THE ROLE OF LIPID PEROXIDATION END PRODUCT; 4-HYDROXY-2-NONENAL IN CELL SIGNALLING.

by
Mezher Adli

Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Master of Science

Sabancı University
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Prof. Dr. A. Nazlı Başak : ……

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ABSTRACT

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Intracellular ROS production has been monitored by a florescent probe, DCFH-DA. When cells were treated with 5 μM 4-HNE, a significant increase in florescence intensity has been observed when compared to the control cells. Two structurally different antioxidants; α-tocopherol and resveratrol prevented 4-HNE-induced ROS production to a significant extent when they were used at 50 μM concentration. This finding provided further evidence for the production of ROS. Differential staining by using fluorescent probes and DNA fragmentation analysis results indicated that 10 μM 4-HNE induced apoptosis and the effect was again overcome by the antioxidants; vitamin E and resveratrol. Furthermore, a combination of acridine orange (AO), Hoechst (HO), and propidium iodide (PI) florescent dyes have been used to differentiate viable, apoptotic and late apoptotic/necrotic cells respectively. Differential staining results indicated that when cells were incubated with 25 μM 4-HNE for 24 hr. apoptotic cells have been observed with condensed orange nucleus whereas viable cells were seen with green nucleus.

The results are discussed in the light of cellular signalling mechanisms induced by 4-HNE leading to apoptosis via effecting the overall redox status of the cell.

ÖZET

Lipid peroksidasyonu son ürünleri hücre içi sinyal iletiminde önemli rol aldıkları için son yıllarda özel bir ilgi odağı olmuşlardır. Bu çalışmada, lipid peroksidasyonu son ürünlerinden biri olan 4-hidroksi nonenal (4-HNE) ve oksidan ajan olarak çok iyi bilinen hidrojen peroksit (H_2O_2) in oksidatif ve sitotoksik özellikleri karşılaştırmalı olarak 3T3 fibroblast hücre kültüründe araştırıldı. Hücre morfolojisi ve canlılığı ile ilgili bulgular her iki oksidan ajanın hücre yapısında önemli değişikliklere yol açtığını gösterdi. Hücreler 500 μM H_2O_2 ile 24 s veya daha uzun süre inkübe edildiğinde, apoptotik yapıların oluştuğu ışık mikroskobuyla gözlemlendi. Benzer sonuçlar hücreler 5, 15, 25, ve 35 μM 4-HNE ile inkübe edildiğinde de görüldü. 4-HNE'nin derişimine bağlı olarak hücrelerin kalınlaştığı ve 35 μM 4-HNE ile inkübe edildiklerinde ise tutunma özelliklerini yitirerek solüsyona geçtikleri görüldü. Hücreler 25 ve 50 μM 4-HNE ile 24 saat inkübe edildiklerinde hücre canlılığının sırasıyla % 25 ve % 93 oranında azaldığı tespit edildi.

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Elde edilen bulgular, sinyal iletisi mekanizmaları ışığında, 4-HNE nin hücre içinde oluşturduğu ROT ve buna bağlı olarak indüklenen apoptoziz olarak tartışıldı.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

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ds	Double stranded
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FCS	Foetal calf serum
GSH	Glutathione
GSHPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
4-HNE	4-hydroxy-2-nonenal
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I-κB	Inhibitory κB
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MAPK	Mitogen activated protein kinases
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NLS	Nuclear localisation signal
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Page

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I. INTRODUCTION

A. Free Radicals

A.1. Oxygen and Reactive Oxygen Species (ROS):

Oxygen, a colourless, odourless and tasteless gas, began accumulating in the Earth atmosphere since the evolution of oxygen-evolving photosynthetic organisms 3 billion years ago. Now molecular oxygen (also called dioxygen, O_2) is the most prevalent element in the Earth's crust and composes 21 % of the atmosphere. While oxygen is essential for animal and plant life, ample evidence exists that oxygen can also be toxic. During the process of respiration, cells are constantly subjected to oxidative stress due to the fact that small amounts of semi-reduced species of oxygen are produced when molecular oxygen is reduced to water. Such semi-reduced species of oxygen (reactive oxygen species, ROS) are highly reactive and initiate a series of oxidative reactions, which collectively constitute oxidative stress[1].

One of the earliest studies to explain the toxic effects of the O_2 molecule revealed that O_2 inhibits cellular enzymes[2]. However recent research in this field indicates that the O_2 molecule alone cannot account for all these toxic effects for two reasons. Firstly, the rates of enzyme inactivation by oxygen are too slow and limited to account for the rate by which toxic effects develop. Secondly, some enzymes are not effected by O_2 at all. These findings have led to another explanation that most of the damaging effects of O_2 are attributed to the formations of oxygen free radicals (OFR) [2].

A.2. Free Radicals

A radical, *free radical,* is defined as a species that possesses one or more unpaired 'odd' or 'single' electrons. Many radicals have a zero net charge, however radicals that carry both a charge and an odd electron are called *radical ions*. These free radicals may either be radical cations or radical anions[3]. To indicate the presence of one or more un-paired electron, a radical dot is inserted into molecular formulas. Electrons are more stable when paired together in orbital of an atom because the two electrons in a pair have different directions of spin. Since radicals have unpaired electron(s), they are generally less stable than non-radicals, but the activities of free radicals varies[4].

There are many different types of free radicals. For the sake of consistency, in the literature, generally reactive oxygen species (ROS), reactive oxygen intermediates (ROI), or reactive nitrogen species (RNS) are used.

TableI.1. Free Radicals

Name	Examples
Hydrogen atom	**$H^{\cdot}$**
Oxygen centered radicals	**$OH^{\cdot}$, $O_2^{-\cdot}$, $H_2O_2^{*}$,$RO^{\cdot}$, $RO_2^{\cdot}$ $HO_2^{\cdot}$, singlet oxygen $<^{*}$**
Ozone and oxides of nitrogen	**O_3^{*}, $NO^{\cdot}$, $NO_2^{\cdot}$**
Transition metal	**metals in the first d-block of the periodic table contain unpaired electron; thus, they are free radicals**
Sulfur centered radicals	**$RS^{\cdot}$ thiyl**
Carbon centered radicals	**$CCl_3^{\cdot}$**
Nitrogen centered radical	**NO_2Cl, $C_6H_5N{=}N^{\cdot}$**
Phosphorus centred radicals	

***** Non-radical oxygen derivatives

however, there are free radicals from sources other than oxygen and nitrogen as well. TableI.1 postulates the wide variety of free radicals that can be formed in the human body and in the food systems.

A.3. The Chemistry of Free Radicals

The most common mechanisms for generating radicals are the loss of a single electron from a non-radical or the gain of a single electron by a non-radical. Free radicals can easily be formed by a process known as ***homolytic fission*** when a covalent bond is broken if one electron from each of the pair shared remains with each atom (Thomas, 1995). In homolytic fission, as a consequence of a covalent bond break, two different radicals can be formed as shown below. If A and B are the two atoms covalently bonded (**:** representing the electron pair), then the result is:

$$A{:}B \rightarrow A + B^{\cdot}$$

For example, the homolytic fission of water will yield a hydrogen radical ($H^{\cdot}$) and a hydroxyl radical ($OH^{\cdot}$).

Another common mechanism is ***heterolytic fission in*** which a covalent bond is broken, and one atom receives both electrons. In this case, the extra electron gives A a negative charge, and B is left with a positive charge as indicated below.

$$A{:}B \rightarrow A{:}^{-} + B^{+}$$

For instance, the heterolytic fission of water gives the hydrogen ion (H^{+}) and the hydroxide ion (OH^{-}).

A.4. Generation of Free Radicals

Most free radicals are generated continuously by normal metabolic processes as well as many other extrinsic factors such as ultraviolet lights (UV). Within the cells and tissues of human bodies, free radicals are produced as side- or end products of normal metabolism, mainly during the reduction of oxygen to water by the mitochondrial electron transport chain. The biological process is not 100% efficient: between one and five percent of all oxygen used in metabolism escape as free radical species[5]. For example, an adult with a 70 kg body mass produces at least 1.7 kg of $O_2^{\cdot-}$ per year[2].

In many cases reactive oxygen intermediates (ROIs) are released deliberately in tissues and organs. One of the cases that carry utmost significance is the process of inflammation. During inflammation, leucocytes, namely macrophages and neutrophils, migrate toward the inflamed area upon activation. NADPH oxidase enzyme within these cells catalyse the one electron of oxygen to O_2^{-}. This superoxide free radical has a potential to generate many other ROIs through series of reaction catalysed by metal ions[5]. The iron or other metal contained in the structure of some vital proteins such as haemoglobin can react with OFR through a series of reactions called *Haber-Weiss* or

Fenton reactions to generate some more toxic species such as $HO^{\cdot}$. In Fenton reactions, free radical reactions are initiated when Fe^{2+} comes into contact with H_2O_2.[6] This process is indicated below.

1. $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$ (Fenton rxns)

2. $Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + OH^{\cdot} + OH^{-}$

$O_2^{-} + H_2O_2 \longrightarrow O_2 + OH^{\cdot} + OH^{-}$ (Haber-Weiss rxns)

Transition metal ions, especially iron, are major contaminant for most bichemical reagents. Due to its importance in these reactions, its distribution in the organism should be kept under strict control[7]. Under nonpathological conditions, there is a strict control in the body due to the presence of some iron binding proteins e.g., hemoglobin, transferrin, ferritin, lactoferrin, and some antioxidant enzymes such as catalase and superoxide dismutase (SOD) which remove the hydrogen peroxides in the medium so that iron can not initiate those damaging reactions.

A.5. Detection of Reactive Oxygen Species

The detection of free radicals is a very significant problem since it is diffucult to measure them *in vivo*. In order to overcome this problem, most generally either of the two techniques, Electron Spin Resonance (ESR) or fingerprinting technique, is used.

The only method that can detect free radicals directly is a spectroscopic technique, *elecron spin resonance (ESR).* This technique detects the presence of unpaired electron. Often this technique is insensitive to detect those free radicals that have very short half lives. For this reason, the *spin trapping* method, which relies on the reaction of a radical with a trap molecule to give a more stable and measurable product, is used.

In addition to spin trapping, *fingerprinting* method is also used. The principle behind this method is to measure products of damage by ROS/RNS, i.e. to measure not the species themselves but the damage that they cause. Since ROS/RNS reacts in chareistic ways with DNA, proteins, lipid, and certain low-molecular mass antioxidants (e.g. ascorbate, urate). The products generated can thus be regarded as fingerprints (or 'footprints') of oxidative attack.

Besides these techniques, there are some colorimetric methods used to detect specific ROS/RNS. One of theese methods used to detect general ROS or oxidative stress in the cell culture is the usage of specific florescent dyes that floresce upon activation by ROIs in the cell.

Dichlorofluorescin diacetate (DCFH-DA) is one of the widely used florescent probes for oxidative stress detection. DCFH-DA is uptaken by the cells and de-acetylated by esterases and converted to *2',7'dichlorofluorescin* (non-florescent) (Figure I.1). When this compound is oxidised in the cells it turns in to florescent *2',7'dichlorofluorescein* that can be easily visualised (strong emission at 525 nm with exitation at 488 nm). This 'florescent imaging' is an assay of 'generalised oxidative stress' rather than detection of production of any particular oxidising species[2].

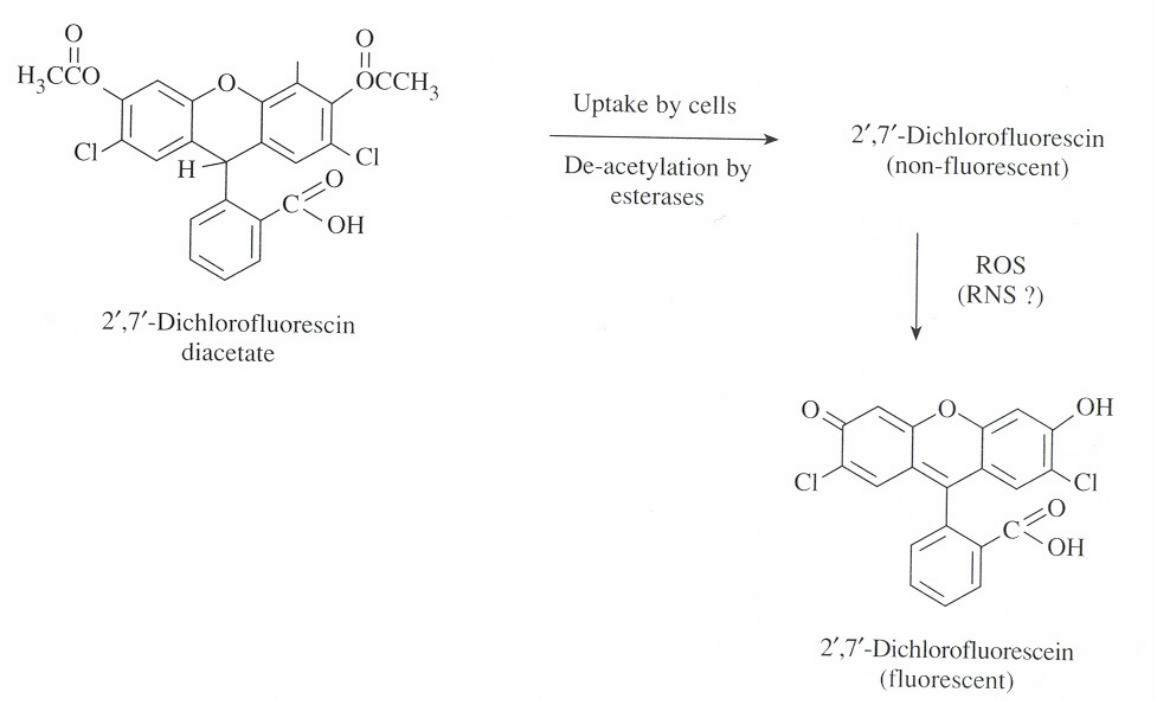


Figure I.1: Structure of 2',7'dichlorofluorescin diacetate (DCFH-DA)

A.6. Biological Targets and Consequences of Free Radicals

Increased free radicals formed as a result of abnormal metabolism are claimed to have a signficant role in over 100 different human diseases. Because free radicals are highly reactive against many biologically important macromolecules, they can damage multiple biological substrates including proteins, lipoproteins, deoxyribonucleic acids, carbohydrates, and polyunsaturated fatty acids (PUFA) (Table I.2). The diseases, that free radicals are associated with, range from rheumatoid arthritis haemorrhagic shock through cardiomyopathy and cystic fibrosis to gastrointestinal ischemia. AIDS, even male pattern baldness, are said to result from free radical implementations (Figure I.2). The increased formation of free radicals (ROS/RNS) is said to accompany tissue injury in most, if not all, human diseases[2]. They make a significant contribution to disease pathology. Moreover, free radicals can also act as second messengers in the induction of molecular process, which is explained in detail in the section B.

Table I.2. Potential targets of free radicals and possible biological consequences[8].

TARGET	DAMAGE	BIOLOGICAL SIGNIFICANCE
Proteins	**aggregation and crosslinking**	**modified enzyme function**
	fragmentation and breakdown	**Increased intracellular Ca^{+2}**
	modification of thiol groups	
	nitration of phenolic compounds	
Lipids	**loss of PUFA**	**decreased membrane fluidity**
	formation of reactive metabolites	
	altered activity of membranes bound proteins, enzymes receptors and transporters	
DNA/RNA	**base damage**	**inhibition of protein synthesis**
	fragmentation	**PARS activation**
	scission of deoxyribose ring	**translational errors**

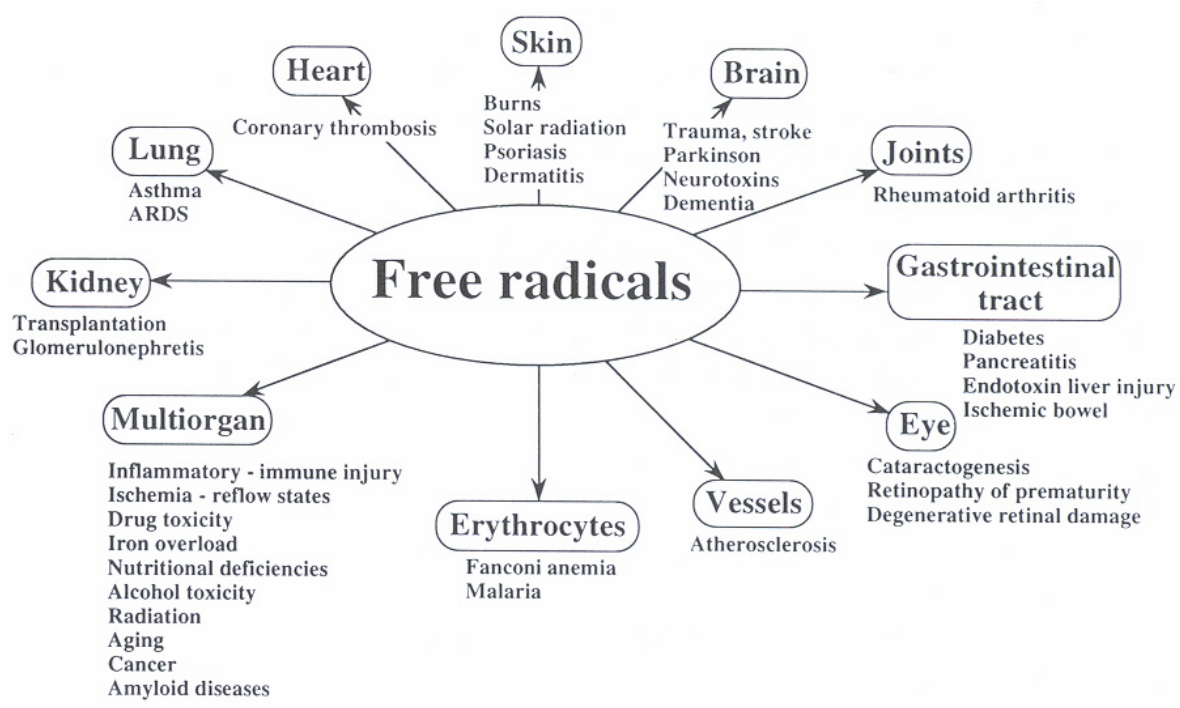


Figure I.2: Free radicals and human diseases: Spectrum of diseases where an excessive free radical production is thought to play a significant role in developing tissue injury.[9]

A.7. Anti-oxidant Defence Mechanism

Cellular response to oxidative stress is a universal phenomenon. Numerous genes and gene products have been identified that protect cells from oxidative stress. In addition to these cellular defences, there are some other vital molecules, called antioxidants, which save cells from free radicals. Antioxidants are defined as any substance that can delay or prevent the oxidation of a substrate when it is present in a small amount relative to the amount of oxidant agent. In order to be an antioxidant a substance should have the following properties[10]:

1. scavenging free radical species, by using either protein catalyst (enzymes) or direct chemical reactions. (In the latter one, the antioxidant is used up as the reaction proceeds.)

2. minimising the formation of free radicals
3. binding metal ions that are essential to convert poorly reactive species such as O_2^- and H_2O_2 into highly reactive species such as $OH^{\cdot}$
4. repairing the damage on the target
5. destroying the damaged target molecules and replacing them with the new ones

The first line of defence mechanism of an organism against free radicals is in vivo antioxidants, which are enzymes and some other low molecular weight compounds. They include glutathione-dependent enzymes, glutathione peroxidase (GPX), glutathione reductase and glutathione transferase, catalase (which breaks down H_2O_2 to oxygen and water), and the enzyme superoxide dismutase (which converts superoxide in to H_2O_2). There are also several low molecular weight compounds, which act as antioxidants such as vitamin C, vitamin E, and carotenoids which are ingested in the daily diet. Also in the human body, there are some intrinsic molecules such as glutathione, biluribin and uric acid. Besides being a substrate for glutathione peroxidase, glutathione is a scavenger of hydroxyl radical and singlet oxygen. Biluribin acts mostly by quenching free radicals while uric acid chelates metal ions so as to protect oxidation of molecules by Cu^{+2}, Fe^{+2}, Fe^{+3} ions.

B. Oxidative Stress and Cell Signalling

In non-pathologic situations, the production of free radicals in an aerobic organism is balanced by antioxidant defence mechanism. This balance is not always perfect; therefore, any disturbance that alters this finely tuned prooxidant/antioxidant balance in favour of prooxidant leads to *oxidative stress*. Elevated concentrations of free radicals relative to antioxidant level results in major cellular and physiological damage, called *oxidative damage.*

In principle, oxidative stress can result from;

1. diminished antioxidants, for instance, a mutation affecting antioxidant defence enzymes such as cupper-zinc superoxide dismutase (CuZnSOD), MnSOD, or glutathione peroxidase (GPX). In addition, depletion of dietary antioxidants can also lead to oxidative stress.
2. increased production of free radicals (ROS/ RNS/RAS) for example, by exposure to high level of O_2^2.

Oxidative stress causes adaptation or cellular injury depending on intracellular concentrations of free radicals. Cells often tolerate to low level oxidative stress by up-regulation of the synthesis of antioxidant defence system. Beyond a certain level, often a cell injury is observed.

B.1. Signalling by Stress: The Role of ROS, RNS, and RAS

Oxidative stress has long been considered as an "accident" of aerobic metabolism: a stochastic process of free radical production and nonspecific tissue damage which is fundamentally unregulated aside from the normal counteract of antioxidant defence mechanisms. In recent years, this paradigm has shifted and certain free radicals (ROS and RNS) have been identified as signalling molecules whose production may be regulated as a part of routine cellular signal transduction.[11]

Increased reactive oxygen species level can act as chemical inducer of the expression of specific genes involved in protecting cells against oxidative damage. This phenomenon was first described in bacteria, which lead to the discovery of several regulatory proteins such as *oxyR* and *soxRS* for oxidative stress in prokaryotes[12]. Similar results were also obtained in eukaryotic system. Today it is accepted without doubt that oxidative stress modulates the expression of number of genes in both eukaryotic and prokaryotic systems. A wide range of protein products from these modulated genes have been identified as antioxidant enzyme, growth arrest, DNA repair, mitochondrial electron transport, cell adhesion, cytokine, and glucose-regulated proteins. It is known that certain transcription factors of NF-κB/ rel family can be activated, not only

receptor-targeted ligand but also by certain oxidising agents such as hydrogen peroxide and ionising radiation.[13] A list of mRNAs induced by oxidative stress is presented in Table I.3. Hydrogen peroxide is one of the best-studied oxidising agents, which is used as endogenous messenger within the cell. The postulated mechanism of peroxide signalling is given in Figure I.3.

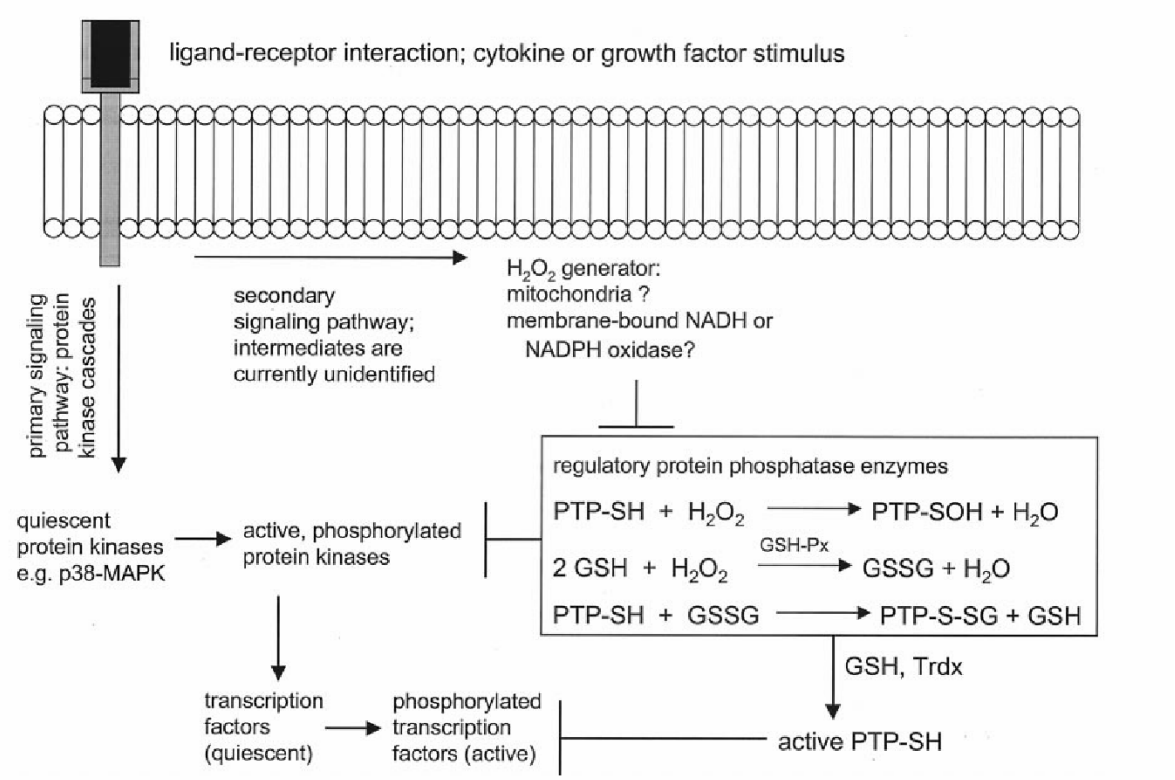


Figure I.3. Postulated mechanism of peroxide-mediated redox signalling. [Note: Arrows indicate stimulatory pathways; ¢ indicate inhibitory pressures. Signalling is initiated by specific ligand-receptor interactions. Typically, a series of protein kinase intermediates propagate the signal toward nuclear TFs][14].

Table I.3. Oxidant induced mRNAs in mammalian cells[15].

Gene	Oxidant stress
c-fos, c-myc	Xanthine/xanthine oxidase
c-fos, c-jun, egr-1, JE	Hydrogen peroxide
C-fos, c-jun	t-butyl hydroperoxide
Heme oxygenase	Multiple oxidants
Gadd45 and gadd 153	Hydrogen peroxide
CL100	Hydrogen peroxide
Interleukin-8	Hydrogen peroxide
Gamma-Glutamyl transpeptidase	Menadione
Vimentine, cytochrome IV, RP-L4	Diethylmalate
c-fos, c-myc, c-jun, HSP	Xanthine/xanthine oxidase
Glucose-regulated proteins	Singlet oxygen, other oxidants
c-fos and zif/268	Nitric oxide
Adapt15, adapt33, adapt78	Hydrogen peroxide
Adapt66(mafG)	Hydrogen peroxide
Adapt73/PigHep3	Hydrogen peroxide
Ref-1	Hydrogen peroxide, hypochlorite
Catalese, MnSOD, GPx	Hydrogen peroxide
MnSOD	Xanthine/xanthine oxidase
Mn-SOD	Hyperbaric acid, multiple oxidants
Numerous redox	p53 overexpression
NKEF-B	Hydrogen peroxide

B.2. Redox regulation of gene expression

In mammalian cells, the mechanisms by which ROSs are sensed and inducibly produced are still not clear, besides the transcription factors that are exclusively activated by ROS or that selectively control the expression of ROS-protective and repair enzymes have yet to be identified. Oxidative stress response involves the activation of

numerous functionally unrelated genes associated with signal transduction, cell proliferation, and immunological defence reactions. In comparison to bacteria, the mammalian oxidative stress response has a protective function. However, most of the genes are activated by both intracellular redox status and by many other physiological signals, such as growth factor and cytokines. These experimental evidences show that both groups of signals converge into the same pathway by sharing the same signalling molecules. For example, both physiological and ROS-triggered signals activate NF-κB and AP-1, two important and widely used transcription factors. The overlapping effects of both physiological and ROS-triggered signals may be explained by the fact that some physiological inducers seem to use ROS as intracellular signalling molecules. Hence, ROS serves as second messenger molecules in the eukaryotic system, and ROS-induced gene expression is not restricted to adverse environmental conditions but has a more widespread and fundamental role in cellular metabolism.

One of the most important regulatory mechanisms in which cells respond to physiological and ROS-triggered signals is the activation of genes via inducible transcriptional activator proteins called *transcription factors*. These activator proteins respond to diverse stimuli such as steroid hormones, heat shock, heavy metals, cytokines, hormones, growth factors and viral infection by binding to specific DNA sequences and either stimulating or inhibiting the transcription of nearby genes. The transcriptional control plays a significant role in a wide variety of biological processes, which result in phenotypic changes in cells and organism. The complexity inherent in the control of gene transcription can be illustrated by examining it at the DNA level. At this level, gene regulation is governed by *cis* regulatory elements. The key to understanding the regulation of gene expression lies in unravelling the functions of the numerous DNA regulatory sequences that reside upstream from the gene itself. The most proximal, upstream regulatory sequence is the TATA box, which is the major component of the gene's promoter. The gene's promoter is a regulatory site on the DNA at which transcription is initiated. The most common conserved sequence found about 30 bp upstream of transcription's start site is 'TAATA/TAA/T' hence called the TATA box, the site of assembly of a number of general transcription factors that are required before a eukaryotic gene can be transcribed by RNA polymerase II. The binding of these transcription factors upstream of the TATA box is essential for the sufficient level of transcription.

In the eukaryotic system, the transcription factors are categorised into two groups: pre-existing or primary transcription factors and secondary transcription factors[16]. The primary transcription factors, already present in a latent form in the nucleus or cytoplasm, require a post-translational modification or interaction with a ligand in order to bind to *de novo* regulatory DNA sequences or should be bound to DNA to acquire transcription activating potential (e.g. NF-κB). Secondary transcription factors require *de novo* synthesis and depend on primary factors for transcriptional activation of their genes (e.g. AP-1) [16]; in other words, these factors require new protein synthesis.

B.2.1. Transcription Factor Nuclear Factor kappa B (NF-κB)

NF-κB was the first transcription factors shown to respond directly to oxidative stress. Initially, it was identified as a nuclear factor of mature B cells that specifically interacts with a decameric enhancer element (5'-GGGACTTTCC-3') of the immunoglobulin κ chain gene[17]. But, it was soon realised that a great variety of stimuli (please refer to Table I.3) can activate NF-κB and initiate transcription of NF-κB dependent and/or responsive genes (Table I.4). The transcription factor NF-κB is believed to play essential role in the regulation of a wide variety of cellular as well as viral genes, particularly those cellular genes involved in immune and inflammatory responses[18]. Relationship of NF-κB in the activation of those genes (Table I.4) leads to the conclusion that this transcription factor is involved in many currently intractable diseases such as AIDS, hematogenic cancer cell metastasis, and rheumatoid arthritis (RA). In addition to this, Nf-κB has been also shown to be involved in the inhibition of programmed cell death (Apoptosis). However, the fine molecular mechanism has yet to be identified[19].

An interesting aspect of NF-κB is that it does not require new protein synthesis during its activation. In non-stimulated resting cells, NF-κB is present in the cytoplasm with some inhibitory proteins, which retain the complex in the cytoplasm and thereby prevent DNA binding. Collectively these inhibitory proteins are called IκBs. Activation and Regulation of NF-κB is controlled by three distinct protein subunits: p50, p65 (Rel

A) and IκBs. Following the activation by extracellular stimuli, the final target of the induction pathway is the releasing of the inhibitory subunit I-κB, by rapid proteolysis, from the cytoplasmic complex with the DNA binding homo/heterodimers of P50 and Rel A[20].

Table I.4. NF-κB activating stimuli[21]

Oxidative stress	Hydrogen peroxide, Antimycin A
	Oxidized lipids, Butyl peroxide
Physical stress	UV light
	Ionizing radiation (x and gamma)
	Photofrin plus red light, Partial hepatectomy
Drug and Chemicals	Phorbol esters, Cycloheximide
	Calyculin A, Okadaic acid
	Ceramide, Pervanadate
	Forskolin, Dibutyril c-AMP
	Anisomycin, Emetine
Cytokines	Tumor Necrosis Factor-alfa (TNF-alfa)
	Lymphotoxin (LT) (TNF-beta)
	Macrophage colony stimulating factor (M-CSF)
	Granulocyte/ Macrophage colony stimulating factor (GM-CSF)
	Interleukin I-α and β (IL - I α and β)
	Interleukin-2, Leukotriene B4
	Leukemia inhibitory factor (LIF)
Mitogenes, Antigens	Allogenic stimulation
	anti-αβ T cell receptor
	anti-CD2, anti-CD3, Lectins,
	Phorbol esters, Calcium ionophores
	Diacylglycerol (DAG)
	Serum, PDGF, anti-surface IgM, p39

Table I.4. *(continued)*

Viruses and viral products	Human immunodeficiency virus-1 (HIV-1)
	Human T cell leukemia virus-1 (HTLV-1)
	Tax, Herpes simplex virus-1 (HSV-1)
	Hepatitis B virus (HBV), HBx, MHBs
	Epstein-Barr virus (EBV), EBNA-2, LMP
	Cytomegalovirus (CMV),
	Newcastle disease virus, Adenovirus 5
	Sendai virus, ds RNA
Bacteria and bacterial products	*Shigella flexneri*
	Mycobacterium tuberculosis
	Toxins: Staphylococcus enterotoxin A and B
	Toxic shock syndrome toxin–1 (TSST-1)
	Cell wall products: LPS
	Muramyl peptides
Eukaryotic parasite	Theilara parva

B.2.1.a. The Rel/NF-κB Protein Family

This widespread protein family is relatively preserved protein groups during the course of evolution. In contrast to most transcription factors, Rel/NF-κB family are activated by an extraordinarily large number of conditions and agents. There are mainly two protein groups in this family. The first group includes p65 (Rel A), Rel B, the proto-oncogenic protein c-Rel, the oncogenic protein v-Rel, and the *Drosophila* morphogenic proteins Dorsal and Dif. The second group is made of p50 (NF-κB1) and p52 (NF-κB2) which are produced from cleavage of the C-terminal part of the inactive precursors p105 and p100, respectively[22]. Proteins belonging to this family have a very conserved Rel domain of homology (RDH), which is about 280 amino acid long and includes the DNA binding domain, the dimerisation domain, the nuclear localisation

signal (NLS), and a potential phosphorylation site. In addition to the RDH domain, there is also transcriptional activation (TA) domain in the first group (Figure I.4). It is believed that combinatorial interactions between different NF-κB subunits, the formation of homo / heterodimers, give rise to dimers with sequence and transactivating specificity. Control of the activation of the multiprotein NF-κB complex depends not only on its subunits but also on the inhibitory subunits.

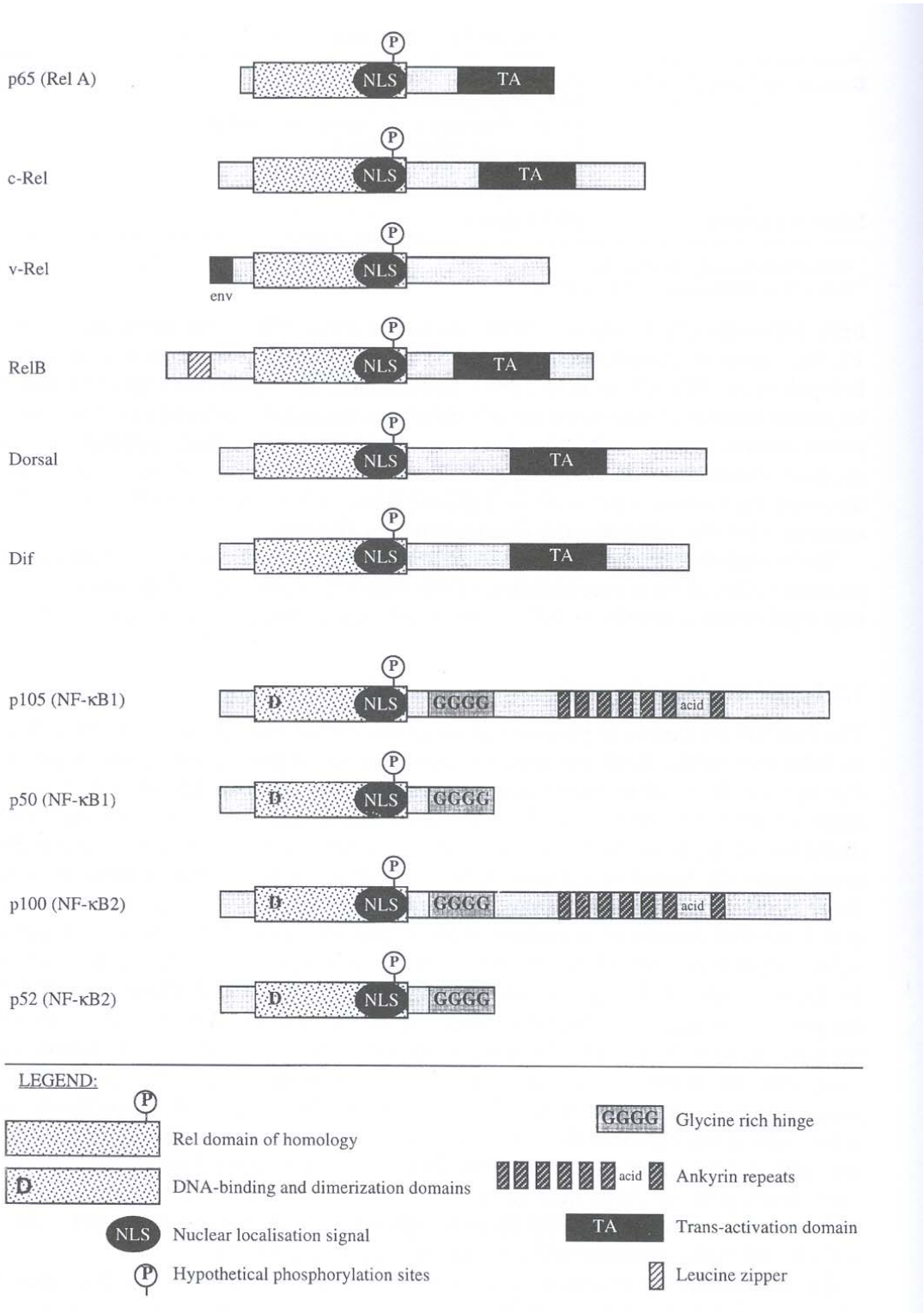


Figure I.4: The Rel/NF-κB family proteins. The members of this family share common conserved region, the Rel domain of homology, a dimerization domain and a nuclear localisation signal (NLS), some members have trans-activation domain (TA) as well[25].

Table I.5. NF-κB responsive genes[21]

Transcription factors and subunits	NF-κB precursor p105
	c-rel, IκB-α, c-myc, A20
	Interferon regulatory factor 1 and 2
Cytokines and growth factors	Interleukin-1β (IL-1β), IL-2, 6, 8
	Interferon β, Interferon γ
	Tumor necrosis factor alpha (TNF-α)
	Lymphotoxin (TNFβ)
	Transforming growth factor β2 (TGF-β2)
	IP-10, MIP-1α, MPC-1/JE, RANTES
	Macrophage colony stimulating factor (M-CSF)
	Granulocyte/ Macrophage colony stimulating factor (GM-CSF)
	Erythropoietin, Proenkephalin
	Melanoma growth stimulating activity (gro α-γ/MGSA)
	Immunoreceptors Immunoglobulin κ light chin (Ig-κ-LC)
	T cell receptor α chain (human) and β chain
	Major histocompatibility complex class I and II (MHC-I and II), B2- microglobulin
	Invarient chain Ii, Tissue factor-1
	Interleukin-2 receptor α chain
Cell Adhesion	Intracellular cell adhesion molecule-1(ICAM-1)
	Vascular cell adhesion molecule-1 (VCAM-1)
	Endothelial –leukocyte adhesion molecule-1 (ELAM-1)
Acute phase proteins	Complement factor B and C4
	Angiotensinogen, Serum amyloid A precursor
	Urokinase-type plasminogen activator

Table I.5. *(continued)*

Viruses	Human immunodeficiency virus-1 (HIV-1)
	Human immunodeficiency virus-2 (HIV-2)
	Cytomegalovirus (CMV)
	Simion virus 40 (SV40)
	Herpes simplex virus 1 (HSV-1)
	Human neurotropic virus (JCV)
Others	NO- synthase, Vimentin
	Apolipoprotein III, perforine, Decorine

B.2.1.b. The IκB Protein Family

IκBs plays a very essential role in the regulation of Nf-κB activity. Untill today, seven different IκB family members have been reported: IκB-α, IκB-β, IκB-γ, IκBR, bcl3 (a protooncogenic protein found only in mammals), p100 (encoded by NFκB2), and p105 (encoded by NFκB1)[23]. IκB-α, which prevents DNA binding to p65, c-Rel, and RelB, is the most extensively studied member of the family. All IκB family members have multiple closely located adjacent copies of a characteristic repeat structure of 30 amino acids, called the ankyrin repeats. (Figure I.5) These repeat are implied to have significant role in protein-protein interaction and are necessary for DNA binding prevention.

IκB-α can associate with Rel/NFκB family to form dimeric, inactive cytosolic complexes. In vivo experiments has been shown that IκB-α binds to NFκB (p50-p65) through the p65 subunit, and preferentially require the presence of dimeric structure (Beg at al, 1992)[24]. IκBs are involved in the retention of NFκB in the cytoplasm. Mutational deletion analysis reveal that IκB-α interacts with p65 by a flexible region (linker); however, an addition to this linker region, ankyrine (ANK) repeats and C-terminal domain plays an important role in the binding.

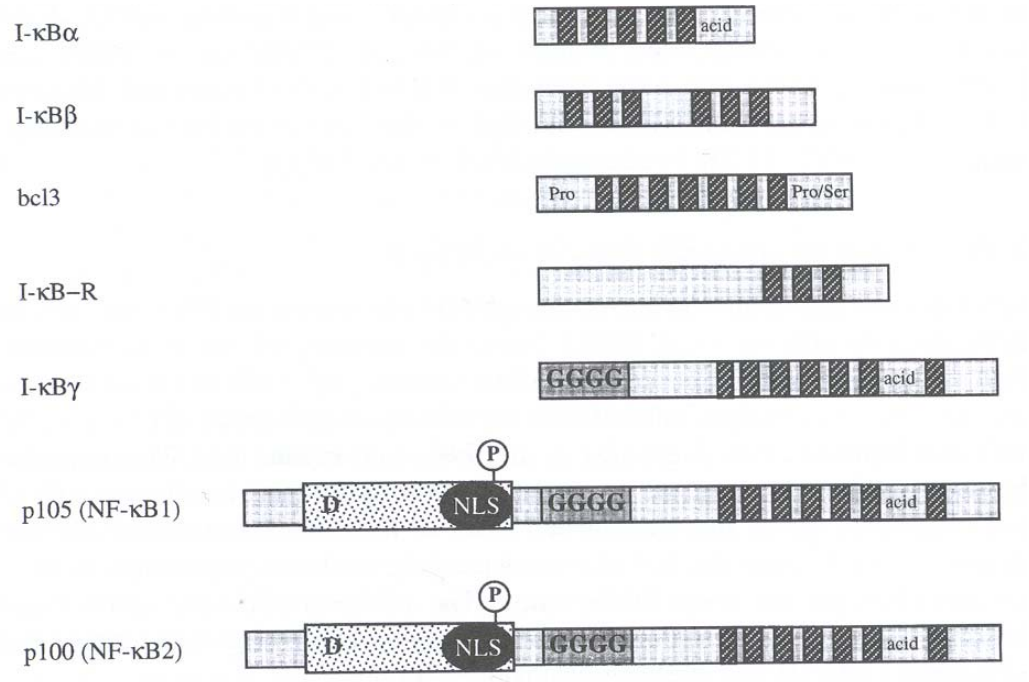


Figure I.5: The IκB family proteins. The members of this family share the presence of ankyrine repeat motifs[25].

It is assumed that IκB-α retains p65 in the cytoplasm by masking the nuclear localisation sequence (NLS) of p65; hence preventing the recognition of p65 NLS by protein(s) involved in the nuclear translocation of p65. This mechanism is thought to be similar in other IκB family members as well since all NfκB/ Rel proteins have similar NLS. Besides sequestering Nf-κB in the cytoplasm, also IκB inhibits the transcription of p50-p65 by migrating into nucleus and blocking the binding of transcription factor to DNA there.[23]

Upon the activation by external NFκB inducing signals such as hydrogen peroxide, UV light, inflammatory cytokines (IL-1 and TNFα), LPS, IκB-α is rapidly phosphorylated at serine 32 and 36 site by a serine/ threonine kinase which is yet to be identified. After phosphorylation, IκB-α is degraded by ubiquitin-proteosome system without being dissociated from NF-κB. In addition to phosphorylation at serine 32 and 36 sites, ubiquitynilation at lysine 21 and 22 is thought to play important role in the regulation of IκB dissociation from NFκB (Chen et al, 1995)[25] (Figure I.6)

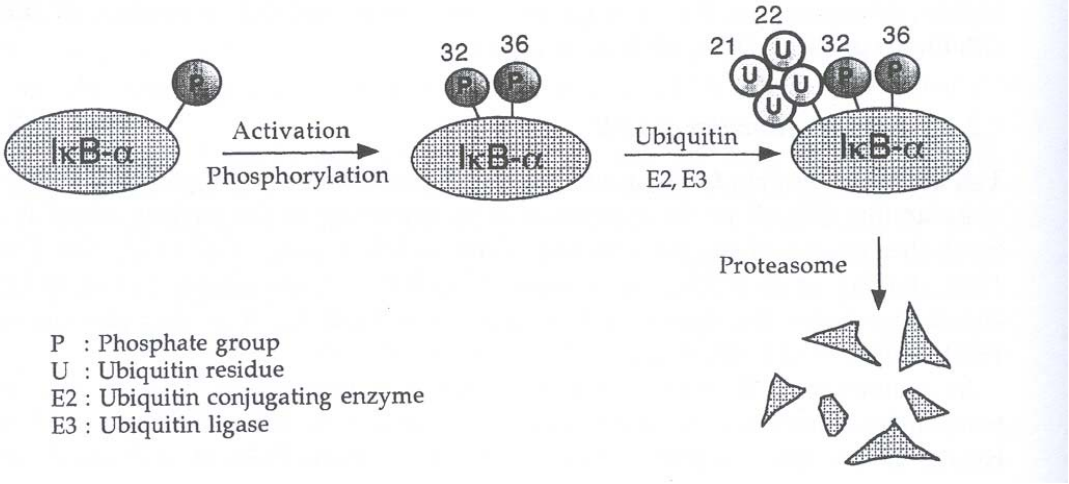


Figure I.6: IκB-α phosphorylation and proteolysis. In non activated cells, IκB-α is phosphorylated at serine 293, activation by some stimulus leads to phosphorylation of serine 32 and 36 and ubiquitination of lysine 21 and 22 that induce the degradation of IκB-α by proteosomes[25].

B.2.2. Redox Regulation of NF-κB DNA Binding activity

There are several evidences that indicate reactive oxygen intermediates (ROIs) play a significant role in the activation of NFκB DNA binding. Firstly, all inducers of NFκB tend to trigger the formation of ROIs or are oxidant themselves. Secondly, a broad range of antioxidants can inhibit NFκB activation. Nf-κB inhibitors are categorised into two main groups: one encompasses scavengers that can directly react with ROIs thereby neutralising their activity; the other groups are compounds that interfere indirectly with the production of ROIs. Thirdly, NFκB activation can be triggered by hydrogen peroxide or organic hydroperoxide in some cell lines in the absence of any other physiological stimulus.

The exact target molecules subject to redox regulation of NFκB remain to be elucidated. Several reports have pointed out that the prooxidant conditions alone may not be sufficient for NF-κB-DNA binding activity. Although, the presence of ROIs in

the cytoplasm favour the activation and translocation of NFκB to the nucleus, the reducing conditions in the nuclei are required to favour the binding of NFκB to DNA. The Rel proteins are not likely to be directly activated by oxidations; however, degradation of IκB, which is crucial for NfκB activation, is assumed to be facilitated by oxidation. Though the mechanism is not yet clear, but ROIs are the key modulators of IκB-α phosphorylation. Putting all these evidences together with the fact that cytokines activate NFκB more rapidly than ROIs, an appealing way to reconcile all presently known data is to assume that ROIs are modulators or costimulatory agents in the signalling pathway that activate NFκB (Figure I.7).

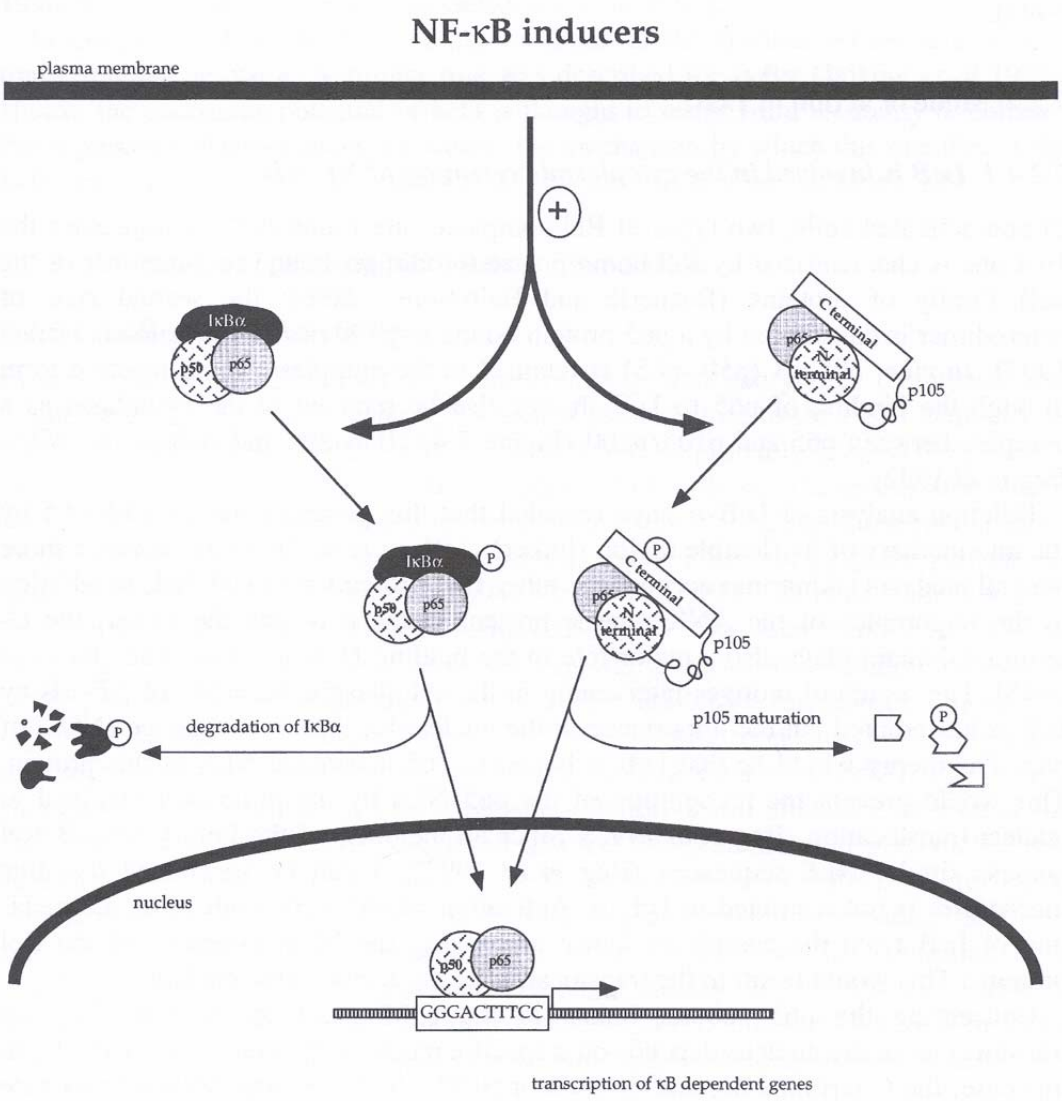


Figure I.7: The general NF-κB activation pathway. NFκB (p50-p65) activation requires IκB-α degradation while p105 maturation is necessary for p105-p65 heterodimer activation[25].

B.3. The Role of ROS in Apoptosis

Apoptosis, programmed cell death, is one of the hottest topics of modern biology and medicine. This death mechanism describes the very orchestrated collapse of cell, membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation, and

DNA degradation that is followed by rapid engulfment of these corpses by neighbouring cells. In contrast to apoptosis, *necrosis,* un-programmed cell death occur as a catastrophic accident to whole cell areas or tissues when they are exposed to severe physical, chemical, or osmotic injury. Necrosis finally results in total dissolution of the cell and induction of inflammatory reaction in the adjacent viable cells or tissue in response to the released cell debris (Figure I.8). Apoptosis is very essential for the good of the organism; when it malfunctions, the results may be catastrophic: cancer and autoimmune diseases when there is too little apoptosis and neurodegenerative diseases such as Alzheimer's and possible stroke damage when there is too much apoptosis[26]. When we think optimum body maintenance, which requires the renewal of 10 billion dead cells by new cells that arise through mitosis, we better understand the importance of apoptosis. Apoptosis sculptures our body, shapes our organs, and carves out fingers and toes during embryonic development. Both the nervous system and the immune system is optimised through overproduction of cells followed by the apoptotic death of those that fail to function properly.

Most of the apoptosis-specific morphological changes during cell death are caused by the activation of set of intracellular cysteine proteases called *caspases.* These highly conserved proteases exist in an inactive form in the cytosol of most cells as single polypeptide that is activated by caspase-mediated cleavage to produce active protease[27]. One of two important events takes place upstream of caspase activation. One is the activation of the receptor mediated death-signalling pathways that ultimately triggers caspase-8 and are exemplified by the interaction of CD95 receptor with its ligand. The other is activated by *intracellular alterations*, which result in the release of set of molecules from stressed mitochondria (e.g, cytochrome c, Apaf-1, apoptosis inducing factor) that ultimately activates caspases-9. This latter pathway is influenced profoundly by pro-apoptotic and anti-apoptotic Bcl-2 family members[28].

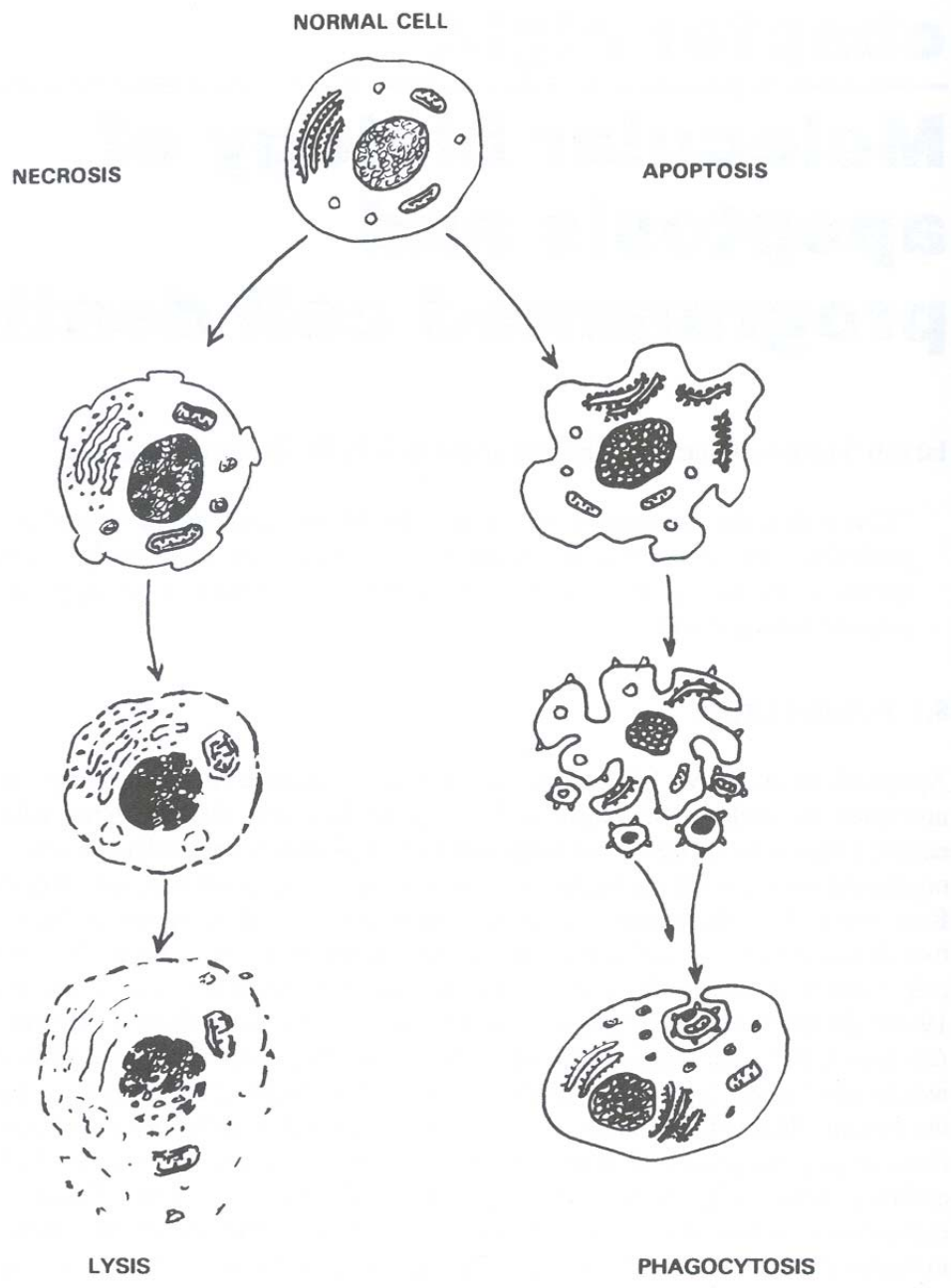


Figure I.8: The morphological differences between necrosis and apoptosis. In necrosis swelling of cytoplasm and organelles including mitochondria is observed where as in apoptosis cytoplasm shrinks and nucleus coalesces into several masses and breaks up into fragments[29]

There is well-established correlation between intracellular ROS level in cells and the induction of apoptosis. Two lines of evidence show that ROS are mediator of apoptotic pathways. Firstly, elevated level of ROS or oxidative damage markers is

detected in cells that are undergoing apoptosis and secondly, antioxidants have been shown to protect cells from undergoing apoptosis induced by diverse stimuli. In many experimental situations, it has been elucidated that apoptosis is preceded by the accumulation of intracellular ROS or the depletion of intracellular antioxidants. In a cell undergoing apoptosis, there are many potential sources of ROS (Figure I.9) one of the most important of such sources is mitochondria. When cytochrome c is released from mitochondria, electron transport chain is blocked and consequently superoxide and other ROIs are generated. Depending on the stimulus inducing apoptosis, NADPH oxidase, cytochrome p 450, and lipooxygenase/cyclooxygenase may also contribute to elevated levels of intracellular ROS generation[30]

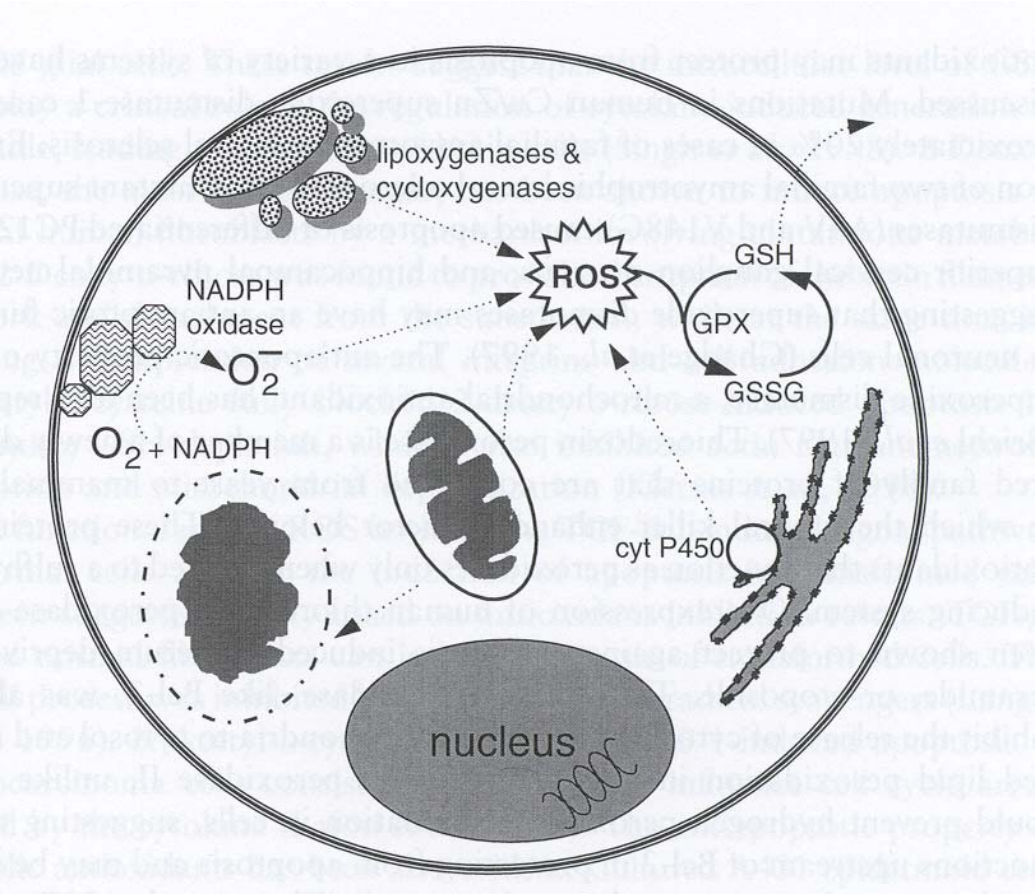


Figure I.9: Possible sources of ROS in a cell undergoing apoptosis. [Note: Dotted arrows represent events that may be induced by apoptosis-triggering signal.][30]

B.3.1. Detection of apoptosis

There are a number of ways to determine that a cell has undergone apoptosis; however, it is relatively difficult to measure this inside out death mechanism. Most techniques used to detect apoptosis are based on *apoptosis-specific* morphological changes measured by light microscopy, electron microscopy, and flow cytometry. In addition to this, some techniques rely on DNA fragmentation in which endonuclease activity, DNA content by flow cytometry, and DNA strand breaks labelled with specific fluorochrome are measured. There are also some techniques based on membrane alterations (e.g., measurement of dye exclusion) and cytoplasmic changes (e.g., measurement of changes in intracellular enzyme activity or measurement of calcium flux which is a result of apoptosis).

DNA fragmentation assay and dye exclusion methods are the two of the most widely used techniques to detect apoptosis. During apoptosis, genomic DNA is fragmented by caspase-activated endonucleases at inter-nucleosomal sites. In almost all circumstances of morphologically well characterised apoptosis, inter-nucleosomal DNA cleavage has been the biochemical event used as the definitive apoptotic marker.[31] This pattern of DNA degradation, by endogenous endonucleases that cleave DNA at linker region between nucleosomes, reveals 180-200 bp fragments or multiples of this fragment since the DNA wrapped around histones is about 180-200 bp. The cleavage can be assessed by the appearance of a ladder of bands, called *the apoptotic-ladder,* on a conventional agarose gel electrophoresis. However, this technique is not sensitive enough to study spontaneous apoptosis and its need of large number of cells makes it inconvenient to use in vivo. It should be also noted that not all kinds of cells exhibit the characteristics of DNA fragmentation in internucleosomal fragments, but the cells do show typical morphological signs of apoptosis.[29]

Another widely used technique is the dye exclusion method, which is based on membrane integrity alterations during apoptosis. Since the integrity of cytoplasmic membrane and most of its biological functions, such as active transport, remain intact apoptotic cells exclude non-vital dyes such as Trypan blue and Probidium iodide while necrotic cells do not. Using a combination of vital dyes and DNA stain simultaneously overcomes the difficulty of erroneously classifying apoptotic cells as vital cells.

Differential staining of healthy, apoptotic and/or necrotic cells in the same sample is possible by using combination of dyes such as Acridine Orange (AO), Hoechst 33342 (HO), and Propidium iodide (PI), respectively.[32]

B.4. Lipid Peroxidation End Products and Cell Signalling

Lipid peroxidation is defined as "the oxidative deterioration of polyunsaturated fats and unsaturated fatty acids (PUFA)"[33]. Lipid peroxidation may be initiated by any free radical species that has a sufficient reactivity to abstract a hydrogen atom from the methylene (-CH_2-) group of polyunsaturated fatty acid side chain such as those in arachidonic acid and linoleic acid in membrane lipids. Since hydrogen atom has one electron, its abstraction leaves behind an unpaired electron on the carbon atom of the fatty acid, which results in the formation of a *carbon-centred radical.* In the presence of O_2, these carbon radicals combine with O_2 and leads to the formation of *peroxyl radical* ($ROO^{\bullet}$). Peroxyl radicals have the potential to abstract the hydrogen atom from the adjacent PUFA and lead formation of lipid hydroperoxide (LOOH), which is shortened as *lipid peroxide*. This step is called the *termination* step; however, it involves the second round of initiation as well, therefore the lipid peroxidation process is a type of radical chain reaction. Since animal cell membranes contain large amounts of PUFA side chains, cellular membranes are much more susceptible to peroxidation, which is proportional to the number of double bonds in a PUFA. Lipid peroxidation products have many harmful effects on cellular membranes (Table I.6).

Most biological studies of lipid peroxidation involve transition metal ions added to, or contaminating the reaction mixtures. When ferrous ion, cuprous ion, or certain chelates of these ions are added to liposomes, lipoproteins, or isolated biological membranes such as microsomes, mitochondria, or plasma membrane fractions, peroxidation occurs. The oxidised form of these metal ions (e.g. Fe^{+2} and Cu^{+2}) can also accelerate peroxidation if there is a reducing agent (e.g. ascorbate, cysteine) added to the medium[34]. In contrast to these non-enzymatic processes, lipid peroxidation can also be initiated by some enzymes (such as cyclooxygenase and lipoxygenase) that catalyse

the controlled peroxidation of fatty acid substrate to give hydroperoxide and endoperoxide as well. Decomposition of lipid peroxides by heating at high temperatures or by exposure to iron and copper ions generates an enormous complex mixture of products, including epoxides, saturated aldehydes (e.g. hexanal), unsaturated aldehydes, ketones (e.g. butanones, pentanones, octanones), and hydrocarbons[33].

Table I.6: The overall effects of lipid peroxidaion on cellular membranes

A. Changes on the lipid bilayer:
Decreased membrane fluidity
Increased leakiness of the membrane
Increase in negative surface charge
The appearance of proton condcutivity
B. Changes on the biomembranes and organelles
The inactivation of membrane bound enzymes
Thiol group oxidation and increase in ionic permeability
The swelling of mitochondria
The uncoupling of oxidative phosphorylation
The loss of cytochrome c from mitochondria
Respiration chain inhibition
Damage to liver hydroxylation system
The release of lysosomal enzymes
The activation of membrane bound phospholipases
The solubilization of membrane proteins
C. Changes in cell metabolism and behaviour
The destruction of tocopherols, steroids, thyroxine
The decrease of thiol compounds
The redistribution of ions
Cell motility inhibition
Cell division deceleration

B.4.1. Role of Reactive Aldehyde Species (RAS) in Cell Signalling

Lipid peroxidation proceeded by a radical chain reaction mechanism, yields hydroproxide. Decomposition of hydroperoxide generates number of reactive end *products* that display a wide variety of damaging actions on biomolecules, including proteins, DNA, and phospholipids. Among lipid peroxidation end products, aldehydes have special characters. Compared to other free radicals (ROS, RNS), reactive aldehyde species are stable and can diffuse within or even escape from the cell and attack targets far from the site of the original event. Therefore, reactive aldehyde species are not only end products and remmants of lipid peroxidation but also may act as *second messenger* for primary reactions[35].

In addition to peroxidative degradation of polyunsaturated fatty acids (lipid peroxidation), glucose-protein or glucose lipid interactions (glycation) and oxidative modification of amino acids(amino acid oxidation) also generates reactive aldehydes in biological systems as by products of non-enzymatic reactions[35] (Figure I.10).

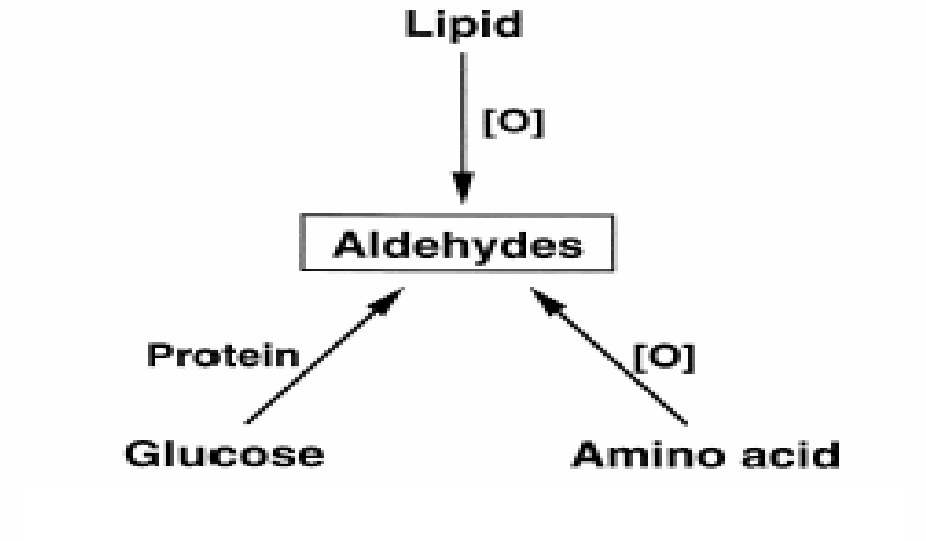


Figure I.10: Potential sources of reactive aldehydes associated with oxidative stress

A great diversity of aldehydes is formed during the breakdown of lipid hydoperoxides in biological systems. A key feature of this process is the fragmentation of PUFAs to yield a broad array of smaller fragments: three to nine carbons in length,

including aldehydes such as 4-hydroxy-2-alkenals, and 2-alkenals (Figure I. 11). 4-hydroxy-2-*trans* nonenal (HNE) is one of the extensively studied and prominent aldehye substances generated during lipid peroxidation (See the next section for a detailed explanation). There is increasing evidence that aldehydes generated endogenously during the process of lipid peroxidation are casually involved in many pathophysiological effects associated with oxidative stress in cells and tissues. These aldehydes react with nucleophilic sites in DNA and proteins and generate various types of adducts.

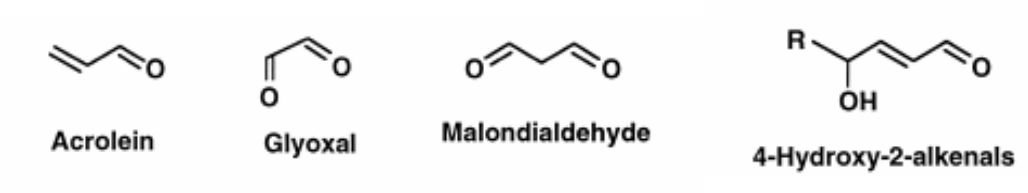


Figure I.11: The structure of reactive aldehydes generated from lipid peroxidation

Glycation is a complex series of reactions between reducing sugars (glucose) and amino groups of proteins. Although most glucose in a solution is present in non-aldehydic ring structure, glucose in its straight –chain form is an aldehyde. Thus it slowly reacts with proteins, amino-lipids such as phosphotidylethanolamine and DNA, modifying them in a process referred to as *non-enzymic glycation.* Products of non-enzymic glycation undergo further reactions, including oxidation, to advanced glycation end products (AGEs). AGE formation is irreversible, occurring over periods of months to years and causing tissue damage. Thus, AGE formation is suggested to contribute much to the pathophysiologies associated with aging and long term complications of diabetes[36]. Glycation reactions produce numerous reactive aldehydes and ketones

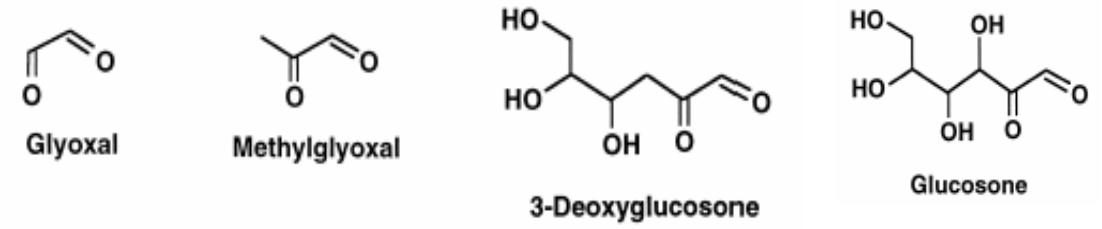


Figure I.12: The structure of reactive aldehydes generated from glycation

Reactive aldehydes species generated from lipid peroxidation or glycation reaction may have no cell surface receptors and directly react with tissue and cell surface proteins, causing the alteration of the structure and function of these proteins; therefore, the major effects of aldehydes are damage to proteins [35]. In addition to these effects, reactive aldehyde species has been shown to increase the intracellular ROIs level and therefore, cause oxidative stress. Lipid peroxidation-derived aldehydes, such as HNE and other α, β-unsaturated aldehydes, have been shown to induce intracellular peroxide production in cultured hepatocytes[37] and inhibit glutathione peroxidase activity[38] in vitro. The fact that one of the potential targets of oxidative stress is mitogen-activated protein kinases (MAPK), and since reactive aldehydes generate oxidative stress, one may conclude that reactive aldehydes modulate gene expression of various redox-response genes (Figure I.13).

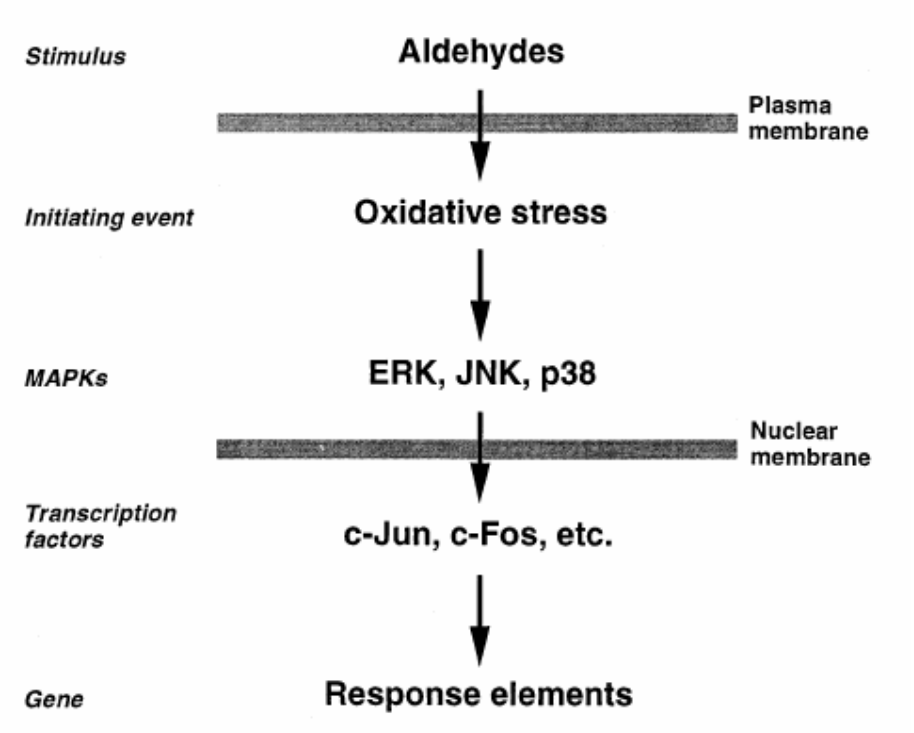


Figure I.13: Cellular response to reactive aldehydes mediated by oxidative stress[35]

B.4.2. HNE and Its Role on Cellular Events

4-Hydroxy-2-nonenal (HNE) is a major product of lipid the peroxidation end product that is generated by β-scission of alkoxyl radicals in polyunsaturated fatty acids such as arachidonic, linoleic, and linoleic acids present in low density lipoproteins (LDL)[39]. HNE is a component of oxidised LDL (ox-LDL), which is initiated by hydrogen abstraction from the double bond of PUFA in LDL, due to a reaction with a reactive oxygen species. Consequently, molecular rearrangements in PUFAs lead to the formation of conjugated double bonds called Conjugated Dienes (CD). Abstraction of $H^{\cdot}$ by PUFA-peroxyl radical ($LOO^{\cdot}$) from another PUFA results in lipid hydroprexides and finally generating reactive aldehydes [4] (Figure I.14). Oxidation of LDL has a significant contribution to the pathophysiology of cardiovascular diseases, especially atherosclerosis. HNE, a component of ox-LDL, is found in atherosclerotic lesions and it is shown that immunoreactive HNE is present in all stages of human atherosclerotic plaques but not in normal arteries [40] (see also [41]); hence HNE is assumed to contribute a lot to the formation of atherosclerotic plaques. HNE may accumulate in membranes at concentrations of 5 to 10 mM in response to oxidative stress[42].

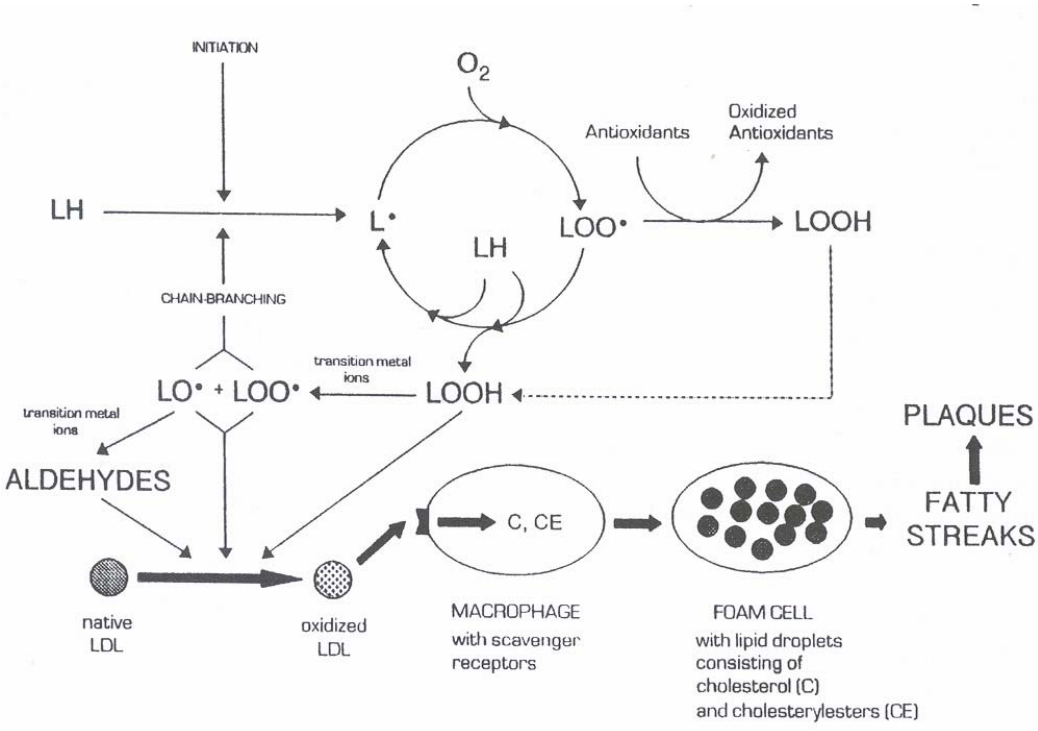


Figure I.14: The scheme of LDL oxidation mechanism

The harmful effects of HNE are attributed to its strong nucleophilic property, it preferentially reacts with cysteine, histidine and lysine residues of proteins[35]. The extensive studies of the mechanism of modifications of these amino acids by HNE established that HNE primarily form adducts having a hemiacetal structure via the Michael reaction (Figure I. 15). These adducts are relatively more stable and do not undergo further reactions often observed in other non-enzymatic modifications of protein with aldehydic compounds. When HNE reacts with lysine residues, both pyrrole and florescent HNE-lysine cross-linking adducts are formed in addition to hemiacetal formation. Due to the formation of these adducts, when added exogenously to the cells or when endogenously generated within the cells, HNE binds to different proteins and impairs their function to a great extent. For instance, it has been shown that HNE impairs the function of Na+, K+-ATPase[43], neuronal glucose transporter (GLUT3)[44], the astrocyte glutamate transporter (GLT-1)[45], and GTP-binding protein $G\alpha_{q/11.}$[46].

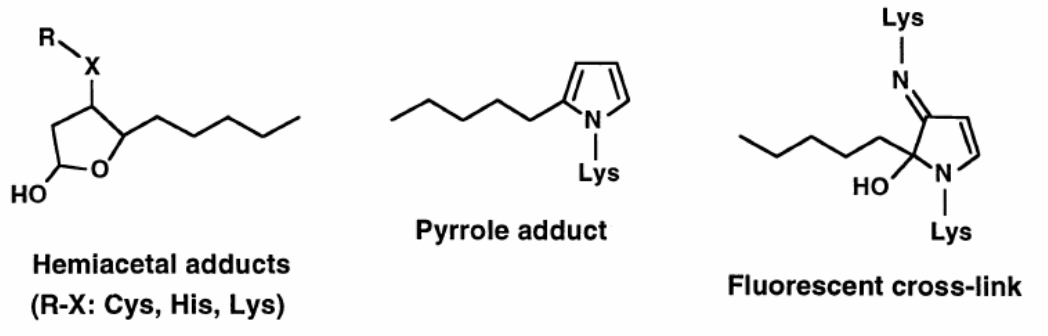


Figure I.15 : The structure of amino acid adducts with HNE [35]

The mechanism of HNE induced signal transduction and cell injury is well studied in many cell lines. It is well established that exposure to certain doses of HNE activate aortic smooth muscle cell[47] and vascular smooth muscle cell (VSMC) growth and causes ERK, JNK, and p38 MAP kinase activation as well as the induction of c-fos and c-jun gene expression[48]. In contrast to these findings, it has been also shown that it induces apoptosis in human leukemic T-and B-cell lines[49], RKO cell line[50], and VSM cell line[31]. These contrasting results are interpreted, as the effect of HNE on a cell line is concentration dependent; at different concentrations HNE exerts different effects. The molecular mechanism of HNE induced signal trunsduction is yet to be clarified. AP-1 is

well studied and has shown to be up regulated by HNE; however, NF-κB, another redox sensitive transcription factor, is shown to be down regulated by HNE. Recently Page et al. demonstrated that HNE counteracts the lipolysaccharide (LPS)-induced activation of NF-kB, at least in the macrophagic cell line THP-1, by inhibiting the phosphorylation and subsequent proteolysis of the NF-kB inhibitory subunit (IkB)[51]. Consistant with the above finding, it has been shown that HNE causes the loss of proteasome activities by the direct attachment to the protein during oxidative stress[52]. The overall cellular effects of HNE can be summarised in three different groups[33]. Depending on the concentration, HNE: a) induces acute toxic effects, mitochondrial damage, apoptosis or necrosis, at high concentrations (100 uM or above) b) inhibits cell proliferation, DNA and protein synthesis at intermediate concentrations (1-20 uM); and c) stimulates several different processes, including phagocyte chemotaxis, activities of many enzymes including phospholipase c and adenylate cyclase (0.1 uM or lower). This is the basal level of HNE in healthy tissues.

II. PURPOSE

Cell signalling by reactive oxygen species and lipid peroxidation end products has become one of the important topics of molecular biology and biochemistry in recent years. Especially lipid peroxidation end products gained special interest and attention since they have been shown to play a significant role in mediation and induction of signal transduction. Among the many different reactive aldehydes generated during lipid peroxidation, 4-HNE is one of the major product and has been shown to have a number of adverse biological effects in different cell lines.

In this study we aimed to;

- investigate the role of 4-HNE in cell signalling and cellular responses, optimize the experimental conditions and establish the cytotoxic and signalling concentrations of 4-HNE in 3T3 fibroblast cell line,
- investigate the mechanism of 4-HNE induced cell signalling and establish a possible link between the production of ROS and the events leading to cell death,
- identify the mechanism of cell death, whether apoptosis/necrosis which has a profound implication on the development of new therapeutic strategies.
- establishe a model for possible effects of 4-HNE on redox regulated transcription factors and gene expresion for future studies.

III. MATERIALS

A. Chemicals

All chemicals and growth mediums were purchased from Sigma (Germany), Merck (Germany), and Biological industries (Israel) unless otherwise indicated. HNE was purchased from Calbiochem (USA). Hydrogen peroxide was purchased from Reidel de Haen®.

B. Solutions and Buffers

HNE……... 6,4 mM stock solution in Ethanol
α-Tocopherol……20 mM stock solution in DMSO
DCFH-DA……10 mM stock solution in Ethanol
Propidium Iodide dye……..1 mg/ml stock solution in PBS
Hoechst dye……... 1,94 mM stock solution in PBS
Acridine Orange dye…….....10 mM stock solution in PBS
Resveratrol……50 mM stock solution in DMSO

C. Equipments

Autoclave:	CERTOCCLAV® A-4050 TRAUN/AUSTRIA
Automatic Pipette:	PIPETTUS® -AKKU, HIRSHMANN LABORGERATE
Balances:	SARTORIUS® BP 221S
Centrifuges:	EPPENDORF® CENTRIFUGES5415 D EPPENDORF® CENTRIFUGES5415 R HERAEUS® MULTIFUGE 3 S-R
CO_2 incubator:	BINDER CO_2 INCUBATOR
Deepfreezer:	HERAEUS ® HERA FREEZE
Electrophoresis:	BIOGEN
Gel Dryer:	E-C APPARATUS CORPORATION EC 355
Incubator:	MEMMERT® MODEL 300
Laminar Flow:	HERAEUS® HERA SAFE, GERMANY
Magnetic Stirrer:	VELP SCİENTİFİCA
Micro pippets:	GILSON PIPETMANN, FRANCE
Microscopes:	OLYMPUS CK40 OLYMPUS IX 70 OLYMPUS BX 60

Oven:	BOSH
Power supply:	BİO-RAD POWERPACK 300
Refrigerator:	BOSH
Spectrophotometer:	SECOMAM ANTHELİA JUNİAR, FRANCE
Vortex Mixer:	VELP SCIENTIFICA
Water Bath:	HUBER POLYSTAT CC1

D. Others

Culture flasks, multiwell plates, falcon tubes, and sterile pipettes were from TPP (Europe).

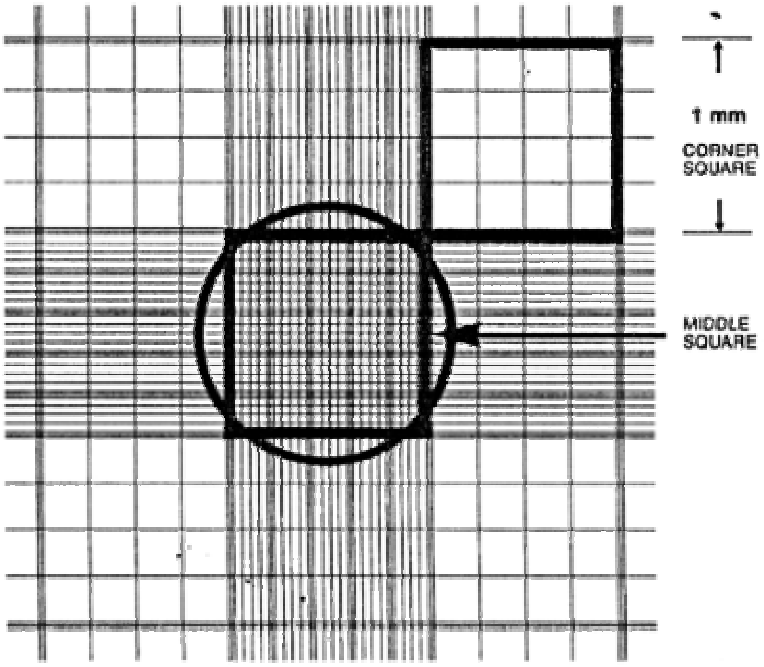
IV. METHODS

A. Cell Culture

Swiss Albino mouse monolayer Fibroblast 3T3 cell line was obtained from Cell culture collection (Hücre Kültürü Koleksiyonu, HÜKÜK, NO: 95021701), Ankara. This cell line was maintained in 90% Dulbecco's Modified Eagles Medium (DMEM) and 10% Fetal Calf Serum (FCS). The medium (500 ml) was supplemented with 2 ml Penicillin-Streptomycin solution (from 10.000 U/ml Penicillin and 10 mgr Streptomycin/ml stock solution), 15 mgr L-methionine, and 290 mgr L-Glutamine. The cells were grown under a humidified atmosphere containing 5 % CO_2 at 37 ^{o}C in 25 or 75 cm^2 culture flasks as an attached monolayer. Throughout all experiments, prior to the treatment, the culture medium containing serum was replaced with either serum free medium (0.2 % FCS) or phosphate buffer saline (PBS) and left for overnight incubation when the cells were at 70-80 percent confluency. Cell viability was checked by Trypan Blue dye exclusion method on a haemocytometer (60-90 per cent viability). The cells used throughout experiments were between passage number 7-25.

B. Determination of cell viability by Trypan Blue Dye exclusion method

After treatment, cells are trypsinised (or scraped, depending on the purpose of the experiment) from the culture flask. After obtaining a homogen cell suspension, a small amount of the suspension (30 ul) is taken to a new eppendorf tube and equal volume of Trypan Blue dye stock solution (0.4 % w/v) is added. Then a drop of (about 25 ul) the stain/culture combination is placed on the haemocytometer for one minute. The cells are observed with low power microscopy. [Trypan Blue is a stain that is actively extruded from viable cells, but readily enters and stains dead cells. Therefore, the cells, which are blue, are dead.] The difference between the total number of cells and the number of dead cells would be the number of viable cells in a given aliquot of culture suspension. The cells in the middle square are counted (refer to Figure IV. 1). The total number of cells/ ml: 2 x number of cells counted x 10^4. The first two comes from dilution factor (since we diluted with trypan blue), and 10^4 comes form the volume factor (since the volume of each square on the haemocytometer is 10^{-4} ml, refer to Figure IV. 1).



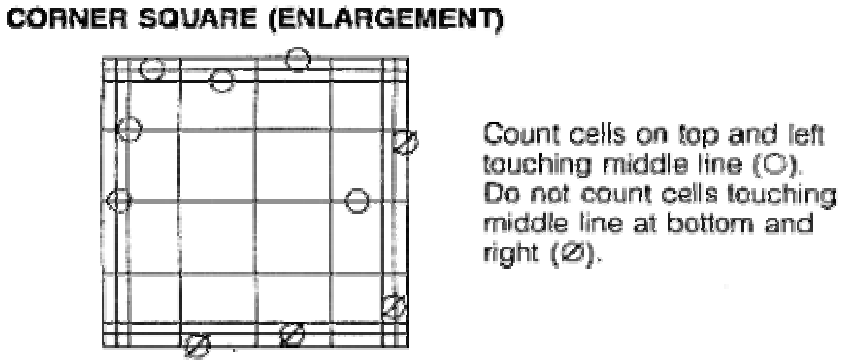


Figure IV. 1: The grids on a haemocytometer.

C. Detection of ROS production by using fluorescence microscopy

Dichlorofluorescin diacetate (DCFH-DA) has been used as fluorescent probe in our experiments. We used hydrogen peroxide (H_2O_2) and lipid peroxidation end product, 4-hydroxynonenal (HNE) in our experiments. Cells grown on coverslips in 6-well plates, were treated with one of the oxidising agents for indicated time period. Then they are incubated with 5 uM DCFH-DA for 20 min. Coverslips were removed from the well and put in beaker of PBS for 5 seconds in order to wash away background florescence. Then a drop of Glycerol was placed on a slide and the coverslip was put on immersion oil in a way that the cells are sandwiched between the coverslip and the slide. The slide is visualised with Olympus Fluorescent microscope.

D. Detection of apoptosis by florescent dyes

For the detection of apoptosis, the method of Foglieni et al.[53] has been used with some minor modifications. We have either fixed cells or we used florescent dyes directly. Briefly, plated cells were fixed in freshly prepared 2% para-formaldehyde in PBS 0.05 M pH 7.4 for 10 min. at room temperature, washed times with PBS and stained either

with AO, HO, or PI, or with one of the following combinations: AO +HO + PI. These dyes respectively show specificity for living, apoptotic and late apoptosis/necrosis states. In order to stain the cells without fixation, cells were grown on coverslips as described in section 'C' of materials part.

E. Analysis of DNA fragmentation

In order to analyse endolytic DNA fragmentation, DNA from 3T3 fibroblast cells has been isolated according to the method described by Herrman et al.[54] with some minor modifications. Briefly, cells were rinsed with ice cold PBS and scraped of the plates. Cells and the rinsing buffer were transferred to the tubes with the medium and cells were pelleted by centrifugation at 500xg for 5 min at ambient temperature. The supernatant was discarded and the pellet gently resuspended in 0.1 ml of lysis buffer (50mM Tris, pH 7.5, 20mM EDTA and 1% Nonidet P-40) for 40 sec. After centrifugation at 2000 rpm for 5 min at room temperature, the apoptotic DNA, remaining in the supernatant, was collected in another tube. After this step, DNA was precipitated by ethanol. For this, the volume of the DNA sample was measured and the salt concentration was adjusted by adding 1/10 volume of sodium acetate, pH 5.2, or an equal volume of 5 M ammonium acetate and mixed well After that, 2 to 2.5 volumes of cold 100% ethanol was added. (Calculated after salt addition). Then it was placed on ice or at -20oC for >20 minutes and spined at maximum speed in a microfuge for 10-15 min. Supernatant was carefully decanted and 1 ml 70% ethanol was added, mixed, and spinned briefly. Supernatant was carefully decanted, pellet was air dried and resuspended in appropriate buffer such as TE or water.

DNA was analysed by running 1,5 % gel electrophoresis at 80 V for 2,5 hr. The DNA was visualised with EtBr under UV light.

V. RESULTS

A. Cell Morphology and Viability Studies

For the studies of cellular morphology phase contrast microscopy, fluorescent microscopy, and light microscopy has been used. Cell viability has been evaluated by using trypan blue dye exclusion method. In time dependent manner significant morphological deformations and membrane blebbings (indicated by arrows) has been detected when cells were incubated with 500 μM hydrogen peroxide (Figure V.3)

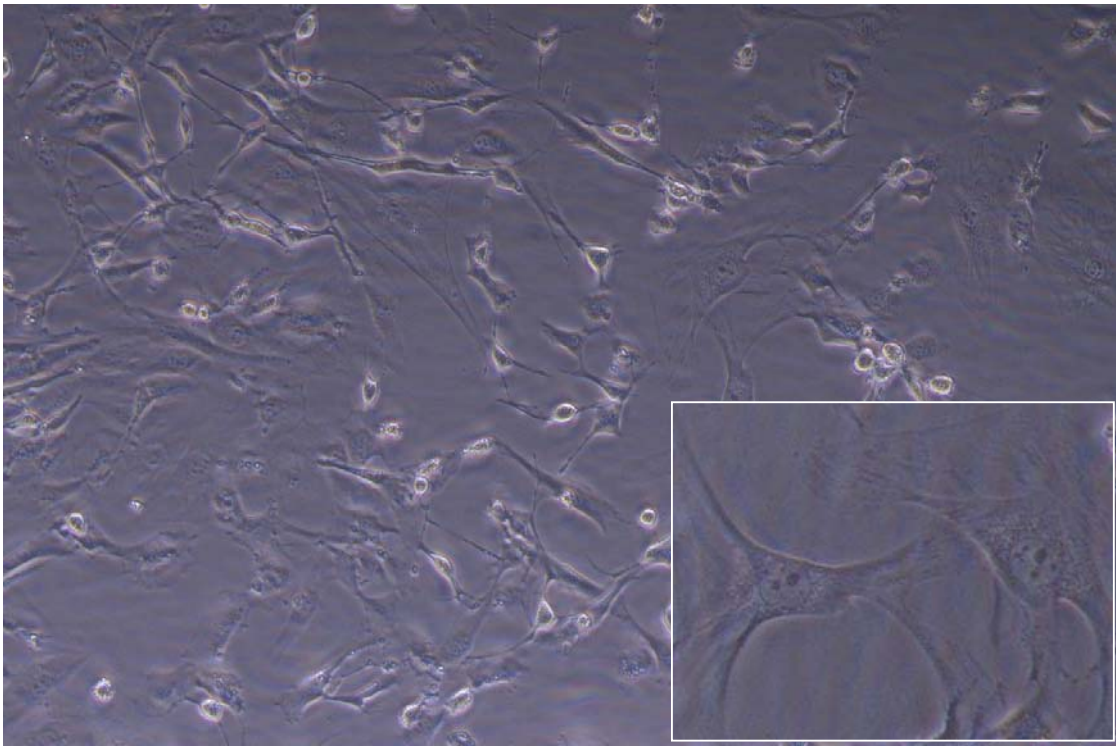


Figure V.1: 3T3 Swiss Albino Mous Fibroblast cell line. Cells are visualised with phase contrast micrpscopy.

AO has **green** emission when bound to double stranded nucleic acids and **red** signal when intercalated into single-stranded nucleic acids. In the above figure, untreated 3T3 Swiss mouse fibroblast cells are seen with green nucleus and partial red cytoplasm which is thought to be due to presence of RNA in the cytoplasm (Figure V.2).

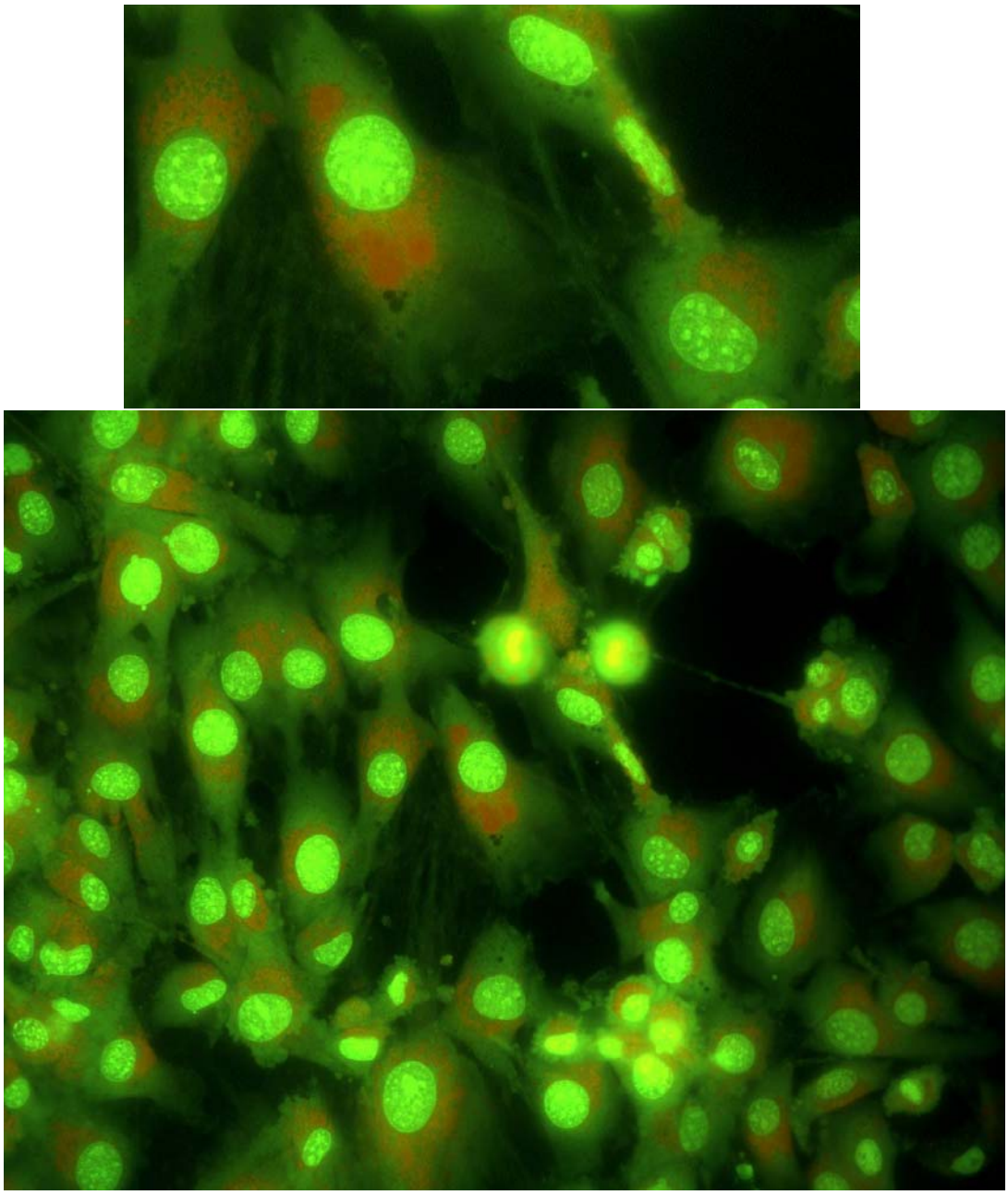


Figure V.2: 3T3 fibroblast cell stained with Acridine Orange (AO). Figure is taken by Olympus BX-60 florescent microscope.

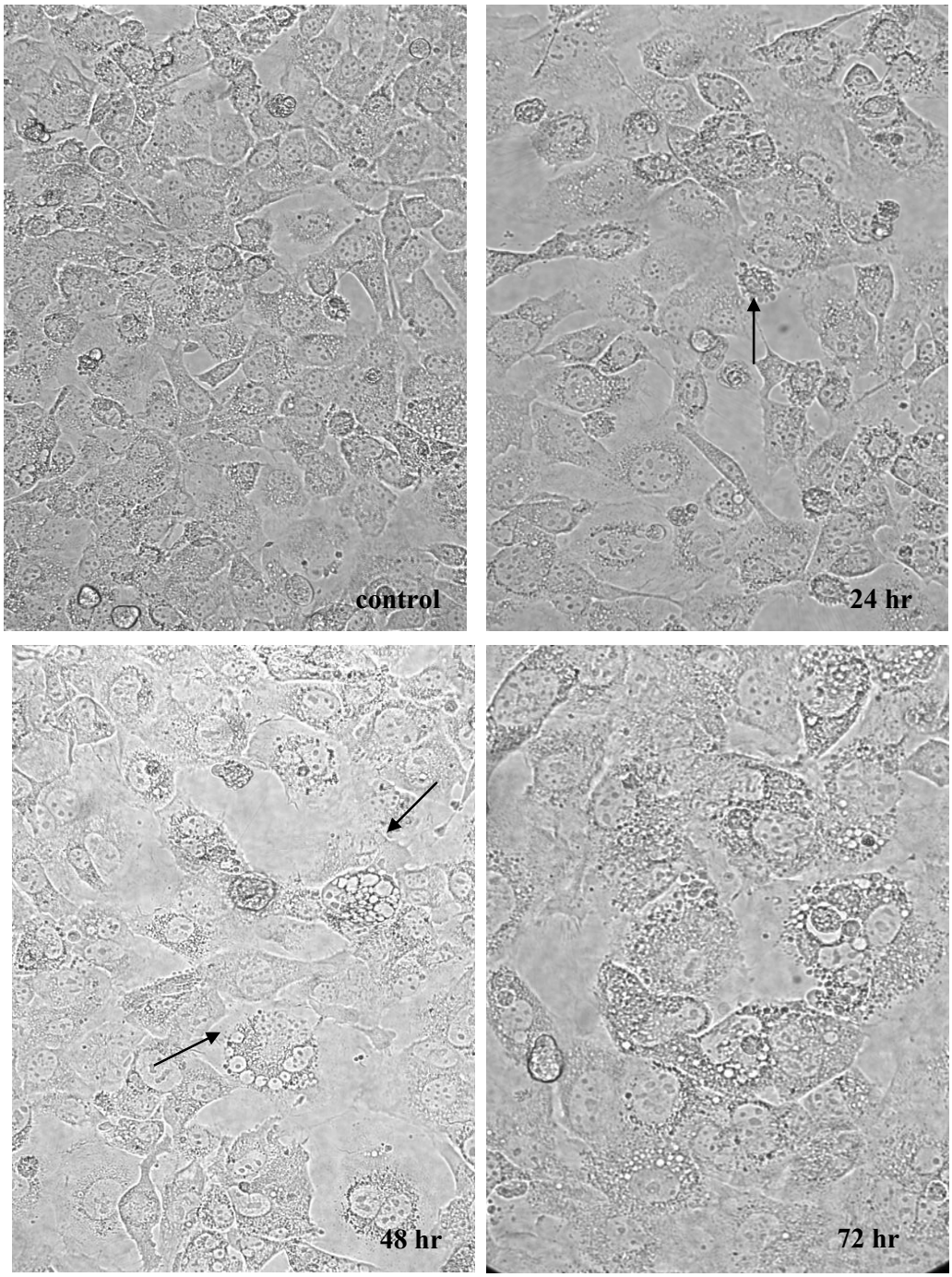


Figure V.3: Effect of hydrogen peroxide on cellular morphology. Figure is taken by light microscope (40X objective)

After incubating with 25, 50, and 75 μM hydrogen peroxide concentrations for 24 hr, cell viability was checked by trypan blue dye exclusion method. Viable cells were seen in bright since they exclude trypan blue dye where as non-viable cells were seen in blue under light microscopy (Figure V.4)

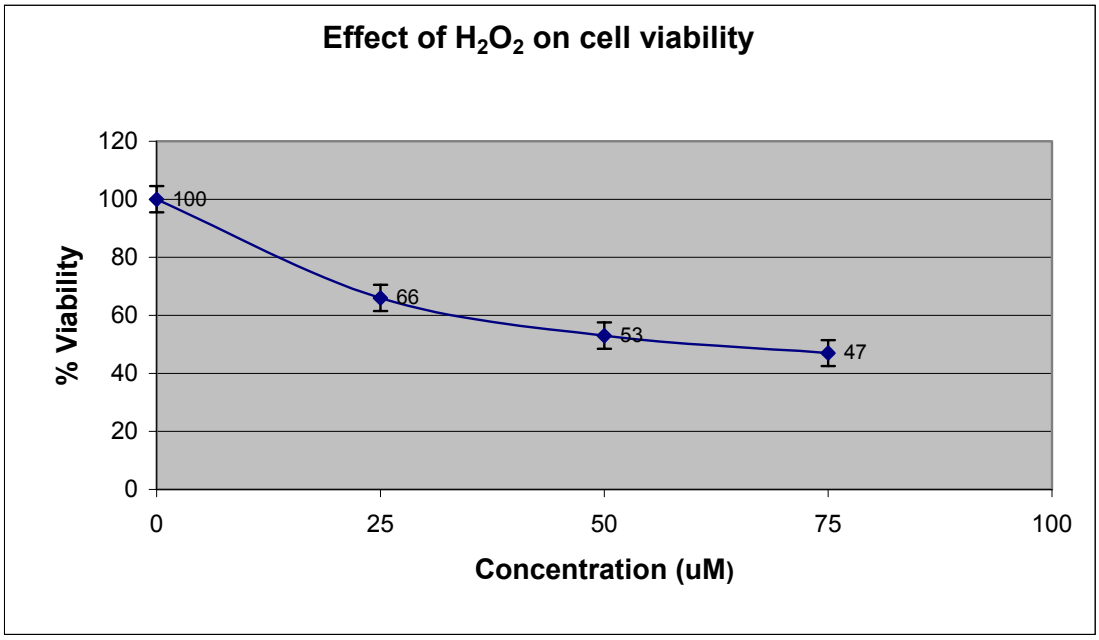


Figure V.4: Effect of hydrogen peroxide on the viability of 3T3 fibroblast cells. Results are mean of two experiments in duplicate.

Cells were incubated with 25 and 50 μM 4-Hydroxy-2-nonenal concentrations for 24 hr; cell viability was checked by trypan blue dye exclusion method. It has been observed that there were respectively 25 % and 93 % loss in cell viability when they were incubated with indicated HNE concentrations.

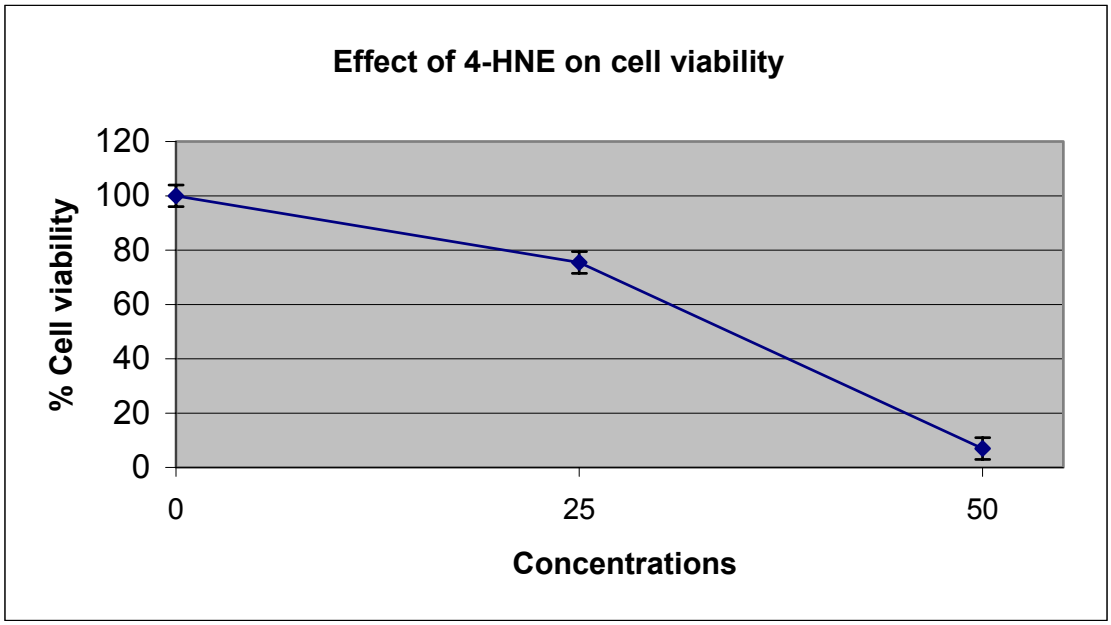


Figure V.5: Effect of 4-hydroxy-2-nonenal on viability of 3T3 cells. The results are mean of two experiments in duplicate.

In order to observe the effect of HNE on cellular morphology, after treatment with indicated concentrations of HNE, they were fixed with 2% paraformaldehyde. In a concentration dependent manner, cells became thicker and after certain concentration detachment has been observed (Figure V.6).

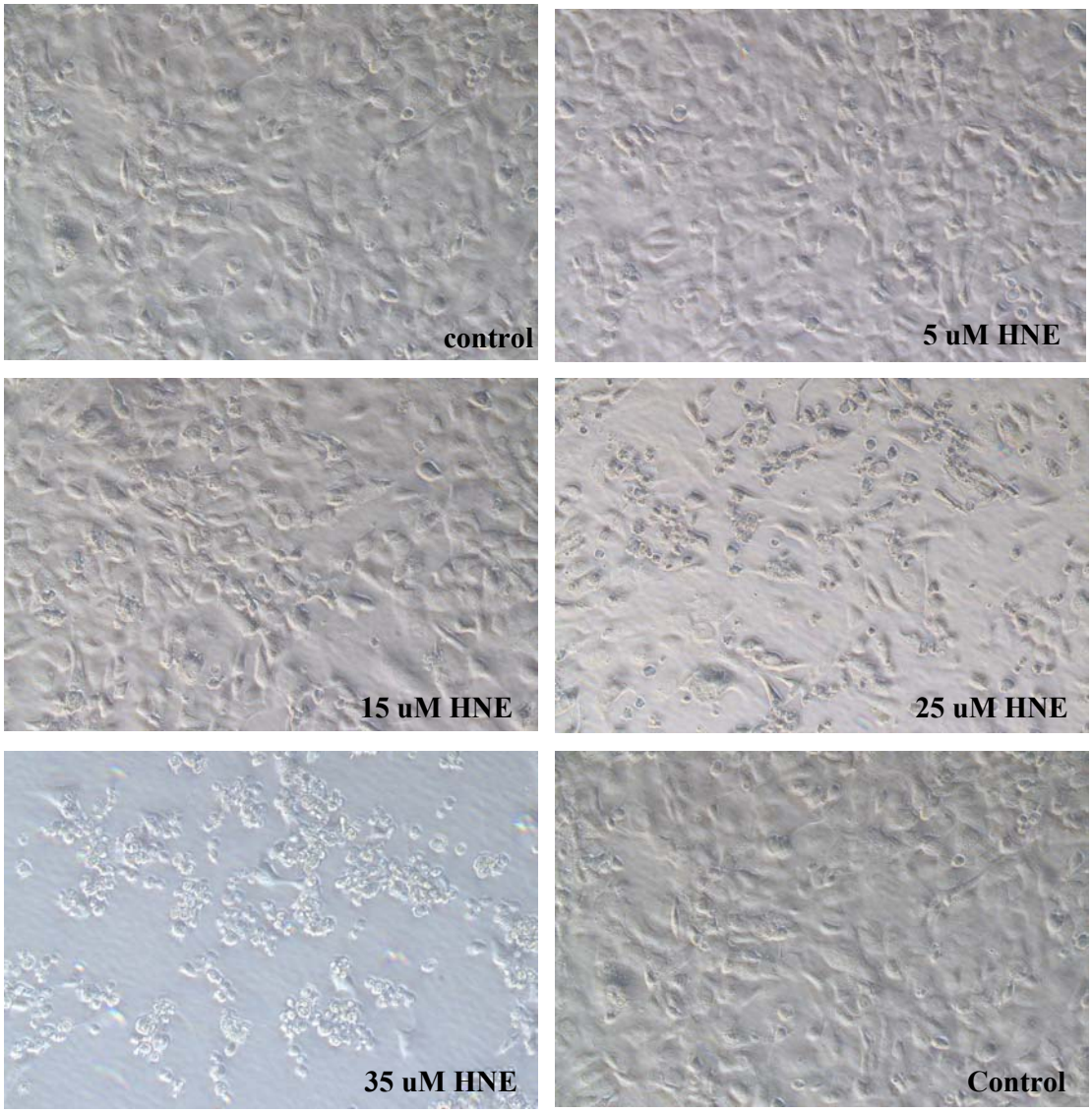


Figure V.6: Effect of 4-hydroxy-2-nonenal on cellular morphology. The figure is taken by phase contrast microscopy.

B. Detection of ROS by florescence microscopy and the effect antioxidants

ROS production has been detected by using fluorescent probe DCFH-DA. Cells were grown on coverslips and indicated concentrations of HNE were applied for 5 hr, after that cells were further incubated with fluorescent probe for 20 min. The cover-slips were removed from plates, washed with PBS and placed on microscope slide. Under fluorescent microscope, with increase in the concentration of HNE, increased fluorescent intensity has been observed (Figure V.7).

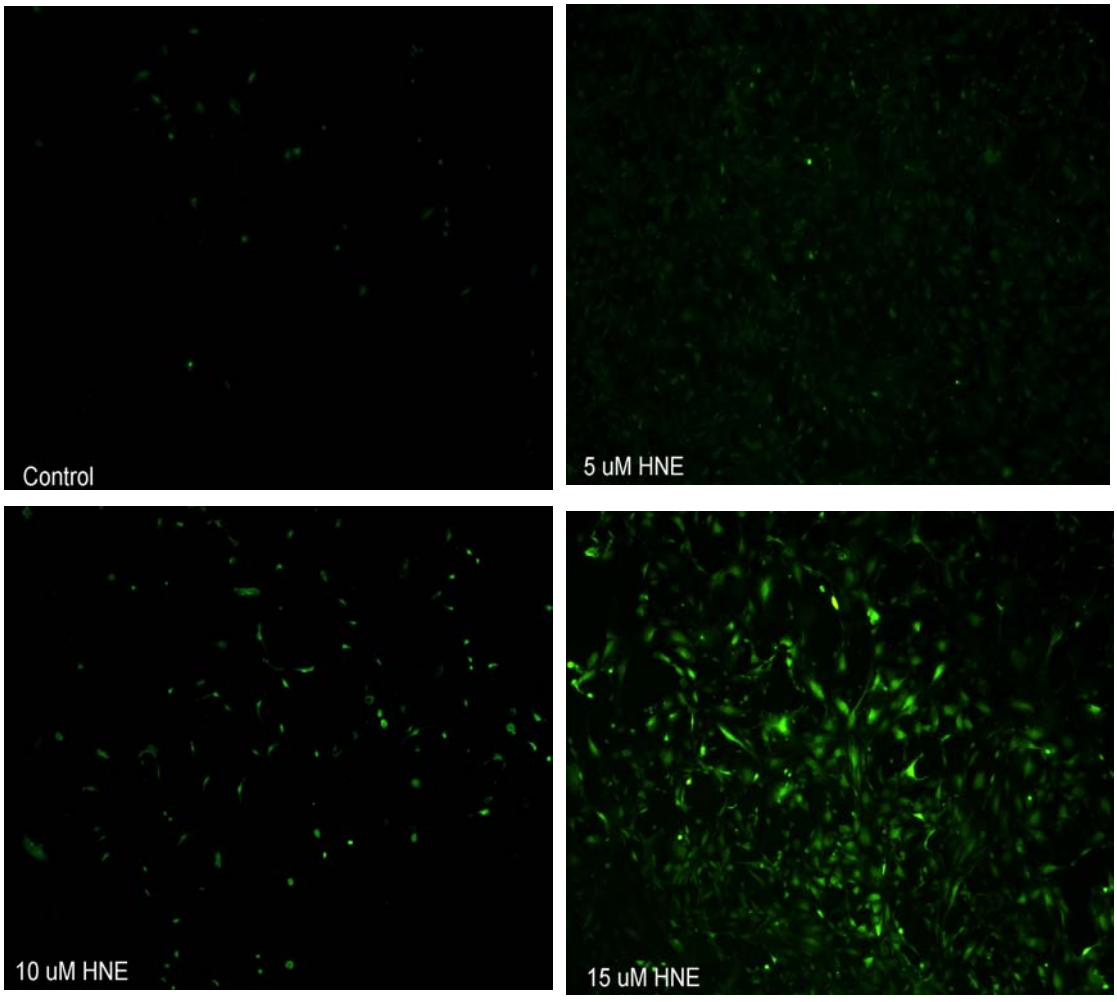


Figure V.7: Effect of HNE on the production of ROS. They are visualised under florescent microscope (Olympus BX-60).

Vitamin E (α-tocopherol) has inhibitory effect on the production of ROS, hence oxidative stress induced by 4-hydroxy-2-nonenal. 3T3 fibroblast cells are pre-incubated with 50 uM α-tocopherol for 12 hr., and indicated HNE concentrations were applied for 5 hr. Vitamin E prevented oxidative stress both at low (5 μM) and (15 μM) concentrationsof HNE (Figure V.8-9).

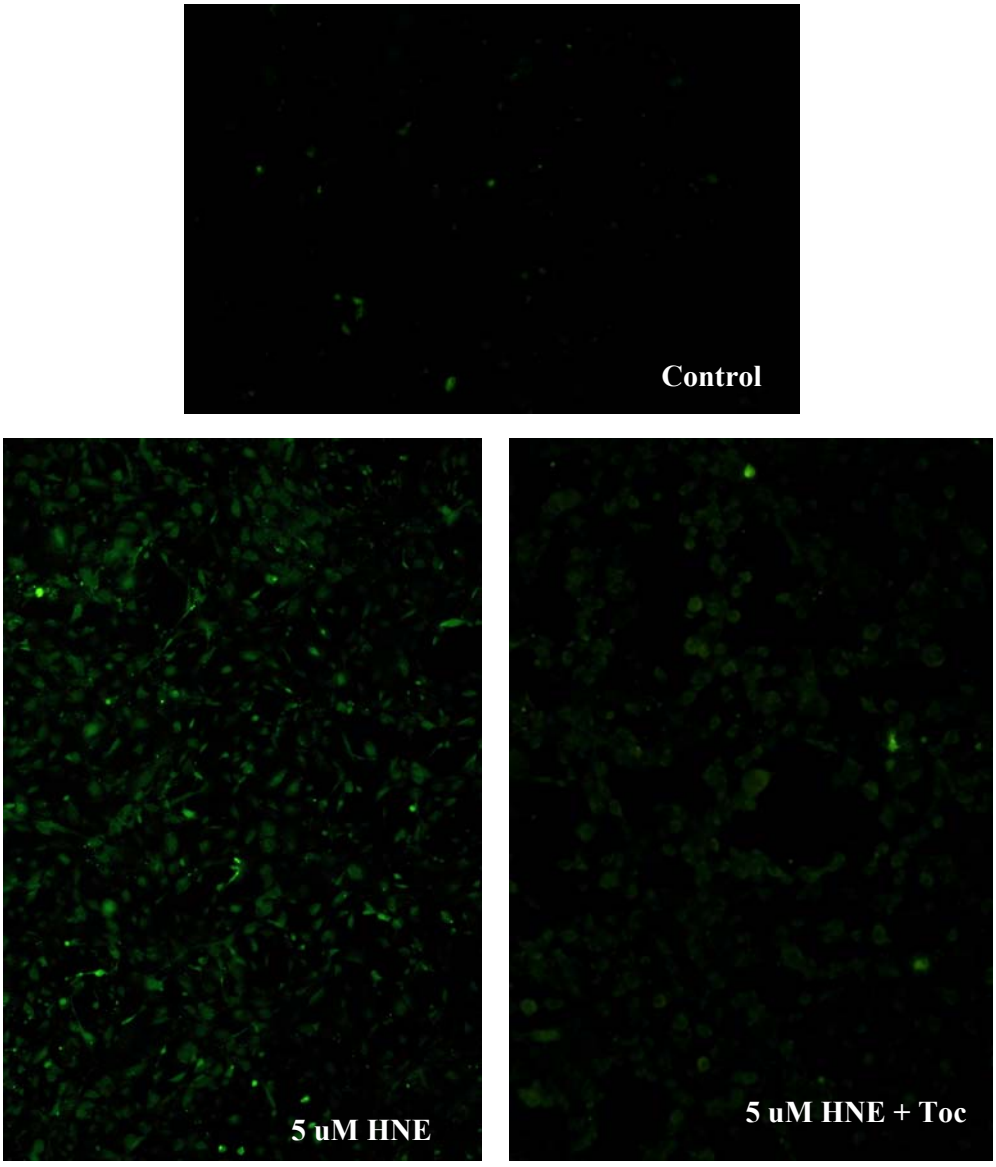


Figure V.8: Effects of α-tocopherol (Vitamin E) on HNE induced ROS production. Cells were visualised under florescent microscope (Olympus BX-60)

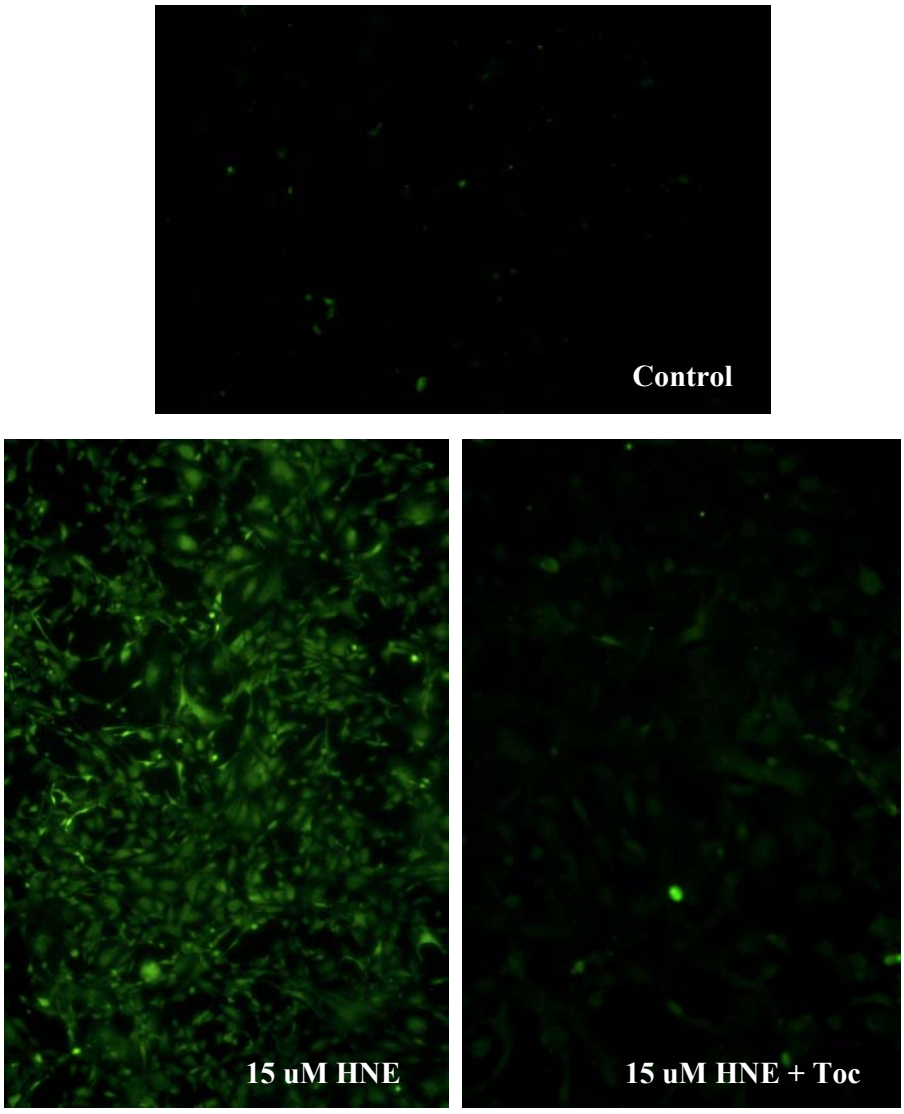


Figure V.9: Effects of α-tocopherol (Vitamin E) on HNE induced ROS production. Cells were visualised under florescent microscope (Olympus BX-60).

Resveratrol, a plant derived antioxidants, like vitamin E, has inhibitory effect on the production of ROS, hence oxidative stress induced by 4-hydroxy-2-nonenal. 3T3 fibroblast cells were pre-incubated with 50 uM resveratrol for 12 hr., and indicated HNE concentrations were applied for 5 hr. It prevented oxidative stress both at low (5 μM) and high (15 μM) concentrations of HNE (Figure V.10-11).

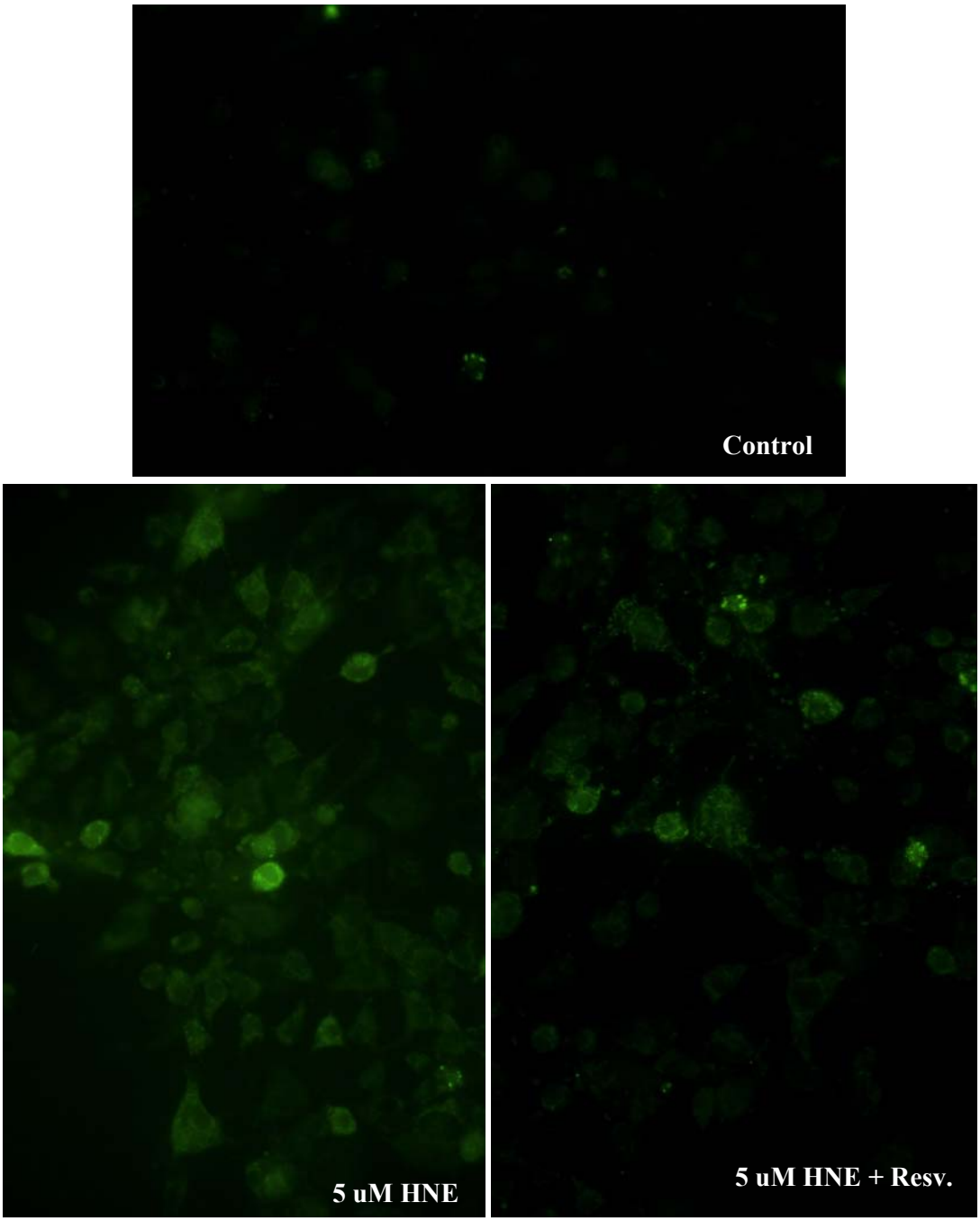


Figure V.10: Effects of resveratrol on HNE induced ROS production. Cells were visualised under florescent microscope (Olympus BX-60).

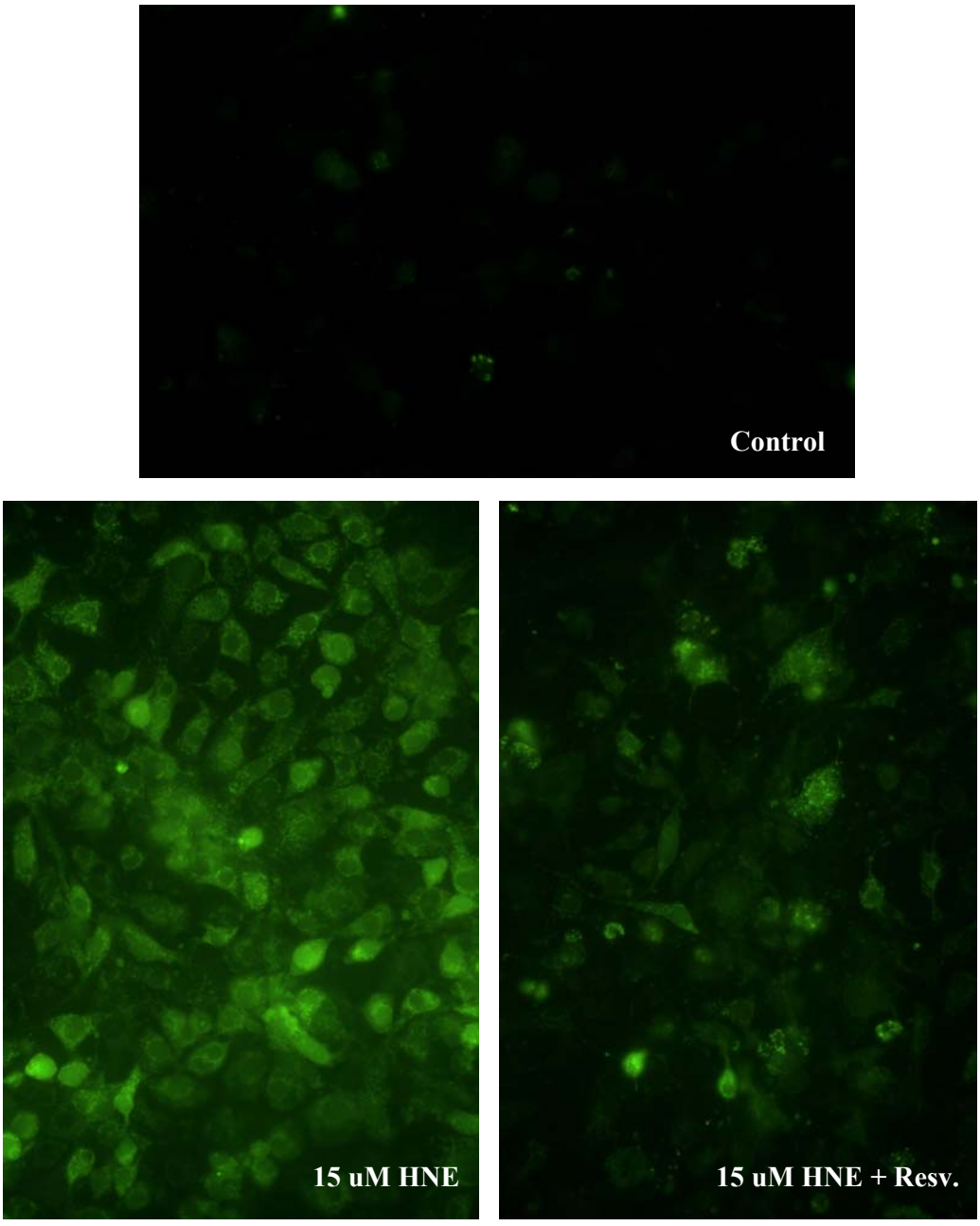


Figure V.11: Effect of Resveratrol on HNE induced ROS production. Cells are visualised under florescent microscope (Olympus BX-60).

C. Detection of HNE induced Apoptosis

Among number of ways for apoptosis detection, we have used differential staining by florescent dyes and DNA fragmentation assay to detect apoptotic cells. Differential staining is the usage of combination of Acridine Orange (AO), Hoechst 33342 (HO) and propidium iodide (PI). They are among the most used fluorescent dyes used to analyse cell culture viability and respectively show specificity for living, apoptotic and late apoptosis/necrosis states. After treatments cells were incubated with three dyes for 20 min and visualised under florescent microscope (FigureV.12). Yellow arrows indicate living cells whereas red arrows indicate apoptotic and necrotic cells. Apoptotic cells have more condensed and bright nucleus.

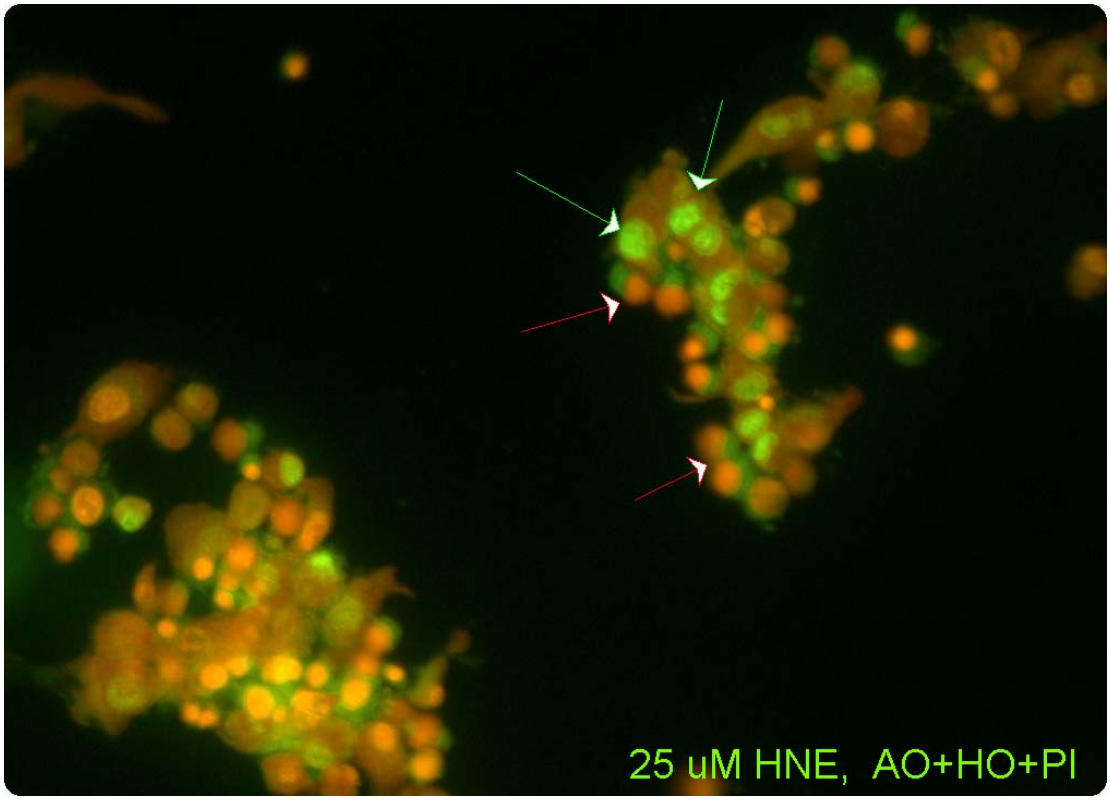


Figure V.12: Differential staining of healthy, apoptotic and/or necrotic cells.

One of the important points in this method is the choice of appropriate solution for the purpose of placing cover slips on the slide. Otherwise the cells will easily dry up and cellular deformations observed will not be due to the agent used in the experiment. We have used both glycerol and immersion oil for 100X objective. Immersion oil gave much better results and its half-life before drying up is much longer than glycerol.

PI stains specifically late apoptotic or necrotic cultured cells. It intercalates into nucleic acids for every 4-5 bp without sequence preferences. In figure V.13, necrotic cells and some cells with condensed nuclei, as indicated by arrows, are seen. They thought to be apoptotic cells.

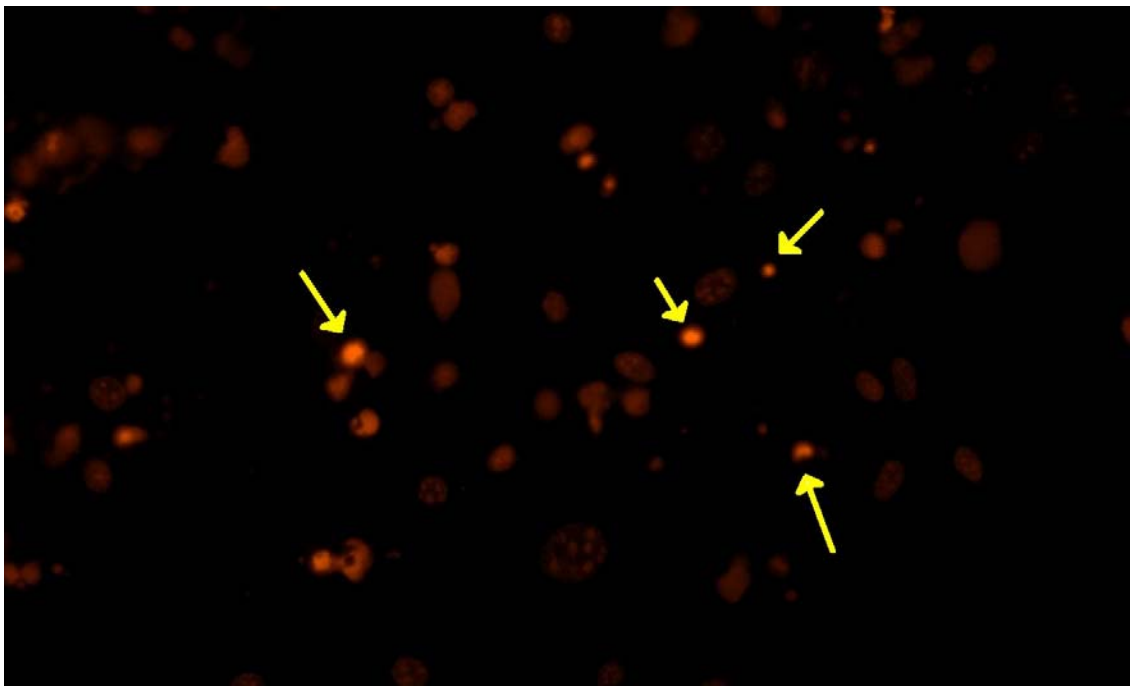


Figure V.13: Detection of late apoptotic and necrotic cells by propidium iodide (PI).

For the detection of apoptosis at the DNA level, genomic DNA has been isolated. Figure V.13 shows that there is decrease in the intensity of intact genomic bands after treatment with different oxidant agents. In gel figure A, DNA was isolated from cells treated with indicated concentrations of H_2O_2 and in B, DNA was isolated from cells treated with indicated concentrations of HNE. Though there is no observable fragmentation, there is decrease in band density as the concentrations of oxidant agents are increasing.

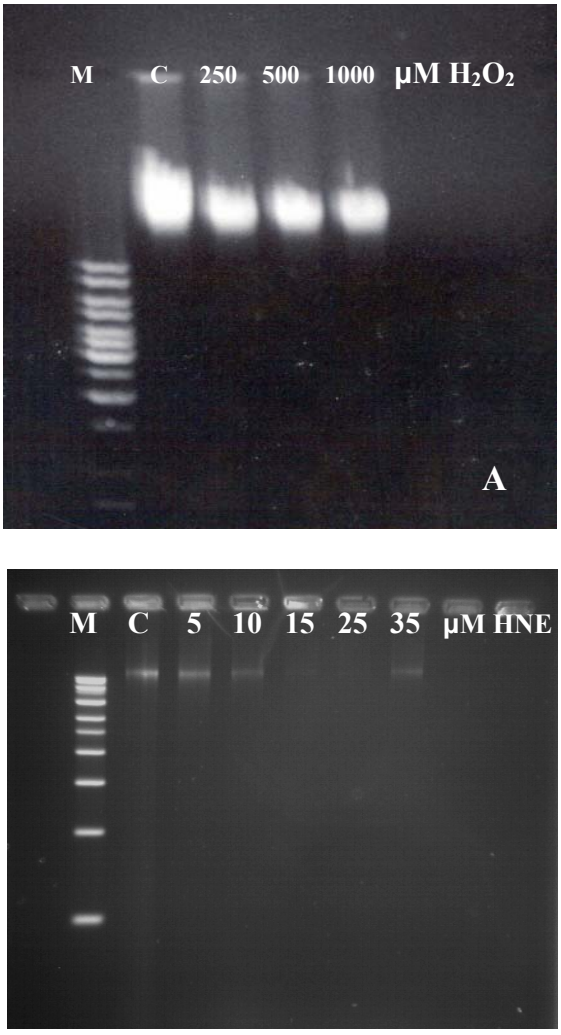


Figure V.14: DNA isolation and analysis for detection of Apoptosis.

Vitamin E has inhibitory effects on DNA fragmentation (Figure V.15). Cells treated with indicated concentrations of 4-HNE for 12 h with/without pre-incubation with 200 μM α-tocopherol succinate (Vit E) for 17 h. Lane 1 through 4 are 1 μM 4-HNE, 5 μM 4-HNE, 10 μM 4-HNE, 20 μM 4-HNE respectively. And lane 6 through 9 are Vit E + 1 μM 4-HNE, Vit E + 5 μM 4-HNE, Vit E + 10 μM 4-HNE, Vit E + 20 μM 4-HNE respectively.

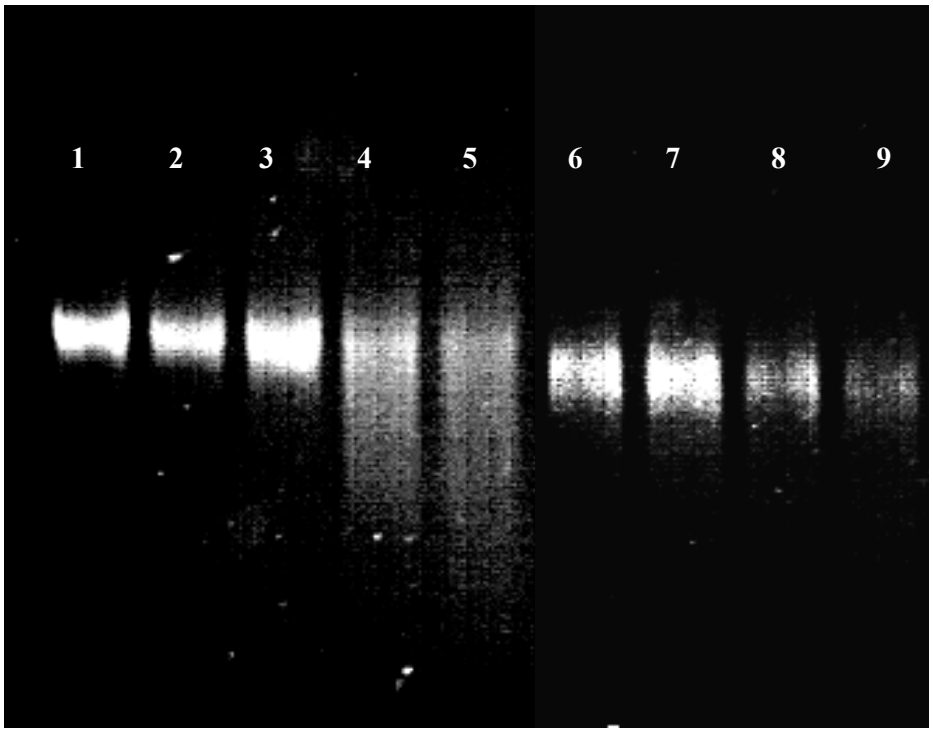


Figure V.15: DNA fragmentation analysis and effect of Vitamin E.

VI. DISCUSSION AND CONCLUSION

In recent years lipid peroxidation end products gained special interest and attention as mediators and inducers of signal transduction pathways, which are known to be important in many clinical conditions. They have a significant contribution to the pathophysiology of cardiovascular diseases, especially to atherosclerosis. At the site of oxidation, the concentration of lipid peroxidation end products reaches a significant level; for example, in membranes 4-HNE concentration reaches 5-10 mM in response to oxidative stimuli. 4-HNE is even detectable in plasma of healthy individuals up to 1-4 μM and is present in tissues at concentrations up to 20 μM[42]. 4-HNE accounts for up to 95 % of the unsaturated aldehydes generated during the peroxidation of ω 6-polyunsaturated fatty acids and have been shown to be highly reactive[55]. Of the numerous intermediates generated during lipid peroxidation, unsaturated aldehydes, such as 4-HNE is particularly important because of the high concentrations (up to 10mM) in which they are generated and their propensity to react with most cellular constituents such as DNA, proteins, and lipids.

Lipid peroxidation end products such as oxidised LDL or the bioactive aldehyde 4-HNE has been shown to exert many diverse effects on different cell lines[41]. Despite the fact that lipid peroxidation is the most studied biologically relevant free radical chain reaction, and despite the known effects of 4-HNE on several cell types, little is known about the effects of 4-HNE on 3T3 fibroblast cell line. In this study the possible cytotoxic, morphologic and signalling effects of 4-HNE in 3T3 fibroblast cell line has been investigated. Throughout the experiments trypan blue exclusion method has been used to check cell viability and 4-HNE concentration has been chosen accordingly. At subtoxic concentrations and as low as 5-10 μM 4-HNE is able to increase the production of ROS which could be overcome by antioxidants. A very well documented

and a very potent oxidant H_2O_2 can induce a similar effect albeit at higher concentrations. Our results clearly indicate the oxidative potential of 4-HNE in 3T3 fibroblast cell lines, an effect, which has been demonstrated at different cell lines at much higher concentrations[59]. 4-HNE at higher concentrations (20-30 μM) was able to induce apoptosis as observed by several different techniques. The differential fluorescent staining techniques as well as DNA fragmentation results indicated that apoptosis could be induced by incubation of cells for longer periods with 4-HNE. Further evidence for ROS triggered apoptosis is provided from the results obtained by preincubating the cells with antioxidants such as vitamin E and resveratrol, prior to the treatment of 4-HNE (Figure V.15). The concentration of 4-HNE that has been chosen to study cell death mechanism is lower than the concentration used by other researchers that has studied apoptotic effects of 4-HNE on different cellular systems[59].

H_2O_2 has been used for the purpose of having a representative oxidant agent whose effect can be evaluated as a marker of oxidative stress in our experimental system. It has been found that when cells were incubated with 25, 50 and 75 μM H_2O_2 for 24 hr, a dose dependent decrease in cell viability has been observed (Figure V.4). We have shown that H_2O_2 can induce ROS production as low as 25 μM in 3T3 cell line as detected by increased fluorescence intensity. In order to ensure that the fluorescence is coming from endogenously produced ROS but not exogenously given hydrogen peroxide, cells has been washed three times with PBS prior to detection under fluorescent microscopy. H_2O_2 can initiate apoptosis at concentrations higher than 500 μM when incubated for longer periods (48-72 hrs), as assessed under light microscopy from morphological analysis. Incubation of cells for 48 hr up to 72 hr causes significant cellular deformations such as membrane blebbings, which is indicative of apoptosis (Fig. V.3). To provide further evidence, light microscopy results has been supported by isolating genomic DNA from treated as well as non-treated cells and studying DNA fragmentation known to be the hallmark of apoptosis. Although clear DNA fragments cannot be observed in agarose gel electrophoresis there is apparent decrease in band density in a concentration dependent manner (Figure V.13). Our results clearly show that there is a loss of intact genomic DNA. There are several reports indicating that DNA fragmentation is not absolutely necessary for apoptosis and in some cell types it is relatively difficult to observe DNA fragmentation[56].

Cytotoxicity studies of 4-HNE showed that it is much more potent than H2O2. Dose-response studies showed that 4-HNE caused 25% and 93 % viability loss at concentrations 25 and 50 μM respectively (Figure V.5) hence, concentrations in further studies were chosen accordingly. Florescent microscopy results showed that 4-HNE stimulates oxidative stress and production of ROS at concentrations as low as 5 μM (Figure V.7). At higher concentration (25 μM or higher) 4-HNE causes morphological deformations and cellular detachment though incubated for 4-5 hr (Figure V.12). Further support for 4-HNE induced endogenous ROS production has been provided by studying structurally two different antioxidants, namely α-tocopherol (Vitamin E) and resveratrol, a plant derived antioxidants. We observed that they both prevented 4-HNE-induced ROS production significantly especially at low concentrations (5-10 μM) of 4-HNE (Figure V. 8-10). The oxidative effects of 4-HNE are attributed to its property that results in depletion of intracellular glutathione (GSH) and in the formation of protein bound 4-HNE in the plasma membrane. In addition 4-HNE strongly induces intracellular peroxide production[35], therefore it can be envisaged that stress is followed by a genotoxic stress which inturn activates cellular signalling pathway(s) that ultimately leads to apoptosis.

Although, much has been published on the cytotoxic effects of reactive aldehydes and 4-HNE, the discovery that 4-HNE induces apoptosis is recent. It has been shown that while apoptosis can be caused by several lipid aldehydes, produced during lipid peroxidation, 4-HNE is the most potent inducer of apoptosis[57]. These pathophysiological effects of 4-HNE, such as apoptosis, are considered to be redox related since these effects are reversed by antioxidants[58]. Our results provide further evidence for the hypothesis. Hence, the identification of 4-HNE as an inducer of programmed cell death suggests a possible causative linkage between oxidant stress-induced lipid peroxidation and redox signalling or modulation of apoptosis. We have shown precisely that 4-HNE induces intracellular ROS production which inturn induces apoptosis. Our florescence microscopy and DNA fragmentation analysis results show that 4-HNE triggers apoptosis when incubated at 10 μM or higher concentrations. In order to detect apoptosis with florescence microscopy, several florescent probes such as acridine orange (AO), hoechst 33342 (HO), and propidium iodide (PI) have been used either alone or in combination. These florescent probes respectively show specificity for living, apoptotic and late apoptosis/necrosis states. AO enters only into viable cells and has green emission when bound to double stranded nucleic acids, and red signal when

intercalated into single-stranded nucleic acids. Hoechst dye is specific for the detection of Apoptosis. PI can enter only in late apoptotic and necrotic cultured cells, intercalating nucleic acids every 4–5 bp without sequence preference. We have used three of these probes together in order to differentially stain viable, apoptotic and late apoptotic/necrotic cells at the same time.

In order to understand whether there is any involvement of ROS production in the induction of apoptosis and DNA fragmentation, a well-known antioxidant, vitamin E has been used. It has been observed that vitamin E inhibits the DNA fragmentation, which also indicates that there is induction of apoptosis by 4-HNE (Figure V.14). This result suggests that the apoptotic pathway induced by 4-HNE is mediated through the production of ROS. As claimed by many authors, our data supports the idea that 4-HNE-induced apoptotic signalling events are through mitochondrial pathway, which is mediated by the production of ROS.

The oxidative and apoptotic effects of 4-HNE are attributed to several properties of this prominent reactive aldehyde.

First, it is very well established that 4-HNE results in depletion of intracellular glutathione (GSH), formation of protein bound 4-HNE in the plasma membrane and it strongly induces intracellular peroxide production[35]. This oxidative stress is most probably followed by a geno-toxic stress which inturn activates cellular signalling pathway that ultimately leads to apoptosis.

Secondly, being a strong electrophile, 4-HNE can react with many amino acids such as cysteine, lysine, and histidine in the structure of proteins. The extensive studies of the mechanism of modifications of these amino acids by 4-HNE established that 4-HNE primarily forms adduct having a hemiacetal structure via the Michael reaction. These adducts are not the initial product but the end product of serial reactions. In addition to this, it is known that 4-HNE impairs the function of many biologically important enzymes such as ATPase. One of the possible explanations for these adverse effects could be due to the presence of one of these amino acids in the enzymes active site. If 4-HNE binds to amino acids in the active site, it will block the active site for the coming substrate of that enzyme.

The third plausible explanation for oxidative and apoptotic effects of 4-HNE is that it could be modifying the mitochondrial membrane proteins leading to the changes in the mitochondrial membrane potential[59], which is considered to play very significant role during the initiation of apoptosis. Several authors have suggested that 4-HNE and related 4-hydroxy-2-alkenals of different chain length may act not only as toxic and mutagenic mediators of oxidative stress-related injury but also as biological signals such as chemotaxis, signal transduction, gene expression, cell proliferation and cell differentiation in normal and pathological conditions.

The major outcomes of the study can be summarised as;

- 4-HNE induces production of intracellular ROS in 3T3 fibroblast cell line at concentrations as low as 5μM and this effect can be overcome by structurally two different antioxidants, namely vitamin E and resveratrol.
- 4-HNE induces apoptosis at concentrations as low as 15-25 μM in our experimental system and this phenomena can also be inhibited by vitamin E as assessed by DNA fragmentation anlysis and differential fluorescent staining techniques.

In view of our findings the following points need to be clarified in order to better understand the underlying mechanism of 4-HNE signalling.

- The effects of 4-HNE on redox regulated gene expression. For this, time and concentration dependent effects of 4-HNE on the activation of redox regulated transcription factors NF-kappa B and AP-1 needs to be investigated.
- The molecular mechanism of 4-HNE induced apoptosis. For this purpose, the effects of 4-HNE and the production of ROS on the release of cytochrome c and caspase activation needs to be investigated.

By understanding the underlying molecular mechanisms for 4-HNE induced signalling leading to ROS production which in turn triggering apoptosis, better preventive and therapeutic strategies can be designed.

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