). Later, during the 80's, liver was reported as a cytotoxic product due to peroxidation of microsomal lipids (Benedetti et al., 1980). 4-Hydroxyalenes produced during membrane lipid peroxidation have a genotoxic effect in humans (Brambilla et al., 1986). 4-hydroxyalkenes are the most important products of peroxidation, since they are produced in extreme amounts with MDA compared to other oxidative products during lipid peroxidation; they function as "secondary reporters of free radicals" and are very reactive aldehydes. In particular, much research has been done on 4-HNE and is thought to be one of the most toxic product in lipid peroxides. The toxic effect of 4-HNE can be explained by its rapid reaction with thiols and amino groups (Schaur, 2003).

1.4.1.1.1 Primer Lipid Peroxidation Products- Lipid Hydroperoxides. Hydroperoxides are produced at the stage of progress and constitutes the main primary product of the lipid peroxidation reaction (Miyamoto et al., 2007). The hydroperoxide group can be bonded to free fatty acids, triacylglycerols, phospholipids and sterols. In contrast to free radicals, lipid hydroperoxides are more stable products under moderate conditions including low temperature, metal deficiency (Girotti, 1998). After formed, lipid hydroperoxides may be the target of different reduction reactions. These reactions result in the inhibition or induction of peroxidative damage. Hydroperoxides can be degraded in vivo by two e^- reductions which can prevent peroxidative damage. Essentially, the enzymes responsible for the two e^- reduction of hydroperoxides are selenium dependent glutathione peroxidases (GSHPx) and selenoprotein P (SeP). If hydroperoxides are degraded by an e-reduction in vivo and incorporated into initiation/propagation steps, it may induce the formation of new lipid hydroperoxides and support the lipid peroxidation process (Yin et al., 2011; Girotti, 1998; Kanner, 1987). Lipid hydroperoxides can be converted to ROS derivatives such as lipid peroxyl or alkoxyl radical. This

mechanism is caused by the decomposition of hydropeoxides and the oxidation and reduction of the transition metals present in the environment. Lipid peroxyl and alkoxyl radicals attack other lipids and promote lipid peroxidation. Lipid hydroperoxides also react with peroxynitrite or hypochlorous acid to produce $^1O_2$ (Miyamoto et al., 2007; Miyamoto et al., 2006) and then $^1O_2$ reacts with amino acids and proteins, resulting oxidation of side chain, dimerization and aggregation, conformational changes, and enzymatic inactivation (Gracanin et al., 2009; Davies, 2003).

The major substrates for lipid peroxidation are polyunsaturated fatty acids (PUFAs), which are a family of two or more paired lipids, also classified as omega-3 (n-3) and omega-6 (n-6) (Yin et al., 2011; Girotti, 1998; Kanner et al., 1987). Arachidonic acid, the most common n-6 fatty acid, can be reduced by enzymatic peroxidation to prostaglandin, leukotriene, thromboxane and cytochrome P-450 products or depending on oxidative pathways, oxidized to MDA, 4-HNE, isoprostanes. Continuous oxidation of fatty acid side chains, released PUFAs and fragmented peroxides leads to loss of membrane integrity and inactivation of membrane-bound proteins. Unlike radicals, aldehydes derived from lipid peroxidation can be easily diffused into membranes and alter cytoplasm and any protein in the nucleus even far away from the areas in which they are produced (Negre- Salvayre et al., 2008).

1.4.1.1.2 Seconder Lipid Peroxidation Product MDA. MDA is a final product formed by degradation of arachidonic acid and larger PUFAs (Esterbauer et al., 1991) via enzymatic or non-enzymatic reactions. Their possible biological and dose-dependent dual role has not been investigated although its chemical stability and membrane permeability are higher than radicals. Additionally MDA is less toxic than 4-HNE and methylglyoxal (MG). However, it is noted that MDA may be a signal transducer and can regulate gene expression in very few studies; the researches showed that MDA acts as a signal transducer and as an islet glucose-stimulated insulin secretion (GSIS) regulator (Robson-Doucette et al., 2011). Moderate and high levels of MDA (5 and 10 μM) increased ATP/ADP ratio, GSIS and cytosolic Ca^{2+}

levels in islet, and affected the GSIS key regulators' gene expression (Wang et al., 2014). On the other hand, MDA production with non-enzymatic processes is not well understood despite its potential therapeutic value. Excessive MDA production has been associated with different pathological conditions because MDA occurs under stress conditions and is capable of causing protein or DNA damage (Zarkovic et al., 2013; Blair, 2008; Garcia et al., 2013; Li et al., 2012).

1.4.2 Oxidative Damage in DNA

The widespread presence of ROS-mediated DNA damage in different pathological conditions such as cancer, aging, neurodegenerative diseases, rheumatoid arthritis causes an increase in interest in connection between ROS and DNA (Kirkinezosa & Moraesa, 2001; Petersen et al., 2005; Waris & Ahsan, 2006; Wiseman & Halliwell, 1996). Free radicals, single electron oxidants, radical-generating chemicals react with different components of DNA to produce many different DNA lesions. These reactive species can change the structure of bases (Jena & Mishra 2005; Jena & Mishra, 2006; Shukla et al., 2011), induce intra- and inter-chain cross-links (Bauer & Povirk, 1997; Minko et al., 2008), cause DNA-protein cross-linking (Johansen et al., 2005; Perrier et al., 2006; Xu et al., 2008) chain breakage (Yermilov et al., 1996; Balasubramanian et al., 1998).

Among all DNA bases, guanine has the lowest oxidation potential and it is the reason for exposing to continuous attack of different reactive species. Modification of guanine formed by oxidation, nitration, halogenation and alkylation are the causes of fatal lesions (Jena & Mishra, 2012). Some of these lesions are shown in Figure 1.4. The different types of guanine lesions can cross-link with DNA chains and proteins and affect DNA chain structure and transcription. (Abdulnur & Flurry, 1976; Niles et al., 2006). Among the many guanine oxidation products, 8-oxoguanine (8-oxoG) (Figure 1.4a) is the most common one in cells. An electron oxidation of guanine (G) forms a guanine cation radical (G^+). It has been suggested that this radical produces a relatively more stable 8-hydroxy-7, 8-dihydroguanyl radical (G-OH) when it reacts with a water molecule (Jena & Mishra, 2012). These

intermediates produce 8-oxoG in the case of advanced oxidation (Matter et al., 2006; Jena & Mishra, 2012). Additionally, in high concentrations of HO• radicals, guanine can be oxidized directly to 8-oxo G. It may also occur due to oxidation from other reactive species such as ONOO-, HOCl (Ravanat & Cadet, 1995, Whiteman et al., 1997; Cheng et al 2003; Yu et al., 2005; Niles et al., 2006; Yadav & Mishra, 2012).

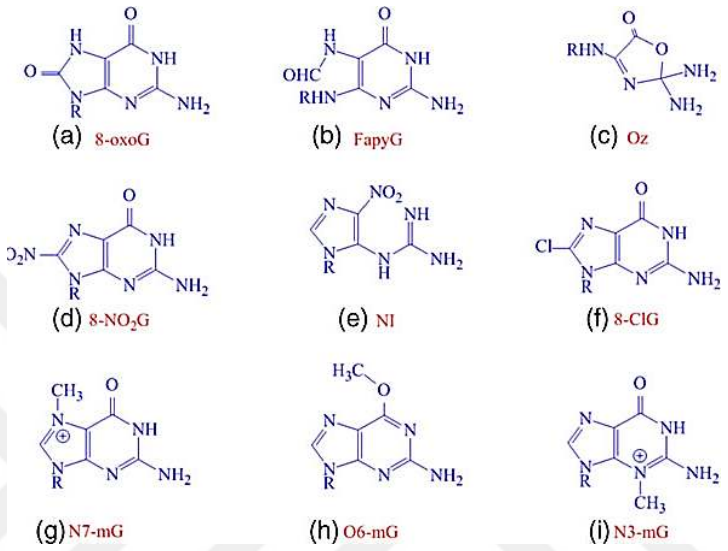


Figure 1.4 Structures of some of the guanine lesions formed due to oxidation (a–c), nitration (d,e), halogenation (f) and alkylation (g–i).

Among the many nitration products of DNA, 8-nitroguanine (8-NO_2G) is a significant and highly toxic nitrated one. As discussed previously, nitric oxide synthases lead to NO• release by macrophages in high amounts to kill pathogens. However, the conversion of the NO• radical to ONOO- or its derivatives increases the reactivity significantly. It was also found that the NO• radical did not react directly with G. But when converted to ONOO- and $ONOOCO_2$-, it reacts with G to produce 8-nitroguanine (8-NO_2G) and 5-nitro-guanidinohydantoin (NI). Additionally, Halogenation of DNA bases is also carcinogenic and harmful to tissues under inflammatory conditions. HOCl, N-chloroamines, HOBr are strongly halogenated compounds that significantly degrade DNA structure and function (Henderson et al., 1999; Jiang et al., 2003; Sasa et al., 2011). Among the various DNA halogenating agents, HOCl is the most abundant in cells and reacts with guanine to form 8-chloroguanine (8-ClG) (Henderson et al., 1999; Masuda et al., 2001; Stanley et al., 2010).

In DNA, inter and intra-chain cross-linking occur in the order of cross-linking in the opposite chains and in cross-linking in the same chain. However, it is difficult to determine precise structures of these crosslinking products. It has been determined that nucleotide modifications facilitate the formation of different DNA cross-linking products by reactive species or UV light effects (Wang, 2008). Interactions with DNA of some anticancer drugs and chemical agents may also lead to the formation of various DNA cross-linking products (Coste et al., 1999; Hofr et al., 2001). These products are believed to be mutagenic and are known to block DNA replication and transcription (Hong et al., 2007). Additionally, DNA-protein cross-linking usually refers to the formation of a covalent bond between a base or sugar of DNA and an amino acid of protein. There are 3 known ways to do this; (a) DNA and proteins are exposed to reactive species and chemotherapeutic drugs, (b) at the processing of DNA with replication and recombination proteins, and (c) during base excision repair of DNA damage. These lesions may inhibit DNA replication and transcription and may cause dysfunction in cells.

Apurinic/apyrimidinic (AP) sites are one of the most common spontaneous lesions in DNA (Boiteux & Guillet, 2004). These are potentially mutagenic and fatal lesions that can interfere with DNA replication and transcription. Furthermore, cleavage of AP sites by AP endonucleases or AP lyases produces DNA single strand breaks (SSBs) with 5'- or 3'-ends, respectively. It has been suggested that more than 10,000 bases per day have been lost per mammalian cell (Lindahl & Nyberg, 1972). These sites can be formed by spontaneous hydrolysis of the N-glycosyl bond. Damaged, inappropriate bases were removed by DNA N-glycosylases and AP sites are formed as a consequence (Krokan et al., 1997; Schärer & Jiricny, 2001). In addition to being very high in the cell, AP sites are cytotoxic and block DNA replication and transcription (Lindahl, 1993; Hoeijmakers, 2001; Friedberg, 2003). The AP units are also mutagenic and include the base pairs in DNA of *E. coli* and yeast cells in particular (Loeb, 1985; Otterlei et al., 2000; Prakash & Prakash 2002).

Given the broad spectrum of species damaging to DNA and its effect on lethal processes, it is surprising that the vast majority of cellular components do not contain

faults under normal conditions (Boiteux, 2004). The fact that healthy cells protect DNA from erroneous and dangerous effects by performing about 1016-1018 repairs per cell per day depends on the fact that enzymes evolve (Schärer, 2003). These DNA repair mechanisms are thought to occur in many ways and classified in two group; (a) direct reversal of the reaction responsible for DNA damage, and (b) removal of damaged bases and replacement with newly synthesized DNA (Friedberg et al., 1995; Hoeijmakers, 2001, Jaruga & Dizdaroglu, 1996; Sancar et al., 2004). One of the examples to direct reversal of the damage reaction can be the reaction of photodeactivating enzyme. Although the repair mechanisms of pyrimidine dimers by this enzyme are found in bacteria, yeast and some of animal and plant cells, humans are lack of this mechanism. The excision repair mechanism is more efficient than the direct reversal repair mechanism. In the excision repair mechanism, damaged DNA is removed as free bases and nucleotides. The gaps are filled with a new DNA strand synthesis using the undamaged complementary strand as a template. Three types of excision repair-base-excision repair, nucleotide-excision repair, and incompatibility repair-allow cells to deal with different types of DNA damage. However, the way of enzymes how performing DNA repair is still mysterious. Two important factors in DNA repair; first the damaged lesion is found among millions of undamaged DNA fragments and then the enzymes begin to work to make it into normal DNA.

1.4.3 Oxidative Damages in Protein

ROS causes the oxidation of amino acid residue side chain, formation of cross linkages between two amino acids either of the same or two different proteins and protein fragmentation with the result of oxidation of the protein (Davies, 2016). In a result of this effect the structure of proteins can be differentiated, and the function of proteins can be disrupted and the catalytic activity of enzymes can be reduced or vanished (Stadtman, 1993; Naskalski & Bartosz, 2000).

All amino acid residues of proteins are exposed to oxidation by HO• radical as well as sulfur-containing amino acid residues are notably sensitive to oxidation by all forms of ROS (Stadtman, 1993). In order to disintegrate these residues to their unmodified form, disulfide reductase and MeSOX reductase exist in most biological

systems (Berlett & Stadtman, 1997). Most biological systems contain disulfide reductases and MeSOX reductases that can convert oxidized forms of cysteine and methionine residues to undamaged forms. Only cysteine and methionine can be repaired when they are undergoing protein oxidation. In advanced studies, methionine units are thought to have established the ROS scavenger system to protect proteins from irreversible oxidation.

Direct oxidation of lysine, arginine, proline and threonine residues may generate carbonyl derivatives besides α-amidation pathway and oxidation of glutamil side chains.. In addition, the carbonyl groups can be produced by the reaction with aldehydes (4-HNE, MDA) caused by lipid peroxidation and with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyzones) caused by glycation and glycoxidation reactions (Schauenstein & Esterbauer 1979). If aliphatic side chains of amino acid residues are removed via β-scission, carbonyl groups bound to proteins can be stabile as the residual structures. Therefore protein carbonyls are suitable as valid biomarkers for determination of protein oxidation.

Oxidation of protein is initiated with the abstraction of α- hydrogen atom from an amino acid residue by hydroxyl radical and this abstraction results in formation of C-centred radical (Liu et al., 2017). The formed C-centred radical rapidly reacts with O_2 to form an alkylperoxyl radical which cause the formation of alkoxyl radical and alkoxyl radical may be converted to hydroxyl protein derivatives. In the absence of oxygen, alkylperoxyl radical cannot be formed and the carbon-centred radical may react with other carbon-centred radical in order to form a protein-protein cross-linked derivatives. The generation of alkoxyl radical can cause the cleavage of peptide bond through both diamide and α-amidation pathways.

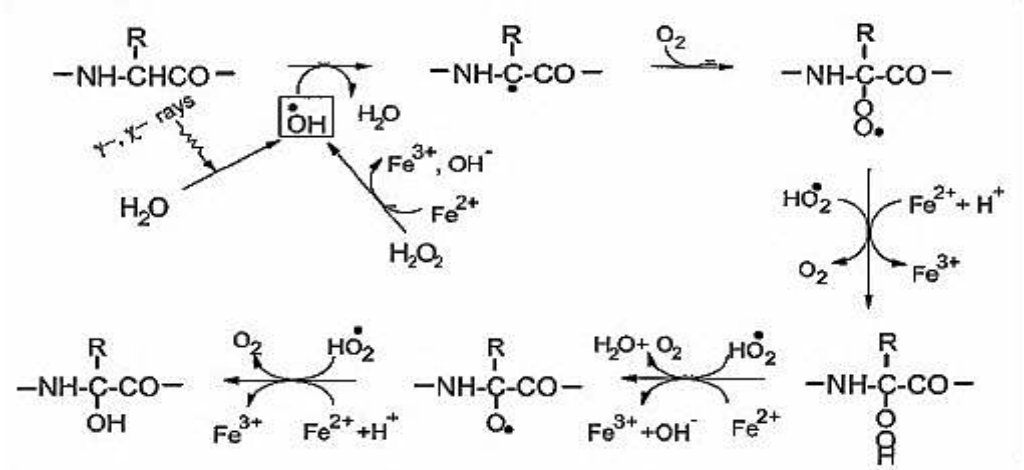


Figure 3.5 General aspects of protein oxidation

The intracellular level of protein oxidation indicates the balance between the protein oxidation formation and the degradation rate of the oxidized protein (Berlett & Stadtman, 1997) (Figure1.6). This balance is influenced by a number of factors affecting intracellular ROS and protein-degrading protease concentrations or activities. Due to the accumulation of oxidized proteins in aging, it can be seen as a degenerative process involving in the progression of degenerative diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis, diabetes. It is very clear that there are significant increases in protein oxidation in these diseases and aging process.

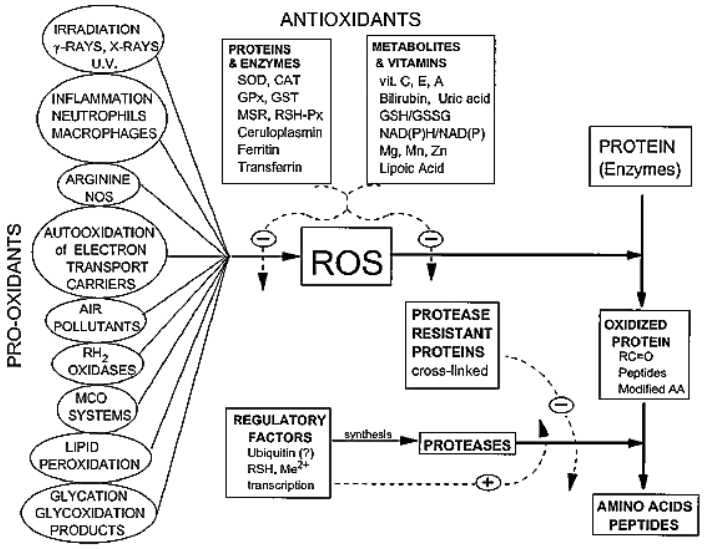


Figure 1.6 The imbalance between pro-oxidant, antioxidant, and proteolytic activities causes accumulation of oxidized protein.

The cells which lost their function caused by cumulative oxidative damage, must die with some control mechanism in order to protect other cells' and whole organism's health (Nita & Grzybowski, 2016).

1.5 Cell Death

Cell death is defined as a failure to carry through functions of a biological cell (Fink & Cookson, 2005). Cell death types can be classified by their morphological appearances (apoptotic, necrotic, autophagic, associated with mitosis), functionally (programmatically or randomly, physiologically or pathological), enzymatic criteria (involving different class proteases such as nucleases or caspases, calpains, cathepsins and transglutaminases) and immunological characteristics (immunogenic or non-immunogenic). In 2005, it is decided that cell death should be classified based on morphological criteria by The Nomenclature Committee on Cell Death (NCCD) (Kroemer et al., 2005). According to this classification, the main cell death types are considered as apoptosis, autophagy, cornification and necrosis.

Apoptosis occurs as a homeostatic mechanism to protect the cell population in tissues during development and aging in physiological conditions (Norbury & Hickson, 2001). Apoptosis is also seen as a defensive mechanism in cells that become unhealthy or damaged by harmful agents and also in immune reactions. Although there is wide range of stimuli that induces apoptosis both physiologically and pathologically, it has been observed that not all the cells are affected in the same way when they triggered with the same stimuli (Fulda et al., 2010; Kalogeris et al., 2012). Irradiation or medicine used in cancer chemotherapy cause DNA damage in some cells and can induce apoptotic cell death through the p53-dependent pathway (Borges et al., 2008; Midgley et al., 1995). Some hormones, like corticosteroids, cause apoptotic cell death in some cells such as thymocyte, but may not affect other cells (Elmore, 2007). Some cells may express Fas or TNF receptors that induce apoptosis via ligand binding and protein cross-links (Hirsch et al., 1997; Zeiss, 2003). Other cells have defective death pathways that must be blocked by life factors

such as hormones or growth. In some cases, beside the type of stimulators, their concentrations affect the decision of which cell death is occurred.

Autophagy is another mechanism of programmed cell death, and this death type plays an important role in cellular responses in human diseases and in the absence of nutrients in developmental processes, similar to apoptosis (Gozuacik and Kimchi, 2004; Debnath, 2005). Autophagic cell death is characterized by the lysis of cells by their own lysosomes (Noda et al., 2002). Similar to apoptosis, autophagy mechanisms and morphology has strong similarities between various organisms such as animals, plants and yeast. The autophagy process depends on both protein synthesis and ATP. Molecular mechanisms have been extensively studied in mammals and mammalian orthologs and continue to be elucidated (Ohsumi, 2001; Huang & Klionsky, 2002). In autophagic cell death, some cells go to death by gene expression independent of caspase and show very little ultrastructural characteristics of the apoptotic cell death and does not show DNA fragmentation (Cohen, 1991). However, these cells require de novo gene expression along with an increase in the expression of the polyubiquitin gene in a similar manner to apoptosis (Ohsumi, 2001; Gozuacik & Kimchi, 2004). Autophagy occurs specifically in all eukaryotic cells and requires the dynamic rearrangement of cell membranes in order to isolate cytoplasm and organelles to lysosome or vacuole.

Autophagy occurs in four stages: (1) induction, (2) autophagosome formation, (3) fusion with lysosomes or vacuole, and (4) autophagic cell disruption and recycling. Despite the fact that molecular details are still elucidated, several kinases, phosphatases and guanosine triphosphatases (GTPases) are involved in the regulation of this process. Mitochondria have been suggested as central organelle that regulates both autophagy apoptosis and (Elmore et al., 2001). Moreover, some of the same signal pathways involved in apoptosis may also participate in autophagic pathways. For example, there is coordinated regulation of both apoptosis and autophagy, Akt (protein kinase B) and p70S6 kinase. Other proteins including DAPK, Beclin 1, BNIP3, HSpin1, or protiimosin-α that may be parts of a network that are thought to

combine two types of cell deaths (Klionsky & Emr, 2000). Malign transformation is another link between autophagy and apoptosis (Ohsumi, 2001).

The role of genetic alterations in pathways controlling cell proliferation and apoptosis in cancer development has been well established (Lowe & Lin, 2000). Similarly, a correlation between autophagy and cancer has also been identified. Studies have shown that autophagic activity is reduced by regulating various proteins and pathways involved in autophagy signalling during malign transformation (Gutierrez et al., 2004; Gozuacik & Kimchi, 2004). This suggests that autophagy may function as a protection mechanism that limits uncontrolled cell growth. Autophagy is also thought to be a protective mechanism against apoptosis. Lemasters et al. (1998) observed that depolarized mitochondria, a characteristic of apoptosis, was rapidly abolished by autophagy in primary hepatocytes. Removing the damaged mitochondria also prevents apoptosis by inhibiting the release of pro-apoptotic substances from the mitochondria. There are some studies shows that apoptotic and autophagic cell death are not largely different from one to another and have partially different morphologic, biochemical, and molecular events (Elmore et al., 2001). In some cells at under certain conditions, the morphological characteristics of autophage may be similar with apoptotic cell death process, which represents the early phase of apoptosis. Interest in autophagic cell death is constantly increasing due to the possible effects on programmed cell death and cancer cell transformation. With the emergence of new tests and markers to illuminate the molecular basis of autophagia is developed but the debate is still ongoing.

Body surfaces of living creatures on land are exposed to air and mechanical stress (Elias et al., 1977). Although terrestrial arthropods have developed a mechanism to protect themselves from desiccation and stress, terrestrial tetrapod uses a layer of dead cells as an outermost skin barrier from the environment. Corneocytes, the building blocks of this barrier, are formed by a unique form of programmed cell death referred as cornification.

Necrosis is considered pathological cell death because it causes local inflammation and promotes tumor growth (Vakkila & Lotze, 2004). This cell death type causes the breakdown of plasma membrane without apoptotic markers and autophagic vacuolization. The most important feature of necrosis is an increase in cell volume, disruption of the plasma membrane and eventual distribution of unorganized and swollen organelles into tissue spaces. Since there is no permeabilization of the plasma membrane, necrosis does not have specific biochemical markers and can only be monitored by electron microscopy.

1.5.1 Morphology of Apoptosis

Light and electron microscopy images have showed that during apoptosis same type of morphological changes are occurred in each cell (Hacker, 2000). In early apoptosis, cell shrinkage and chromatine condensation can be observed by light microscopy (Kerr et al., 1972). When the cell is shrunk, the size of the cell gets smaller, cytoplasm condenses and the organelles become denser. Picnosis is defined as shrinkage of the cell nucleus. It is a consequence of chromatin condensation and the most characteristic feature of apoptotic cell death (Savill & Fadok, 2000). Histological experiments, done with haematoxylin and eosin, have shown that apoptosis is involved in single cells or small cell clusters. On the other hand, electron microscopy describes better the subcellular changes. In the early phase of chromatin condensation, the dense nuclear material characteristically aggregates underneath the nuclear membrane and homogeneous dense nuclei may also be present.

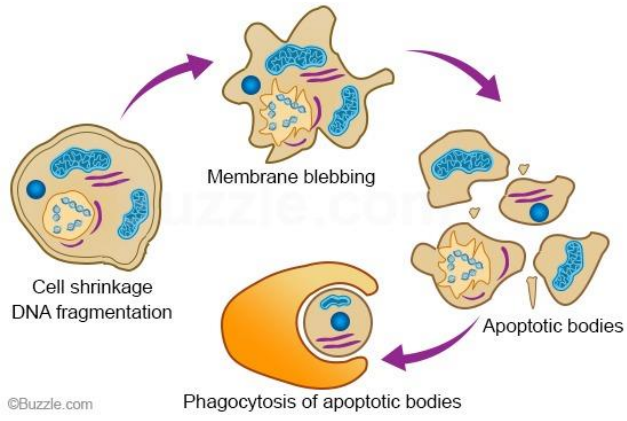


Figure 1.7 Morphology of Apoptosis

Plasma membranes are formed by karyorrhexis (cell nucleus explosion) followed by separation of cell fragments into apoptotic bodies called "budding". Apoptotic bodies consist of cytoplasm, in which tightly packed organelles, with or without nuclear fragments (Majno & Joris, 1995). These organelles are all present in a robust plasma membrane, with the integrity of the organelles are being preserved. These bodies are then phagocytized by macrophages, parenchymal or neoplastic cells and degraded in phagolysosomes. Macrophages that recognize and digest apoptotic cells are called "tingible body macrophages" and are often found in the regenerative germinal centres of the lymphoid follicles, or sometimes in the thymic cortex. Tingible bodies are nucleus debris from apoptotic cells. In the apoptotic process and during the removal of apoptotic cells, inflammation does not occur because: (1) apoptotic cells do not release their cellular components into the interstitial tissue; (2) apoptotic cells are rapidly exposed to phagocytosis by cells around them thus avoiding possible secondary necrosis; and (3) other cells that remove apoptotic cells do not produce anti-inflammatory cytokines (Savill & Fadok, 2000; Kurosaka et al., 2003).

1.5.2 Biochemical Features of Apoptosis

Apoptotic cells show a number of biochemical alterations such as protein breakdown, protein cross-linking, DNA fragmentation, and phagocytic recognition (Hengartner, 2000).

Caspases are commonly found in most of the cells in the form of an inactive proenzyme (Cohen, 1997). After activation, these enzymes activate other procaspases to initiate a protease cascade. A proteolytic cascade, where a procaspase activates the other caspases, induces apoptotic signalling pathway that leads to rapid cell death. Caspases with proteolytic activity are capable of degrading proteins from aspartic acid units in mammals, although they have different specificity for the identification of neighbouring amino acids. Once caspases are activated, an irreversible process for cell death begins. Till now, ten main caspases have been identified. They are classified as starter caspases (caspas-2, -8, -9, -10), effectors or

executers (caspas-3, -6, -7) and inflammatory caspases (caspas-1 , -4, -5) (Cohen, 1997; Rai et. al., 2005). Other caspases that identified includes; caspase-11 which regulates apoptosis and cytokine maturation during septic shock, caspase-12 which is responsible for endoplasmic reticulum-specific apoptosis, caspase-13 which is known to be found in bovine genes and caspase-14 which is known to be overexpressed in embryonic tissues but cannot be expressed in adults. (Hu et al., 1998; Nakagawa et al., 2000, Koenig et al., 2001; Kang et al., 2002).

Protein cross-linking is another common feature of apoptotic cells and is characterized by the expression and activation of tissue trans-glutaminase (Nemes et al., 1996). DNA fragmentation occurred by Ca^{+2} and Mg^{+2} dependent endonucleases and triggers the formation of 180 to 200 base pairs of DNA fragments (Bortner et al., 1995). These DNA fragments can easily be observed by agarose gel electrophoresis with ethidium bromide staining and ultraviolet illumination.

Another biochemical feature is defined as cell surface markers that result in early recognition of apoptotic cells by phagocytes and it triggers rapid phagocytosis which prevents damage to surrounding tissue. This is achieved by moving the lipid bilayer of the cell, which is called phosphatidylserine, to the outer layers of the membrane (Bratton et al., 1997). Although the orientation of phosphatidylserine is a well-known ligand for phagocytes on apoptotic cell surface, recent studies have shown that other proteins such as Annexin I and calreticulin are also located on the cell surface during apoptotic cell death. Annexin V is a recombinant phosphatidylserine-binding protein that specifically interacts with phosphatidylserine units and can be used for detecting apoptosis (Arur et al., 2003). Calreticulin binds to proteins linked to an LDL receptor on the digestive cells and is thought to work with phosphatidylserine as a recognition signal (Gardai et al., 2005). Adhesive glycoprotein thrombospondin-1 can also be expressed on the outer surface of activated microvascular endothelial cells and with the help of CD36 and caspas-3 like proteins, triggers the receptor mediated apoptosis (Jimenez et al., 2000).

1.5.3 The Mechanisms of Apoptotic Cell Death

The apoptotic cell death mechanism is quite complex and an energy dependent cascade (Elmore, 2007). Studies have shown that apoptotic cell death is related with two main pathways; one of them is extrinsic (death receptore pathway) pathway, another is intrinsic (mitochondrial) pathway (Landgraeber et al., 2008). However, it is now known that these two pathways are connected to each other and a molecule in one pathway can affect the other pathway (Igney & Krammer, 2002). Perforin / granzim pathway is another pathway beside extrinsic and intrinsic pathways. All these pathways converge on the cell death pathway. The death pathway generally begins with the activation of the enzyme called caspase-3 and results in 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 et al., 2005).

The extrinsic pathway is initiated through a subgroup of the Tumor Necrosis Factor receptors, TNFR, Fas and TRAIL (Nair et al., 2014). Activation of these death receptors facilitates the accumulation and activation of initiator caspases such as caspases 8 and 10. The process continues with the formation and activation of complexes like "Death inducing signalling complex, DISC". The DISC activates the effector caspases, mainly caspase-3. 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 also interacts with intrinsic pathway of apoptosis, as well as necrosis. The intrinsic pathway is largely regulated by mitochondria (Wang & Youle, 2009). Intrinsic apoptosis is the well-known form of apoptosis, release of cytochrome c from mitochondria in the oxidative stress condition, followed by apoptosis, activation of the initiating caspases such as caspase 9 which later activate death caspases (Arama et al., 2006). In response to any apoptotic stimulation, pro-apoptotic members of the Bcl-2 protein family (Bax and Bak) are activated and act on mitochondria to induce cytochrome c release. In addition to cytochrome c, other pro-apoptotic factors such as Smac / Diablo (Second

Mitochondrial derived activator of Caspase / Direct IAP-Binding protein with a Low pI), the serine protease Omi / HtrA2, endonuclease G (EndoG) and apoptosis inducing factor (AIF) proteins are also released into the cytosol from the mitochondria. As emphasized above, activation of the mitochondrial pathway may also occur following activation of the extrinsic pathway. It has been shown that caspase-8, which is activated via extrinsic pathway, hydrolyses the pro-apoptotic Bcl-2 member Bid protein and transforms it into the active form tBID (Lutter et al., 2000).

Increased permeability of the mitochondrial membrane is one of the most important events observed in apoptotic and necrotic deaths (Green & Evan, 2002).. At mitochondrial membrane permeability transition (MPT) process, mitochondrial swelling, disruption of the outer mitochondrial membrane, and finally release of apoptotic mediators based on Ca^{2+} occurs (Kroemer et al., 1995). MPT is thought to take place after the opening of a channel known as MPTP. This channel is known to consist of voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT), cyclophilin D, and other molecules (Crompton, 2003).

Deletion of ADP/ATP carrier (AAC) protein in yeast protects cells from toxic effect of acetic acid and diamide (Kokoszka et al., 2004). Absence of AAC proteins has been shown to disrupt mitochondrial outer membrane permeabilization and cytochrome c release (Scorrano et al., 2002).

In recent years, *S. cerevisiae* have been used as eukaryotic model organism in the studies concerning the clarification of apoptosis mechanisms induced by various environmental stresses because of having similarity with the mammalian cells, especially in terms of intrinsic pathway (Madeo et al., 1999; Ludovico et al, 2001; Apel & Hirt, 2004; Eisenberg et al., 2007; Carmona-Gutierrez, 2010). With the increasing interest in researches conducted with yeast apoptosis, many genes, which express the homolog proteins with mammalian cells, were found (Table 1.2).

Table 1.2 Homologue Proteins with Mammalian in Yeast

Proteins in Human	**Homologue in Yeast**	**Function of protein in Apoptosis**
Valosin-containing protein (Cdc48/VCP)	Cdc48p/p97	Anti-apoptotic Protein
Caspase Family (Caspase 1-14)	Metacaspase (Yca1p)	Protease activity
High-temperature requirement (HtrA2/Omi)	Nuclear mediator of apoptosis (Nma111p/Omi)	Serine protease, aggregates in the nucleus
Apoptosis Inducing Factor (AIF)	Apoptosis Inducing Factor (AIF)	Aif1p translocate to the nucleus from the mitochondria
Endonucleas G (EndoG)	Nuc1p	Apoptotic nucleus
Cytochrome c	Cytochrome c	Release into cytoplasm and provide apoptotic signals
AIF homologous mitochondrion-associated inducer of cell death (AMID)	External NADH dehydrogenase (NDE1) and internal NADH dehydrogenase (NDI1)	NAD(P)H oxidase activity
Inhibitor of Apoptosis Proteins(IAP)	Inhibitor of apoptosis protein (Bir1p)	Inhibitor-of-apoptosis

Some of these proteins are metacaspase (Yca1/Mca1) (Madeo et al., 2002), homologous of htra2 / OMI; Nma111 (Fahrenkrog et al., 2004), the two different homologous of AIF/AMID; Aif1 and Ndi1 (Wissing et al., 2004), homologous of inhibitor of Apoptosis Proteins (IAP); Bir1p and the homolog of proapoptotic Endo G; Nuc1 (Büttner et al., 2001; Wissing et al., 2004) and the pathways are shown in (Figure 1.8).

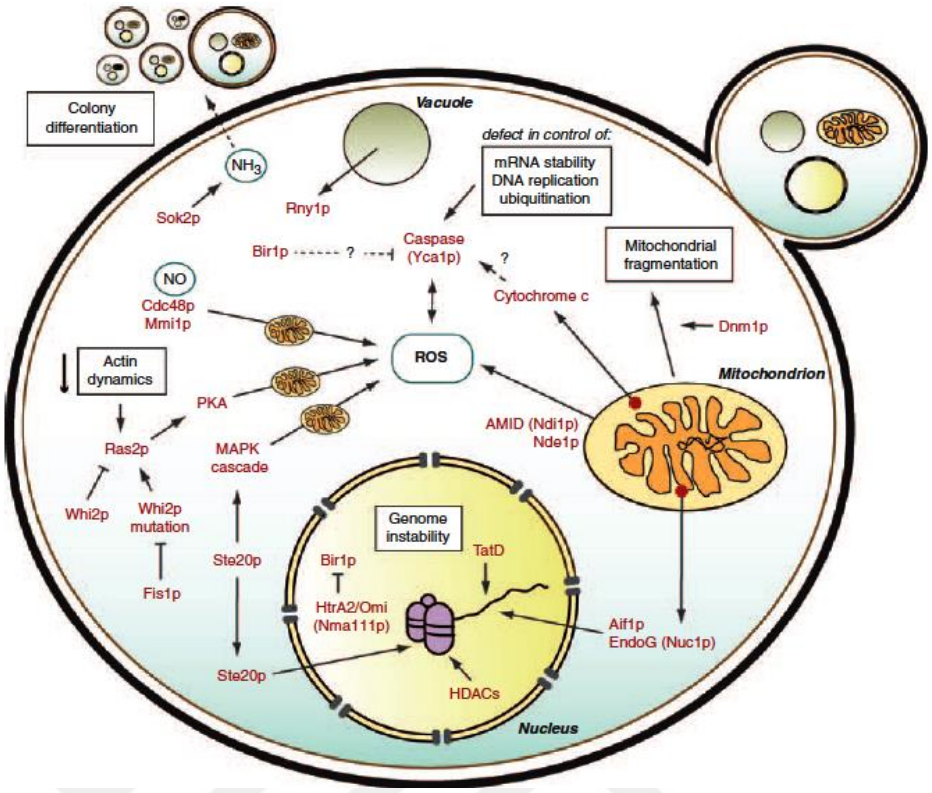


Figure 1.8 Apoptotic Proteins and Pathways in yeast apoptosis

1.5.3.1 Valosin-containing protein (Cdc48/VCP) / Cdc48p/p97

Evolutionally conserved Cdc48/VCP protein one of the most important protein which participates in lots of cellular processes such as protein degradation, membrane fusion, chaperone activity, cell growth and death (Wójcik et al., 2006). A significant correlation between Cdc48 / VCP level and cancer has been suggested, and this protein seems has a pathological effect in protein deposition diseases.

Cdc48p is the firstly found apoptotic mediator in *S. cerevisiae* (Madeo et al., 1997). The replacement of a conserved serine residue in the second ATPase domain (D2) of the yeast CDC48 (glycine, S565G of serine 565) resulted that increased susceptibility to cell death in yeast cells. In the CDC48 mutant cells, cell death was effectively triggered by aging, radiation, heat shock (37 °C), and when they were growing in late exponential or stationary phases (Madeo et al., 1999; Markkanen et al., 2004). The apoptotic yeast cells showed morphological features of apoptosis: (1) nuclear degradation, (2) chromatin condensation and fragmentation, (3) DNA

fragmentation, (4) cytoplasmic vacuolysis, (5) abnormally shaped cells and (6) phosphatidylserine externalisation. Furthermore, cycloheximide treeatment, which inhibits protein synthesis in CDC48 mutant cells, prevented the development of apoptotic properties of cells (Madeo et al., 1999). Since gene expression and protein synthesis are required for programmed cell death scenarios, these data demonstrate that CDC48 (CDC48S565G) mutant yeast cells are killed by programmed cell death.

In addition to ROS production, cells expressing CDC48 also have other functions of mitochondria during apoptosis. These cells released cytochrome c to cytosol and identified caspase-like enzymatic activity (Braun et al., 2006). In cytochrome c mutant yeast strains, hyperosmotic stress conditions resulted in a decrease in apoptosis compared to wild type strains (Silva et al., 2005). Degradation of cytochrome c resulted in apoptosis-delayed cell death induced by yeast pheromone without affecting ROS production (Severin & Hyman, 2002; Pozniakovsky et al., 2005). This suggests that the cytochrome has an important pro-apoptotic role during apoptosis and is therefore considered to be an important factor during cell death triggered by CDC48 (cdc48S565G) mutation.

1.5.3.2 Caspase Family (Caspase 1-14)/ Metacaspase (Yca1p)

The discovery of cysteine proteases, called caspases, has led to significant improvements in the understanding of the molecular control mechanism of apoptotic cell death in human (Thornberry, 1997). They can hydrolyse caspase substrates and related proenzymes from their certain aspartic acid residues in mammalian cells. Mammalian caspases may be divided into three functional groups, apoptotic initiator caspases (Caspase-2, -8, -9, -10), apoptotic effect caspases (Caspase-3, -6, -7) and caspases associated with inflammatory cytokine processing (caspase -1, -4, -5, 11, and -12L / 12S) (Chang & Yang, 2000). All caspases consist of zymogens with a variable length pro-domain and a large (20 kDa) and a small (10 kDa) subunit. Apoptotic caspases are involved in both intrinsic and extrinsic pathway (McIlwain et al., 2013). The extrinsic pathway is activated by ligand binding to the cell death receptor (TNF RI, Fas/CD95, DR3, TRAIL R1/DR4, TRAIL R2/DR5) and then

receptors form oligomers and cleavage of pro-caspase -8 and -10 occurs. Initiator caspases in intrinsic pathways are activated after the formation of apoptosomes by cytochrome c releasing and then they activate executioner caspases, once one executioner caspase is activated, it also activates other caspase and cascade begins (Cullen & Martin, 2009). In addition, in plants, fungi, Dictyostelium and metazoa, paracapases and metacaspases are defined which show similar homology to mammalian caspases but showing different substrate specificities (Vercammen et al., 2007). In *S. cerevisiae,* a gene (MCA1/YCA1) encoding metacaspase involved in cell death induction was detected during the improved pattern-based sequence homology search (Madeo et al., 2002). The presence of caspases in the analysis of the *S.cerevisiae* genome could not be demonstrated, but with sequence comparison ORA YOR197w was identified as the codon for a caspase-like protein of the type I category of metacaspase and was termed MCA1. Metacaspases are homologs of caspases interms of sequence and function but not biochemically (Carmona-Gutierrez et al., 2010). The caspases shows proteolytic activity at C-terminus of aspartate whereas metacaspases show proteolytic activity at the C-terminus of arginine or lysine (Vercammen et al., 2004). Genetic deletion of this protein leads to completely different types of death from apoptosis. Degradation of YCA1 during oxygen stress and aging reduces cell death, apoptotic phenotype formation and increases the level of oxidized proteins (Madeo et al., 2002).

Physiological conditions that cause yeast cell programmed cell death; (1) the presence of conjugated pheromone (mating pheromone) in small quantities, (2) the development of colonies on solid media, (3) chronological aging, and (4) killer toxin (Falcone & Mazzoni, 2016). An elder yca1-null mutant strain, after a hunger period, when it reaches the point where it can use nutrients, cannot go to normal level and leads to a high percentage of damaged and elderly cells. This demonstrates the physiological importance of apoptosis in cell death associated with senescence in yeast and in making a selection for healthy cells (Fabrizio et al., 2004; Herker, 2004). The killer phenotype, first described in *S. cerevisiae*, is a common phenomenon among various yeast species (Bendová, 1986). Killing is typically associated with the secretion of low molecular weight protein or glycoprotein toxin (killer toxin) that

kills sensitive cells of the same or related yeast strain, without direct cell-cell contact, in a two-stage receptor mediated process.

Three different killer toxins (K1, K2, and K28) have recently been identified in *S. cerevisiae* that are encoded as alpha-beta-toxins secreted by cytoplasmic double-stranded RNA viruses (Becker & Schmitt, 2017). At low concentrations, three viral-encoded yeast toxins cause apoptotic cell death accompanied by apoptotic markers. Yca1p and ROS production mediate this process. Conversely, high concentrations of killer toxins induce non-apoptotic necrotic cell death independent of Yca1p and ROS (Reiter et al., 2005). In a natural environment where the toxic concentration is generally low, killer yeast can remove sensitive, competing yeast by induction of apoptosis.

YCA1 activation seems to be associated with numerous basic cellular process defects such as DNA replication, mitochondrial function, RNA and protein stability (Falcone & Mazzoni). The origin recognition complex (ORC) is a protein complex with six subunits required to initiate DNA replication in all eukaryotic origin replication sites (Diffley, 2001). Cdc6p, a component of the pre-replicative complex, necessary for the initiation of eukaryotic DNA replication, rapidly induces apoptosis of cells in a protease-dependent manner, and this mechanism is conserved from the human to the yeast (Blanchard et al., 2002). The activation of both ROS and Yca1p, defective control points and chromosomal breaks are associated with DNA replication defects in the cells and are likely to be induced by DNA damage (Thompson & Parker, 2009).

It has been reported that apoptotic cell death is observed in cells following the treatment of *S.cerevisiae* with acetic acid at low doses and Yca1p has been shown to be the apoptotic protein that causes acetic acid in yeast (Ludovico et al., 2001). On the other hand, Guaragnella et al. (2006) showed that acetic acid-induced apoptosis was observed in the YCA1-deleted yeast cells in a similar manner compared to normal cells and the inhibition of caspase by z-VAD-FMK did not affect apoptosis caused by acetic acid in the yeast. Thus, it has been shown that Yca1p is involved in

another effect, not related to caspase-like activity in acetic acid-induced apoptosis. At low doses, acetic acid inducing apoptosis in yeast has caused mitochondrial degradation, cytochrome c release, alteration of mitochondrial membrane potential, and decreases in COX activity (Ludovico et al., 2002). However, acetic acid does not induce apoptosis in cells with respiratory insufficiency (Rho), and cell death is partially inhibited in cells which unable to express cytochrome c.

1.5.3.3 High-Temperature Requirement (HtrA2/Omi) /Nuclear Mediator of Apoptosis (Nma111p/Omi)

HtrA (high-temperature requirement A) proteases have been first described as heat shock-induced serine proteases in *E. coli* and are commonly found in almost all bacterial and eukaryotic genomes (Laskowska et al., 1996). HtrA is ATP-independent serine proteases. They can behave as one of the stress sensors and regulators of the unfolded protein response signal pathways; they can mediate repair, assembly or removal of damaged, fragmented and misplaced proteins. As a result, HtrA proteases play a role in many important cell processes and dysfunction of mammalian HtrA correlates with severe diseases such as Parkinson and Alzheimer's disease, myocardial ischemia and cancer (Walle et al., 2008, Chien et al., 2009).

S. cerevisiae encodes an HtrA-like protein called NMA111p (also known as Ynm3p, Nma is a nuclear mediator of apoptosis). Nma111p has an N-terminal repeat HtrA-like sequence containing catalytic triple units of the HtrA-like sequence (Clausen et al., 2002; Pallen & Wren, 1997; Ponting, 1997). However, it contains an incomplete serine protease region that is expected to be nonfunctional with the C-terminal repeat. Nma111p plays a role in lipid homeostasis and shows chaperone activity (Tong et al., 2006; Martins et al., 2002).

Nma111p has been described by a binary hybrid interaction with a yeast nuclear pore protein (Fahrenkrog et al., 2004). In contrast to Omi / HtrA2, which is localized to the mitochondria in metazoa, it is localized in the nucleus (Hegde et al., 2002). Yeast cells lacking Nma111p are more resistant to elevated temperatures and, after

being treated with H_2O_2, a known trigger of apoptosis in yeast, cells do not show apoptotic characteristics such as chromatin condensation, phosphotidyl serine externalisation or ROS accumulation (Fahrenkrog et al., 2004). Conversely, overexpression of NMA111 triggers apoptotic cell death in yeast, and this activity is dependening on the integrity of the serine protease domain. A cysteine mutation (S235C) from Ser235 significantly reduces the pro-apoptotic properties of NMA111p compared to non-mutated NMA111p. However, controversially, the chaperone activity of NMA111p appears to be dependent on Ser236 (Padmanabhan et al., 2009).

Omi / HtrA2 induces apoptosis by binding and disrupting cellular IAPs (apoptosis inhibitor proteins) (Martins et al., 2002; Srinivasula et al., 2003). IAPs are characterized by the presence of BIR (baculovirus IAP repeat) domains (Vaux & Silke, 2005) and the yeast genome contains only one BIR protein called Bir1p. Bir1p was known for its role as a chromosomal passenger protein (Li et al., 2000; Uren et al., 1999), but the later, appeared to be a substrate for Nma111p and an IAP in *S. cerevisiae* (Walter et al., 2006). Bir1p degradation occurs in yeast cells overexpressing Nma111p, but not in cells expressing a S235C mutant of Nma111p. In vitro, recombinantly expressed exogenous Bir1p interacts physically with Nma111p and N-terminal HtrA repeats, but does not interact with the C-terminal HtrA of Nma111p (Walter et al., 2006). However, the molecular mechanism of the degradation of Bir1p by NMA111p, which triggers yeast cell death, has not yet been elucidated. Nma111p is a nuclear protein like Bir1p. This suggests that some events that occur at cytoplasm during the apoptosis of mammals may occur in nucleus of yeast. However, the regulation of Nma111 is not known in a great extent, but some evidence has shown that it is provided by phosphorylation.

1.5.3.4 Apoptosis Inducing Factor (AIF)

AIF is encoded as a 67-kD protein containing the mitochondrial localization signal (MLS) at the N-terminus (Sevrioukova, 2011). With moving to the mitochondria, the MLS is cleaved by mitochondrial peptidases and the 62-kD mature

protein occurs (Otera, 2005). The N-terminal of AIF is bound to the internal mitochondrial membrane and it exhibits NADH oxidase activity in physiological conditions (Otera et al., 2005). After apoptotic stimuli, AIF can be hydrolysed by proteolytic cleavage from the membrane and 57 kDa soluble AIF fragment occurs in the cells. This fragment can be released into cytosol through the increases in the mitochondrial membrane permeabilization. The AIF protein contains two nuclear localization signals (NLS) and translocate to the nucleus when released into the cytosol. AIF participate in DNA fragmentation and chromatin condensation in the nucleus, and the net mechanism of AIF on the nucleus is not fully known (Candé et al., 2002).

Apoptosis inducing factor (AIF) is a mitochondrial flavoprotein with NADH oxidase activity (Miramar et al., 2001). This protein has been discovered as a soluble protein in mitochondria which the permeability of mitochondria increased and it has been shown to have the ability to translocate to the nucleus. AIF causes apoptotic phenotype such as chromatin condensation and DNA fragmentation in the cell (Daugas et al., 2000). Cloning full-length cDNAs corresponding to mouse AIF (612 amino acids) and human AIF (613 amino acids) revealed that all of the eukaryotic and prokaryotic organisms in which AIF was conserved (92% identity for the entire protein) oxidoreductases and extremely high homology (Lorenzo et al., 1999). Recombinant AIF treated by microinjection to cytoplasma of living cells has led to several apoptotic properties such as chromatin condensation, DNA loss, reduction of $\Delta\Psi m$, and the conversion of phosphatidylserine to the outer surface of the plasma membrane (Susin et al., 1999). In addition, no type of AIF is inhibited by z-VAD-FMK, a broad-spectrum caspase inhibitor, indicating that AIF activity is caspase independent. AIF is seen as a key protein to caspase-independent cell death pathways (Lorenzo et al., 1999).

Similar to mammalian AIF, the yeast AIF homologue, Ynr074cp, also controls yeast apoptosis (Wissing et al., 2004). In the AIF1 deleted yeast strain, H_2O_2 or acetate depending apoptosis has disappeared and aging-related apoptosis is delayed. Exposure of cells that overexpress AIF1p to low-dose H2O2 resulted in cell death

that was much greater than with H_2O_2-treated isogenic controls. However, resistance to oxidative stresses is observed in AIF protein damaged yeast. Mammalian AIF has been reported to be present in the intermembrane space, as a soluble protein or as an internal mitochondrial membrane in the intermembrane space (Sevrioukova, 2011). Several experiments with fluorescence microscopy and 35S-labeled AIF1p precursor have been carried out to determine the localization of AIF in the yeast, and as a result, the yeast AIF has been found to localise to the mitochondrial inner membrane (Wissing et al., 2004).

In healthy cells, AIF has a vital role in bioenergetics and redox metabolism (Elguindy & Nakamaru-Ogiso, 2015). Upon induction of various apoptotic agents, both recombinant and endogenous AIF are released into the cytoplasm from mitochondria (Daugas et al., 2000). In contrast to cytochrome c staying in the cytosol, AIF moves to the nucleus to take place at the initial stage of chromatin condensation. A two-stage process is required for the release of AIF from the mitochondria: In the first step, the protease cleaves AIF at a region near 100 amino acids and removes it from the inner membrane; in the second stage, AIF is released into the mitochondrial outer membrane through the permeabilization of the mitochondrial membrane (Modjtahedi et al., 2006)

Furthermore, cyclophilin (CypA) may interact with AIF in humans, and a molecular complex of AIF and CypA has been shown in vitro to have DNase activity on purified DNA (Cande et al., 2004). AIF mutants lacking CypA binding domain have been shown to be unaffected to apoptosis sensitizers in transfection experiments. In yeast, apoptotic induction was not observed even in conditions where CypA homologue CPR1 was damaged although AIF was overexpressed. This demonstrates the importance of CPR1 at the apoptosis process of AIF.

1.5.3.5 Endonucleas G (EndoG)/Nuc1p

Chromatin condensation and DNA fragmentation are both key features of apoptosis, and caspase-active DNase (CAD) is the best characterized endonuclease

responsible for the caspase-dependent apoptotic process (Nagata et al., 2003). Endo G, another endonuclease involved in caspase-independent apoptosis, is a mitochondrial enzyme thought to participate physiologically in the replication of mitochondrial DNA (Hong et al., 2006). Endo G is involved in the apoptotic process by a change in intracellular localization: it is found in mitochondrial membrane space in normal cells and is released into the cytosol from mitochondria and then to the nucleus upon induction of apoptosis, which causes fragmentation of oligonucleosomal DNA (Varecha et al., 2009). The whole process takes place independent of caspase activation. Genetic studies have shown that an Endo G mutation results in less efficient DNA degradation and it delays in vivo apoptosis (Zhang et al., 2003). Additionally, the endo G-mediated apoptosis pathway is unknown, and many contradictory aspects and data of endo G are available in mammalian cell studies.

The role of Endo G in mammalian development and apoptosis in vivo investigated in cells and mice of which End G gene deleted (Zhang et al., 2003). In this study, it was shown that the Endo G mutation caused premature embryo death. Cells in the endoG mutant mice were resistant to both tumor necrosis factor α and staurosporin-induced cell death. In addition, DNA fragmentation on activation of apoptosis was reduced in mutant thymocytes and splenocytes compared to wild-type cells. These findings showed that Endo G plays a critical role during early embryogenesis and apoptosis. In research related with yeast, valuable data insight into how EndoG regulates cell death provided (Buttner et al., 2007). Yeast Nuc1p was found to be homolog for the mammalian Endo G by sequence comparison. Nuc1p is one of the best conserved yeast homolog which is apoptosis regulatory proteins of mammalian. Overexpression of EndoG (Nuc1p) in yeast resulted in increase of apoptosis compared with control when treated with both acetic acid and H_2O_2, and this effect was found to be dependent on the nuclease activity (Rocha, 2013; Buttner et al., 2007). Similar to human EndoG, Nuc1p is predominantly localized in mitochondria at physiological conditions and is transferred to the nucleus when treated with H_2O_2. On the other hand, Nuc1p-mediated death is independent to Yca1p and Aif1p (Burhans & Weinberger, 2007). Interestingly, deletion of the NUC1 gene inhibits

apoptotic cell death in the glycerol medium, but strongly enhances necrotic death in the glucose containing medium (Dzierzbicki et al., 2012). It means that Nuc1p in the respiratory phase functions as an apoptosis inducer, while at the oxidative phosphorylation suppressed conditions, it protect the cell from non-apoptotic death. It is known that overexpression of Nuc1p does not result in increased cell death, and deletion of the karyopherin (KAP123) gene has been observed to significantly inhibit Nuc1p-mediated death in H_2O_2 treatment at low concentrations (Büttner et al., 2007). On the other hand, neither karyofenin KAP114 nor deletion of KAP120 inhibited Nuc1p-mediated death, so it was suggested that KAP123 played a specific role in this process. In a study using aging as a more physiological stimulant for apoptosis, overexpression of Nuc1p resulted in very high cell death, whereas damages in KAP123 completely inhibited cell death. In addition, there are some data that Yca1p-mediated cell death is not dependent on KAP123 (Liang & Zhou, 2008).. In addition, cell death is much higher with deletion of the AIF1 gene in cells without the NUC1 gene. For this reason, NUC1 and AIF1 proteins can partially retain each other, although they are in two independent ways. This is thought to be due to mitochondrial-nuclei localization and their nuclease activities may have similar functions, since AIF and EndoG have a relatively wide specificity for nucleic acids.

1.5.3.6 AIF homologous mitochondrion-associated inducer of cell death (AMID)/NDE1 and NDI1

The AIF family has two more members, AIFL (AIF analogue) and AMID proteins in human (Elguindy & Nakamaru-Ogiso, 2015). When human AIFL is heterologously expressed, it induces apoptosis in a caspase-dependent manner (Xie et al., 2005). On the other hand, since AMID-induced apoptosis cannot be inhibited by the caspase inhibitor z-VAD-fmk or CrmA, it is thought that AMID-induced apoptosis is not caspase dependent. AMID, a flavoprotein with NAD(P)H oxidase activity, and catalyse NAD(P)H reductions of cytochrome c and other electron acceptors including molecular oxygen (Wu et al., 2002). Similar with AIF, AMID has significant homology with NADH oxidoreductases/flavoproteins in many organisms, from bacteria to mammalian species. AMID does not contain a

recognizable mitochondrial localization signal (MLS) but is bound to the outer membrane of mitochondria and localized in the cytoplasm (Wu et al., 2002).

The association of AMID with the mitochondria outer membrane is important for AMID-induced apoptosis (Li et al., 2006). It is clear that the transcription of the human AMID gene is regulated by p53 and genotoxins (Mei et al., 2006). While embryonic fibroblasts lacking the amide protein exhibit normal proliferation, they are resistant to genotoxin-induced inhibition of growth (Modjtahedi et al., 2006). The mechanism by which AMID induces apoptosis is still unknown in mammalian cells and further investigation is needed. Last yeast studies have been a little enlightening in this regard. There are two genes closely related to human AMID in yeast, NDE1 and NDI1, which encode enzymes for external NADH dehydrogenase and internal NADH dehydrogenase, respectively (Li et al., 2006). Overexpression of the internal NADH dehydrogenase (NDI1) gene in the yeast AMID homologue causes apoptosis-like cell death, but this effect is not apparent in the external NADH dehydrogenase (NDE1) enzyme. The apoptotic effect of NDI1 can be suppressed by increased respiration in the glucose-restricted medium (0.1% glucose). Overexpression of NDI1 in Sod2Δ mutant cells causes cell death in both fermented and semi-fermented media (Braun, 2012). In addition, degradation of the NDE1 or NDI1 genes reduced ROS production and prolonged the chronological life of the yeast even though it reduced the health of the cells (Perrone et al., 2008). Thus, the apoptotic effect of NDI1 overexpression, when glucose used as a energy source, is associated with increased ROS production, which is probably produced as a byproduct of Ndi1p's NADH dehydrogenase activity, resulting in the suppression of intramitocondrial SOD2 activity by 5-10 fold (Cui et al., 2012). In this process, it has been determined that ROS is an important intermediate product that damages mitochondria and triggers mitochondrial membrane permeabilisation, resulting in cell apoptotic death. Nd1p has also been shown to be effective in Cu and Mn-induced yeast apoptosis; on the other hand, AIF1Δ strains did not show a significant difference in the viability of yeast cells (Liang & Zhou, 2007) when incubated with Cu^{2+}, so it is suggested that Ndi1p's role in apoptosis is significantly different from AIF1p.

1.5.3.7 Cytochrome C

It is found that cytochrome c releases from the mitochondria during apoptotic cell death and induces important effector mechanisms in the apoptotic caspase. This knowledge provides considerable advances in mechanism for apoptotic cell death activation and control. Cytochrome c is localized in the space between the mitochondrial outer and inner membranes under normal conditions, which participates in the oxidative phosphorylation process. When cells are exposed to apoptotic stimulation, cytochrome c is released into cytosol from mitochondria as one of the factors involved in caspase-9 mediated proteolytic activation of caspase-3. On the other hand, the effect of cytochrome c to YCA1p in yeast is still unclear.

1.5.3.8 Histone H2B

Although significant progress has been made in connection between upstream apoptotic regulators and effectors, the molecular details of downstream events are relatively unclear (Cheung et al., 2003). Posttranslational histone modifications are very important in the DNA modelling process Phosphorylation by Mst1 kinase, a mammalian sterile 20-like kinase, in the serine 14 (S14) region of H2B of human, chicken, and Xenopus cells has been shown to be closely related to apoptosis (Ahn et al., 2005). Ahn et al. (2005) have discovered a pathway between the caspase-independent H2BS10 phosphorylation (H2BS10ph) and lysine 11 acetylation (H2BK11Ac) in the yeast. First, the amino terminus of histone H2B in the budding yeast was found to be associated with an apoptotic-like role. However, this is not the case at the N-terminal of any other nuclear histones. In addition, the phosphorylation of S10 in H2B is required for the induction of apoptotic cell death, and this process is catalysed by sterile 20 kinase (Ste20p). Upon apoptotic induction with H_2O_2, the acetyl marked on K11 is removed by HDAC Hos3 and at the same time, Ste20p translocate to the nucleus from cytoplasm, where S10 is phosphorylated and causes chromatin condensation (Carmona-Gutierrez & Madeo, 2006).

1.5.4 Yeast Apoptosis Studies Based on Environmental Stress and Drug

Many exogenous factors such as hyperosmotic stress, high temperature, mating type pheromone, amiodarone, low dose H_2O_2 or acetic acid, osmotin, a kinase inhibitor SFK1 have been reported to induce apoptosis in yeast, as in metazoan (Ribeiro et al., 2006).

1.5.4.1 High temperature

Qi & Zakian (2000) observed phosphatidylserine externalisation, ROS accumulation and yeast caspase activation were increased when the cdc13-1 mutants of *S. cerevisiae* were treated at high temperature (37 °C). However very few apoptotic markers were examined in this study and the yeast caspase activity was not extensively studied. Shortly thereafter, Wysocki and Kron (2004) showed that cell death triggered by CDC13-1 is independent of caspase-like proteases, including Yca1p, and ROS, and directly related to cell cycle arrest. The consequence of this study is that cell death of Cdc13-1 mutants occurs as a result of loss of cell wall integrity in large budding cells by another active process such as autolysis and / or autophagy induction rather than apoptosis. However, no direct evidence has been produced for these claims. Although the cdc13-1 mutant cell death at 37 ° C is not caspase dependent, the death type may not be clear and may be an atypical apoptosis, as well as apoptosis in these cells.

1.5.4.2 Acetic acid

It has been reported that apoptotic cell death is observed in cells following the treatment of S.cerevisiae with acetic acid at low doses and Yca1p has been shown to be the apoptotic agent that causes acetic acid in yeast (Madeo et al., 2002; Ludovico et al., 2001; Giannattasio et al., 2005). On the other hand, Guaragnella et al. (2005) showed that acetic acid-induced apoptosis was observed in the YCA1-deleted yeast cells in a similar manner compared to normal cells and the inhibition of caspase by z-VAD-FMK did not affect apoptosis caused by acetic acid in the yeast. Thus, it has

been shown that Yca1p is involved in another effect, not related to caspase-like activity in acetic acid-induced apoptosis. At low doses, acetic acid inducing apoptosis in yeast has caused mitochondrial degradation, cytochrome c release, alteration of mitochondrial membrane potential, and decreases in COX activity (Fahrenkrog et al., 2004). However, acetic acid does not induce apoptosis in cells with respiratory insufficiency (RhoO), and cell death is partially inhibited in cells which unable to express cytochrome c.

1.5.4.3 NaCl Stress

There are some evidences that exposure of yeast to high salinity induces apoptosis (Huh et al., 2002). Deletion of the SOP1 / SRO7 gene with the isoenzyme SRO77 has been shown to impair intracellular ionic balance in NaCl stress and increase cell sensitivity (Larsson et al., 1998). Wadskog et al. (2004) showed that active caspases significantly increased in wild-type cells after 4 hours exposure to NaCl stress, and the survival rate significantly increased at high salinity in YCA1-deleted cells. This suggests that Yca1p is involved in NaCl-induced apoptotic cell death. However, when treated with 1.2 M salt stress, sRo7Δ exhibited more caspase activity than wild-type cells, while the caspase activity observed in sro77Δ mutants did not change. The findings suggest that sro77Δ and sro77Δ yca1Δ mutants exhibit NaCl-induced nuclear degradation and strong DNA chain breaks, suggesting caspase-independent apoptotic pathway as well as caspase-dependent pathway.

1.5.4.4 Cu and Mn Stress

Liang & Zhou (2007) investigated the effect of Cu and Mn stress on yeast apoptosis. In non-toxic levels, both metals have shown to be beneficial to yeast cells. However, in mild toxic levels, both metals have been shown to induce apoptosis in yeast cells. At much higher concentrations, the cells undergo necrotic death. In petite yeast, very little apoptotic marker was observed in Cu and Mn stress and cell viability was higher than control cells. This is evidence that mitochondria is associated with both Cu and Mn-induced apoptosis. ROS is produced in large

quantities in Cu treated cells, but not in Mn-treated cells, and Cu toxicity is reduced by overexpression of SOD2. This is an indication that ROS is not effective on Mn-induced apoptosis while being effective on Cu-induced apoptosis. Yeast caspase has different roles in Cu and Mn-induced apoptosis. Deletion of YCA1 did not contribute to the survival of wild-type cells exposed to Cu^{2+}. In contrast to the findings of Cu^{2+} treatment, the yca1Δ mutant showed more viability than the wild-type cells after Mn^{2+} treatment. This suggests that Yca1p is effective in Mn-induced apoptosis. In summary, yeast metacaspase plays an important role in the Mn-induced process but is not effective in Cu-induced apoptosis.

1.5.4.5 Sphingolipids

It has been suggested that syphingolipid metabolism plays an important role in apoptosis (Spincemaille et al., 2014). Recently, it has been reported that two major sphingolipid bases, named as dihydrofigosin (DHS) and phytosivinosin (PHS), have a strong fungicidal activity with a high structural and stereochemical specificity against *Aspergillus nidulans* (Cheng et al., 2003). With apoptotic activity of DHS and PHS in *A. nidulans*, induction of DNA condensation, large amounts of DNA fragmentation and phosphatidylserine externalization have occurred. The metacaspase gene in *A. anidulans* called casA, was cloned based on the homology of Yca1p (YOR197w). Overexpression of CasA inhibited the growth of *A. nidulans* and caused morphological changes consistent with apoptosis. The casA gene appears to be ineffective in PHS-induced apoptosis so it is thought that this apoptotic induction is independent of YCA1. However, further studies are needed to determine the mechanism of action.

1.5.4.6 Dermaseptin S3 (1-16)

Dermaseptins are a family of cationic, lysine-rich, amphipathic antimicrobial peptides isolated from the tree frog *Phyllomedusa sauvagii* (Brand et al., 2002). Morton et al. (2007) showed that the antifungal effect of dermaseptin S3 (1-16) was due to the apoptotic cell death induction in a study conducted with *S. cerevisiae* as a

model organism. As a result of treatment with Dermaseptin S3 (1-16), the production of ROS has been triggered and in the presence of DsS3 (1-16), the ratio of TUNEL positive nuclei was increased from about 1% to about 30% compared to control cells, and phosphatidylserine externalization was significantly increased. However, the deletion of YCA1 did not provide resistance against the death caused by DsS3 (1-16). In contrast, AIF1p is involved in apoptosis due to dermaseptin S3 (1-16) (Morton et al., 2007).

1.6 Classification of Anticancer Drugs

Most of the used cancer drugs are wanted to be able to activate the apoptotic cell death due to preventing inflammation and loss of health cells (Hu & Kavanagh, 2003). Anticancer agents can be classified into 6 classes which are alkylating agents, antimetabolites, plant alkaloids, antitumor antibiotics, monoclonal antibodies and hormone regulators.

Alkylating agents interact with proteins responsible for formation of the double-helix structure of DNA and add alkyl groups to some or all of these proteins (Wheeler, 1962). This disturbs the formation of DNA helix structure and the cancer cell to die. Nitrogen mustards, nitrosoureas, alkyl sulfonates, triazines and ethylenimines are involved in alkylating agents.

Antimetabolites are defined as drugs that inhibit normal metabolic processes in cells (Kaye, 1998). Potential targets of antimetabolites can be ordered as dihydrofolate reductase, nucleosides, thymidylate synthase, and glycinamide ribonucleotide formyltransferase and aminoimadazole carboxamide ribonucleotide formyl transferase. Plants are natural sources for drug discovery against various diseases and alkaloids derived from plants have anticancer activities (Mohan et al., 2012).

Additionally plant alkaloids are investigated in terms of being anticancer drugs; vinblastine and vincristine are two of the important plant alkaloids (Kong et al.,

2003). Antitumor antibiotics cause intercalation in DNA and stop DNA and RNA synthesis. Additionally, they modify membrane fluidity, disrupt ion transport and cause the formation of semiquinone radicals.

Hormone therapy (HT) is a form of cancer therapy that slows or arrests the growth and spread of cancer-dependent hormones, such as breast and prostate cancer (Grodstein et al., 1997, Powe et al., 2010).

Interestingly, most of the currently used antitumor drugs were firstly used as antimicrobial agent (Umezawa et al. 1965; Kauh & Bjomsti, 1995; Fukushima et al., 1999; Snell & Arora 2012; Gaspar et al. 2014). However, the characterisation and efficiency studies of these drugs have been increased after realising their antitumor properties.

1.7 Ketoconazole as a Potential Anticancer Drug

Ketoconazole (1-[4-[4-[[(2R,4S)-2-(2,4-dichlorophenyl)-2-(imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy] phenyl] piperazin-1-yl]ethanone), which is investigated in terms of its toxic and apoptotic properties within the scope of this thesis, is one of the imidazole antifungals used in the treatment of various fungal infections (Maertens, 2004). The most prominent feature of ketoconazole is the inhibition of cytochrome P450 (Moody et al., 2004). The CYP17 enzyme, which catalyzes the last step of testosterone production, has become a target for the systemic inhibition of androgen production (Payne & Hales, 2004). Because of its ability to inhibit CYP 450 enzymes, ketoconazole has been clinically used in advanced hormone refractory prostate cancer and shown to be effective in patients who do not respond to the anti-androgen flutamide, (Liebertz & Fox, 2006; Trachtenberg, 1983; Trachtenberg, 1984, Heyns et al., 1985, Mahler et al., 1993, Liebertz & Fox, 2006). Ketoconazole was reported to have apoptotic effect on colon, liver, osteosarcoma, cervical cancer, lymphoblastic and lymphoma as well as prostate cancer. (Ho, 1998; Lin, 2009; Robles-Escajeda, 2013; Giuliano, 2003). However, the effects on antioxidant system and apoptotic pathways were not clearly demonstrated in these researches and the

molecular mechanism of the *in vivo* effect of ketoconazole is not yet clear. In other studies, it was shown that ketoconazole inhibited drug metabolism by blocking the activation of nuclear receptors (Huang et al., 2007), involved in the regulation of Ca^{+2} activated K^{+} channels (Power et al., 2006; Andersson et al., 2000) and it was found that ketoconazole induced Ca^{+2} transport in renal tubular cells and inhibited steroid aromatization and progesterone hydroxylation. Furthermore, studies in recent years have indicated that ketoconazole treatment effects the genes depending on aryl hydrocarbon receptors (AHRs) which is involved in numerous cellular processes, including xeno-protection, cell cycle, immune system and apoptosis in human cancer cell lines, rats and aquatic species (Abel & Haarmann-Stemmann, 2010; Hasselberg et al., 2005; Tay et al., 2009; Korashyet al., 2007). Additionally, ketoconazole has been shown to inhibit hepatic metastasis of human pancreatic adenocarcinoma in the nude mouse model (Tzanakakis et al., 1990), as well as the effect of pulmonary metastases in the mouse melanoma model (Nardone et al., 1988). But the mechanism of ketoconazole on hormone-dependent cancer cell lines has not been determined clearly. Although most of the studies focused on the effect of ketoconazole on prostate cancer, it has been demonstrated that cervical adenocarcinoma cells (HeLa) are more effected than the prostate cancer line (PC-3) and comprehensive studies about the mechanism of ketoconazole in the cellular processes has been suggested (Giuliano, 2003).

Ketoconazole is also known to be effective in the treatment of acne and/or excessive hairlessness in women (Venturoli et al., 1990) and in the first stages of Leishmania braziliensis (Saenz et al., 1990). It was declared by the European Medicine Agencies and the Food and Drug Administration not to be used orally for antifungal purposes due to its hepatoxicity in 2013. However, it is still widely used to reduce the level of cortisone in the circulatory system (Feelders et al., 2010; Bertagna & Guignat, 2013; Barbot et al., 2014; Costenaro et al., 2015). According to the results of a study conducted with Cushing's disease (CD), although many pharmacological agents have been developed for CD, the efficacy of these drugs is low and their side effects are high (Castinetti et al., 2014). The benefit/risk balance favours the use of ketoconazole in patients who require medical treatment for CD,

since ketoconazole is highly effective and side effects are acceptable and hepatoxicity is usually mild and when the medicine is discontinued, the side effect of ketoconazole is disappeared.

CHAPTER TWO
MATERIAL AND METHODS

2.1 Microorganism and Culture Conditions

All chemicals used were at analytical grades or higher where appropriate. A wild type yeast strain *S. 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.

S. cerevisiae yeast strains, BY4742 (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) was used for all experiments. For routine spore formation, BY4742 cells incubated 5 days in YPDA medium at 30 ^{0}C in petri dishes. For suspension culture cells were inoculated in YPD broth medium at 30^{o}C. Inoculation was performed with 95 mL medium and 5 mL cell suspension in 250 mL erlenmeyer flask with 180 rpm agitation speed at 30 ^{0}C. To optimise inoculum cell concentration, initial cell concentration at OD_{660nm} as being 0.005, 0.01 and 0.02 were tested. The initial OD_{660nm} did not affect the time to reach to stationary phase and the inoculation cell concentration was decided as OD660 nm: 0.01. The cell number at OD660 nm: 0.01 was approximately $1.5x10^5$.

2.2 Ketoconazole Treatment Condition

Cells were harvested at the mid exponential phase (15th hour after inoculation) and resuspended in 20 mL conditioning medium to obtain the A_{660nm}=0.240. Conditioning medium was used in all experiments in order to protect the cells from the adaptation process caused by the fresh medium. Different concentrations of ketoconazole (0-250 μM) were added to the medium that containing $3x10^6$ cell/mL

S. cerevisiae in 100-mL erlenmeyer flask. Incubation was performed at 30 °C with agitation at 180 rpm for 1–6 h. Control samples were incubated with 0.2% dimethyl sulfoxide (DMSO) which is the solvent of ketoconazole.

2.3 Crude extract preparation

In the experiments that crude extract needed, the ketoconazole-treated and control cells were harvested by centrifugation with 1h intervals and washed 3 times with 20 mM potassium phosphate buffer (pH 7.4) at 4 °C, and then the crude extracts were prepared by breaking the cells with glass beads using a Retsch Bead Mill Homogenizer for 14 min with 5 min interval at 4°C and centrifuged at 15000 rpm.

2.4 Measurement of Cell viability

The FUN-1 test was used to determine the effects of ketoconazole on cell viability of *S. cerevisiae* BY4742. To measure the viability of the cells, the ketoconazole-treated and control cells were harvested by centrifugation. The cells were resuspended in 10 mM Na-HEPES buffer (pH 7.2), containing 2% D-(+)- glucose at the cell concentration of $1x10^7$ cell/mL (Millard et al., 1997). 100 µl cell suspension was mixed with 100 µl, 4 µM FUN-1 dye and incubated at 30 °C with shaking in dark. Fluorescence was read at $\lambda_{exc/ems}$: 485nm/530 nm (green) and 485 nm/ 620 nm (red) for 150 min with 10 min intervals. Time-dependent red/green fluorescence intensity for both ketoconazole treated and control groups were calculated and the viability of these groups was compared.

2.5 Annexin V-PI Staining

To execute Annexin V- PI staining, yeast cells were harvested and washed with sorbitol buffer (Madeo et al., 2002). Totally $3x10^6$ cells were washed twice with binding buffer and resuspended. 2 µL AnnexinV-FITC and 2 µL propidium iodide (PI) were added to 38 µL cell suspension, incubated for 20 min at room temperature and analysed under flow cytometry at FL1 and FL2 channels.

2.6 DAPI Staining

The protocol for 4,6-Diamidino-2-phenylindole (DAPI) nuclei staining was used, as described by Streiblova & Hasek (1996). The cells were collected, resuspended in 70% ethanol for brief fixation and permeabilization, and then stained with DAPI solution. Cell images were recorded at room temperature from a fluorescence microscope (model BX53; Olympus, Tokyo, Japan). A 100× objective lens was used.

2.7 TUNEL Staining

DNA fragmentation is one of the most important features of apoptotic cell death and is often used to separate apoptotic cells from necrotic cells (Denton & Kumar, 2015). To perform this test, yeast cells were fixed with formaldehyde as described by Madeo et al. (2002), and the cell wall was digested with 15 U/mL 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 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. Cell images were recorded at room temperature from a fluorescence microscope (model BX53; Olympus, Tokyo, Japan). A 100x objective lens was used.

2.8 Mitochondrial Membrane Potential

100 µL (6x106 cells/mL) cell suspension was incubated with 100 µL of 2 µg/mL Rhodamine-123 (or tetramethylhodamine ethyl ester, TMRE, Rh123) solution for 30 min at 37 °C in a light-free conditions. Later, cells were washed twice with PBS then analysed by microplate reader and flow cytometry at Ex / Em = 485 nm / 535 nm and FL-1 channel (Mathur et al., 2000).

2.9 Yeast Caspase Activity

5.10^6 yeast cells were harvested, washed once in 1 mL PBS, and resuspended in 200 µL staining solution containing FITC-VAD-fmk (Madeo et al., 2002). After incubation for 20 min at 30 °C, cells were washed in 1 mL PBS and resuspended in 200 µL PBS. For flow cytometric analysis, stained cells were counted with excitation and emission settings of 488 nm and 525–550 nm. Cell images were recorded at room temperature from a fluorescence microscope (model BX53; Olympus, Tokyo, Japan). A 100× objective lens was used.

2.10 Determination of ROS Levels

2.10.1 Measurement of the $O_2^{\bullet-}$ level

Lucigenin (bis-N-methylacridinium nitrate, is an acridine-derived probe that has been used widely to detect superoxide anion radical (Skatchkov, et al, 1999). Lucigenin (5 µM) was added to the 96 well plate that contained 250 µl cell suspensions ($1.3x10^6$ cell/mL) and luminescence was measured with luminescence counter after the incubation of plate at 37 °C for 1 h (Skatchkov, et al, 1999).

2.10.2 Measurement of the H_2O_2 Level

To measure intracellular H_2O_2 level, homovanillic acid was used as a fluorescence probe. It forms dimer when react with H_2O_2 with the presence of horseradish peroxidase (Barja, 2002). Briefly, the cell homogenate was incubated at 30 °C with 1.5 mL phosphate buffer. After 15 min, the reaction was stopped with 0.5 mL stop solution 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.

2.10.3 Determination of Total ROS Level

Total ROS levels were measured with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). The total ROS levels were determined according to the method of Kirkland et al. (Wu and Yotnda, 2011), cells were loaded with dye by replacing medium with 10 μM CM-H2DCFDA (C6827, Invitrogen, Grand Island, NY) containing conditional medium for 1 h at 28°C. After loading, media were removed and replaced with the medium with or without ketoconazole. Fluorescence measurements were made using microplate reader, using a 485/20 excitation, 528/20 emission.

2.10.4 Measurement of HO• Level

HO• radical level was determined fluorometrically (Ruenroengklin et al., 2009). For the measurement of level of HO• radical, thiobarbituric acid reactive substance (TBARS) products were measured. The fluorescent intensity was measured at the excitation wavelength of 532 nm and the emission wavelength of 553 nm against the reagent blank solution. The amount of hydroxyl radical was calculated by equation of the calibration curve that was plotted with MDA as standard.

2.10.5 Measurement of NO• Level

Nitric oxide (NO•) was measured by using the Griess reaction (Giustarini et al., 2008). 100 μL of supernatant was applied to a microtiter plate well, followed by 100 μL of Griess reagent (1 g/L sulphanilamide, 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.

2.11 Enzyme activity assays

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 et al., 1970). The decrease in the A_{340} value was recorded of two 5-min intervals. A millimolar extinction coefficient of 6.22 $M^{-1}cm^{-1}$ was used to calculate the NADH disappearance.

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 et al., 1970). A millimolar extinction coefficient of 6.22 $M^{-1}cm^{-1}$ was used to calculate the volume activity of the enzyme.

2.11.3 SOD Activity Assay

The SOD enzyme activity was determined spectrophotometrically at 490 nm. The SOD activity assay was based on the measurement of autoxidation of 6-hydroxydopamine (6-OHDA), which is inhibited by SOD (Crosti et al., 1987). One unit is the amount of SOD required to inhibit the initial rate of 6-OHDA autoxidation by 50%.

2.11.4 CAT Activity Assay

CAT activity was measured spectrophotometrically (Shimadzu UV-1800 UV–VIS) at 25 °C by following the absorbance decrease at 240 nm caused by the disappearance of H_2O_2 according to the Aebi method (1974). The molar extinction coefficient at 240 nm for H_2O_2 was 39.4 $M^{-1}cm^{-1}$. The reaction mixture contained appropriate amount of H_2O_2 and enzyme extract in 50 mM phosphate buffer (pH

7.0). The reaction was run for at least 1 min and the initial linear rate was used to estimate activity. One unit of enzyme activity was defined as the amount of enzyme decomposing one μmole of H_2O_2 under standard assay conditions.

2.11.5 GSH-Px Activity Assay

The GSH-Px enzyme activity was measured as reported by Paglia & Valentine (1967) with modifications. The change in absorbance at 340 nm was recorded for 5 min, and the activity was defined as the pmol NADPH oxidised per min per mg of protein.

2.11.6 GR Activity Assay

The GR activity was assayed as reported by Massey & Williams (1965), with slight modifications. NADPH oxidation was monitored at 340 nm, and the enzyme activity was expressed as IU per mg of protein.

2.11.7 GST Activity Assay

The GST activity was measured as reported by Habig et al. (1964), using chlorodinitrobenzene (CDNB) as a substrate. The formation of the GSH-CDNB conjugate was monitored by the change in absorbance at 340 nm, and the specific activity was expressed as IU per mg of protein.

2.12 Intracellular GSH and GSSG Determination Assay

Intracellular 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 5, 5′-dithio-bis (2-nitrobenzoic acid). The formation of the product is followed by measuring absorbance at 412 nm.

2.13 Total Protein Assay

Bradford method (A_{595}) was used for the measurement of the total protein concentration in the samples (Bradford, 1976).

2.14 Membrane LPO Level Assay

Cells were homogenized and transferred into 2.5 mL 10 % trichloroacetic acid (TCA), incubated 90 ºC for 15 min, cooled and then centrifuged at 3900 rpm for 10 min. 2 mL supernatant was added into 1 mL % 0.675 thiobarbituricacid (TBA) solution (Schmedes & Hølmer, 1989). The mixture was incubated 90 ºC for 15 min. After cooling, the absorbance was measured 532 nm. Malondialdehyde (MDA), an end product of fatty acid peroxidation, reacts with TBA and forms a colored complex. MDA values in micromoles were calculated from the absorbance coefficient of MDA-TBA complex at 532 nm, 1.56×10^5 M^{-1} cm^{-1}.

2.15 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
RESULT AND DISCUSSION

In the scope of this thesis; it was aimed to clarify the effect of ketoconazole on the viability and the induced cell death types in *S. cerevisiae* BY4742. Cell viability, phosphatidylserine externalisation, DNA fragmentation, mitochondrial membrane potentials of *S. cerevisiae BY4742* treated with ketoconazole were determined in order to decide the cell death types triggered by ketoconazole in a time (1-6 h) and dose (150-250 μM) dependently. After reaching optimal apoptotic conditions, the apoptotic pathways were started to be investigated by determining caspase enzyme activities and using the mutant *S. cerevisiae* BY4742 cells in which the genes related with main apoptotic proteins (yca1p, nma11p, endoG, aifp) were deleted. In the second step of the thesis, in order to determine oxidative stress status caused by ketoconazole treatment to *S. cerevisiae* BY4742, intracellular ROS ($O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$, total ROS and $NO^{\bullet}$) levels, the response of enzymatic and non-enzymatic antioxidant response system and lipid peroxidation levels as oxidative stress indicator were investigated in ketoconazole treated cells and compared with their controls.

3.1 Cytotoxicity of Ketoconazole in *S. cerevisiae* BY4742

Ketoconazole targets the ergosterol biosynthetic pathway leading to cells depleted in ergosterol and with elevated levels of toxic intermediates which prove fatal to the cell (Mukhopadhyay et al., 2004). It is also inhibitor of cholesterol, adrenal steroid synthesis in human (Buttke & Chapman, 1983; Deuschle, 2003).

To explore the cytotoxicity of ketoconazole to *S. cerevisiae* BY4742, the viability test was evaluated by FUN-1 staining test which is a unique two-colour fluorescent viability probe for yeast and fungi and stain passively diffuses into cell and initially stains the cytoplasm with a green fluorescence which is converted to red fluorescence by live cells (Gupta et al., 2003). FUN-1 staining test is highly sensitive compared to the colony forming unit method, giving 2-colour fluorescent light.

Healthy and viable cells can metabolise FUN-1 dye which normally gives green fluorescent light into another derivative which gives red fluorescent (Millard et al., 1997). Dead or metabolically damaged cells cannot metabolize the dye and it will continue to give green fluorescent light. Hence, the ratio of red fluorescence to green fluorescence is an indicator of viability. The exponentially growing cells exposed to ketoconazole showed 27%, 52% and 68% cell death compared with the controls after 200 µM treatment for 3, 6 and 9 h, respectively (Figure 3.1). According to these results, the IC_{50} value of ketoconazole for *S. cerevisiae* BY4742 was decided as 200 µM, 6h.

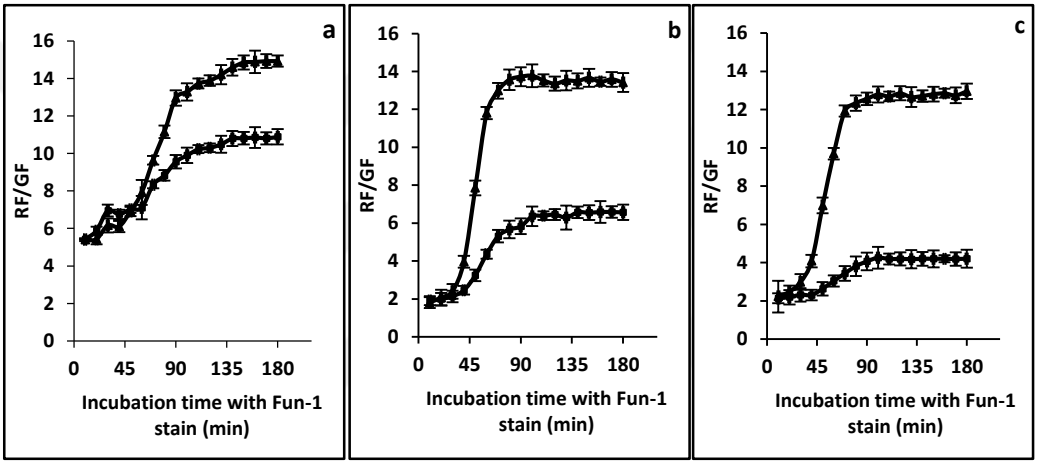


Figure 3.1 The viability of *S. cerevisiae* BY4742 cells after treatment with 200 µM ketoconazole (---♦---) compared to 2% DMSO treated controls (---▲---) for 3 (a), 6 (b) and 9 h (c). The cells were incubated with FUN-1 stain up to 180 min (as described in Material and Method section)

The cancer cell lines human osteosarcoma and prostate cancer treated with ketoconazole at the dosage of 100 µM and 20 µM, respectively, showed 50% viability at the end of 24 and 120 h (Dixon et al., 1997; Lin et al., 2009). In the study of Kaur et al. (2004), the treatment of 100 µM fluconazole, one of the azole antifungals, to *Candida glabrata* for 24 h caused 50% cell death. Additionally, auranofin which is used for clinical treatment of severe rheumatoid arthritis decreased the cell viability after 24 h treatment at 25 µM concentration while there was no impact on cell viability after 6 h (Gamberi et al., 2015). The IC_{50} value of miltefosine, one of the anti-leishmanial drugs, for *Leishmania donovani* cells was found as 25 µM for 48 h (Paris et al., 2004). At this point, it must be explained that

human cell lines are seen more sensitive than microorganisms to chemotherapeutics in the literature data (Ządzinski et al., 1998; Verrax et al., 2004; Marullo et al., 2013). Although dosage of ketoconazole which caused 50% cell death seemed higher than fluconazole and auranofin, the treatment time was rather shorter.

3.2 The Cell Death Types in *S. cerevisiae* Exposed to Ketoconazole

In yeasts, as in mammalian cells, phosphatidylserine has an asymmetric distribution in the lipid bilayer of the cytoplasmic membrane (Cerbón & Calderón, 1991). Phosphatidylserine (PS) externalisation was detected by Annexin V-FITC-PI co-staining.

The IC50 value of ketoconazole was determined as 200 µM treatment for 6 h. To understand which cell death type induced by ketoconazole treatment, Annexin V-PI co-staining was experimented in the presence of ketoconazole at the concentration range of 100-200 µM for 6 and 9 h by flow cytometry (Figure 3.2).

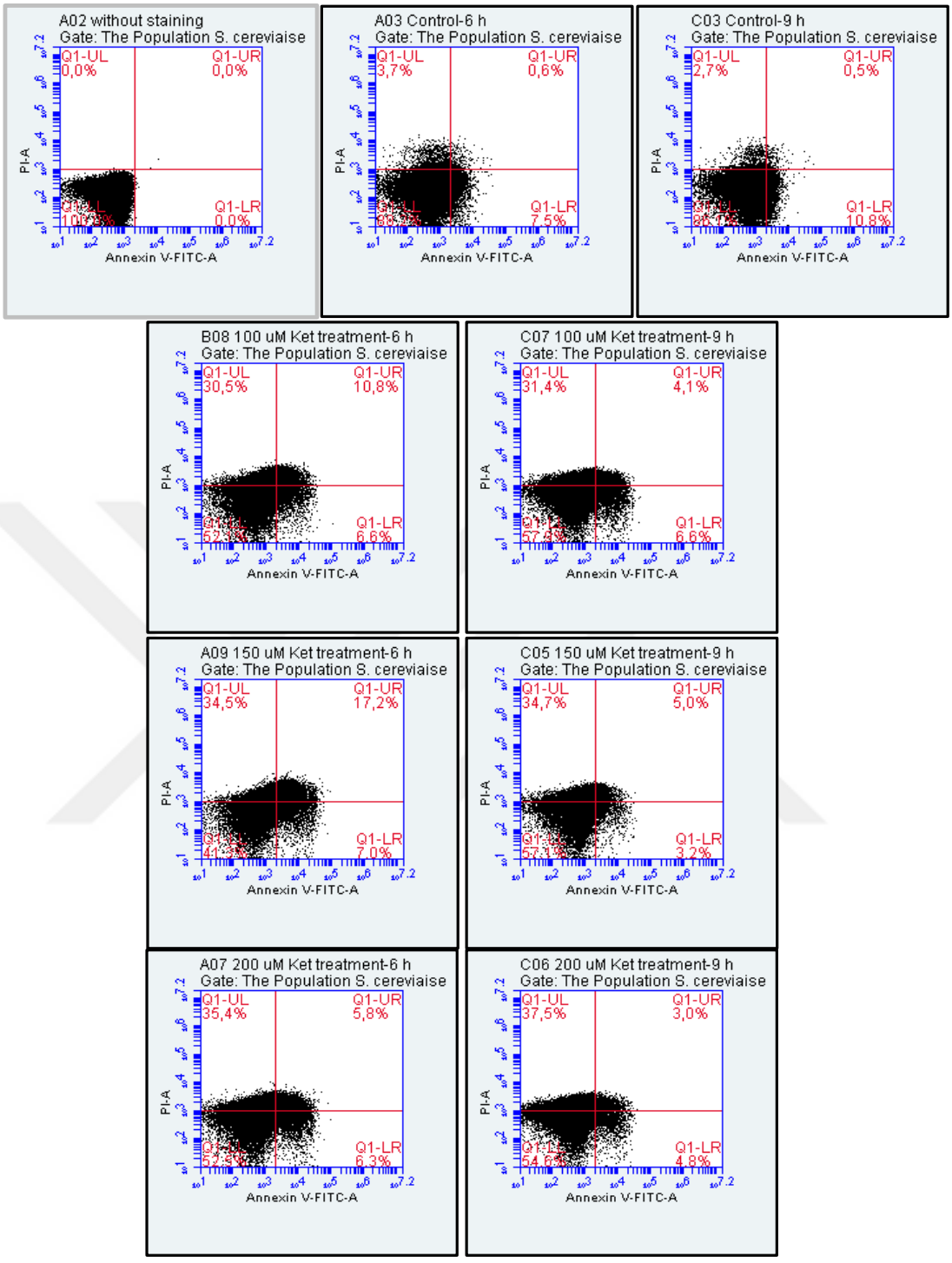


Figure 3.2 The percentages of live, early, late apoptotic and necrotic population of S. cerevisiae cells after treatment with 100-200 µM ketoconazole for 6 and 9 h compared to 2% DMSO treated controls. (Q1-LL; Healthy cells Q1-LR; Early apoptotic cells, Q1-UR; Late apoptotic cells, Q1-UL; necrotic cells)

There was totally 11.8% and 14.0% cell death in 6 and 9 h's controls, respectively. Although apoptotic cell death induced up to 2.98 fold compared to its control by 150 μM treatment for 6 h, the necrosis was seen at the range of 30-37.5% in all ketoconazole treatments. Additionally, apoptotic cell death levels significantly decreased with 9 h treatments in all concentrations. According to these results, there were two possible ways to lead cell death by ketoconazole; primary or secondary necrosis. Secondary necrosis occurs when apoptotic cells are not digested by phagocytic cells and completes the apoptotic cell death by releasing of all cell components (Silva, 2010) and a typical result of apoptosis in unicellular eukaryotes. To understand the type of cell death results deeply, concentration of ketoconazole were decreased to 10- 75 μM and treatment time were still 6 and 9h. The lower doses of ketoconazole did not increased necrosis, except 75 μM treatment for 9 h while apoptotic cell death of *S. cerevisiae* BY4742 increased 1.67 fold of its control with the treatment of 75 μM for 6 h (Figure 3.3). These results showed that the observed necrosis with higher doses (100-200 μM) and 6-9 h treatment were most probably secondary necrosis and ketoconazole can induce apoptotic cell death if the concentration and treatment time is adjusted correctly. Otherwise the cells treated with lower doses of ketoconazole would have been also died by necrosis, instead of apoptosis. The results depending on time also showed secondary necrosis. Although the cells showed apoptotic character with 6 h treatment, with the increasing time, the necrotic cell death was also increased.

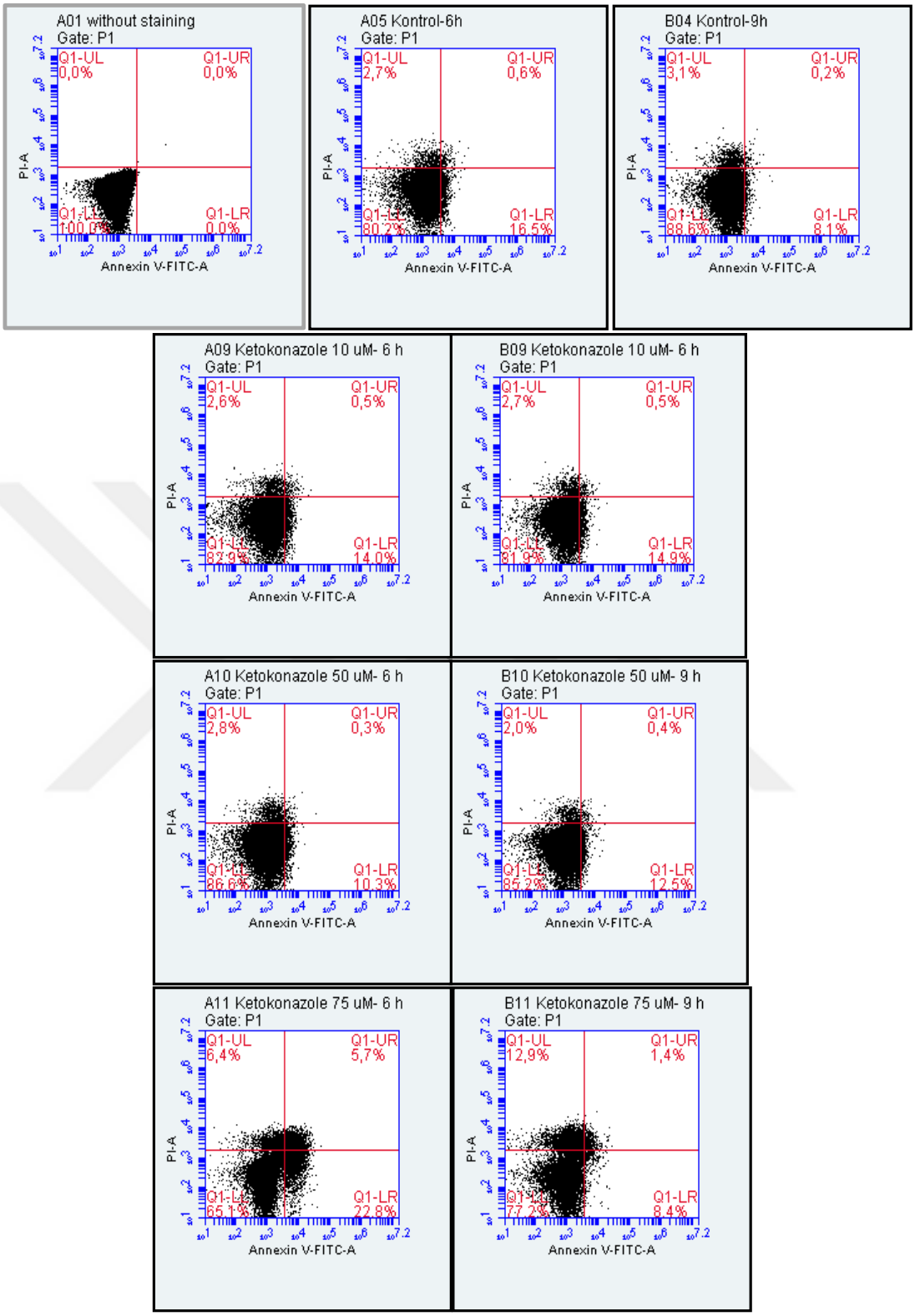


Figure 3.3 The percentages of live, early, late apoptotic and necrotic population of S. cerevisiae cells after treatment with 10-75 µM ketoconazole for 6 and 9 h compared to 2% DMSO treated controls. (Q1-LL; Healthy cells Q1-LR; Early apoptotic cells, Q1-UR; Late apoptotic cells, Q1-UL; necrotic cells)

In order to increase the apoptotic population from totally 28.5% to higher levels, the cells were treated with 200-250 μM ketoconazole in the shorter incubation times (2-4 h) and the results were quite promising that necrosis decreased to approximately 15-20% values and apoptosis induced at the range of 46.2- 68.2% in all treatments and totally cell death were only 16% for control cells (Figure 3.4). According to these results, the optimum apoptotic condition was decided as 225 μM ketoconazole treatment for 4 h. 250 μM treatment was not chosen because there was not significant differences between the dosage of 225-250 μM.

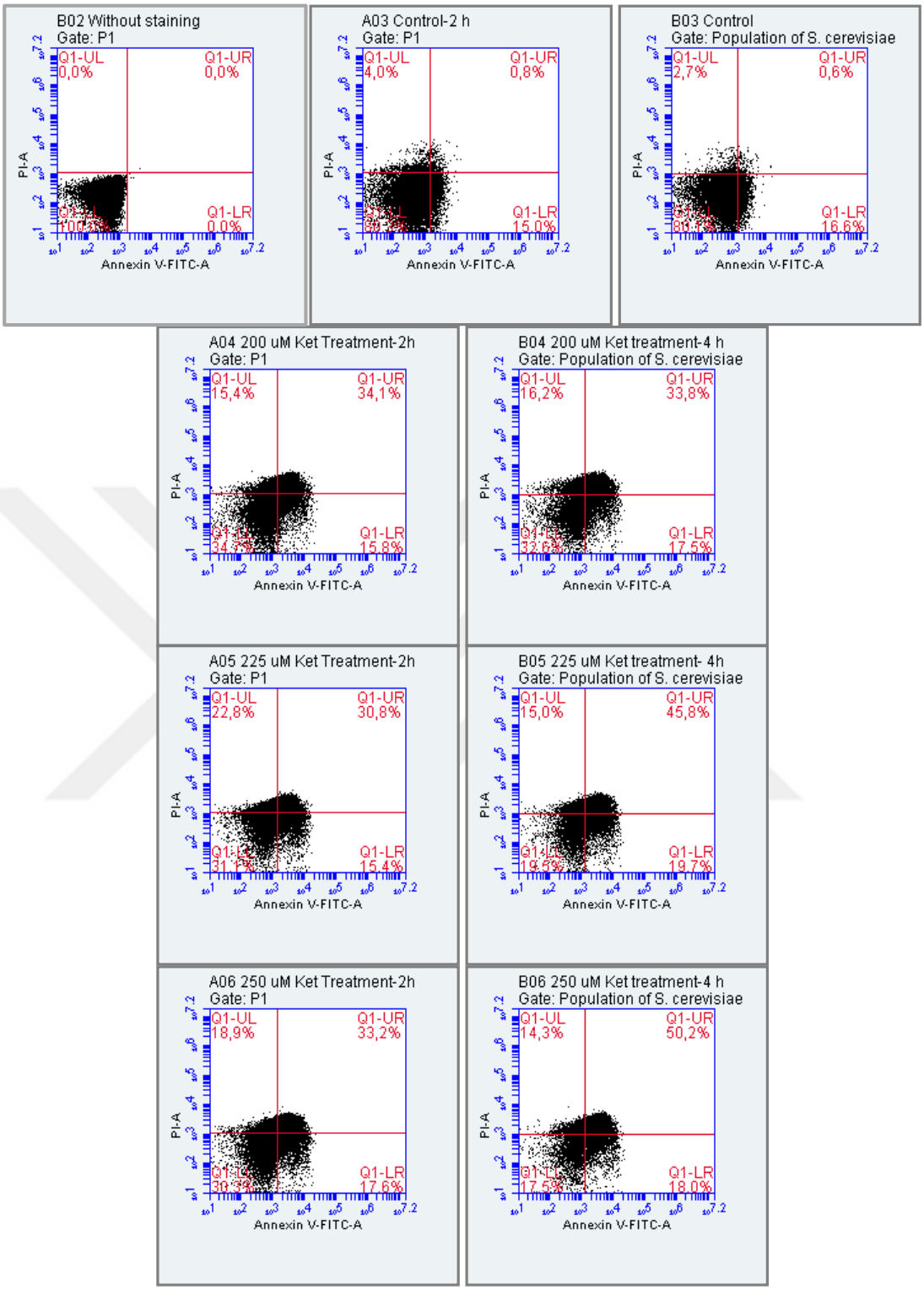


Figure 3.4 The percentages of live, early, late apoptotic and necrotic population of *S. cerevisiae* BY4742 cells after treatment with 200-250 µM ketoconazole for 2 and 4 h compared to 2% DMSO treated controls. (Q1-LL; Healthy cells Q1-LR; Early apoptotic cells, Q1-UR; Late apoptotic cells, Q1-UL; necrotic cells)

In the study of Lin et al. (2009), apoptosis in human osteosarcoma cell line exposed to 100 μM ketoconazole for 24 h increased up to 70% of the cell population, similar with our results. On the other hand, 15 μM ketoconazole treatments to lymphoblastic lymphoma cell line only increased the apoptotic cell line up to 20% of cell population when the apoptotic cell percentage was 10% in the population of untreated control cells (Robles-Escajeda et al., 2013). In addition, the apoptosis induction in *Mucorales* after the treatment of posaconazole and itraconazole, which are also azole antifungals, increased up to 40% with the co-treatment of Tacrolimus (Shirazi and Kontoyiannis, 2013). In the study of Augustin et al. (2013), although EC50 value of C-1311 (topoisomerases I and II inhibitor) for hepatoma cells was 1.8 μM, there was not any induction of apoptotic cell death.

3.3 Alterations of Mitochondrial Membrane Potential in *S. Cerevisiae* Exposed to Ketoconazole

Mitochondrial dysfunction has been shown to participate in the induction of apoptosis and has even been suggested to be central to the apoptotic pathway (Ly et al., 2003). Rhodamine-123 (or tetramethylhodamine ethyl ester, TMRE) is a widely used a cell-permeable cationic dye for determining the changes in mitochondrial membrane potential by both flow cytometry and fluorescence microscopy. Rh123 directly dye mitochondria without dyeing endocytotic vesicles and lysosomes (Chen et al., 1981) and is distributed in mitochondrial matrix in response to the potential difference of mitochondrial membranes and the more fluorescence means the more decreases in mitochondrial membrane potential (Emaus et al., 1986). The decrease in ΔΨm which is a marker of mitochondrial dysfunction and apoptotic cell death, was also detected in *S. cerevisiae* BY4742 treated with ketoconazole at the dosage range of 150-250 μM for 2, 4 and 6 hour. ΔΨm were decreased with increasing ketoconazole concentration and incubation periods, except for the 250 μM treatment for 4 and 6h (Figure 3.5). This exception is most probably caused by the destruction of mitochondria in the cells exposed to 250 μM. The highest decrease was observed in 225 μM treatment at 4^{th} h of incubation as 3.50-fold of its control. The increases in ROS levels with the increasing ketoconazole concentration may be the reason of

dysfunction on the mitochondria. In the study of Du et al. (2007), the ΔΨm *S. cerevisiae* cells exposed to 3 mM Arsenic for 12 h decreased approximately 6-fold of its control.

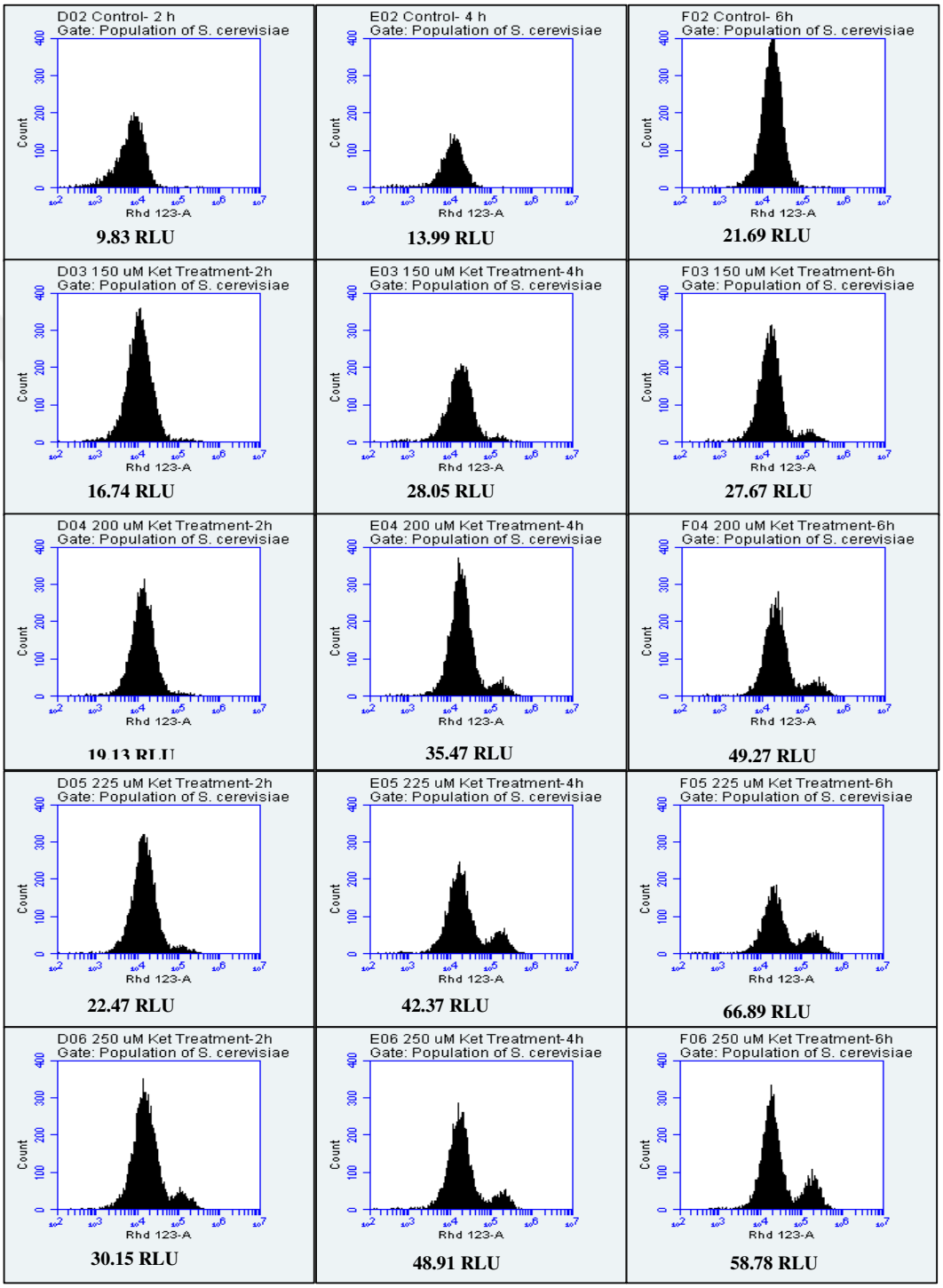


Figure 3.5 Mitochondrial membrane potentials in *S. cerevisiae BY4742* cells after treatment with 150-250 µM ketoconazole for 2 and 4 h compared to 2% DMSO treated controls

3.4 DNA Fragmentation in *S. cerevisiae* Exposed to Ketoconazole

According to the results from Annexin V-PI staining and mitochondrial membrane potentials, the optimum condition for the induction of apoptosis at *S. cerevisiae* BY4742 was considered as 225 µM ketoconazole and 4 hour treatment. DNA fragmentation represents a characteristic hallmark of apoptosis. TUNEL-staining method, which is the most established DNA nick formation, was examined in *S. cerevisiae* exposed to optimum apoptotic condition. As can be seen in Figure 3.6, DNA fragmentation was observed in the cells exposed to 225 µM ketoconazole for 4 h while little or no fragmentation was found in the control cells. Dependency of ketoconazole induced apoptosis to yeast caspase activity in *S. cerevisiae* BY4742.

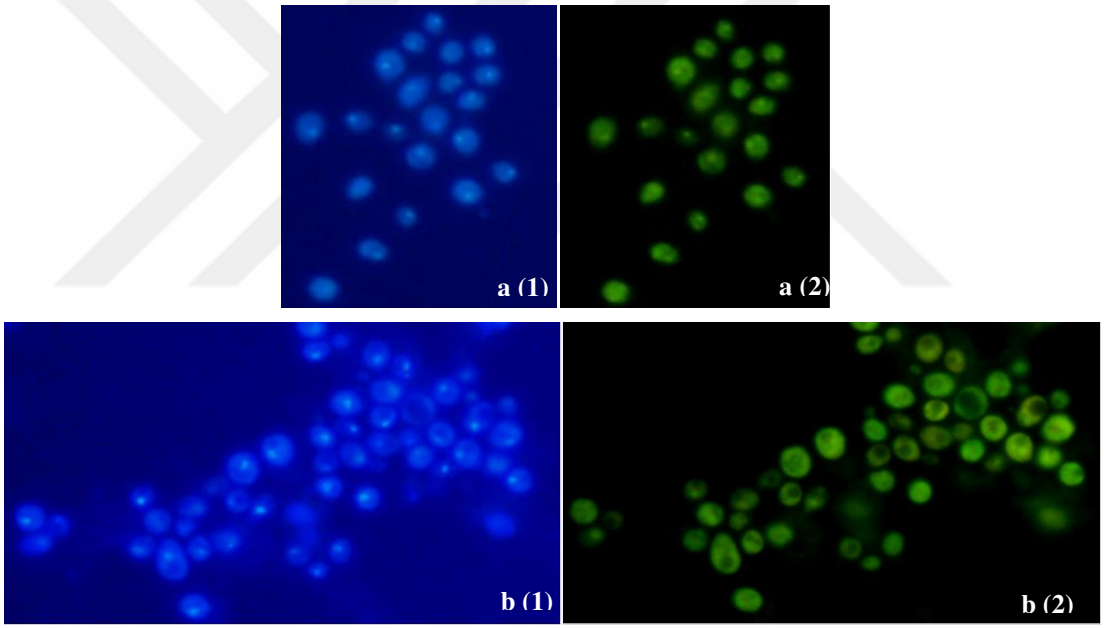


Figure 3.6 Ketoconazole-induced *S. cerevisiae* BY4742 cell death exhibits features of apoptosis. DAPI staining of 2% DMSO control (a1) and 225 µM ketoconazole treated cells for 4 h (b1). TUNEL staining of 2% DMSO treated control (a2) and 225 µM ketoconazole treated cells for 4 h (b2)

3.5 Clarification of Apoptotic Pathways Participated in Ketoconazole Induced Apoptosis via Mutant Cells

To determine the apoptotic proteins which are participated in ketoconazole induced apoptosis, nma111p, aifp, endoG and yeast caspases were investigated by

using the mutant *S. cerevisiae* BY4742 cells in which these genes were deleted. These four proteins are thought to work independently from each other and the homologues of these proteins are also the ones of the most responsible protein in human apoptosis Liang, et al., 2007).

3.5.1 The Dependency of Apoptosis Induced by Ketoconazole on Nma111p

Nma111p is Htr2/Omi homologues nuclear serine protease and can hydrolyse Bir1p which is only known as inhibitor protein of apoptosis in *S. cerevisiae*. Contrary to Htr2/Omi, which is located in mitochondria and translocate to nucleus in apoptotic conditions, Nma111p is located in nucleus in also physiological conditions. In the study of Fahrenkrog et al. (2004), the yeast cells which do not contain Nma111p were more resistant to elevated temperature and despite the induction of apoptosis in wild type; H_2O_2 did not induce apoptosis in NMA111 mutants.

To understand the effect of NMA111p on ketoconazole induced apoptosis, the lack of NMA111 gene cells exposed to ketoconazole at optimum apoptotic conditions (225 μM, 4 h) and compared with its control (Figure 3.7). As seen in Figure 3.7, apoptotic cell death could not be induced by ketoconazole treatment and necrotic cell death also did not significantly increase ($p>0.01$) although in wild type BY4742 cells, apoptotic cell population was increased as 65.5 %. This result showed that ketoconazole based apoptotic induction is directly related with nma111p and absence of this protein prevents apoptosis in *S. cerevisiae* BY4742. According to literature view, there has not been any data about the relation between ketoconazole and nma111p. On the other hand, the effect of cisplatin, one of the widely used anticancer drugs, on colon cancer was found based on HtrA2/Omi protein (Pruefer et al., 2008).

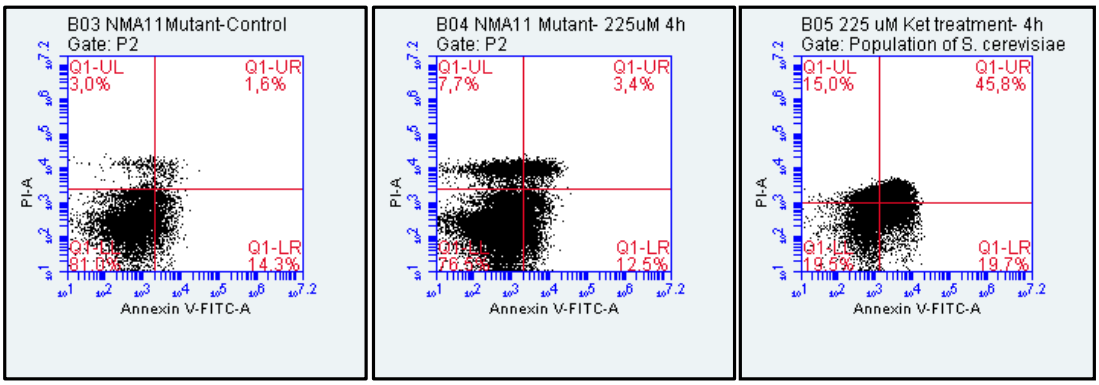


Figure 3.7 The percentages of live, early, late apoptotic and necrotic population of ΔNma111 and wild type *S. cerevisiae* BY4742 cells after treatment with 200-250 µM ketoconazole for 2 and 4 h compared to 2% DMSO treated controls. (Q1-LL; Healthy cells Q1-LR; Early apoptotic cells, Q1-UR; Late apoptotic cells, Q1-UL; necrotic cells)

3.5.2 The Dependency of Apoptosis Induced by Ketoconazole on Aif1p

Apoptosis inducing factor (AIF) is a mitochondrial flavoprotein with NADH oxidase activity (Wissing et al., 2004). This protein is located in mitochondria in physiological conditions and translocated to nucleus when apoptosis occurs. The AIF pathway is described as the main caspase independent pathway and very important for development and neurodegeneration (Susin et al., 1999; Cregan et al., 2004). When AIF deleted mutant treated with ketoconazole at the optimum apoptotic condition, apoptotic cell death decreased to 11.6 % from 65.5 %. On the other hand, necrotic cell death increased to 24.6 %. It can be clearly said that ketoconazole-induced apoptosis pathway is related to AIF1.

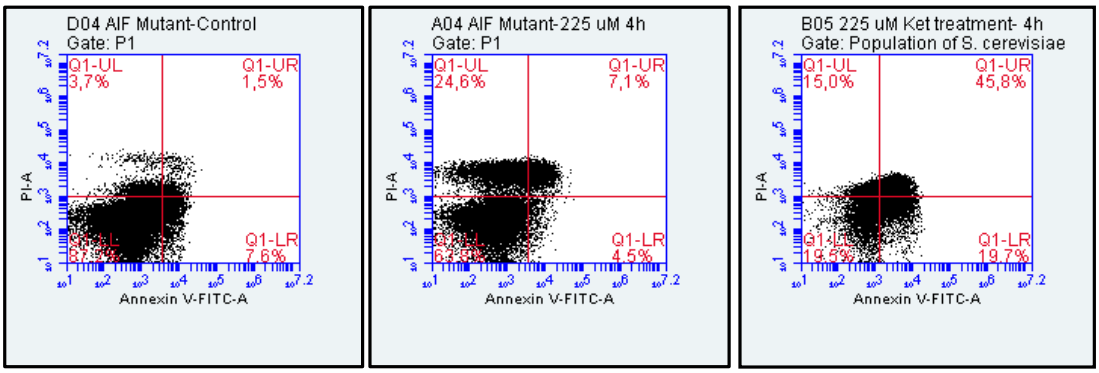


Figure 3.8 The percentages of live, early, late apoptotic and necrotic population of ΔAif and wild type *S. cerevisiae* BY4742 cells after treatment with 200-250 µM ketoconazole for 2 and 4 h compared to 2% DMSO treated controls. (Q1-LL; Healthy cells Q1-LR; Early apoptotic cells, Q1-UR; Late apoptotic cells, Q1-UL; necrotic cells)

In the study of Wissing et al. (2004), apoptosis induced by the treatment with 0.6 mM H_2O_2 and 200 mM acetic acid to *S. cerevisiae* and its aging process were to be found dependent to aif1p and . Additionally, while there was a role of aif1p on Mn induced apoptosis in *S. cerevisiae,* Cu, selenite and hyperosmotic stress-induced apoptosis seemed to be independent from aif1p (Izquierdo et al., 2010, Silva et al., 2005). In ascorbic acid, luteolin treated human breast cancer cell lines, apoptosis seemed to be dependent to aif1p (Kim et al., 2012). Dependency of apoptosis induced by cisplatin, granzyme B and taxol is also showed in some cancer cell lines (Yang et al., 2008; Rousalova et al., 2010; Kim et al., 2012, Ofir et al., 2002).

3.5.3 The Dependency of Apoptosis Induced by Ketoconazole on yeast EndoG

EndoG is one of the mithocondrial endonucleases and translocates to nucleus and attacks to nuclear DNA in apoptotic process (Burhans & Weinberger, 2007). In addition, EndoG-mediated apoptosis is independent from yca1p and aif1p but dependent to transition pore components, Histone H2B and karyopherin. To determine the role of EngoG in ketoconazole induced apoptosis, mutant S.cerevisiae BY4742 cells that do not contain endoG protein treated with ketoconazole at the optimum apoptotic conditions which was determined for wild type and the induction of apoptosis was not observed in ΔEndoG mutants and necrotic cell death was not as much as ΔAif mutants, similar with ΔNma111 (Figure 3.9).

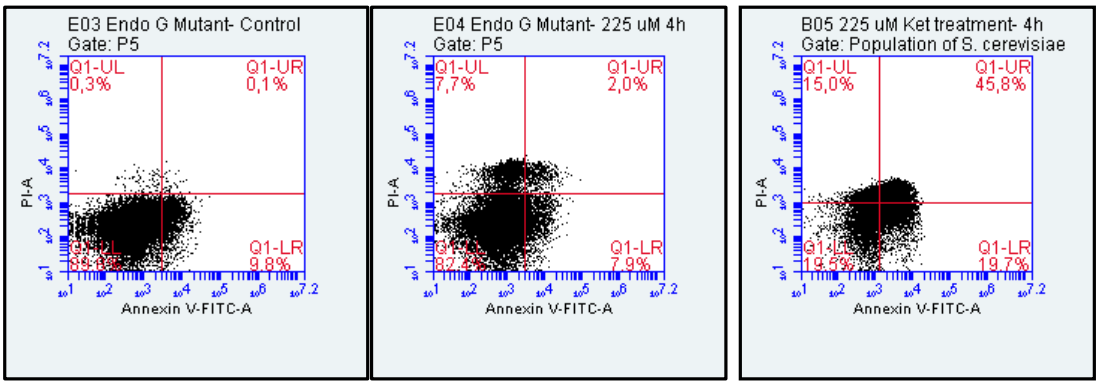


Figure 3.9 The percentages of live, early, late apoptotic and necrotic population of ΔEndoG and wild type *S. cerevisiae* BY4742 cells after treatment with 200-250 µM ketoconazole for 2 and 4 h compared to 2% DMSO treated controls. (Q1-LL; Healthy cells Q1-LR; Early apoptotic cells, Q1-UR; Late apoptotic cells, Q1-UL; necrotic cells)

The toxicity of α-synuclein, an intracellular trigger of Parkinson's disease, was found as related with EndoG was shown in S. cerevisiae (Buttner et al., 2013). On the other hand, Carmona-Gutierrez et al. investigated the relation between yeast EndoG and HOCl-induced apoptosis and EndoG is found irresponsible for apoptosis in *S. cerevisiae* (Carmona-Gutierrez et al., 2013). Additionally, in the apoptosis induced by propolis, *Mentha piperita* essential oil was determined as unrelated with EndoG pathway (Buttner et al., 2008, Ferreir et al., 2014).

3.5.4 The Dependency of Apoptosis Induced by Ketoconazole on Yeast Caspase

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, were often dependent on the activity of the yeast caspase (Yca1) which is a homologous of mammalian caspases (Reiter et al., 2005). The caspases are members of a family, or structurally related group, known as cysteine proteases (Mazzoni and Falcone, 2008). The apoptotic events observed in *S. cerevisiae*, due to external stimuli as well as internal signals, were often dependent on the activity of Yca1 which is a homologous of mammalian caspases (Reiter et al., 2005). In order to determine the connection of the apoptotic cell death induction caused by ketoconazole and Yca1 enzyme, the activity in *S. cerevisiae* treated with 225 µM ketoconazole for 4 h was detected with flow

cytometry and fluorescence microscopy. Although the induction of apoptotic cell death in *S. cerevisiae* was rather higher than the control, the Yca1 activity was only induced 1.61 fold of its control (Figure 3.10). When the 65.5% apoptotic cell death was considered, the 1.61 fold induction of Yca1p seemed inadequate to induced apoptotic cell death alone. In order to clarify this situation, the Yca1p deleted mutant BY4742 cells were treated with 225 µM ketoconazole for 4 h and cell death ratios were detected by Annexin V-PI staining (Figure 3.11) and the mutant cells showed 30.4 % apoptotic cell death. According to these results, it can be said that yca1p participated in ketoconazole induced apoptotic process but not responsible for predominantly and the deletion of gene may also slow down the apoptotic process because there is only 2% necrotic cell death and as discussed above, the observed necrotic cells are most probably secondary necrosis.

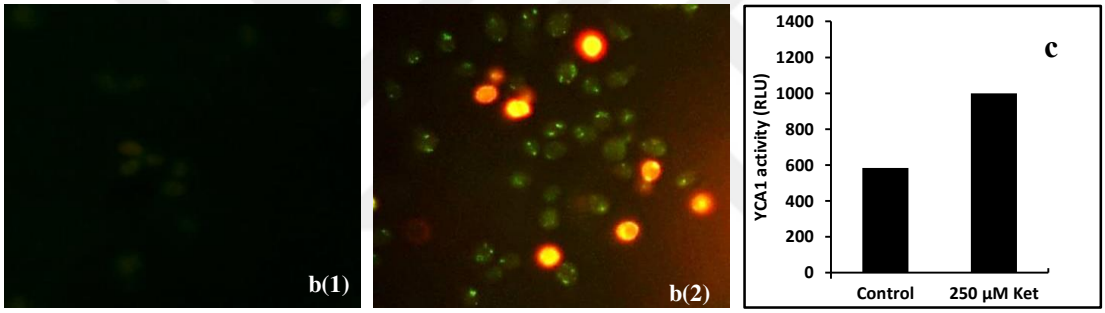


Figure 4.10 Yeast caspase activities in *S. cerevisiae* BY4742 cell after treatment with 225 µM ketoconazole for 4 h and 2% DMSO control. Flow cytometric analysis of YCA1 (a1,2); Fluorescence microscopy images (b1,2); The relative fluorescence of the activities(c)

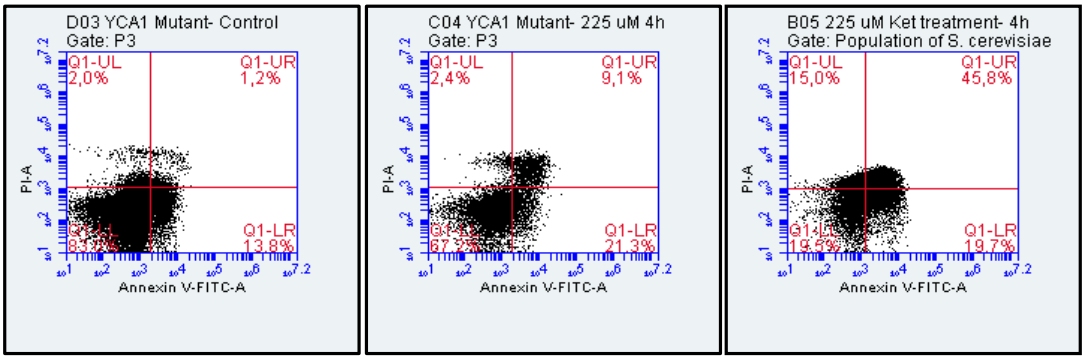


Figure 3.11 The percentages of live, early, late apoptotic and necrotic population of ΔYCA1 and wild type *S. cerevisiae* BY4742 cells after treatment with 200-250 µM ketoconazole for 2 and 4 h compared to 2% DMSO treated controls. (Q1-LL; Healthy cells Q1-LR; Early apoptotic cells, Q1-UR; Late apoptotic cells, Q1-UL; necrotic cells)

3.6 Alterations in Intracellular ROS Levels and the Antioxidant System of *S. cerevisiae BY4742* Depending on Ketoconazole Treatment

One of the most important reasons of diseases and aging is ROS production and the imbalance between antioxidant response system and ROS (Perrone et al., 2008). On the other hand the amount of ROS determines the cell death types in much cases and if it promotes apoptotic cell death, it predominantly induces intrinsic pathway of apoptosis. In this part of thesis, the levels of $O_2^{\bullet -}$, H_2O_2, total ROS, $HO^{\bullet}$ and $NO^{\bullet}$, the activities of the $O_2^{\bullet -}$-producing enzymes NADH and NADPH oxidases and the antioxidant enzymes SOD, CAT, GR, GSHPx, GST and G6PDH, the levels of GSH, GSSG and membrane LPO levels in eukaryotic model *S. cerevisiae* were investigated. We have found IC_{50} value of ketoconazole on *S. cerevisiae* as 200 µM after 6 h treatment and this study performed in the concentration range of 150–250 µM and time range of 1-6 h, including the determined IC_{50}. All of the results were compared with related controls.

3.6.1 Variation in Intracellular ROS levels in ketoconazole-treated S. cerevisiae BY4742

3.6.1.1 $O_2^{\bullet-}$ level

The most important reaction of $O_2^{\bullet-}$ is participation to dismutation reaction that produces H_2O_2 and O_2 (Waris & Ahsan, 2006). After ketoconazole treatment, $O_2^{\bullet-}$ in *S. cerevisiae* BY4742 cells were higher than their controls, except in the last hour of incubation time (p<0.05) (Figure 3.12). The levels increased with increasing ketoconazole concentration up to the 4th hour except 250 µM at which the highest level was seen at the 1st h of incubation as 45.32±2.88 relative light unit (RLU), 2.23 times its control, and showed decreasing tendency in the following period. Although we cannot encounter with the literature about the effect of ketoconazole on $O_2^{\bullet-}$ production in any cell type, in the studies conducted with *Vibrio cholera* and rat testis exposed to 300 µM paraquat for 1 h and 200-mg/kg methoxychlor for 7 days, respectively, the formation of $O_2^{\bullet-}$ was less induced than the result obtained from 250 µM ketoconazole treatment for 1 h (Abrashev et al., 2011; Latchoumycandane & Mathur, 2002). While ketoconazole resulted in the formation of $O_2^{\bullet-}$ in *S. cerevisiae,* it was shown that patulin and citrinin could not alter the $O_2^{\bullet-}$ levels in another eukaryotic yeast model *Schizosaccharomyces pombe* (Pape et al., 2016). In another study, it was shown that exposure to 60 µM myclobutanil and 70 µM cyproconazole fungicides for 24 h induced the formation of $O_2^{\bullet-}$ in *Tetrahymena thermophila* cells (Huang et al., 2016).

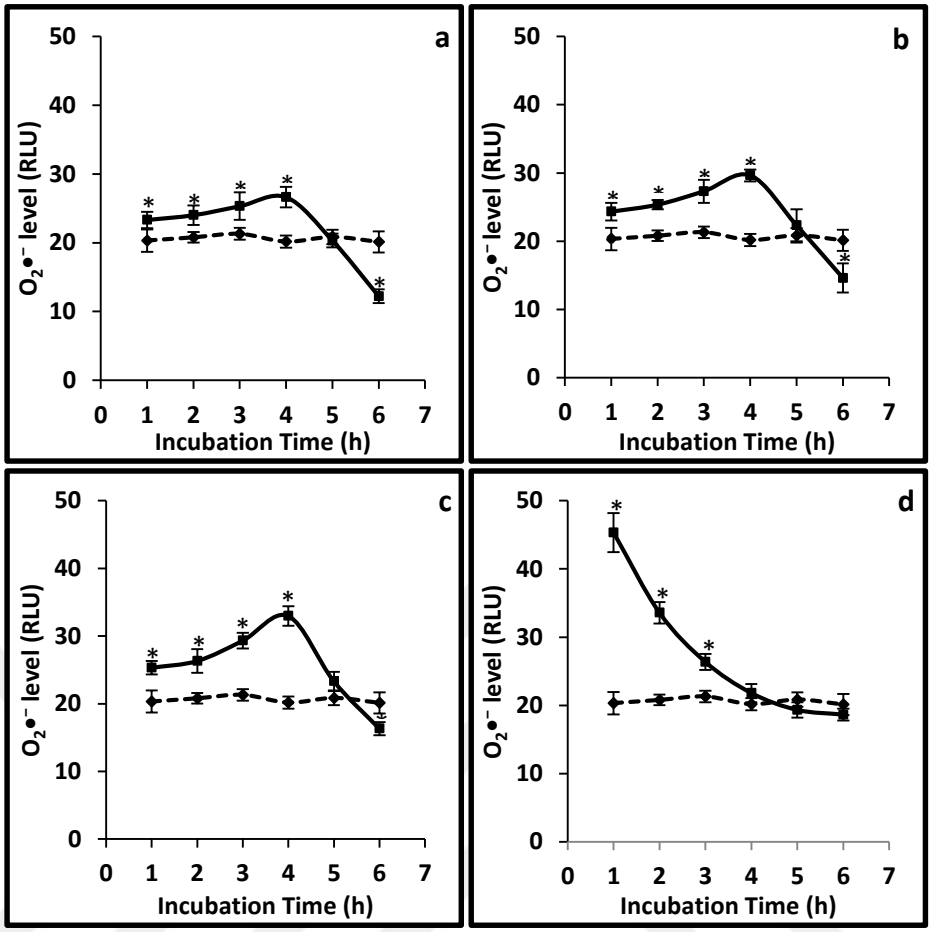


Figure 3.12 Variation in $O_2^{\bullet-}$ levels depending on the incubation period and concentration of ketoconazole; control (–♦–) , and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d). The results were obtained from three independents experiments and expressed as mean±SD. * Significant difference ($p<0.05$) from control

3.6.1.2 H_2O_2 level

H_2O_2 is one of the non-radical ROS that can cross cell membranes easily. Although H_2O_2 seems to be harmless, it is the precursor of the $HO^{\bullet}$ radical (Waris & Ahsan, 2006). As can be seen from Figure 3.13, ketoconazole treatment caused induction of H_2O_2 formation, similar with $O_2^{\bullet-}$ in *S. cerevisiae* BY4742 compared to the controls ($p<0.05$) and the levels maintained in certain levels throughout at all investigated concentrations. The results showed that increased ketoconazole concentration caused more H_2O_2 production and the highest increases were determined as approximately 4.3 fold of their controls. Despite the lack of any data about the effect of ketoconazole on the production of H_2O_2, similar to $O_2^{\bullet-}$, 200 µM

menadione was found to increase H_2O_2 level in *Phanerochaete chrysosporium* to 1.35 times within 3 h, while a 2.65-fold increase was observed in *S. cerevisiae* for the same concentration of ketoconazole and incubation time (Tongul & Tarhan, 2014). In addition, H_2O_2 level in *V. cholera* exposed to 300 µM of paraquat for 1 h increased by only 2.03 times (Abrashev et al., 2011).

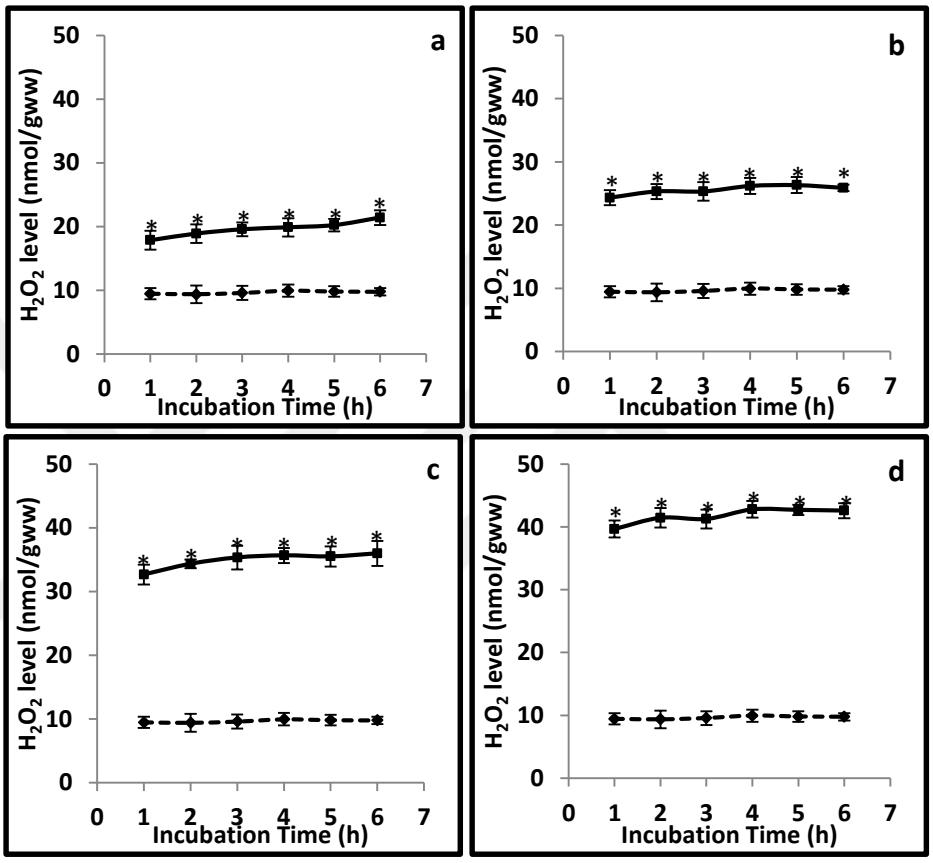


Figure 3.13 Variation in H_2O_2 levels depending on the incubation period and concentration of ketoconazole; control (–♦–), and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d). The results were obtained from three independents experiments and expressed as mean±SD. * Significant difference ($p<0.05$) from control

3.6.1.3 Total ROS level

The total ROS levels in all ketoconazole treatment were higher than their control levels and increased with increasing concentrations ($p<0.05$) (Figure 3.14). The highest total ROS level was 2.68 ± 0.07 RLU, 4.24 fold of its control. In addition, while the levels increased up to the 4th hour at 150–225 µM, they did not change

significantly in the following period (p>0.05). This situation may reflect that total ROS formation caused by ketoconazole treatment slowed down or the total ROS levels could not be measured properly because of more the effective participation in cell damage process. The variations of total ROS levels in 250 µM ketoconazole-treated *S. cerevisiae* increased continuously throughout incubation with the decreased acceleration after the 3rd hour that may be due to similar reasons.

The increases in total ROS, despite the general stability of H_2O_2 in *S. cerevisiae*, may be a proof for the formation of other hydroperoxides besides H_2O_2. In the study conducted with another antifungal, the total ROS level in *C. albicans* after the treatment with 10 µM amphotericin B for 200 min was found as 2.00 times its control (Phillips et al., 2003). Although the minimum investigated ketoconazole dose was rather higher than 10 µM, only a 2.52-fold increase was observed in *S. cerevisiae* at the end of 180 min. On the contrary, 163 µM fluconazole treatment for 2 h resulted in a 1.44-fold increase in *C. albicans* that was similar to the result of the 150 µM ketoconazole treatment (Kaur et al., 2004). In another study, total ROS level in *Cryptococcus gattii* exposed to 104 µM fluconazole increased 2.5 times compared with related control after 1 h at which only a 1.3-fold increase was observed in 150 µM ketoconazole-treated *S. cerevisiae* (Ferreira et al., 2013). While the total ROS level was found 2.08-fold higher than its control in 250 µM ketoconazole-treated *S. cerevisiae* for 1 h, only a 1.39-fold increase was observed in *S. pombe* cells treated with patulin and citrinin mycotoxins at the same conditions in the study by Papp et al (Papp et al., 2016).

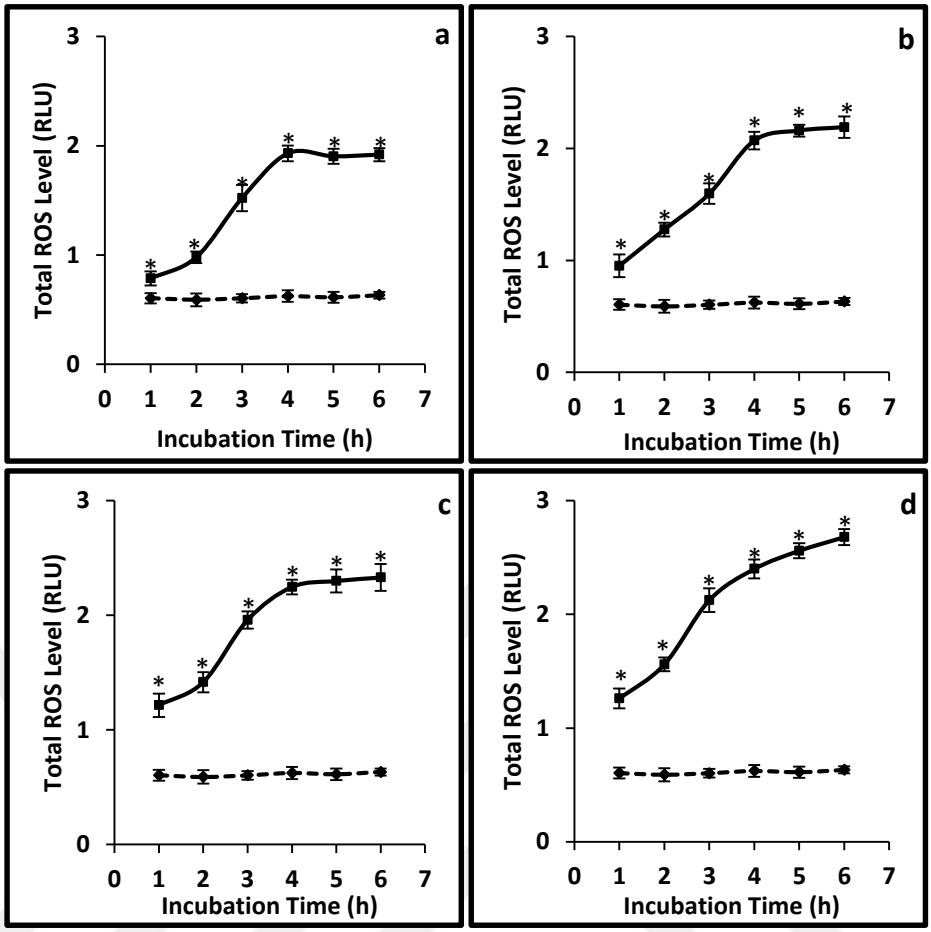


Figure 3.14 Variation in Total ROS levels depending on the incubation period and concentration of ketoconazole; control (–♦–), and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d). The results were obtained from three independents experiments and expressed as mean±SD. * Significant difference ($p<0.05$) from control

3.6.1.4 HO• level

The HO• levels in ketoconazole treated *S. cerevisiae* increased with increasing concentration and time that the levels were over the control ($p < 0.05$), except at 150 µM for the first 2 h ($p>0.05$) (Figure 3.15). The highest HO• levels was found in 225 and 250 µM ketoconazole-treated cells that was approximately 2.4-fold higher than their controls at 6th h. the observed negative correlation between HO• and $O_2^{\bullet -}$ ($r_{150\mu M} = -0.77$; $r_{200\mu M} = -0.65$; $r_{225\mu M} = -0.70$; $r_{250\mu M} = -0.76$) most probably caused by the Haber-Weiss reaction that produced HO•. It was shown that HO• levels increased to approximately 4.37 times their control in *C. albicans* treated for 2 h with 9 µM arenicin-1 that was extremely lower than the minimum examined ketoconazole

concentration (Cho & Lee, 2011). Sampson et al. (2012) found approximately 2.50- and 2.00-fold increases in HO• levels of *Acinetobacter baumannii* treated with 5-ppm kanamycin and 2-ppm colistin for 30 min in comparison with their controls. In addition, HO• levels in HepG2 cells exposed to 200 µM adriamycin, which is an anticancer drug, was 30 times its control within 100 min (Liu et al., 2012). On the contrary, we only observed a 1.05-fold increase even in cells treated with 150 µM ketoconazole for 60 min.

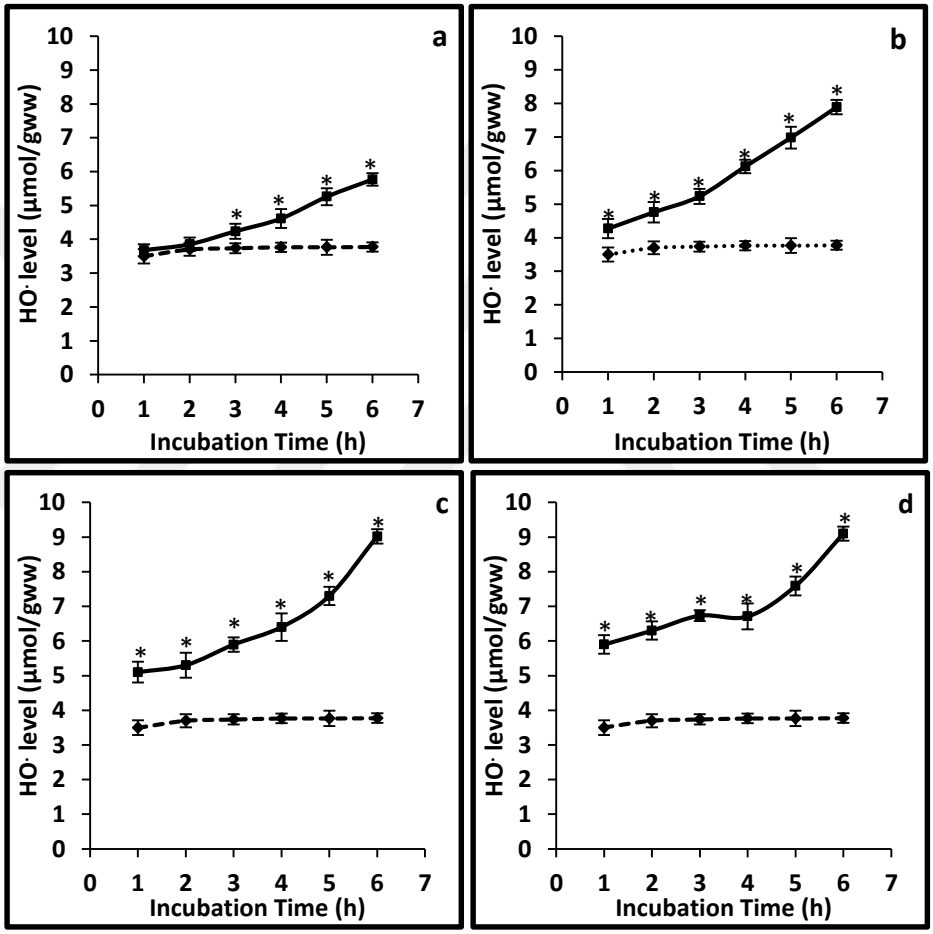


Figure 3.15 Variation in HO• levels depending on the incubation period and concentration of ketoconazole; control (–♦–), and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d). The results were obtained from three independents experiments and expressed as mean±SD. * Significant difference ($p<0.05$) from control.

3.6.1.5 NO• levels

NO• is present at the bottom of the 'reactivity scale' of free radicals and reacts slowly with most biological molecules, including thiols, cytochrome oxidase and

ribonucleotide reductase (Cooper, 1993). The NO• levels in all ketoconazole-treated *S. cerevisiae* were higher than the controls up to the fifth hour of incubation time (p<0.05). The levels at 150–225 μM were increased with increasing ketoconazole dose (p<0.05) without showing significant differences in a time-dependent manner up to the fourth hour (p > 0.05). The highest NO• level was found as 3.23 ± 0.11 μmol/gww in 250 μM ketoconazole-treated *S. cerevisiae* at the first hour, which was 3.43-fold higher than its control, but then showed decreasing tendency continuously as in the case of $O_2^{\bullet-}$ and dropped to 54% of its control at the end of incubation. The literature on the free-radical reaction of NO• has largely focused on its reaction with $O_2^{\bullet-}$ to give ONOO. In this study, the trends in the change of NO• and $O_2^{\bullet-}$ levels were similar after the treatment of ketoconazole and showed positive correlations ($r_{150\mu M}$ = 0.89; $r_{200\mu M}$ = 0.84; $r_{225\mu M}$ = 0.79; $r_{250\mu M}$ = 0.94). It may reflect that the observed decreases of NO• and $O_2^{\bullet-}$ at the same incubation periods may be caused by the reactions of these two radicals to form mainly $ONOO^-$. RNS level in *C. gatti* treated with 0.27 μM amphotericin B for 1 h was 1.52-fold higher than its control (Ferreira et al., 2013). Although the investigated ketoconazole doses were extremely higher, only the levels in *S. cerevisiae* treated with 225 and 250 μM ketoconazole for 1 h reached the values higher than these values.

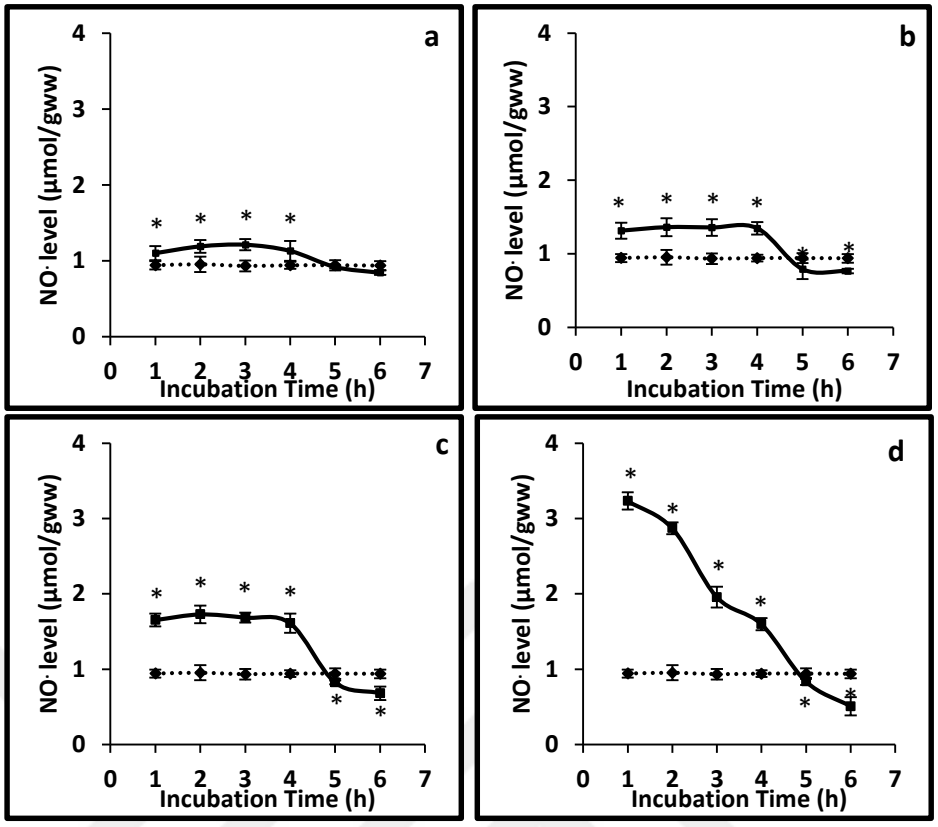


Figure 3.16 Variation in NO• levels depending on the incubation period and concentration of ketoconazole; control (–♦–), and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d). The results were obtained from three independents experiments and expressed as mean±SD. * Significant difference ($p<0.05$) from control

3.6.2 *Variations in NADH and NADPH oxidase activity levels in ketoconazole-treated S. cerevisiae*

NADH and NADPH oxidases are known as the $O_2^{\bullet -}$ producer enzymes as endogenous sources of ROS and transfer electrons from NAD(P)H across the membrane and when this electrons encounter O_2, they generate $O_2^{\bullet -}$ (Miramar, 2001). NADH oxidase levels in ketoconazole-treated *S. cerevisiae* were higher than the control in all concentrations and incubation times ($p < 0.05$), except the last 2 h of the 250 µM treatment ($p > 0.05$) (Figure 3.17). While NADH oxidase activities at 150 µM generally showed similar values throughout the incubation period, the levels increased in a time-dependent manner at 200 and 225 µM. Although the initial NADH oxidase activity was 4.31-fold higher than its control at 250 µM, it showed decreasing tendency from the beginning of incubation time and dropped to less than the control values.

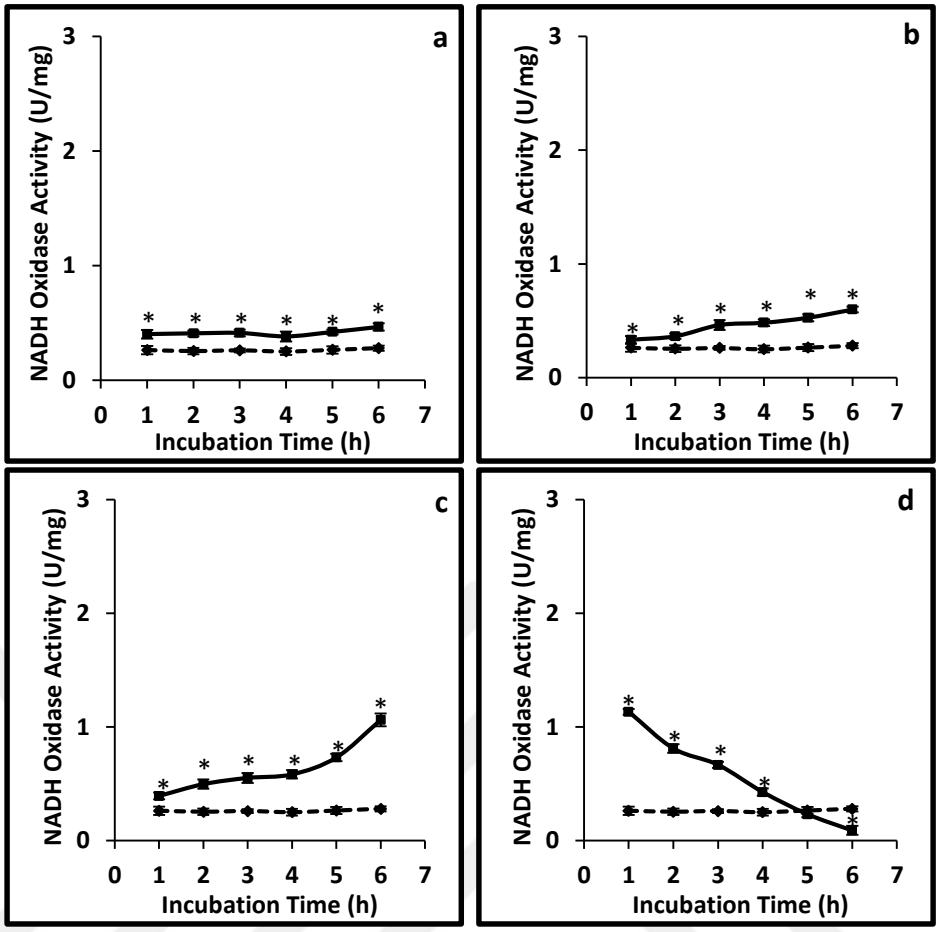


Figure 3.17 Variation in NADH oxidase activities depending on the incubation period and concentration of ketoconazole; control (–♦–), and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d). The results were obtained from three independents experiments and expressed as mean±SD. * Significant difference ($p<0.05$) from control.

In all treatments, NADPH oxidase activities were also significantly higher than their controls ($p<0.05$) (Figure 3.18). The activities increased time- and dose-dependently at the range of 150–225 µM. While there was a 13.39-fold induction in 250 µM ketoconazole-treated *S. cerevisiae* in the first hour of incubation, the activities continuously decreased and dropped to nearly control values at the end of incubation. There were strongly positive correlations between $O_2^{\bullet -}$ levels and NAD(P)H oxidase activities in 250 µM treatment (for NADH oxidase $O_2^{\bullet -}$: $r_{250\mu M}$ = 0.96; for NADPH oxidase $O_2^{\bullet -}$: $r_{250\mu M}$ = 0.97). On the contrary, positive correlation was observed up to the fourth hour of incubation at the other concentrations of ketoconazole. The induced antioxidant system in *S. cerevisiae* may prevent the observation of the correlations after the 4th hour.

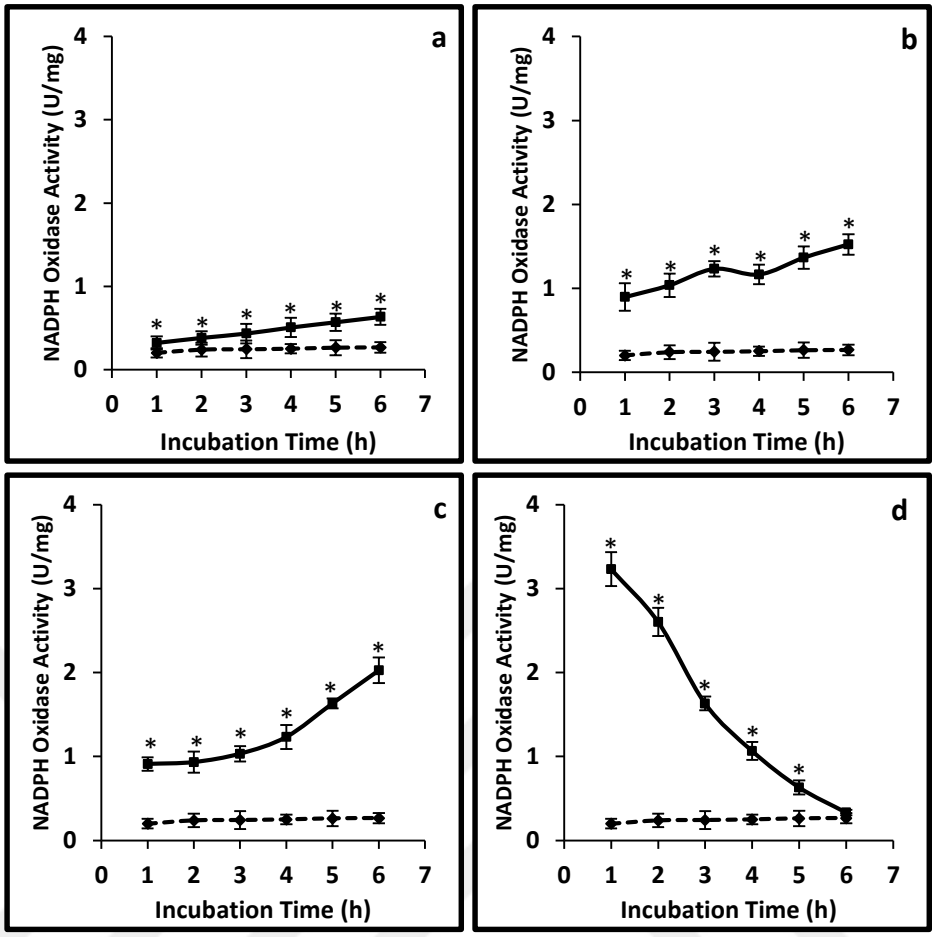


Figure 3.18 Variation in NADPH oxidase activities depending on the incubation period and concentration of ketoconazole; control (–♦–), and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d). The results were obtained from three independents experiments and expressed as mean±SD. * Significant difference ($p<0.05$) from control

3.6.3 Variation in antioxidant response system in ketoconazole-treated S. cerevisiae

3.6.3.1 SOD activity

The SOD activities in all ketoconazole treatments were higher than the controls ($p<0.05$) (Figure 3.19). The activities at the range of 150–225 µM showed increasing tendency more effectively after the third hour of incubation. On the contrary, the observed highest activity was 38.32 ± 1.59 U/mg, which was 3.11-fold higher than its control at the beginning of the period in 250 µM treated *S. cerevisiae*, and this value continuously decreased up to the fourth hour. Although SOD activity

inductions were observed at the range of 150–225 µM during all periods, only the accelerated activity increases after the third hour could be sufficient to prevent the increase of $O_2^{\bullet-}$ levels in the following incubation periods. In addition, there were negative correlations between $O_2^{\bullet-}$ level and SOD activity after treatment with 150–225 µM ketoconazole, while positive correlations were found at 250 *µM* ($r_{150\mu M}$ = −0.71; $r_{200\mu M}$ = −0.67; $r_{225\mu M}$ = −0.68; $r_{250\mu M}$ = 0.98). In this study, there were also highly positive correlations between SOD activities and H_2O_2 levels in ketoconazole-treated *S. cerevisiae*, except 250 µM treatment ($r_{150\mu M}$ = 0.93; $r_{200\mu M}$ = 0.94; $r_{225\mu M}$ = 0.73; $r_{250\mu M}$ = −0.90). This situation may reflect that the dismutation reaction is one of the main factors increasing H_2O_2 levels at the range of 150–225 µM, while other ways to produce H_2O_2 may be triggered at the highest investigated dose of ketoconazole. In addition, there were highly positive correlations between NAD(P)H oxidases and SOD activities (for NADH oxidase and SOD $r_{150\mu M}$ = 0.62; $r_{200\mu M}$ = 0.94; $r_{225\mu M}$ = 0.97; $r_{250\mu M}$ = 0.88; for NADPH oxidase and SOD $r_{150\mu M}$ = 0.99; $r_{200\mu M}$ = 0.91; $r_{225\mu M}$ = 0.99; $r_{250\mu M}$ = 0.93). It was clear that ketoconazole treatments induced NAD(P)H oxidase activities and consequently the $O_2^{\bullet-}$ levels. Therefore, it can be certainly said that the antioxidant system of *S. cerevisiae* is triggered to protect the cells from ketoconazole-based ROS production and exposure of oxidative stress. Although 150 µM ketoconazole treatments increased the activity 2.1-fold higher than its control within 6 h, 104 µM fluconazole did not affect and 0.7 µM itraconazole increased only 1.12 times in *C. gattii* (Romeo et al., 2012). In the study of Ahmad et al. (2011). SOD activities in *C. albicans* treated with 133 µM thymol and 67 µM carvacrol reached 2.04- and 2.12-fold higher values, similar to the findings of theirs.

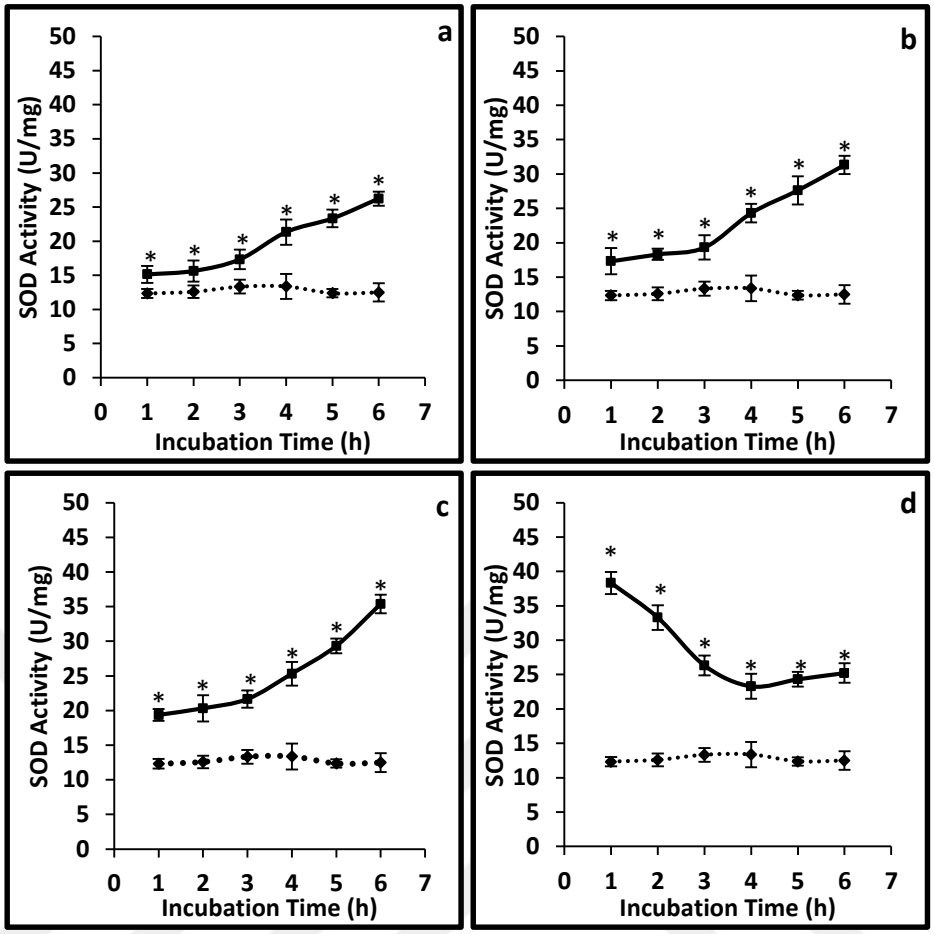


Figure 3.19 Variation in SOD oxidase activities depending on the incubation period and concentration of ketoconazole; control (–♦–), and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d). The results were obtained from three independents experiments and expressed as mean±SD. * Significant difference ($p<0.05$) from control.

3.6.3.2 CAT activity

CAT is a very important enzyme in protecting the cell from oxidative damage by ROS and mainly catalyses decomposition reaction of H_2O_2 (Deisseroth & Dounce, 1970). CAT activity in *S. cerevisiae* varies depending upon the microbial growth phase. While the activities observed at stationary phase were at between 7 and 8 U/mg in control cells, this value decreased to 0.78-U/mg protein at exponential phase during ketoconazole treatment.

CAT activities in all ketoconazole-treated *S. cerevisiae* were higher than control from the beginning of incubation time ($p < 0.05$) and also increased with increasing

concentrations (Figure 3.20).While the activities continuously increased at 150 µM throughout the investigated incubation period, they did not significantly change at 200–250 µM from the fourth hour of incubation. The CAT activities reached their observed highest levels from the fourth hour with approximately 3.57-fold increase. In addition, there were highly significant correlations between CAT activities and H_2O_2 levels in all treatments ($r_{150\mu M}$ = 0.96; $r_{200\mu M}$ = 0.98; $r_{225\mu M}$ = 0.95; $r_{250\mu M}$ = 0.95). This situation may be explained by the fact that the inductions in CAT activities were due to the production of H_2O_2, and these inductions could not be sufficient to decrease the levels of H_2O_2 but only to maintain them at a certain level. SOD and CAT activities also showed positive correlations, except 250 µM, and it is possible that the increasing production of H_2O_2 caused by the SOD induction took part in the induction of CAT activity ($r_{150\mu M}$ = 0.99; $r_{200\mu M}$ = 0.90; $r_{225\mu M}$ = 0.81; $r_{250\mu M}$ = −0.97). On the contrary, the stable H_2O_2 and total ROS levels besides the observed decreasing levels of $O_2^{\bullet -}$ showed that there were also other ways to produce H_2O_2 and other hydroperoxides. CAT activities in *C. albicans* treated with 133 µM thymol and 67 µM carvacrol were increased 2.61 and 2.11 times, respectively, and these agents seem to have produce effects similar to ketoconazole in terms of induction of SOD and CAT (Ahmad et al., 2011).

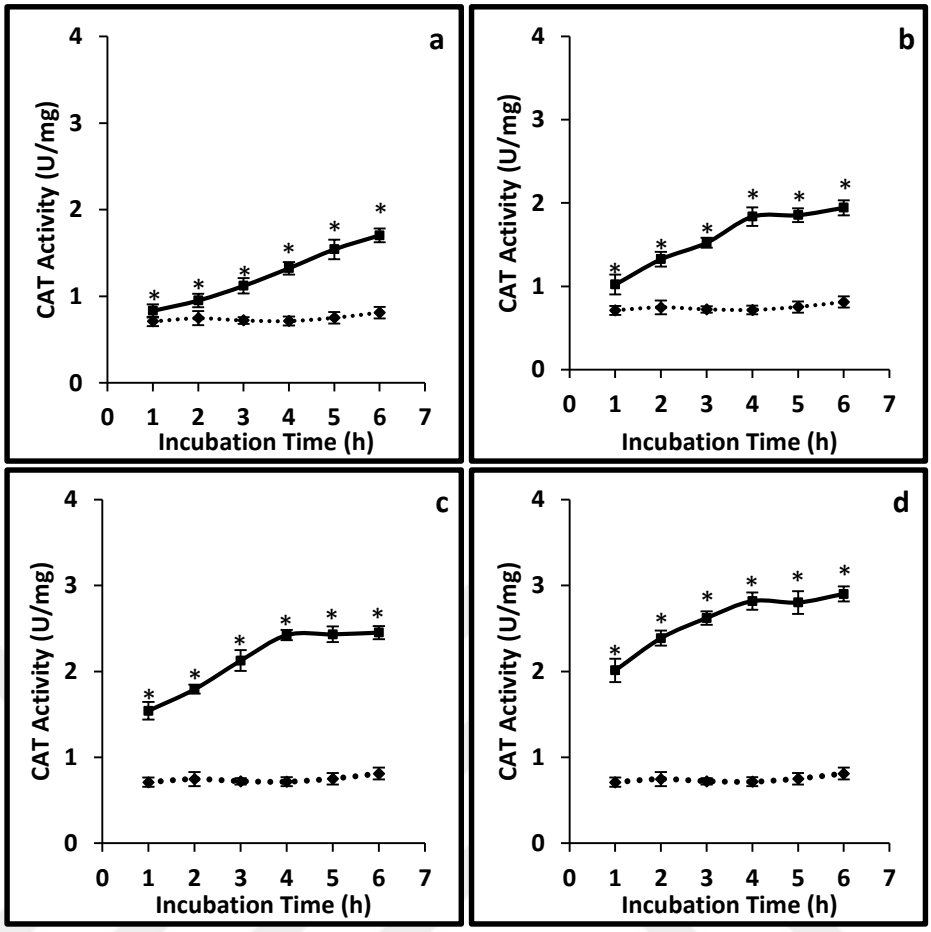


Figure 3.20 Variation in CAT oxidase activities depending on the incubation period and concentration of ketoconazole; control (--♦--), and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d). The results were obtained from three independents experiments and expressed as mean±SD. * Significant difference ($p<0.05$) from control

3.6.4 The Response of Glutathione System in S. cerevisiae Treated With Ketoconazole

GSH- and GSH-dependent enzymes are known to be important for the detoxification of xenobiotics and drugs (Blokhina et al, 2003). Therefore, this multicomponent system is essential for cell resistance against oxidative stress. The variations of the intracellular GSH and GSSG levels and the activities of GSH-Px, GST, and GR determined depending on ketoconazole concentrations and treatment times and compared with the non-treated control cells.

3.6.4.1 The variation in GSH levels in ketoconazole treated S. cerevisiae

GSH is the most abundant antioxidant in the cell. Its protective effect is dependent upon the oxidation of the thiol group of its cysteine to form oxidized glutathione (GSSG) (Reed, 1990). The GSH levels in 150-225 µM ketoconazole treated *S. cerevisiae* cells increased from the beginning of incubation time and the increasing rate was correlated with increasing ketoconazole concentration. The reached highest GSH level was 6.36 µmol/gww at 225 µM treatment for 6 h that was 2.74 fold of its control. On the other hand, the levels in 250 µM treated cells were under the control levels from the beginning of incubation time and decreased to 4.31% of its control at the end of incubation time. The increased GSH levels, except 250 µM treatment showed the induction of antioxidant system in *S. cerevisiae* in response the ketoconazole treatment. Although GSH levels and CAT activities increased in all incubation time, these increases could not be enough to decrease H_2O_2 levels. In the study of Rodriguez & Buckholz (2003), the 90 mg/kg ketoconazole treatment to Sprague–Dawley rats caused 2.1-fold GSH depletion after 24 h in hepatic cells. In many studies about the toxicity of ketoconazole pointed out the depletion of GSH (Ozyazici et al., 2007; Buckholz, 2003). On the other hand, ketoconazole treatment for infected mice with *S. mansoni* increased the GSH levels in liver tissue up to their uninfected values (Saif el-Din, 2013). Lee et al (1992) reported that the treatment of *Schizosaccharomyces pombe* with 1 mM menadione, one of the superoxide anion radical producer agents, for 3 hours decreased the intracellular GSH level to 41% of the control. Melanoma cells were also treated with doses of 5-20 µM of 1,4-naphthoquinone, which resulted in 12-77% drops in the levels of intracellular GSH (Kumar, 2009). Additionally, fluconazole, one of the azole antifungal, caused 25% decrease in GSH of *C. albicans* at the dosage of 5 mg/L. 50 µM (Maras, et al., 2014). Fumonisin B1, the most prevalent member of a family of toxins, exposure to Pig kidney cells for 24 h decreased GSH levels from 56 to 42.7 pmol/mg protein (Kang & Alexander, 1996). In the study of Fekete et al. (2007), GSH levels increased 1.33 fold with 6 mM tert-butylhydroperoxide treatment. In addition, *C. albicans* cells treated with 200 µM farnesol, one of the antifungal, for 4 h decreased approximately

2-fold of its control (Zhu et al., 2011). The levels in menadione treated *S. cerevisiae* decreased up to 3.33% of its control at the dosage of 20 mM within 1 h.

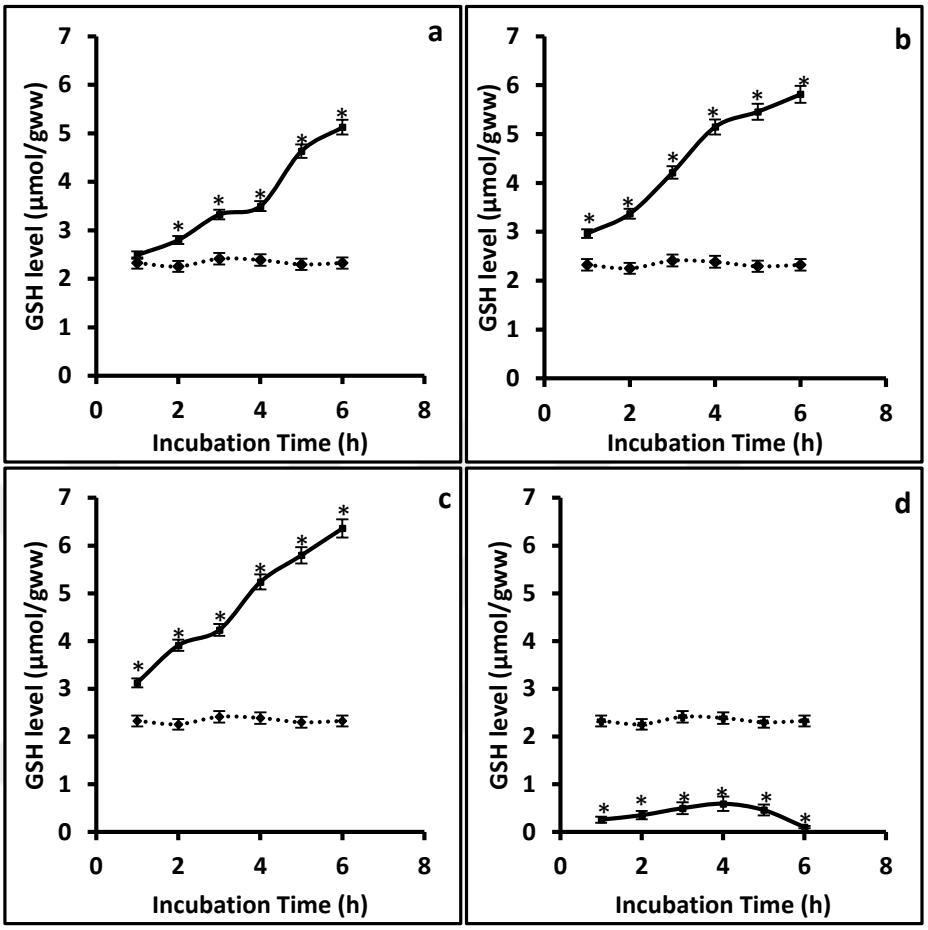


Figure 3.21 Variation in GSH levels depending on the incubation period and concentration of ketoconazole; control (--♦--), and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d).

3.6.4.2 The variation in GSSG levels in ketoconazole treated S. cerevisiae

Excessive GSSG is toxic to biological molecules because of its high reactivity with free sulfhydryl groups (Patel et al, 2011). Under the physiological conditions of living systems, the GSSG level is expected to be lower than the GSH level. The effects of exogenous factors are important in maintaining this balance. GSSG levels in all ketoconazole treatment, except 150 µM were increased within the first hour of incubation time in *S. cerevisiae* BY4742. In addition, the increases in the levels of initial GSSG concentrations were correlated with increasing ketoconazole

concentrations (r=0.98) and the observed highest value was 4.31-fold of its control. While GSSG levels in 200-250 μM ketoconazole treated cells started to decrease after first hour up to 4th h, the levels in 150 μM treated cells increased from the beginning of incubation time but did not reached the highest levels that observed in other treatments. The levels in 250 μM treated cells decreased more rapidly than other treatments and decreased to 37.32 nmol/gww from 109.32 nmol/gww. The rapid increases in GSSG levels at up to first hour showed the toxic effect of ketoconazole and the reason of observed decreases in the following incubation time may be related with the induced antioxidant response systems and also releasing from the cell. 6 mM tert-butylhydroperoxide treatment to *C. albicans* caused 5.2-fold increases in GSSG levels (Fekete et al., 2007). Additionally, fluconazole treatment did not affect GSSG levels in *Candida albicans* at the dosage of 5 mg/L (Maras, et al., 2014). Farnesol treatment to *C. albicans* did not change GSSG levels even at the dosage of 200 μM. The levels in menadione treated *S. cerevisiae* increased 4.74 fold of its control at the dosage of 20 mM within 1 h (Castro et al., 2008).

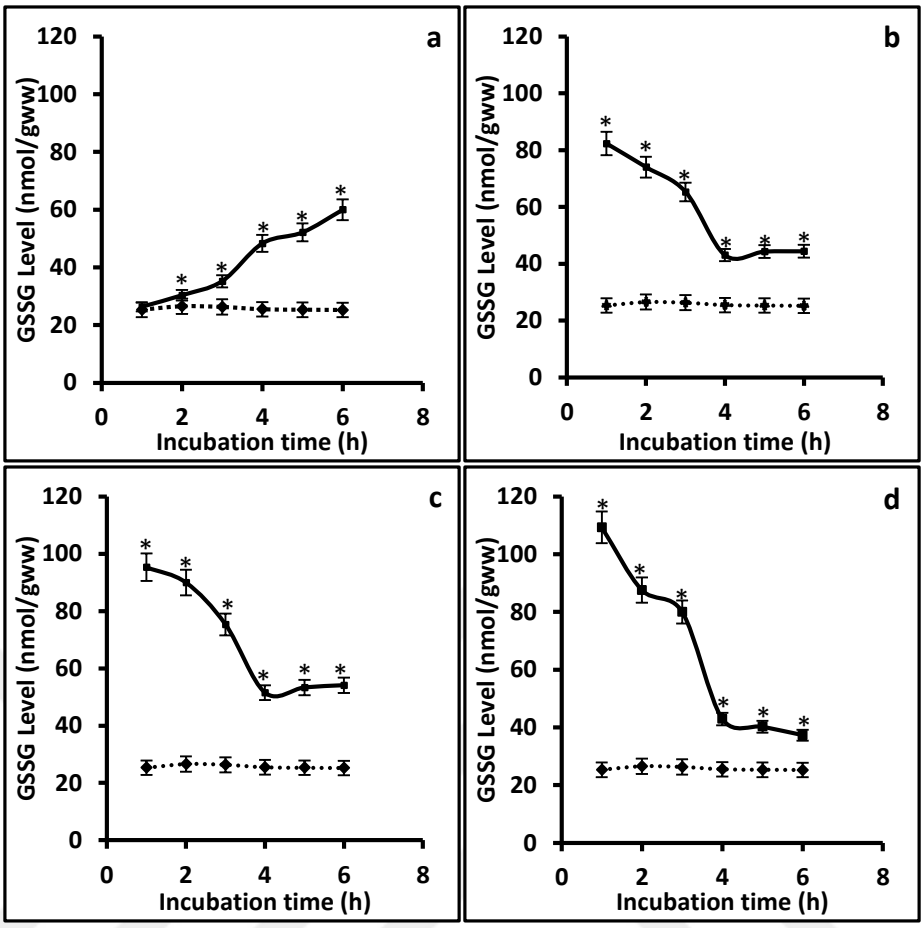


Figure 3.22 Variation in GSSG levels depending on the incubation period and concentration of ketoconazole; control (–♦–) , and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d).

3.6.4.3 The variation in GSH-Px activities in ketoconazole treated S. cerevisiae

The GSH-Px is one of the important enzymes in GSH system that reduces H_2O_2 to H_2O. GSH-Px activities induced by ketoconazole treatment in all concentrations and increased with increasing ketoconazole treatment. The observed highest activity was 195.32 U/mg that was 4.61 fold of its control. Although the activities were higher than their controls, they decreased up to 4th h at 150-200 µM and all incubation time at 225-250 µM. According to these results, the reason of increasing GSSG levels seemed to be induced GSH-Px which uses GSH as co-substrate in the reduction of H_2O_2 to H_2O, except 150 µM treatment. In addition, the negative correlation was observed between GSH-Px and CAT activities in all ketoconazole treated cells

($r_{150\mu M}$= -0.94; $r_{200\mu M}$=-0.99; $r_{225\mu M}$ =-0.95; $r_{250\mu M}$=-0.90). In the study of Emri et al.(1997), The activity of GSH-Px in *Penicillium chrysogenum* treated with 0.5 mM tert-BOOH, for 5 hours was 1.52-fold higher than in the control cells (Emri et al., 1997). Additionally, the activities in fluconazole treated *C. albican* and Fumonisin B1 treated pig kidney cells did not change significantly (Maras, et al., 2014, Kang and Alexander, 1996).

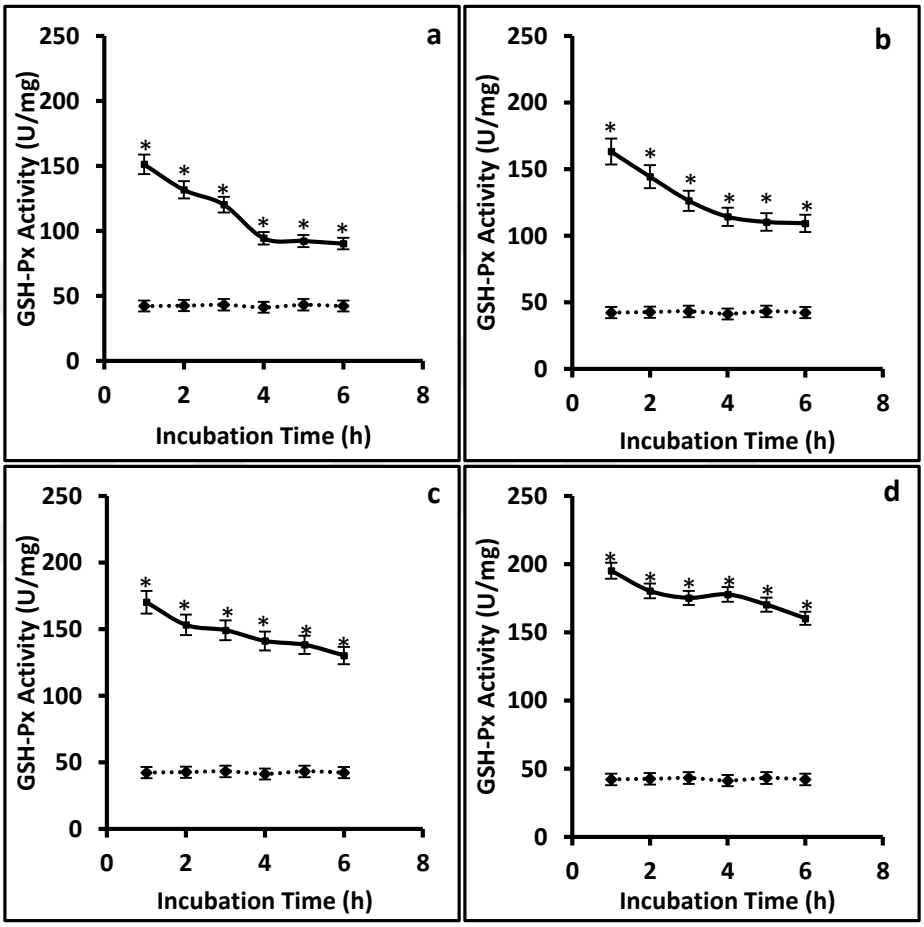


Figure 3.23 Variation in GSH-Px activities depending on the incubation period and concentration of ketoconazole; control (--♦--) , and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d).

3.6.4.4 The variation in GR activities in ketoconazole treated S. cerevisiae

GR, one of the enzymes in the glutathione system, is one of the major regulators of the GSH/GSSG ratio in cells (Zitka et al., 2012). The GR activities in ketoconazole treated *S. cerevisiae* cells showed different response to ketoconazole

compared to other glutathione related enzymes. GR activities in 150 µM treated cells were over the control in all incubation time and increased up to 4th hour of incubation. On the other hand, the activities in other treatments were under the control up to 4th hour but showed increasing tendency from the beginning of detected incubation times. The increased GSSG levels at the beginning of incubation time in all ketoconazole treatments, except 150 µM may be caused by the decreased GR activities besides the induced GSHPx. In the rat liver tissues exposed to 5-methoxytryptamine at 5 mg/kg body weight, the GR activity was increased only 1.11 fold of its control. According to a study by Lee at al (1997), the activity of GR in *Schiz. pombe* increased 2.72-fold over the control after the first hour of 200 µM menadione treatment, it was 2.8 fold lower in S. cerevisiae treated with 200 µM ketoconazole. In *Arabidopsis thaliana callus* exposed to 10 µM abscisic acid, GR activity increased 1.5 fold at the end of 8th day (O'Kane et al., 1996).

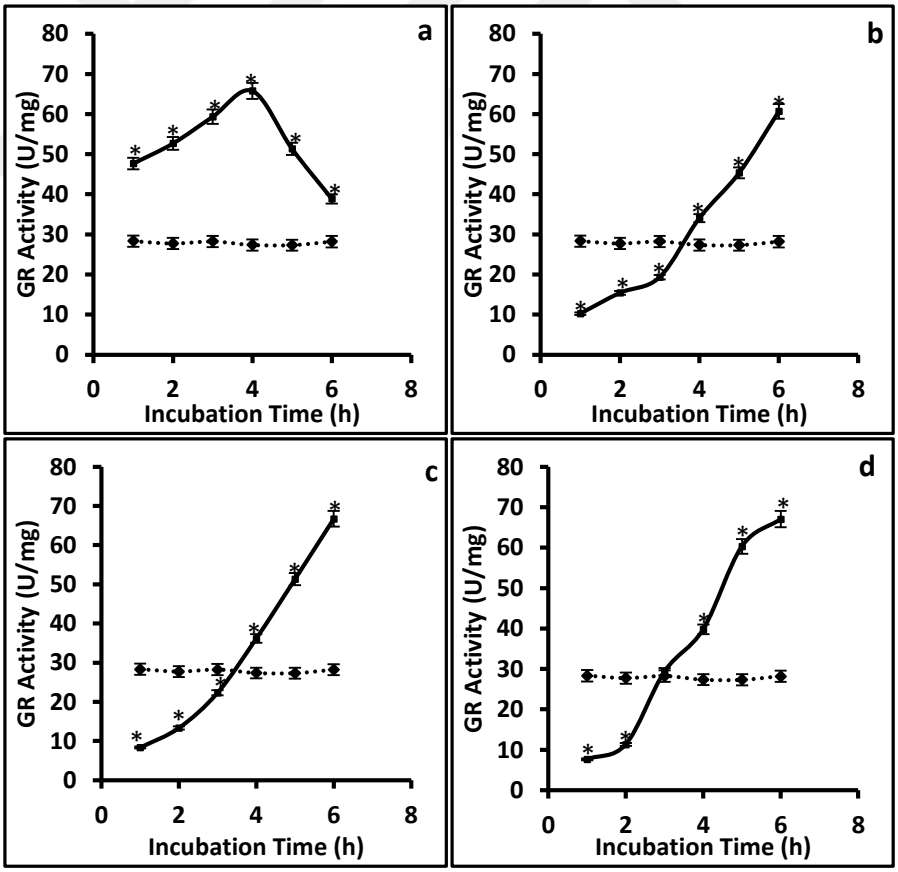


Figure 3.24 Variation in GR activities depending on the incubation period and concentration of ketoconazole; control (--♦--) , and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d)

3.6.4.5 The variation in GST activities in ketoconazole treated S. cerevisiae

GSTs are the enzymes of the glutathione system that conjugate xenobiotics or their metabolites to GSH, and they are important for protection against oxidative stress, the regulation of gene expression, and signal transduction (Bonham et al, 2014). GST activities *in S.cerevisiae* BY4742 increased within the first hour of incubation and the initial activities were negatively correlated with ketoconazole concentration (r=-0.99). Although the activities decreased at the following incubation periods, they never decreased to control values. The observed inductions in GST activity showed the effectiveness of the enzyme in the antioxidant response of *S.cerevisiae BY4742* cells against ketoconazole by making conjugation with ketoconazole or its metabolites.

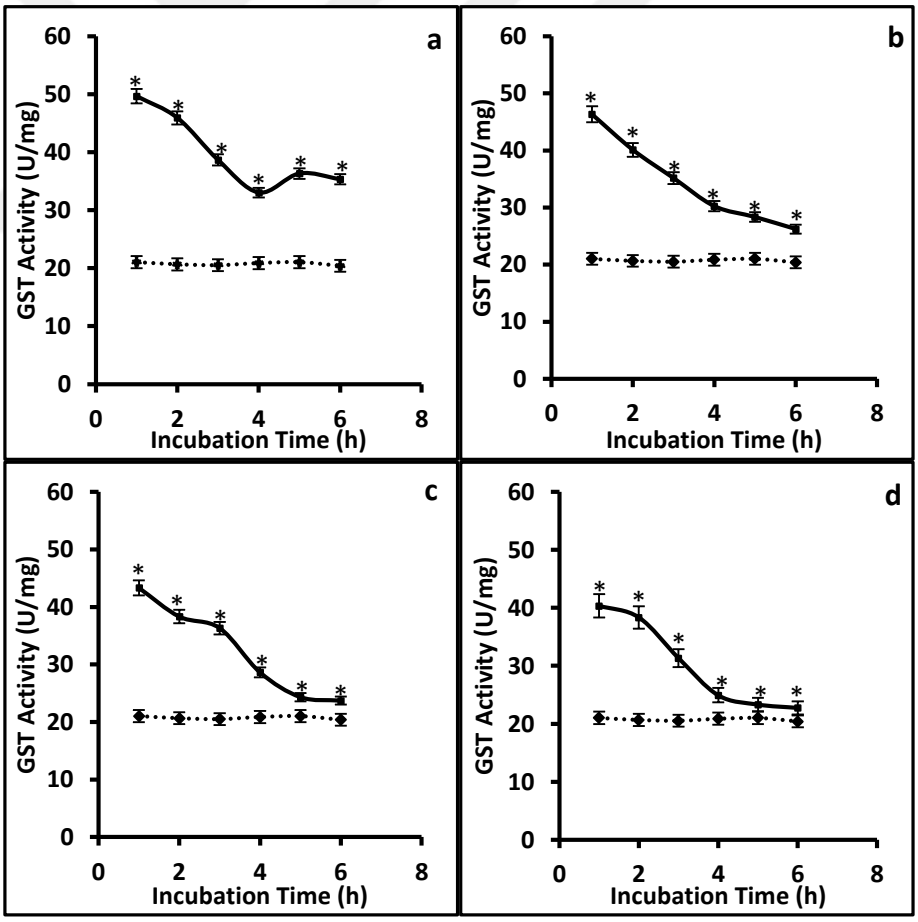


Figure 3.25 Variation in GST activities depending on the incubation period and concentration of ketoconazole; control (--♦--) , and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d)

3.7 Oxidative Damages in Biomolecules

3.7.1 LPO Levels in Ketoconazole-Treated S. cerevisiae

LPO is one of the molecular mechanisms in living organisms involved in toxicology, which can occur as a result of oxidative stress.[56] LPO occurs when free radicals attack the unsaturated fatty acid, causing cell membrane damage, affecting its structure and function. The membrane LPO levels in all ketoconazole-treated *S. cerevisiae* were over the controls in all incubation periods, except 150 μM at the first 2 h (Figure 3.26). Although LPO levels at 150 μM increased slowly, the significant and rapid increases were observed in all of the other studied concentrations throughout the incubation period ($p<0.05$). The observed highest membrane LPO level was 5.69 ± 0.14 nmole MDA/gww, which is 2.28-fold higher than its control, at 250 μM after 6-h treatment. In addition, a similar LPO level as 5.21 ± 0.22 nmole MDA/gww was found in cells treated with 225 μM for 6 h. In the study conducted with two monoterpene phenols having antimicrobial effects, the LPO levels in *C. albicans* treated with 133 μM thymol and 67 μM carvacrol were 4.75- and 5.13-fold higher than their controls, respectively (Ahmad et al., 2011). The level in *S. cerevisiae* exposed to 250 μM ketoconazole was lower than those levels, although the dose was relatively higher than that in the study of Ahmad et al (2011). It was shown in an another study that while LPO levels in fluconazole-treated *C. gattii* cells did not change at 104 μM for 1 h, 0.7 μM itraconazole caused a threefold increase (Korashy et al., 2007) In our study, the levels were not significantly higher than its control at 150 μM within the first 2 h and ketoconazole showed similar effect with fluconazole in terms of LPO.

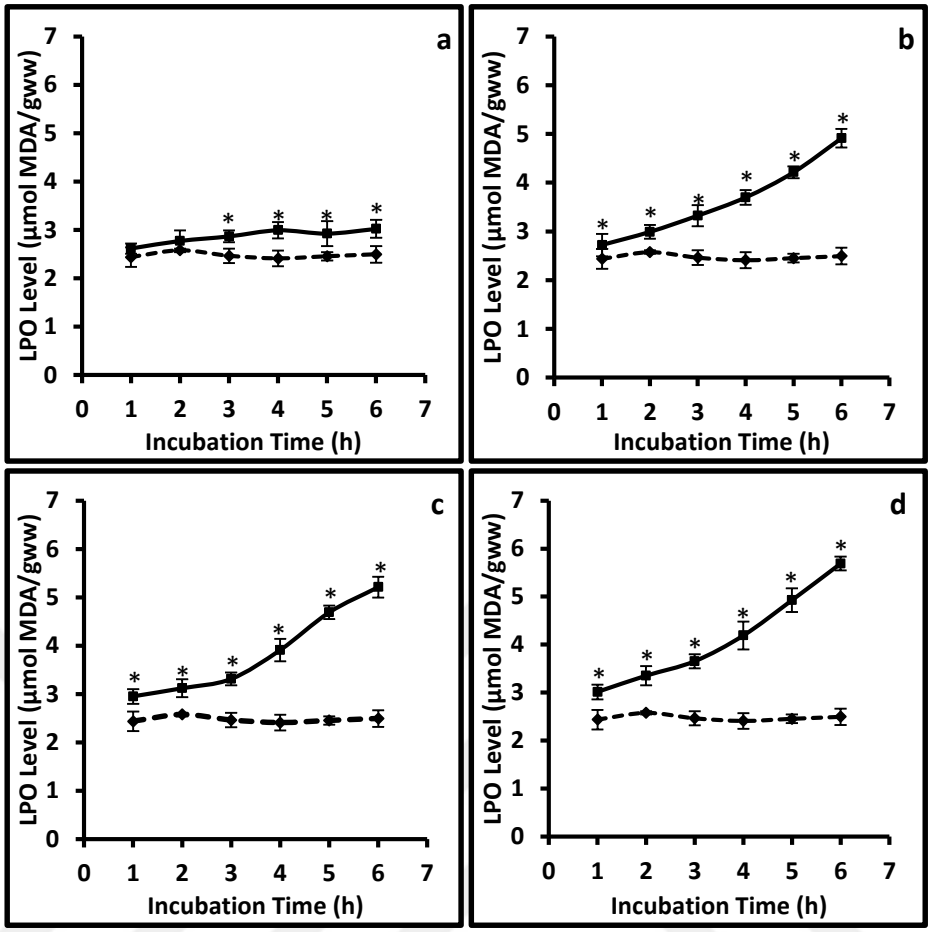


Figure 3.26 Variation in LPO levels depending on the incubation period and concentration of ketoconazole; control (--♦--) , and ketoconazole treatments (–■–); 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d)

3.7.2 Protein Carbonyl Levels in Ketoconazole-Treated S. cerevisiae

Protein carbonyl levels in all ketoconazole treated *S. cerevisiae* cells were over the control from the beginning of incubation time and increased time dependently. While the levels observed in 150-200 µM ketoconazole treatment were similar with each other, the levels in 225-250 µM treatment reached to the similar values at the end of incubation time. The observed higher protein carbonyl level was 4.25-fold of its control. The increased protein carbonyl levels showed the oxidative effect of ketoconazole on *S. cerevisiae* despite the induction of antioxidant system.

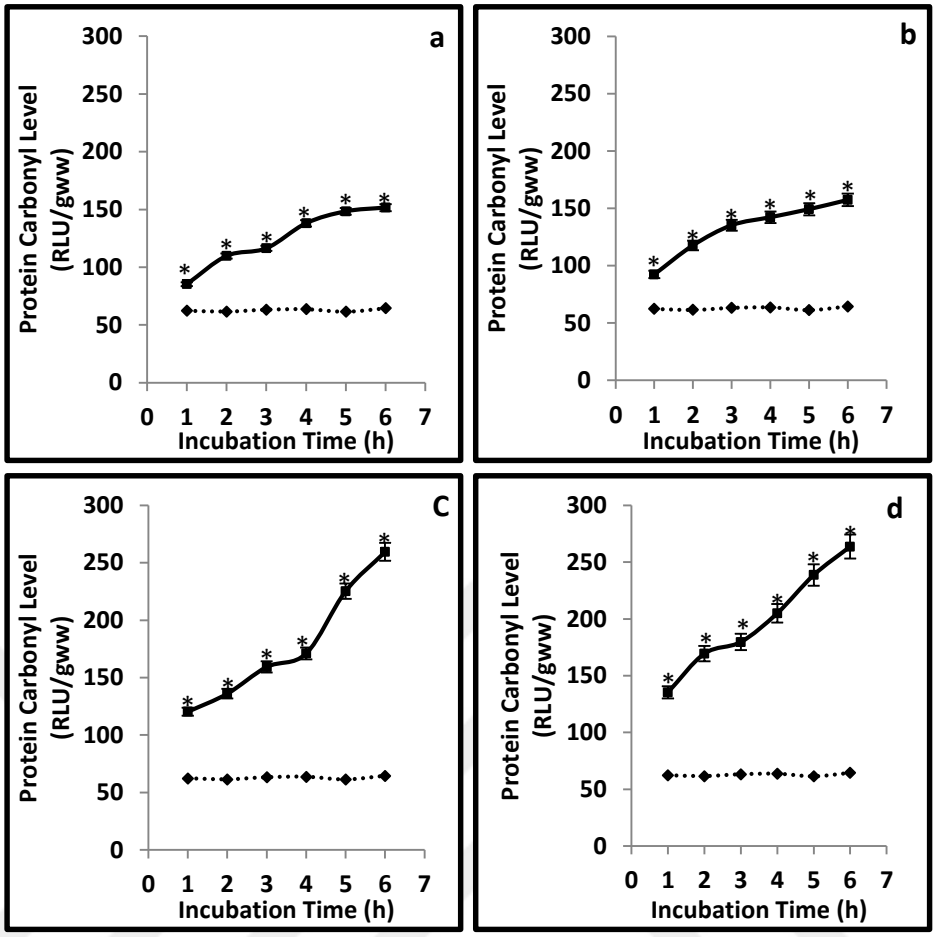


Figure 3.27 Variation in protein carbonyl levels depending on the incubation period and concentration of ketoconazole; control (--♦--) , and ketoconazole treatments (–■–) 150 µM (a), 200 µM (b), 225 µM (c), 250 µM (d)

CHAPTER 4
CONCLUSION

Ketoconazole treatment to *S. cerevisiae* effectively induced apoptotic cell death up to 65.3% and necrotic cell percentage was quite low. The seen high necrotic cell death with treatment of ketoconazole for 6 and 9 h caused by secondary necrosis which is also the results of apoptotic cell death. One of the most important data obtained from this thesis is that the apoptotic cell death induced by ketoconazole is related with four different investigated apoptotic proteins. In most cases, yca1p is the most important regulator of apoptosis but in ketoconazole treatment, this protein was seen less important than other apoptotic proteins.

ROS levels in *S. cerevisiae* treated with ketoconazole at the range of 150-250 µM increased significantly compared to non-treated control cells. Although two main antioxidant enzymes SOD, CAT and glutathione system were also induced, this induction could not be sufficient to decrease ROS levels to physiological conditions, except $O_2^{\bullet -}$ and NO•. The observed results showed that one of the ROS formation ways caused by ketoconazole treatment is the induction of NADH and NADPH oxidases and also dismutation reaction catalysed by SOD participated in the formation of H_2O_2. The results showed that $O_2^{\bullet -}$ and NO• levels and NADH, NADPH oxidases and SOD activities in 250 µM ketoconazole treated *S. cerevisiae* showed different tendencies when compared to other investigated concentrations. It is possible to say that the different metabolic ways took part in the induction of the enzymes and also in the production of $O_2^{\bullet -}$ and NO• at this concentration. It was observed in all ketoconazole treatments that although observed decreases in $O_2^{\bullet -}$, ·OH levels increased throughout the incubation period. This situation showed that ·OH was produced except for Haber-Weiss reaction, such as Fenton mechanism. Although the antioxidant enzymes SOD and CAT induced up to 3.11- and 3.57-fold of their controls, respectively, the inductions could not prevent the lipid peroxidation and protein carbonylation dose- and time-dependently.

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