CYTOTOXICITY OF PEROXYNITRITE AND NITRIC OXIDE INDUCED OXIDATIVE STRESS ON 3T3 FIBROBLAST CELL LINE

by

Dilber Ece GAMSIZ

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

Spring 2003

...to my family and my fiancé

ACKNOWLEDGEMENTS

First, I would like to express my deepest appreciation to my research advisor Prof. Dr. Huveyda BASAGA for her guidance, support and endless encouragement during this study.

I am thankful to Assoc. Prof. Zehra Sayers and Yusuf Menceloglu for devoting their precious time in evaluating this work. Also I would like to thank to Dr. Alpay Taralp for his advice and support in this work.

Special thanks to my undergraduate advisor Prof. Dr. Guldem Ustun for encouraging me for a career in academic life.

I am thankful to Dr. Ozgur Kutuk for his guidance in the laboratory, time and patience throughout this study.

Special thanks to my roommate Evren Burcu Kivanc and Ozkan Ozturk for their support and comrade during these two years we spent together, day and night.

I would like to thank to Gozde Ozturk, Aylin Eksim, Mehmet Kayhan, M.A. Umut Soydaner and Sinan Cirpici for their help during writing my thesis. Also I would like to thank to all of my friends from Biological Sciences and Bioengineering program at Sabanci University, İstanbul-Turkey for their friendship and help.

To my parents and my sister.. From the bottom of my heart, I would like to express my special thanks to them for their encouragement and support during my MSc and all of my life.

Finally, I am grateful to my fiancé for everything....

ABSTRACT

In the present study, the oxidative and cytotoxic effects of two biologically important molecules, $NO^{\cdot}$ and $ONOO^{-}$ have been studied in 3T3 Fibroblast cell line. $NO^{\cdot}$ is a key molecule in many physiological pathways, but also its reactivity gives it the potential to cause considerable damage to cells and tissues. $NO^{\cdot}$ reacts rapidly with superoxide anion ($O_2^{\cdot -}$) to form $ONOO^{-}$ which is a powerful oxidant. Cell morphology and viability studies showed that both $NO^{\cdot}$ and $ONOO^{-}$ caused significant loss of viability in 3T3 fibroblast cell line. Intracellular ROS production has been monitored by a fluorescent probe, DCFH-DA. When cells were treated with of $NO^{\cdot}$, a significant increase in fluorescence intensity has been observed when compared to the control cells and this was supported with flourometric analysis results. Similar results have been observed when cells were incubated with $ONOO^{-}$ at the same concentration range. To further studies, cells were stained by a fluorescent probe Hoechst 33342 (HO) to identify apoptotic cells; both $NO^{\cdot}$ and $ONOO^{-}$ induced apoptosis.

In molecular studies, cells were incubated with the oxidant molecules, $NO^{\cdot}$ and $ONOO^{-}$ for different time periods and at all relevant doses. In all conditions, DNA remained intact; indicating that cytotoxic effect of $NO^{\cdot}$ and $ONOO^{-}$ were merely due to a mechanism other than apoptosis. This phenomena requires further mechanistic studies.

A potential antioxidant molecule, Catechin has been studied as a preventive molecule against cytotoxicity. In chemical model system Catechin was left to react with $ONOO^{-}$ and FT-IR and NMR analysis of the end product gave us preliminary information about its structure. In cellular studies, the effect of Catechin on $NO^{\cdot}$ and $ONOO^{-}$ induced cytotoxicity was investigated. The optimum determined conditions for preventive effect of Catechin were 5 μM of Catechin for 50 μM of $NO^{\cdot}$ and 200 μM of $ONOO^{-}$.

These results were discussed in the light of ROS induced cytotoxicity in cellular signaling mechanisms.

ÖZET

Bu çalışmada biyolojik olarak önem taşıyan iki molekül; $NO^{\cdot}$ ve $ONOO^{-}$ ‘nun 3T3 fibroblast hücreleri üzerindeki oksidasyon ve toksik ozellikleri incelenmiştir. $NO^{\cdot}$ pek çok fizyolojik olayda anahtar görevi taşimasinin yaninda, reaktif olmasi sebebiyle pek çok hücre ve dokuya zarar vermektedir. $NO^{\cdot}$ kisa bir surede $O_2^{\cdot -}$ ile reaksiyona girerek çok kuvvetli bir oksidan olan $ONOO^{-}$‘ yu oluşturur. Hücre morfolojisi ve canliliği ile ilgili bulgular $NO^{\cdot}$ ve $ONOO^{-}$‘nun 3T3 Fibroblast hücrelerine büyük oranda zarar verdiğini göstermiştir. Hucre içindeki reaktif oksijen türleri (ROT)’nin oluşumunu tesbit için DCFH-DA floresan boyasi kullanilmiştir. Hücreler $NO^{\cdot}$ ile inkübe edildiğinde floresan ısığın yoğunluğunun kontrole göre önemli miktarda arttığı saptanmıstır ve bu bulgu florometrik analizlerle de desteklenmiştir. Benzer sonuçlar hücrelerin aynı konsantrasyon aralığında $ONOO^{-}$ ile inkübasyonuyla da elde edilmiştir. Bulguları genişletmek amacıyla apoptotik hücrelerin görüntülenmesini sağlayan floresan boya Hoechst 33342 (HO) kullanılmış ve hem $NO^{\cdot}$ hem de $ONOO^{-}$‘nun apoptozize sebep olduğu saptanmıştır.

Molekuler çalışmalarda, hücreler oksidan moleküller $NO^{\cdot}$ ve $ONOO^{-}$ ile değisik zaman aralıklarında ve benzer dozlarda inkübe edilmiştir. Tüm şartlarda, DNA ‘nın parçalanmadan kalmış olması $NO^{\cdot}$ ve $ONOO^{-}$‘nun yol açtığı sitotoksik etkinin apoptozizden başka bir mekanizmaya dayandığını göstermektedir. Bu olay daha ileri mekanistik çalışmaları gerektirmektedir.

Potansiyel bir antioksidan molekül olan Catechin sitotoksiteye karşi koruyucu molekül olarak calışılmıştır. Kimyasal model sistemde Catechin $ONOO^{-}$ ile reaksiyona sokulmuş ve elde edilen ürünün FT-IR ve NMR analizleri bize ürünün yapısı hakkında ön bilgi vermiştir. Hücre çalışmalarında, Catechin’nin $NO^{\cdot}$ ve $ONOO^{-}$‘nin yol açtığı sitotoksite üzerindeki etkisi incelenmiştir. Catechin’nin koruyucu etkisini sağlayan optimum koşullar 5 μM Catechin, 50 μM $NO^{\cdot}$ ve 200 μM $ONOO^{-}$ içindir.

Elde edilen bulgular, ROT’un yol actığı sitotoksitenin sinyal iletisi mekanizmalari ışığında değerlendirilmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AO	Acridine Orange
AP-1	Activator protein 1
DCFH-DA	Dichloroflourescin diacetate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
FCS	Fetal calf serum
GSH	Glutathione peroxidase
HO	Hoeschst 33342
I-κB	Inhibitory κB
JNK	c-Jun amino terminal kinases
MAPK	Mitogen Activated Protein Kinases
NF-κB	Nuclear factor κB
$NO^{\cdot}$	Nitric Oxide
NOS	Nitric Oxide Synthase
$ONOO^{-}$	Peroxynitrite
PARP	Polo(ADPribose) polymerase
PI	Propidium Iodide
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
SOD	Superoxide Dismutase
TNF-α	Tumor necrosis factor α

CYTOTOXICITY OF PEROXYNITRITE AND NITRIC OXIDE INDUCED OXIDATIVE STRESS ON 3T3 FIBROBLAST CELL LINE

by

Dilber Ece GAMSIZ

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

Spring 2003

...to my family and my fiancé

ACKNOWLEDGEMENTS

First, I would like to express my deepest appreciation to my research advisor Prof. Dr. Huveyda BASAGA for her guidance, support and endless encouragement during this study.

I am thankful to Assoc. Prof. Zehra Sayers and Yusuf Menceloglu for devoting their precious time in evaluating this work. Also I would like to thank to Dr. Alpay Taralp for his advice and support in this work.

Special thanks to my undergraduate advisor Prof. Dr. Guldem Ustun for encouraging me for a career in academic life.

I am thankful to Dr. Ozgur Kutuk for his guidance in the laboratory, time and patience throughout this study.

Special thanks to my roommate Evren Burcu Kivanc and Ozkan Ozturk for their support and comrade during these two years we spent together, day and night.

I would like to thank to Gozde Ozturk, Aylin Eksim, Mehmet Kayhan, M.A. Umut Soydaner and Sinan Cirpici for their help during writing my thesis. Also I would like to thank to all of my friends from Biological Sciences and Bioengineering program at Sabanci University, İstanbul-Turkey for their friendship and help.

To my parents and my sister.. From the bottom of my heart, I would like to express my special thanks to them for their encouragement and support during my MSc and all of my life.

Finally, I am grateful to my fiancé for everything....

ABSTRACT

In the present study, the oxidative and cytotoxic effects of two biologically important molecules, $NO^{\cdot}$ and $ONOO^{-}$ have been studied in 3T3 Fibroblast cell line. $NO^{\cdot}$ is a key molecule in many physiological pathways, but also its reactivity gives it the potential to cause considerable damage to cells and tissues. $NO^{\cdot}$ reacts rapidly with superoxide anion ($O_2^{\cdot -}$) to form $ONOO^{-}$ which is a powerful oxidant. Cell morphology and viability studies showed that both $NO^{\cdot}$ and $ONOO^{-}$ caused significant loss of viability in 3T3 fibroblast cell line. Intracellular ROS production has been monitored by a fluorescent probe, DCFH-DA. When cells were treated with of $NO^{\cdot}$, a significant increase in fluorescence intensity has been observed when compared to the control cells and this was supported with flourometric analysis results. Similar results have been observed when cells were incubated with $ONOO^{-}$ at the same concentration range. To further studies, cells were stained by a fluorescent probe Hoechst 33342 (HO) to identify apoptotic cells; both $NO^{\cdot}$ and $ONOO^{-}$ induced apoptosis.

In molecular studies, cells were incubated with the oxidant molecules, $NO^{\cdot}$ and $ONOO^{-}$ for different time periods and at all relevant doses. In all conditions, DNA remained intact; indicating that cytotoxic effect of $NO^{\cdot}$ and $ONOO^{-}$ were merely due to a mechanism other than apoptosis. This phenomena requires further mechanistic studies.

A potential antioxidant molecule, Catechin has been studied as a preventive molecule against cytotoxicity. In chemical model system Catechin was left to react with $ONOO^{-}$ and FT-IR and NMR analysis of the end product gave us preliminary information about its structure. In cellular studies, the effect of Catechin on $NO^{\cdot}$ and $ONOO^{-}$ induced cytotoxicity was investigated. The optimum determined conditions for preventive effect of Catechin were 5 μM of Catechin for 50 μM of $NO^{\cdot}$ and 200 μM of $ONOO^{-}$.

These results were discussed in the light of ROS induced cytotoxicity in cellular signaling mechanisms.

ÖZET

Bu çalışmada biyolojik olarak önem taşıyan iki molekül; $NO^{\cdot}$ ve $ONOO^{-}$ ‘nun 3T3 fibroblast hücreleri üzerindeki oksidasyon ve toksik ozellikleri incelenmiştir. $NO^{\cdot}$ pek çok fizyolojik olayda anahtar görevi taşimasinin yaninda, reaktif olmasi sebebiyle pek çok hücre ve dokuya zarar vermektedir. $NO^{\cdot}$ kisa bir surede $O_2^{\cdot -}$ ile reaksiyona girerek çok kuvvetli bir oksidan olan $ONOO^{-}$‘ yu oluşturur. Hücre morfolojisi ve canliliği ile ilgili bulgular $NO^{\cdot}$ ve $ONOO^{-}$‘nun 3T3 Fibroblast hücrelerine büyük oranda zarar verdiğini göstermiştir. Hucre içindeki reaktif oksijen türleri (ROT)’nin oluşumunu tesbit için DCFH-DA floresan boyasi kullanilmiştir. Hücreler $NO^{\cdot}$ ile inkübe edildiğinde floresan ısığın yoğunluğunun kontrole göre önemli miktarda arttığı saptanmıstır ve bu bulgu florometrik analizlerle de desteklenmiştir. Benzer sonuçlar hücrelerin aynı konsantrasyon aralığında $ONOO^{-}$ ile inkübasyonuyla da elde edilmiştir. Bulguları genişletmek amacıyla apoptotik hücrelerin görüntülenmesini sağlayan floresan boya Hoechst 33342 (HO) kullanılmış ve hem $NO^{\cdot}$ hem de $ONOO^{-}$‘nun apoptozize sebep olduğu saptanmıştır.

Molekuler çalışmalarda, hücreler oksidan moleküller $NO^{\cdot}$ ve $ONOO^{-}$ ile değisik zaman aralıklarında ve benzer dozlarda inkübe edilmiştir. Tüm şartlarda, DNA ‘nın parçalanmadan kalmış olması $NO^{\cdot}$ ve $ONOO^{-}$‘nun yol açtığı sitotoksik etkinin apoptozizden başka bir mekanizmaya dayandığını göstermektedir. Bu olay daha ileri mekanistik çalışmaları gerektirmektedir.

Potansiyel bir antioksidan molekül olan Catechin sitotoksiteye karşi koruyucu molekül olarak calışılmıştır. Kimyasal model sistemde Catechin $ONOO^{-}$ ile reaksiyona sokulmuş ve elde edilen ürünün FT-IR ve NMR analizleri bize ürünün yapısı hakkında ön bilgi vermiştir. Hücre çalışmalarında, Catechin’nin $NO^{\cdot}$ ve $ONOO^{-}$‘nin yol açtığı sitotoksite üzerindeki etkisi incelenmiştir. Catechin’nin koruyucu etkisini sağlayan optimum koşullar 5 μM Catechin, 50 μM $NO^{\cdot}$ ve 200 μM $ONOO^{-}$ içindir.

Elde edilen bulgular, ROT’un yol actığı sitotoksitenin sinyal iletisi mekanizmalari ışığında değerlendirilmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AO	Acridine Orange
AP-1	Activator protein 1
DCFH-DA	Dichloroflourescin diacetate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
FCS	Fetal calf serum
GSH	Glutathione peroxidase
HO	Hoeschst 33342
I-κB	Inhibitory κB
JNK	c-Jun amino terminal kinases
MAPK	Mitogen Activated Protein Kinases
NF-κB	Nuclear factor κB
$NO^{\cdot}$	Nitric Oxide
NOS	Nitric Oxide Synthase
$ONOO^{-}$	Peroxynitrite
PARP	Polo(ADPribose) polymerase
PI	Propidium Iodide
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
SOD	Superoxide Dismutase
TNF-α	Tumor necrosis factor α

CHAPTER 1

INTRODUCTION

1.1. Nitric Oxide ($NO^{\cdot}$)

1.1.1. Chemistry of Nitric Oxide

Nitric Oxide ($NO^{\cdot}$) is a radical serving as a messenger binding to the heme of guanylate cyclase and indirectly as a cytotoxic agent. Although it is a radical, $NO^{\cdot}$, which has a solubility of 1.55 mM at physiological ionic strength and temperature, is relatively stable. It is known that NO. reacts with nitrite (NO^{-}) to form dioxodinitrite (1-) ($N_2O_2^{\cdot -}$), even trioxotrinitrite (1-) $N_3O_3^{\cdot -}$ [2].

$$NO^{\cdot} + NO^{-} \Longleftrightarrow N_2O_2^{\cdot -} \qquad (1)$$

$$k_f = 1.7 \times 10^{9}\ M^{-1}s^{-1}$$

$$k_r = 6.6 \times 10^{4} M^{-1}s^{-1}$$

$$N_2O_2^{\cdot -} + NO^{\cdot} \longrightarrow N_3O_3^{\cdot -} \qquad (2)$$

$$k = 3.0 \times 10^{6}\ M^{-1}\ s^{-1}$$

$$N_3O_3^{\cdot -} \longrightarrow N_2O + NO_2^{-} \qquad (3)$$

$$k = 2.35 \times 10^{2}\ s^{-1}$$

Besides these reactions, another important reaction of $NO^{\cdot}$ is formation of ONOO- [2]:

$$NO^{\cdot} + O_2^{\cdot -} \longrightarrow ONOO^- \quad (4)$$

Chemistry of ONOO- is described in detail in Chapter 1.2.1.

The reaction between NO. and thiols to form nitrosothiols, which is very important in biological systems, requires an electron acceptor [2].

$$RSH + NO^{\cdot} + O_2 \longrightarrow RSNO + O_2^{\cdot -} + H^+ \quad (5)$$

Although superoxide formed in this reaction disappears very rapidly, there is a possibility for the formation of ONOO- [2].

In some cases nitrosothiols act like NO. or store NO.:

$$RSNO(aq) \longrightarrow RSNO(g) \quad (6)$$

$$RSNO\ (g) \longrightarrow RS^{\cdot}\ (g) + NO^{\cdot}(g) \quad (7)$$

$$RS^{\cdot}(g) + NO^{\cdot} \longrightarrow RS^{\cdot}\ (aq) + NO^{\cdot}(aq) \quad (8)$$

$$RS^{\cdot}(aq) + e^- + H^+ \longrightarrow RSH\ (aq) \quad (9)$$

$$RSNO(aq) + e^- + H^+ \longrightarrow RSH(aq) + NO^{\cdot}\ (aq) \quad (10)$$

One of the facile reactions of $NO^{\cdot}$ in biological systems is that of $NO^{\cdot}$ reacting with metalloproteins such as guanylate cyclase, cytochrome P450 and NOS containing heme moieties in which a ferrous-nitrosyl complex is formed [15]:

$$Fe_{aq}(II) + NO \longrightarrow Fe_{aq}(II)\text{-}NO \quad (11)$$

1.1.2. Role of Nitric Oxide in Biological Systems

Nitric Oxide ($NO^{\cdot}$), which is a unique diffusible molecular messenger in the vascular and immune system, is catalytically formed by means of NO-synthase (NOS) isoforms during the conversion of L-arginine to citrulline in biological systems. The reaction depends on the presence of NADH and O_2 as co substrates with other redox cofactors such as enzyme bound heme, reduced thiols, FAD, FMN and tetrahydrobiopterin [16].

$$\text{L-Arginine} \xrightarrow{\text{NOS}} \text{Citrulline} + NO\cdot \qquad (12)$$

The first NO-synthase was observed in the $NO^{\cdot}$ synthesis of inflammatory cells as immunoactivation, therefore the corresponding enzyme was called as inducible NO synthase (iNOS, now known as NOS2). The iNOS was different from a constitutive NO synthase activity, which was expressed as cNOS in characteristic cell, types such as neuronal or endothelial cells. These NOS isoforms were then termed as nNOS (NOS1) and eNOS (NOS3). It is known that for full enzymatic activity of NOS isoforms intracellular calcium level is important. Although eNOS and nNOS are affected by changes in calcium level, iNOS seems to become activated even at low calcium levels. Beside calcium level, intracellular localization of the isoforms and phosphorylation are other effective factors to modulate enzyme activity [16].

The most important role of $NO^{\cdot}$ in vivo is the activation of soluble guanylate cyclase, which mediates the neurotransmission and vasodilatation actions of the molecule. $NO^{\cdot}$ binds to the sixth coordination position of the enzyme's iron protoporphyrin IX group to form a nitrosyl-heme. Stimulation of guanylate cyclase causes the synthesis of the biologically important second messenger, cGMP and subsequent activation of cGMP-dependent kinases in responder cells. Even nanomolar concentrations of $NO^{\cdot}$ are enough for the activation of guanylate cyclase leading to the increase in the level of cGMP, which

lowers intracellular free Ca^{2+} and relaxes the muscle, dilating the vessel and lowering blood pressure [17].

The high concentrations of $NO^{\cdot}$ generated by activated macrophages are known to be cytotoxic in the defense against tumor cells and pathogens. The ability of macrophages to kill tumor cells and bacteria by $NO^{\cdot}$ synthesis was noticed in 1987. After this finding, it was indicated that NO. damages naked DNA and induces oxidative DNA damage in activated macrophages. DNA damage involves attachment of poly(ADPribose) polymerase (PARP) to the strand breaks and synthesis of short-lived polymers by the bound enzyme. Although PARP has no direct effect on DNA excision repair, the enzyme binds to DNA strand breaks and in some instances interferes with repair if poly(ADP-ribose) synthesis is prevented. Extensive DNA damage via PARP activation leads to NAD^{+}, the ADP-ribose donor depletion and in order to resynthesize NAD^{+}, ATP becomes depleted, then this leads to cell death due to energy deprivation. In this case, energy depletion is found to be related with neurotoxicity and islet cell death [16].

One of the cytotoxic targets of $NO^{\cdot}$ is known to be mitochondrion in which $NO^{\cdot}$ induced formation of dinitrosyl adducts of aconitase cause the inhibition of mitochondrial activity leading to cytotoxicity. Also it is indicated that $NO^{\cdot}$ derived from S-nitroglutathione (GSNO) inhibits respiration. Besides these, $NO^{\cdot}$ is known to interact with cytochrome c oxidase that is a heme protein forming a Fe-NO adducts to reversibly inhibit respiration. However, it is found that under inflammatory conditions, complex I (NADH:ubiquinone oxidoreductase) and complex II (succinate:ubiquinone oxidoreductase) are irreversibly inhibited by $NO^{\cdot}$ [15].

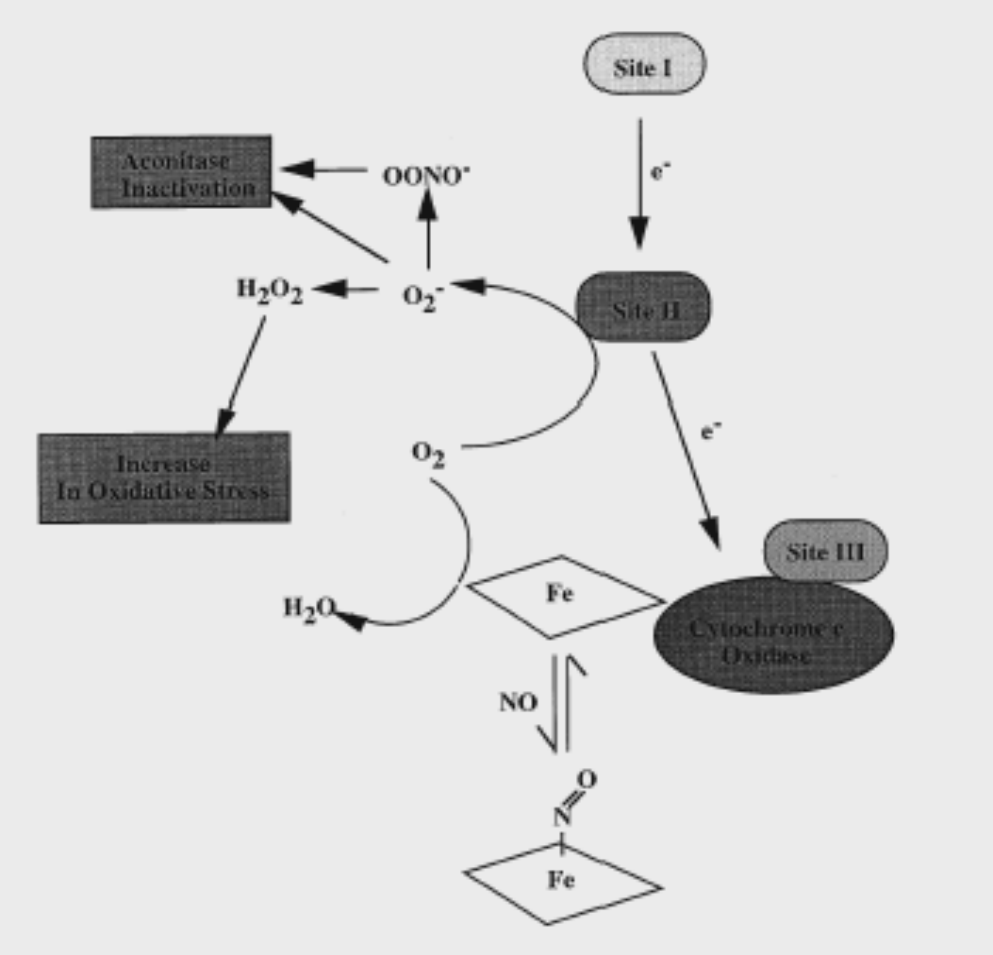

Figure 1.1: $NO^{\cdot}$ and mitochondrial function [15]

Beside direct damage of $NO^{\cdot}$, formation of N_2O_3 (Reaction 13-14) may lead to damage to DNA. N_2O_3 causes damage to DNA by nitrosation of primary amines on DNA bases leading to deamination. Adenin , cytosine, 5-methylcytosine and guanine can also be deaminated to form hypoxanthine, uracil, thymine and xanthine respectively. Xanthine which is formed by deamination of guanine, is unstable in DNA and can depurinate readily leaving an abasic site leading to G:C⟶A:T transversion mutation. Moreover, the abasic site may be cleaved by endonucleases forming single-strand breaks [17].

$$2NO^{\cdot} + O_2 \longleftrightarrow 2NO_2^{\cdot} \quad (13)$$

$$NO_2^{\cdot} + NO^{\cdot} \longleftrightarrow N_2O_3 \quad (14)$$

Besides its cytotoxic effects, $NO^{\cdot}$ has roles in prevention of lipid peroxidation chain reactions. Lipid peroxidation results in the formation of alkoxy and peroxy radicals, which react with $NO^{\cdot}$. Reaction 13 is very important in termination of lipid peroxidation protecting the cells from peroxide induced cytotoxicity.

$$LOO^{\cdot} + NO^{\cdot} \longrightarrow LOONO \qquad (15)$$

Exposure of cells to copper, xanthine oxidase or azo-bis-amidinopropane results in lipid peroxidation, which is mostly terminated by $NO^{\cdot}$. Also it is known that termination of lipid peroxidation protects endothelial and macrophage cells against oxidation of low-density lipoproteins. Lipid peroxidation is one of the important aspects in pathogenesis of cardiovascular diseases like atherosclerosis , thus limiting the lipid peroxidation by $NO^{\cdot}$ is very important [15].

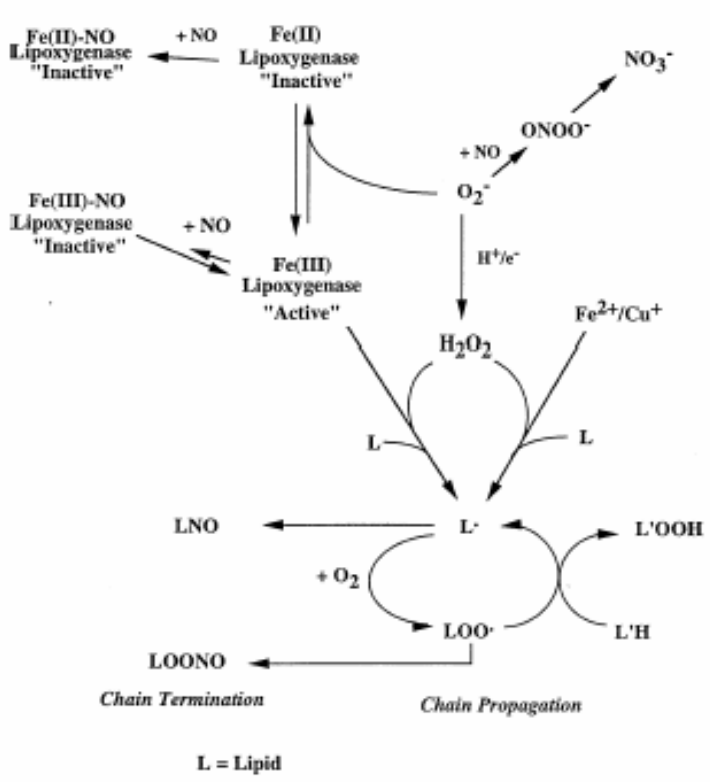

Figure 1.2: Mechanism of termination of lipid peroxidation by $NO^{\cdot}$ [15].

In order to study the effects of $NO^{\cdot}$ on different cell types, $NO^{\cdot}$ releasing compounds, which are generally nitrovasodilators or $NO^{\cdot}$ donors preserving $NO^{\cdot}$ in their molecular structure, are used [16]. Metal nitrosyl complexes are commonly used $NO^{\cdot}$ donors such as sodium nitroprusside (SNP) which has the structure of $Na_2Fe(CN)_5NO$. Reaction 14 indicates the conversion of SNP to $NO^{\cdot}$.

$$[Fe(CN)_5NO]^{2-} \xrightarrow{e^-} [Fe(CN)_5]^{3-} + NO^{\cdot} \qquad (16)$$

Another metal nitrosyl complex used as $NO^{\cdot}$ donor is ruthenium nitrosylpentachloride (RNP) which is $K_2Ru(Cl)_3NO$. S-Nitrothiols are other NO. donors such as nitrocysteine (GSNO) and S-nitroso-N-acetyl-DL-penicillamine (SNAP) which are generated by the reaction of RNS with thiols. SIN-1, organic nitrates and nitrites, NONOates containing $[N(O)NO]^-$ functional groups are the examples of NO. donors [19].

Type of Compound	**Examples**
Metal nitrosyl complexes	Sodium Nitroprusside (SNP) Ruthenium nitrosylpenrachloride
S-Nitrosothiols	Nitrocysteine (GSNO) S-Nitroso-N-acetyl-DL-penicillamine (SNAP)
SIN-1	
Organic nitrates and nitrites	Nitroglycerine (glycerol trinitrate ester) amyl nitrite
NONOates	Spermine:$H_2N(CH_2)_3N^+H_2(CH_2)_4$

Table 1.1: Compounds used as $NO^{\cdot}$ donors.

1.1.3. Nitric Oxide in Cell Signalling

Oxidative and nitrosative stress is known to be associated with the pathogenesis of most of the diseases and aging in which mitogen-activated protein kinase (MAPK) signaling pathways are implicated. MAP kinase signaling pathways transduce extracellular and intracellular stimuli into cellular responses, which consist of phosphorylation of cytosolic or nuclear target proteins and activation of transcription factors modulating gene expression. ERK ½, c-Jun amino terminal kinases (JNK 1/2/3) and p38 kinases are the different regulated groups [24].

Active MAPKs function as modulators for differentiation, proliferation, cell death and survival. Although the activation of ERK 1/2 is implicated for cell survival, JNK and p39 has been associated with cell death. Activation of ERK 1/2 can activate transcription factors and phosphorylate specific kinases such as MAPK-activated protein kinases (MAPKAPKs) like the mitogen and stress activated kinase-1 (MSK1) or the pp90 ribosomal S6 kinase (RSK), which phosphorylates the Bcl-2 family member Bad inhibiting its pro-apoptotic activity [21]. RSK and MSK1 are activators of the camp response element binding protein (CREB) which is transcription factor of Bcl-2 therefore it is important for cell survival [25]. Also, activation of Ras, which is an initiator of ERK 1/2 signaling cascade was found to activate phosphoinositol 3-kinase (PI3-kinase)/Akt pathway, another important factor for cell survival [21].

Active JNKs have various potential phosphorylation targets in both nucleus and cytoplasm. Nuclear substrates of JNKs are the transcription factors such as c-Jun, which is part of the activator protein-1 (AP-1), ATF-2 and ELK-1. Cytosolic substrates for JNKs include cytoskeletal proteins, the tumor suppressor protein p53, glucocorticoid receptors, mitochondria associated anti-apoptotic proteins; Bcl-2 and Bcl-X_L. Also it is known that JNK mediates the release of cytochrome c and other apoptotic factors such as DIABLO/smac from the mitochondria. Caspases are the major executors of apoptosis and it is indicated that the release of cytochrome c promotes the formation of the apoptosome

leading to the activation of caspase-3 (Figure 1.3). It is becoming clear that JNK activation involves in apoptotic processes in vivo and in vitro [21].

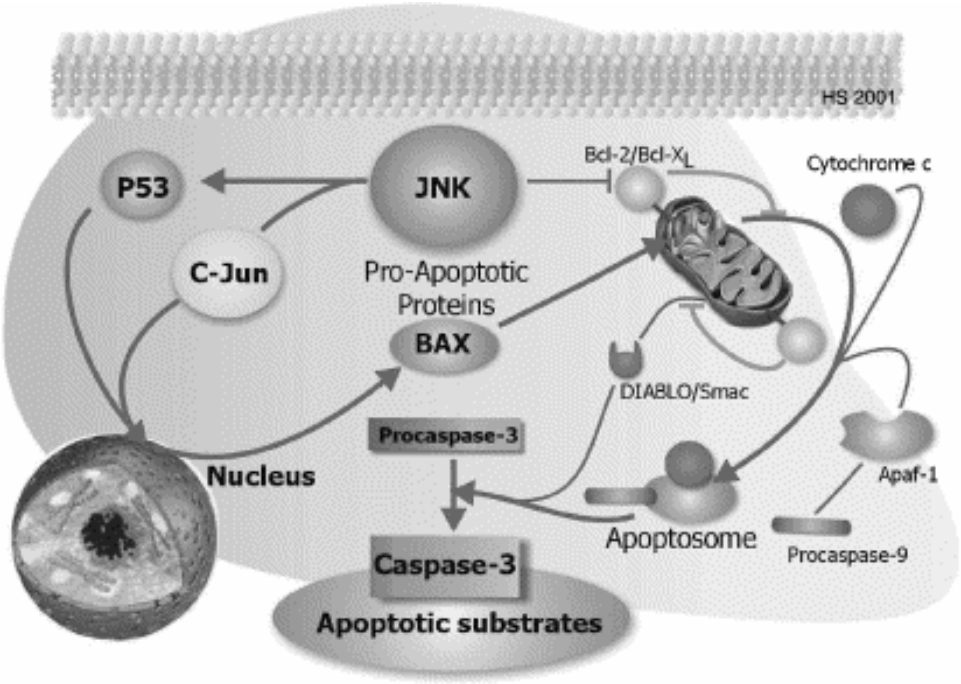

Figure 1.3: Apoptotic pathway in which JNK is involved [21].

The role of $NO^{\cdot}$, which is a activator of guanylate cyclase via binding to the heme group leading to increase in cGMP levels and cGMP-dependent kinases or phosphotases, was firstly identified in the vascular system by the regulation of smooth muscle contraction. NO., which is a neuronal messenger in neuronal cells, promotes Ca^{+2}-dependent neurotransmitter release. Beside its positive effects, $NO^{\cdot}$ is implicated in the pathogenesis of neurodegenerative diseases via neuronal apoptosis when NO. is generated in toxic levels in the cell. Overload of Ca^{+2} in neurons via over-stimulation of NMDA receptor by glutamate leads to activation of Ca^{+2}/Calmodulin-dependent NO synthase (neuronal NOS) resulting in the high intracellular concentration of $NO^{\cdot}$ [21].

$NO^{\cdot}$ has been implicated in the mechanisms protecting against stress-induced cell injury such as the activation of Ras, which is an intermediate in the transduction of signals from membrane receptor tyrosine kinases to MAP kinases, by S-nitrosation of a cystein residue [26]. It was proposed that by Ras activation, ERK 1/2 is activated leading to inactivation of pro-apoptotic Bad protein. Also the activation of Ras may activate the anti-apoptotic protein Bcl-2 via activation if CREB. Beside these, it was indicated that pro-apoptotic protein Bax is inactivated by the activation of Ras via PI3/Akt pathway [27]. Pro-apoptotic JNK is known to be suppressed by $NO^{\cdot}$ via S-nitrosation leading to the inactivation of c-Jun (Figure 1.4) [21].

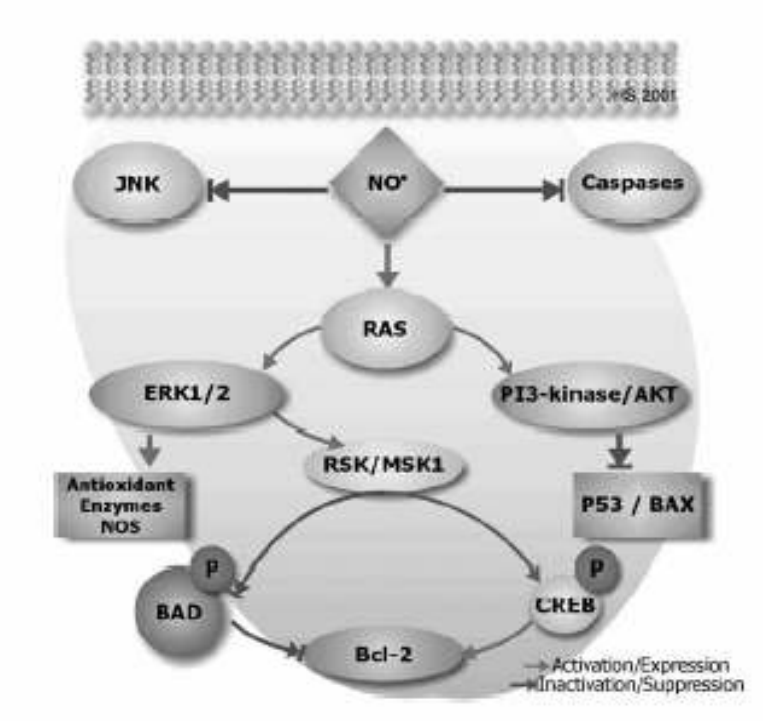

Figure 1.4: Anti-apoptotic pathway mediated by $NO^{\cdot}$ [21].

The tumor suppressor gene p53, which is an important member of DNA-damage response pathway, is able to induce growth arrest or apoptosis in DNA-damaged cells. P53 induces G1 arrest via sequence specific DNA binding and transcriptional activation of target genes such as p21 which is inhibitor of cyclin dependent kinases and blocks cell cycle progression [16]. In a study on RAW 264.7 macrophages, it was indicated that activation of iNOS resulted in the accumulation of NO. and caused p53 accumulation leading to DNA fragmentation [32]. It was also shown that when cells are treated with $NO^{\cdot}$

donors, the level of p53 increased in response. Furthermore, the removal of $NO^{\cdot}$ was observed to cause p53 to decline with a small percentage of cells entering apoptosis [34].

Bcl-2 protein, which is responsible for the protection of cells in the apoptotic pathway, has been found to decrease in the cases of $NO^{\cdot}$ mediated apoptosis. However, it is not known if it is obligatory for initiation of apoptosis. Also it was indicated that upregulation of proapoptotic protein Bax is associated with $NO^{\cdot}$ mediated apoptosis [16]

The diverse properties of $NO^{\cdot}$ are commonly used in drug discovery and development in the diseases of sexual dysfunction, cardiovascular and inflammation. There are three therapeutic approaches for $NO^{\cdot}$ augmentation therapy to drug development. The first one is to potentiate a $NO^{\cdot}$-based regulatory pathway, which is used in the therapy of male erectile dysfunction. The second one is supplementation of $NO^{\cdot}$ in a situation where a functional or quantitative $NO^{\cdot}$ insufficiency may underlie the pathology. The last one is to utilize $NO^{\cdot}$ to improve drug safety and efficacy such as in nonsteroidal anti-inflammatory drug [18].

1.2. Peroxynitrite ($ONOO^-$)

1.2.1. Chemistry of Peroxynitrite

Peroxynitrite is the product of the reaction between $NO^{\cdot}$ and superoxide ($O_2^{\cdot -}$), which is a radical anion formed by reduction of oxygen molecule (O_2) by one electron [1].

$$NO^{\cdot} + O_2^{\cdot -} \longrightarrow ONOO^- \quad K_f = 6.7 \text{ X } 10^9 \text{ } M^{-1}s^{-1} \qquad (17)$$

Superoxide is dismutated by the enzyme, Superoxide dismutase (SOD) to oxygen and hydrogen peroxide (H_2O_2) containing copper ions in its active site, which reacts with superoxide at a fast rate ($k = 2 \text{ X } 10^9 \text{ } M^{-1}s^{-1}$). SOD is present in the cell at concentrations about 10 μM. When the concentrations of $NO^{\cdot}$ approach SOD, they compete with each other in many pathological circumstances. The yield of the reaction between $NO^{\cdot}$ and superoxide depends on the competition between SOD and $NO^{\cdot}$ for superoxide [1].

$$Cu^{+2}\text{-SOD} + O_2^{\cdot -} \longrightarrow Cu^{+1}\text{-SOD} + O_2 \quad k_d = 2 \text{ X } 10^9 \text{ } M^{-1}s^{-1} \qquad (18)$$

One electron oxidation/reduction potential of $ONOO^-$ [$E°(ONOO^-, 2H^+/NO_2^{\cdot}, H_2O)$ = 1.6V at pH =7] shows that it is a highly oxidizing species. It is disprotonated to nitrogen dioxide and nitrosyldioxyl radical, $ONOO^-$ as it is unstable. Peroxynitrous acid, ONOOH has a pK_a value of 6.8. Protonation of $ONOO^-$ to ONOOH allows isomerization to cis or trans form [2].

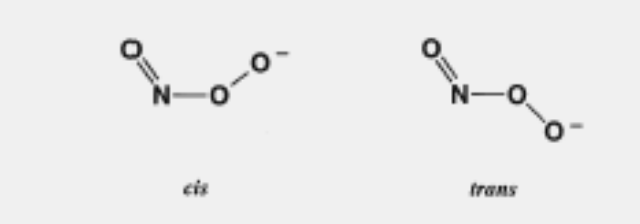

Figure 1.5: Cis and trans forms of ONOO- [2].

Cis-peroynitrite is known to react directly with thiols. The major low molecular weight thiol is GSH which is very important in several cellular processes like maintenance of hydrogen sulfhydryls and the removal of hyroperoxides. Depletion of GSH is known to be toxic due to these important cellular processes. In addition to GSH, $ONOO^-$ also reacts with proteins containing cysteine residues [1].

Nitration of phenolic compounds like tyrosine is another important reaction of ONOO- in which 3-nitrotyrosine is the most often observed product (Figure 1.6). Nitrated proteins are seen in various diseases and pathological conditions, including atherosclerosis, rheumatoid arthritis, septic lung, heart and ischemic brain [1]. Also the nitrated protein may be involved in signal transduction and nitration of tyrosine can modulate phosphorylation then unregulate the signalling pathway [3].Thyrosine phosphorylation of the key enzymes is a nearly ubiquitous mechanism for mediating internal signaling of cells [22]. In a study done on human ductal adenocarcinoma cells, it was shown that the nonreceptor c-SRC tyrosine kinases are tyrosine nitrated and phosphorylated. Also it was found that $ONOO^-$ increased c-Src activity indicating that $ONOO^-$-mediated modification of c-Src may play an important role in the pathogenesis of the disease [23].

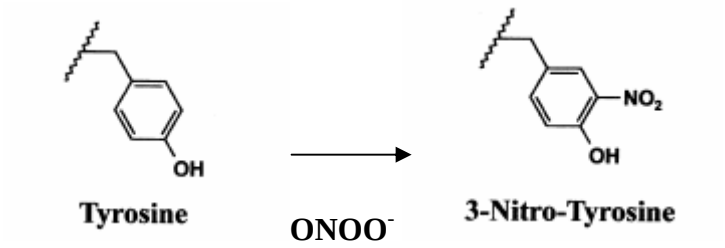

Figure 1.6: Nitration of tyrosine [13].

$ONOO^-$ is also known to react with CO_2. The reaction between CO_2 and $ONOO^-$ in vivo is mediated by ONOO--bicarbonate intermediates. The high reactivity of $ONOO^-$ with aqueous CO_2 (pH-independent $k = 5.8 \times 10^4\ M^{-1}s^{-1}$) and the high biological concentration of CO_2 (about 1mM CO_2) make the reaction between $ONOO^-$ and CO_2 more frequent [3].

$$ONOO^- + CO_2 \longrightarrow ONOOCO_2^- \qquad (19)$$

The most important effect of the reaction between $ONOO^-$ and CO_2 is the protection of the potent targets of $ONOO^-$ like tyrosine. The $ONOOCO_2^-$ adduct is very unstable with a half-life of 3 ms and reacts with tyrosine easily (2×10^5 $M^{-1}s^{-1}$). Since the concentration of CO_2 is very high in vivo, formation of $ONOOCO_2^-$ is a potent protection against $ONOO^-$ reactions [1].

Transition metals are known to catalyze the reaction between tyrosine and $ONOO^-$. Low molecular weight complexes such as ferric-EDTA form a nitronium ion by reacting with $ONOO^-$. Beside this, $ONOO^-$ is known to react with metalloproteins like hemeproteins, iron-sulfur proteins and copper proteins. The reaction of $ONOO^-$ bovine-Cu,Zn SOD is one example of these reactions. The end product of the reaction between bovine-Cu,Zn SOD and $ONOO^-$ is identified to be a nitrated tyrosine residue of the protein [1].

Mitochondrion is known to be a primary source for $ONOO^-$ formation and reactions. It is known that $ONOO^-$ can diffuse from the extramitochondrial compartments into the mitochondria or it is formed intramitochondrially then reacts with various constituents. $ONOO^-$ reactions in mitochondria were firstly proposed by the studies on $NO^\cdot$. As $NO^\cdot$ is not capable of reacting directly with mitochondrial components, $NO^\cdot$-derived secondary species were suggested to be active in mitochondria, such as $ONOO^-$. In addition to this, $NO^\cdot$ can easily diffuse into mitochondria which is a source of superoxide anion ($O_2^{\cdot-}$) [5].

The biological half-life of $ONOO^-$ in extramitochondrial compartments is approximately 10 ms. The exact mechanism of extramitochondrial $ONOO^-$ diffusion into intramitochondrial compartments is not elucidated yet. Half-life of intramitochondrial ONOO- is suggested to be very short (3-5 ms) because of metalloproteins, thiols and CO_2. Although $NO^\cdot$ is a hydrophobic and diffusible compound , diffusion of $O_2^{\cdot-}$ is restricted by mitochondrial SODs. Therefore, $ONOO^-$ formation in intramitochondrial compartments indicate $O_2^{\cdot-}$ formation [5].

In various cell types, formation and reactions of mitochondrial $ONOO^-$ was investigated. In a study on PC-12 cell line, it was shown that the cells which were resistant to $NO^\cdot$ induced cell death over expressed Mn-SOD. As it was indicated before, formation of $ONOO^-$ is inhibited by Mn-SOD as it dismutases superoxide anion as a result of this $ONOO^-$ formation leading to cell death is prevented. This is the evidence of $ONOO^-$ formation in PC-12 cell line [6]. Beside this, in animal and human tissues during chronic and acute inflammation the nitration of Mn-SOD has been detected. This shows the relevance between inflammation and $ONOO^-$ formation. There are many evidences for formation and reactions of $ONOO^-$ in mitochondria [5].

As it was mentioned before, $ONOO^-$ is cytotoxic in many cell types and scavenging of $ONOO^-$ by certain compounds is needed. There are two important compounds, which are capable of scavenging $ONOO^-$ at micromolar concentrations, ebselen ($k = 2X\ 10^6\ M^{-1}s^{-1}$ at 25°C) and 5,10,15,20-tetrakis (N-methyl-4'-pyridyl) porphinatoiron, which is an iron (III) porphyrin ($k = 2.2X10^6M^{-1}s^{-1}$ at 37°C) [2].

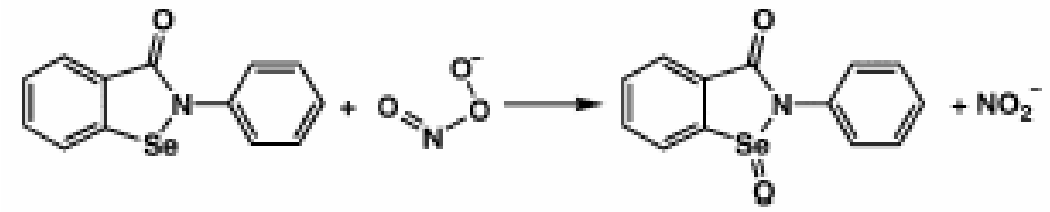

Figure 1.7: The reaction between Ebselen and ONOO- [2]

Pelargonidin is another compound known to be able to scavenge $ONOO^-$. In a chemical model system Pelargonidin reacted with $ONOO^-$ and the end product was detected by high performance liquid chromatography (HPLC) and a reaction mechanism was proposed. The reaction products were identified as p-hydroxybenzoic acid and 4-hydroxy-3-nitrobenzoic acid indicating the peroynitrite scavenging effect of pelargonidin (Figure 1.8) [7].

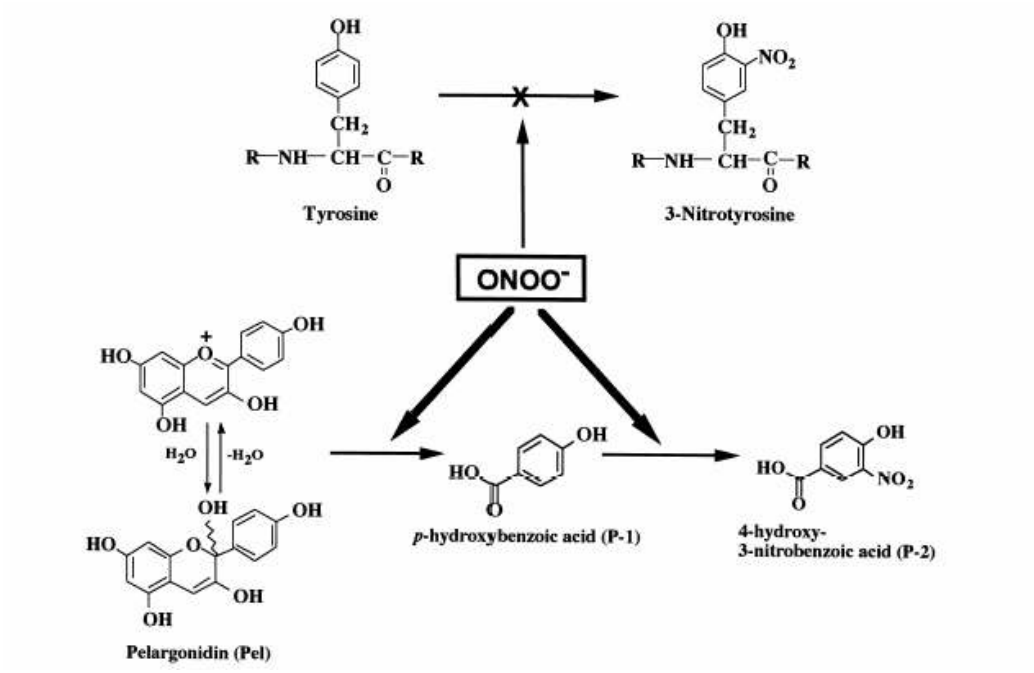

Figure 1.8: Proposed mechanism for $ONOO^-$ scavenging effect of Pelargonidin [7]

1.2.2. Role of Peroxynitrite in Biological Systems

Although $ONOO^-$ is not a free radical by chemical nature, it is a powerful oxidant initiating lipid peroxidation, causing DNA breakages, reacting with aminoacids and causing protein modifications. When $ONOO^-$-induced cell damage reaches a level that cannot be repaired, cells undergo apoptosis or necrosis. Apoptosis, programmed cell death is defined by certain parameters like cell morphology, plasma membrane integrity, mitochondrial polarization, activation of caspases and DNA fragmentation [8]. Necrosis occurs as a result of catastrophic toxic or traumatic events leading to passive cell swelling, injury to cytoplasmic organelles, membrane lysis, release of cellular contents and inflammation. The main difference between apoptosis and necrosis is that in necrosis cellular organelles are released out of the cell and this causes proinflammation in neighbouring cells. In apoptotic cells, the organelles are maintained inside the cell as the membrane is preserved and the neighbouring cell is not affected [10].

$ONOO^-$ induced apoptosis was firstly identified in thymocytes and HL-60 cells in 1995. These reports were followed by other studies on $ONOO^-$ mediated oxidative damage in different cell types like lymphoblastoid cells, human aortic endothelial cells, HaCaT keratinocytes, cardiac myocytes and human islet cells [8].

Although it is known that $ONOO^-$ induces apoptosis in various cell types, the mechanism is not quiet clear yet. In a study done on HL-60 cells, it was shown that reactive oxygen species participate in $ONOO^-$ induced apoptosis. Treatment of HL-60 cells with $ONOO^-$ resulted in ROS production and $O_2^{\cdot -}$ generation in a dose-dependent manner. It was proposed that ROS formation might cause secondary antioxidant –depletion oxidative stress leading to oxidative damage and apoptosis [9].

Mitochondria are important sites for $ONOO^-$ induced apoptosis initiation. Characteristic indicators of apoptosis are opening of mitochondrial permeability transition pore, mitochondrial depolarization and secondary superoxide production. Some of the mitochondria-derived apoptogenic factors are also known to act as nucleases, nuclease activators (e.g.cytochrome c) or serine proteases. Also it is known that $ONOO^-$ inhibits mitochondrial respiratory chain by inactivating complexes I-III [8].

Members of Bcl-2 family are implicated as the regulators of apoptosis. The Bcl-2 family involves pro-apoptotic (bax, bak, bad, bik) and antiapoptotic (Bcl-2, Bcl-x_L, mcl-1) molecules. The ratio of pro-apoptotic and antiapoptotic Bcl proteins is thought to indicate the fate of the cell. The Bcl-2 family members are not only located in mitochondria, but also in nuclear membrane and in the endoplasmic reticulum. In a study done on thymocytes, it was observed that anti-apoptotic Bcl-2 protein is capable of inhibiting $ONOO^-$ induced apoptosis. It was proposed that Bcl-2 reduced the permeability transition then prevents cytochrome c release, caspase activation and most importantly DNA fragmentation [11].

It has been already shown that 3-nitrotyrosine, which is the product of the reaction between $ONOO^-$ and tyrosine, is very important in $ONOO^-$ induced apoptosis. It was indicated that nitrotyrosine induces DNA damage leading to apoptosis in endothelial cells [8].

Although there are proposed mechanisms for $ONOO^-$ induced apoptosis, the exact mechanism is not known yet. The executional phase of apoptosis is known to be carried out by caspases, which are also active in $ONOO^-$ induced apoptosis. Studies showed that caspase-3 like proteases, caspase 2 are required for $ONOO^-$ induced apoptosis [8].

Beside apoptosis, it is known that $ONOO^-$ induces necrosis in different cell types. $ONOO^-$ induced cell death was investigated in Calu-1 cells, which are human lung epithelial cells and it was observed that $ONOO^-$ triggers necrosis in the cells [10]. Also $ONOO^-$ induced cell death in thymocytes was indicated to be necrosis rather than apoptosis. In this study, a paradigm was occurred identifying oxidative stress-induced necrosis. According to this concept, activation of poly (ADP-ribose) synthase (PARS) (also called poly(ADP-ribose) polymerase, PARP) mediates oxidative stress-induced cell death. Active PARS cleaves NAD to nicotinamide and ADP-ribose and catalyses the addition of $(ADP\text{-}ribose)_n$ adducts to proteins, including PARS. Excessive PARS activation exhausts cellular NAD and ATP pools leading to necrotic cell death. Inhibition of PARS prevents loss of energy in the cell and allows the cell to undergo apoptosis. Inhibition of PARS is found to be very useful in various disease models. [11].

1.2.3. Peroxynitrite in Cell Signalling

Exposure of cells to $ONOO^-$ results in the induction of stress genes such as c-fos, heme oxygenase-1 or the growth arrest and DNA damage-inducible (Gadd) proteins 34, 45, 153 and apoptosis that is linked with MAPK activation [12].

The Mitogen-activated protein kinase (MAPK) pathways are activated by various stimuli including oxidative stress. MAPKs are Ser/Thr kinases which phosphorylate their substrates on Ser or Thr residues. The MAPK subfamilies are activated by MKK and in turn phosphorylated by MKK Kinases (MKKKs). Transcription factors like Elk1, Sap, c-Jun or ATF2 are the substrates of MAPKs. The leucine-zipper transcription factor AP-1 is another important substrate of MAPKs. The stress activated protein kinases consisting of p38, c-Jun-N-Terminal kinases (JNK) and the extracellular signal regulated kinases (ERK1/2) are other MAPK subgroups activated by mitogenic stimuli [12].

$ONOO^-$ is indicated to cause activation of all three MAPK family members, p38, JNK and ERK1/2 in various cell types. Studies showed that JNK and p38 play a role in protecting cells against apoptosis, but ERK1/2 is identified as a prerequisite for apoptosis. ERKs are activated via Epidermal growth factor receptor (EGFR),a receptor tyrosine kinase followed by the activation of downstream molecules such as Ras, Raf and MKK 1/2 which are the upstream of ERKs. Beside this, it is known that there is a pathway for activation of ERKs which is independent from EGFR and Raf, but MKK ½ [12]. There is also another EGFR and MKK ½ independent pathway, which is dependent on Ca^{+2}-dependent PKC-isoform [20]. Also further activation of ERK 1/2 results in the activation of transcription factor activator protein-1 (AP-1) (Figure 1.9) [12].

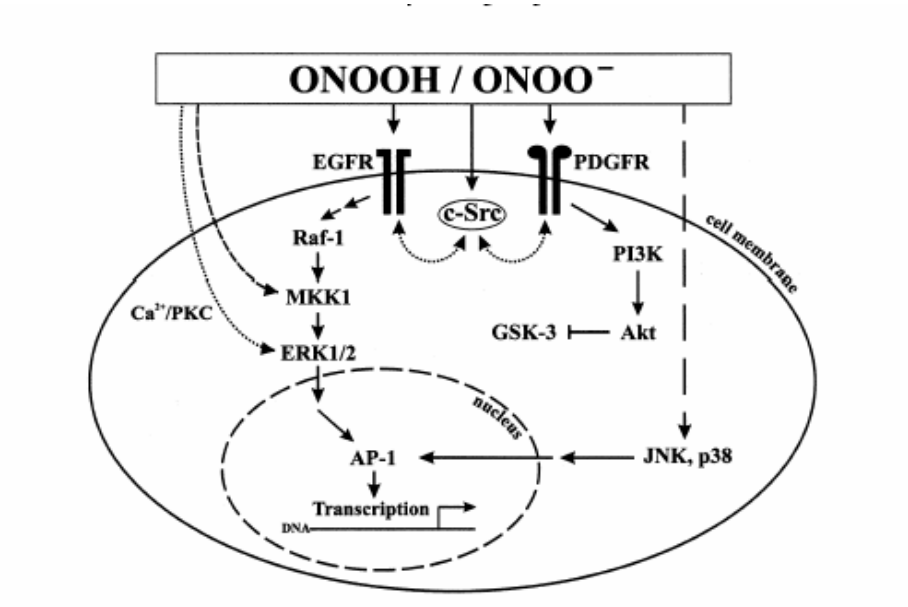

Figure 1.9: ONOO- signaling [12]

In a study on human skin fibroblasts, it was shown that exposure of cells to $ONOO^-$ causes the activation of PI3K/Akt pathway via phosphorylation of platelet-derived growth factor receptors (PDGFR). Activation of PI3K/Akt pathway leads to inactivation of glycogen synthase kinase-3 (GSK-3) (Figure 1.2.4) [12]. Besides these, the nonreceptor tyrosine kinase, c-Src is known to be involved in EGFR and PDGFR activation in various cell types, but the mechanism remains to be resolved [13].

NF-κB, which is a redox sensitive transcription factor regulating the expression of various inflammatory mediator, is known to have roles in $ONOO^-$ cytotoxicity. NF-κB is known to be activated by $ONOO^-$, but the mechanism is still under investigation [8]. In only one study, the role of NF-κB in the regulation of cell death was investigated and it was indicated that $ONOO^-$ treatment did not activate NF-κB in IEC-6 enterocytes but inhibition of NF-κB by transfection with AdIκB, a suppressor of NF-κB via IκB, leaded to $ONOO^-$ induced apoptosis in IEC-6 cells [14].

1.3. Antioxidants

1.3.1. Flavonoids

In recent years there has been a considerable interest to flavonoids because studies have suggested an association between the consumption of polyphenol-rich foods and beverages and the prevention of diseases such as cancer, stroke, osteoporosis and coronary heart diseases [30]. The antioxidant properties of flavonoids are explained with their electron-donating properties in vitro. The structure of the flavonoids is defined to have the hydroxylation pattern, a 3',4'-dihydroxy catechol structure in the B-ring making it an electron-donator [28].

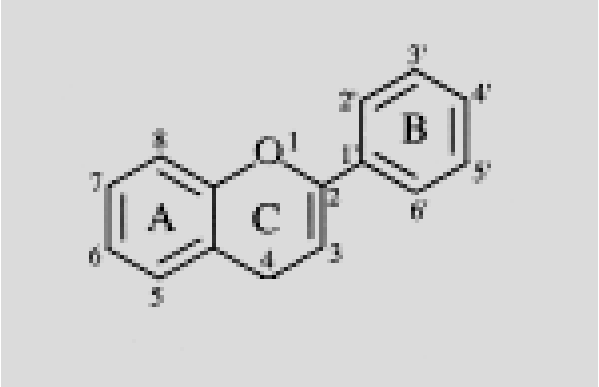

Figure 1.10: General structure of Flavonoids [31]

Many studies have shown that flavonoids are capable of inhibiting the lipid peroxidation and $ONOO^-$ mediated tyrosine nitration which is related with neurodenegerative diseases by a structure-dependent mechanism involving both the oxidation and nitration of the flavonoid ring system. Also flavonoids are known to chelate metals and scavenge singlet oxygen [21]. Besides these, flavonoids are capable of

scavenging reactive oxygen species (ROS) such as hydroxyl radical and superoxide anion [30].

The anticancer effects of flavonoids can be attributed to the prooxidant mechanisms including enhanced apoptosis, growth arrest at one stage and modulation of signal transduction pathways by expression of key enzymes such as cyclooxygenases and protein kinases which is related with MAPK pathway at certain points. Prooxidant toxicity is also thought to be involved in the inhibition of mitochondrial respiration by flavonoids. This may be related with the ability of autooxidation of flavonoids catalyzed by metals to produce superoxide anion that is dismutated to hydrogen peroxide and is converted to hydroxyl radicals via Fenton chemistry. However, this mechanism unlikely happens in vivo as the metals in the plasma make complexes with proteins. It was also found that peroxidases catalyze the oxidation of polyphenols leading to prooxidant toxicity. Plasma myeloperoxidase catalyzes the production of prooxidant phenoxyl radicals, which catalyze lipoprotein oxidation and protein crosslinking leading to formation of atherosclerotic plaque [30].

1.3.2. Catechin

Catechin is a flavane 3-ol (2-[3,4-dihydroxyphenyl]-3,4-dihydro-2H-1-benzopyran-3,5,7-triol) which is a polyphenol, a member of flavonoid family found in fruits, vegetables,wine and green tea abundantly [28].

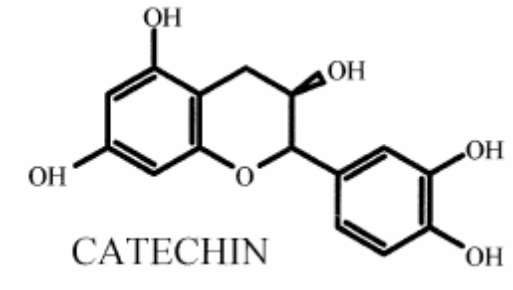

Figure 1.11: Structure of Catechin [29].

As a polyphenolic compound catechin is known to have antioxidant activities such as ROS scavenging, blocking the growth of cancer cells such as breast, prostate or lung, but in some cases it was found that only Catechin is not effective. In a study done on the mouse hippocampal cell line HT-22, the protection of catechin against glutamate cytotoxicity was compared with other flavonoids such as galangin, chrysin, flavonol, luteolin and quercetin. It was found that Catechin is ineffective against glutamate toxicity despite its five hydroxyl groups indicating the number of hydroxyl groups are not correlated with the protective efficacy of a flavonoid [31]. Beside this, it was indicated that Catechin shows intermediate anticarcinogenic efficacy on CD-1 mouse skin cancer cell line when it was compared with three polyphenols; trans-resveratrol, quercetin and gallic acid [28].

In a study catechin and three phytochemicals; naringenin, quercetin and resveratrol have been investigated in a different way, their activity as estrogen antagonists as their structure is similar to the structure of estrogen. Estrogen, which is known to reduce the risk of osteoporosis, heart disease, hyperlipidemia and related illnesses modulate the expression of mRNA stabilizing factor (E-RmRNASF) protecting RNA from endonucleolytic degradation. The antagonistic activity of catechin and other phytochemicals was determined by examining their ability to mimic estrogen in vivo via estrogen cell signaling pathway. The results showed that Catechin has partial agonistic activity, resveratrol has the most and the other two phytochemicals are antagonistic [29].

Also the protective effect of Catechin against lipid peroxidation was compared with its oligomers in liposomes and it was shown that flavonols and procyanidins interact with phospholipid head groups with those containing hydroxyl groups reducing the rate of membrane lipid oxidation. Beside this, it was indicated that the protective effect of flavonols and procyanidins is not related with their ability to induce changes in membrane physical properties, but their chain length. Catechin has been demonstrated to have intermediate protection against lipid peroxidation in liposomes when it is compared with other procyanidins [35].

Studies have been shown that flavonoids containing Catechin are capable of protecting cells against oxidative stress in different conditions by reacting with ROS/RNS and other mechanisms in vitro. However, the reactions of flavonoids are dependent on bioavailability of the compounds. It was shown that after oral ingestion, flavonoids enter the gastrointestinal tract and undergo Phase I/II metabolism. Metabolized derivatives of flavonids enter the portal vein and are transported to the liver, then to the circulation. In the circulation they may be distributed to the peripheral tissues, even to the blood-brain barrier or taken out via kidneys. Flavonoids, which reach to the colon will be digested and the resulting will be absorbed (Figure 1.11) [16].

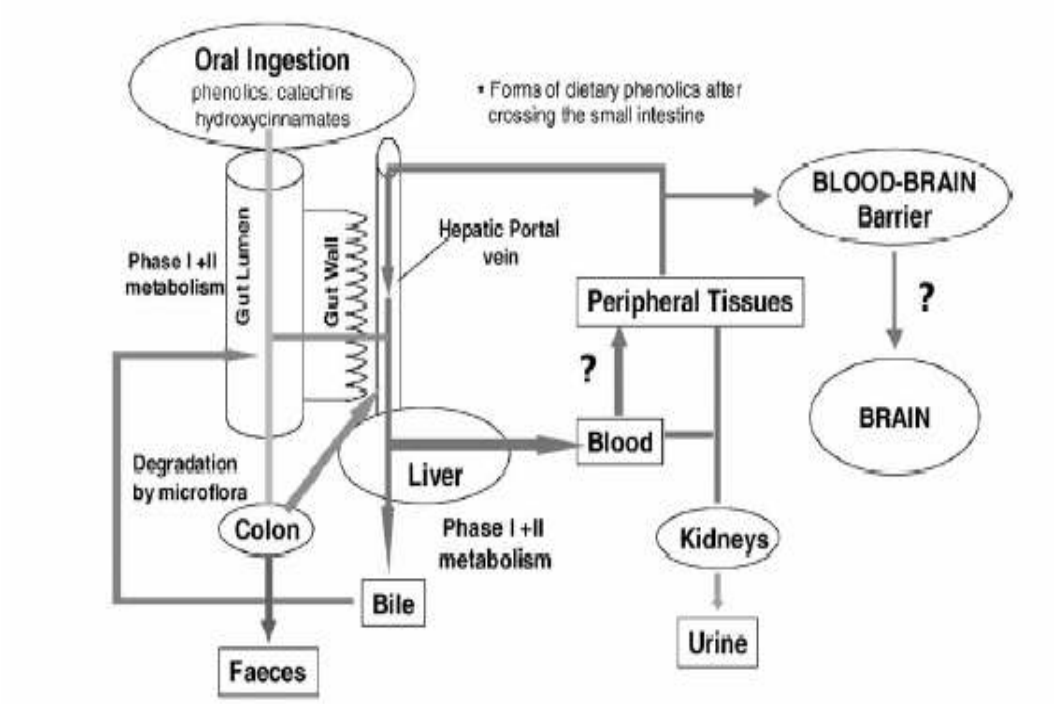

Figure 1.12: Metabolism of Flavonoids [21].

CHAPTER II

PURPOSE

$NO^{\cdot}$, not only a free radical, but also a messenger, has become one of the most important topics of molecular biology in recent years as its role was understood in pathophysiology. So as $ONOO^{-}$, a powerful oxidant has been implicated in the pathogenesis of various diseases such as atherosclerosis, cancer. $NO^{\cdot}$ and $ONOO^{-}$ have been indicated to have a number of adverse biological effects in different cell lines such as inducing ROS/RNS production, DNA damage, apoptosis and necrosis.

The purpose of this study is

- to investigate the molecular mechanism of $NO^{\cdot}$ and $ONOO^{-}$ induced ROS production leading to cell death in 3T3 Fibroblast cell line,
- to investigate the antioxidative and cytoprotective potential of Catechin on $NO^{\cdot}$ and $ONOO^{-}$ induced ROS production and cytotoxicity,
- to obtain mechanistic data with the aim of designing better therapeutic strategies in ROS induced cell signaling leading to cytotoxicity

CHAPTER 3

MATERIALS

3.1. Chemicals

All chemicals and growth mediums were purchased from Sigma (Germany), Merck (Germany) and Biological industries unless otherwise indicated. Catechin was purchased from Extrasynthese (France).

3.2. Solutions and Buffers

Sodium Nitroprusside.....0.336 M stock solution in Water
Catechin.....0.862 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.....94 mM stock solution in PBS
Acridine Orange dye.....10 mM stock solution in PBS

3.3. Equipments

Autoclave:	CERTOCCLAC® A-4050 TRAUN/AUSTRIA
Automatic Pipette:	PIPETTUS®-AKKU, HIRSHMANN LABORGERATE
Balances:	SARTORIUS® BP 221S
Centrifuges:	EPPENDORF® CENTRIFUGES 5415 D EPPENDORF® CENTRIFUGES 5415 R HERAEUS® MULTIFUGE 3 S-R
CO_2 Incubator:	BINDER CO_2 INCUBATOR
Deepfreezer:	HERAUS® HERA FREEZE
Electrophoresis:	BIOGEN
Fluorometer:	SPECTRA MAX GEMINI XS
FT-IR:	BRUKER EQUINOX 55
Incubator:	MEMMERT® MODEL 300
Laminar Flow:	HERAEUS® HERA SAFE, GERMANY
Magnetic Stirrer:	VELP SCIETIFICA
Micro pipettes:	GILSON PIPETMANN, FRANCE

Microscopes:	OLYMPUS CK40 OLYMPUS IX 70 OLYMPUS BX 60
Microplate Reader:	BIORAD MODEL 680
NMR:	VARIANT 11.8 TESLA (500 MHz)
Oven:	BOSCH
Power supply:	BIO-RAD POWERPACK 300
Refrigerator:	BOSCH
Spectrophotometer:	SECOMAM ANTHELIA JUNIAR, FRANCE
Vortex Mixer:	VELP SCIENTIFICA
Water Bath:	HUBER POLYSTAT CCI

3.4. Others

Culture flasks, multiwell plates, falcon tubes and sterile pipettes were from TPP (Europe). Cell Proliferation Kit I (MTT) was purchased from Roche (Germany).

CHAPTER 4

METHODS

4.1. Cell Culture

Swiss Albino mouse monolayer 3T3 Fibroblast cell line was obtained from Cell Culture Collection (Hucre Kulturu Koleksiyonu, HUKUK, NO: 95021701), Ankara. Cell line was maintained in Dulbecco's Modified Eagles medium (DMEM, Sigma) supplemented with 10% Fetal calf serum (FCS, LabTech) 10000U/ml Penicillin, 1mg/ml Streptomycin (P/S, Gibco). The cells were grown in 25 or 75 cm^2 flasks as an attached monolayer under humidified atmosphere containing % 5 CO_2 at 37°C. When confluency has been reached, cells were trypsinized to be detached and one flask is passaged to two flasks. Throughout the experiments, before the treatment 3T3 Fibroblast cells were seeded into 6 well plates (10^6 cells/ml) and treated when approximately 70-80% confluent, in a reduced serum (0.2%) environment for 6 hours. The cells used in the experiments were between passage numbers 6-25.

4.2. Determination of Cell Viability by Trypan Blue Dye Exclusion Method

After treatment, cells were scraped and washed with PBS. Pellet was dissolved in PBS and a homogenous suspension was obtained. A small amount of the suspension (500 µL) was taken and mixed with equal volume of Trypan Blue dye stock solution (0.4 % w/v)

in an eppendorf tube and waited for one minute. Then a drop (20-25 µL) is placed in a haemocytometer and the cells were visualized with a low power microscopy. The cells, which are dead, are seen blue and the viable cells were seen bright white. The percentage of the viable cells was calculated with following method. Each large square equals a volume of 10^{-4} cm^3 or 10^{-4} mL (as 1 cm^3 = 1 mL). This is calculated by multiplying 1 mm (length) × 1 mm (width) × 0.1 mm (depth) and then converting mm^3 to cm^3. The total number of cells per mL (the cell concentration per mL) is Cells/mL = the count per large square × dilution factor (2) × 10^{-4}.

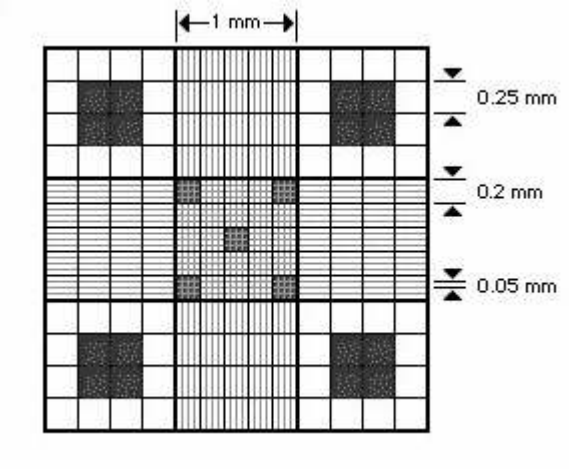

Figure 4.1: Scheme of Haemocytometer

4.3. Detection of ROS Production by Using Fluorescence microscopy

Dichlorofluorescin diacetate (DCFH-DA) was used as fluorescent probe in order to determine ROS production. Cells were grown on the coverslips in 6-well plates and treated with ONOO- for 2 hours at the concentrations of 50-300 µL. Then the cells were incubated with 5 µM DCFH-DA for 20 minutes. Coverslips were removed and placed on a slide and visualised with Olympus Fluorescent Microscopy.

4.4. Detection of Apoptosis by Fluorescent Dyes

For the detection of apoptotic cells, the method of Foglieni et al has been used with minor modifications [4]. Briefly, cells were grown on coverslips in 6-well plates. After treatment, cells were washed PBS twice. Then cells were fixed in 2% para-formaldehyde in PBS for 10 minutes at room temperature, washed with PBS three times and stained either with HO, AO and PI or with one of the combinations. These dyes respectively show specificity for viable, apoptotic and late apoptotic/necrotic cells. Stained cells were visualised under Olympus Fluorescent Microscopy.

4.5. Determination of DNA Fragmentation

3T3 Fibroblast cells (1x106 cell/well) were grown on 6-well plates, starved with Dulbecco's Modified Eagles medium (DMEM, Sigma) supplemented with 0.2% Fetal calf serum for 6 hours. After indicated treatments, cells were harvested by scraping, washed twice with ice cold PBS and lysed in lysis buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA and % 0.2 Triton X-100] on ice for 40 minutes. Cells were subsequently centrifuged at 13000 *g* at 4°C for 10 minutes, supernatant was collected and transferred to a new tube. Supernatant was incubated with RNAse A (200 µg/mL) at 37°C for 1 h and then incubated with Proteinase K (1 mg/mL) with 1% SDS solution at 50 °C overnight. Soluble DNA was isolated by phenol-chloroform-isoamylalcohol extraction and ethanol precipitation. Vacuum dried DNA pellets were dissolved in TE buffer and resolved on 1.5% agarose gel for 2 h. DNA fragments were visualized by staining with ethidium bromide.

4.6. Determination of Cell Viability by MTT Assay

In order to determine cell viability, Cell Proliferation Kit I (MTT) has been used. MTT Assay is designed for the spectrophotometric quantification of cell growth and viability without the use of radioactive isotopes. The assay is based on the cleavage of yellow tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid) to purple formazan crystals by viable cells. The formazan crystals formed are solublized and the resulting solution, which is colored, can be quantified by spectrophotometer.

According to the protocol, 3T3 Fibroblast cells ($1X10^6$) were grown on 96-well plates, starved with Dulbecco's Modified Eagles medium (DMEM, Sigma) supplemented with 0.2% Fetal calf serum for 6 hours. After treatment, the medium was changed and 10 μL of the MTT labeling reagent was added to each well and incubated under humidified atmosphere containing % 5 CO_2 at 37°C for 4 hours. Then 100 μL of the solubilization solution was added into each well and incubated under humidified atmosphere containing % 5 CO_2 at 37°C overnight. Spectrophotometrical absorbance of the samples was measured by Model 680 Microplate Reader (BioRad) at 550 nm.

4.7. Synthesis of ONOO-

ONOO- was synthesized according to the procedure described by Tsuda et al with minor modifications [7]. 10 ml of the acidic solution (0.6 M HCl containing 0.7 M H_2O_2) was mixed with 10 ml of 0.6 M $NaNO_2$ in a cold bath, 20 ml of 1.5 M NaOH was added to the solution immediately. The solution was frozen at -20°C for 2 hours, then thawed on ice and filtered with bacterial filter to be treated to the cells. The concentration was determined by UV absorbance spectrophotometer in 1.2 M NaOH (ε_{302nm}= 1670 M-1 cm-1).

4.8. Freeze Drying

Freeze-Drying, which is also called as Lyophilization, is the process of removal of water content from a solution. In order to analyze accurately in FT-IR, as there is too much water, the end product of a reaction in solution was freeze-dried. In order to freeze-dry the sample, it was frozen in nitrogen to -80°C and by means of a vacuum the product was sublimated, then condensed with a condenser.

4.9. Flourometric Analysis

In order to quantify the ROS production in 3T3 Fibroblast cell line, the protocol described by Lin et al was applied with modifications [9]. Cells were grown in 24-well plate, after treatment cells were washed with PBS twice, and then incubated with DCFH-DA (1 μM) for 30 minutes. Cells were washed with PBS to remove the excess dye and read on Microplate Reader (Spectra Max Gemini XS) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The increase in absorbance indicates ROS production.

CHAPTER 5

RESULTS

5.1. Effect of Nitric Oxide ($NO^{\cdot}$) on 3T3 Fibroblast Cell Line

5.1.1. Effect of $NO^{\cdot}$ on Cell Viability

Cell viability has been analyzed by trypan blue dye exclusion method and MTT assay as well as microscopic examinations.

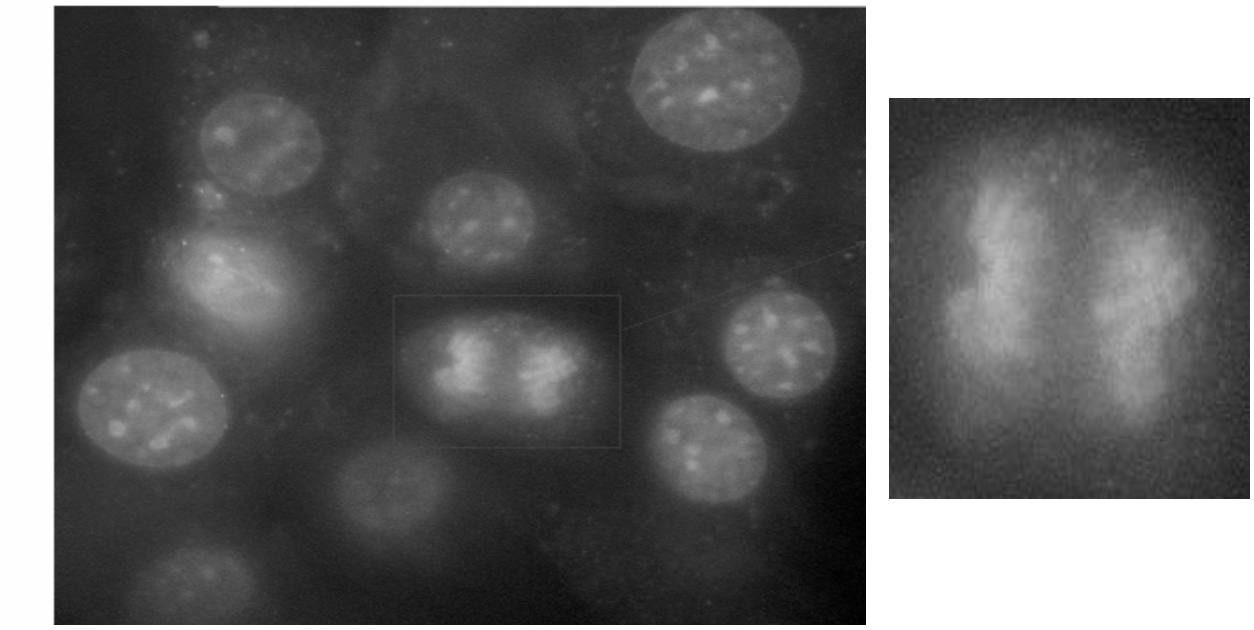

Figure 5.1: Swiss 3T3 Fibroblast cells were treated with Hoechst 33342 (HO). A cell in the anaphase stage of mitosis was visualized. OLYMPUS BX-60 Florescence microscopy (X100 objective) was used.

Cells were incubated with sodium nitroprusside at the concentrations of 50, 100, 200, 300 μ M for 12 hours. Cell viability was checked by trypan blue dye exclusion method. It has been observed that at the highest concentration, 300 μM, cell viability was decreased to % 33.3, which is a significant loss.

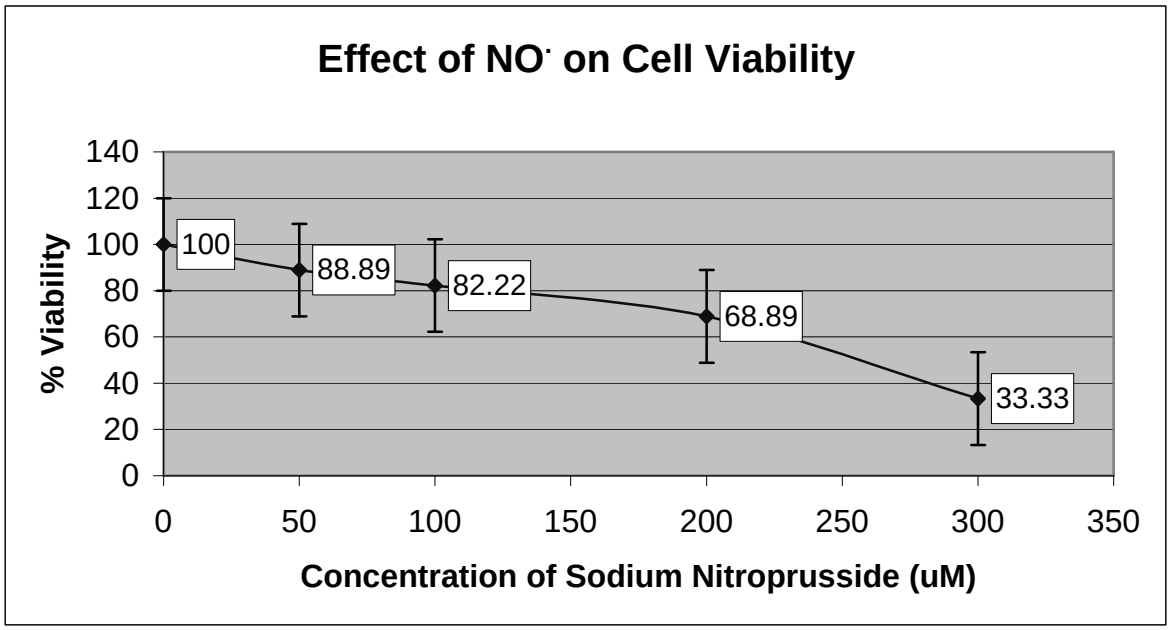

Figure 5.2: Effect of $NO^{\cdot}$ on cell viability. 3T3 Fibroblast cells were treated with sodium nitroprusside for 12 hours at the concentrations indicated. The values are mean (± S.D) of two experiments in duplicate.

Cells were incubated with sodium nitroprusside at the concentrations of 50, 100, 200, 300 μ M for 12 hours. Cell viability was checked by MTT Assay. Decrease in absorbance indicates the cytotoxic effect of sodium nitroprusside.

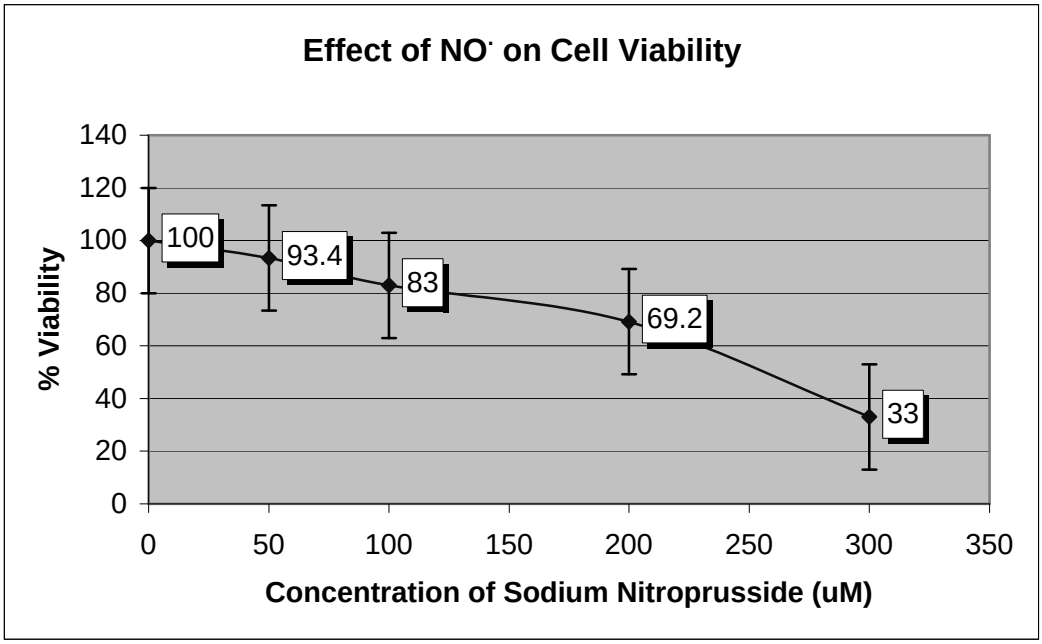

Figure 5.3: Effect of $NO^{\cdot}$ on cell viability. 3T3 Fibroblast cells were treated with sodium nitroprusside for 12 hours at the concentrations indicated. The values are mean (± S.D) of two experiments in duplicate.

5.1.2. Effect of $NO^{\cdot}$ on ROS production

ROS production was detected by using fluorescence probe DCFH-DA. Cells were grown on cover slips and indicated concentrations of the agents were applied. After that, the cells were incubated with the fluorescence probe for 20 minutes. The cover slips were removed from the plates carefully, washed with PBS and placed on microscopy slides. Cells were visualized under fluorescent microscopy.

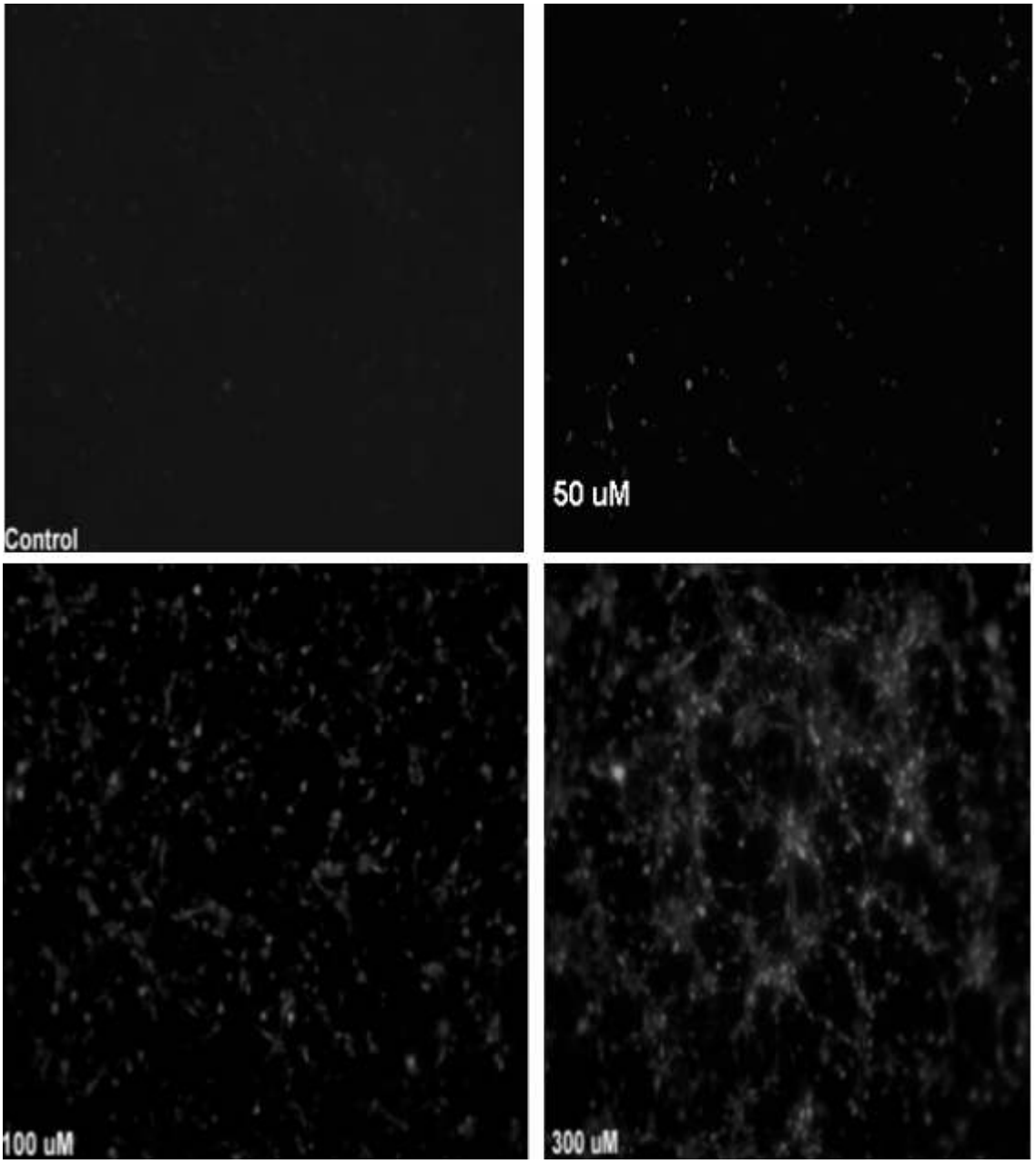

Figure 5.4: Effect of $NO^{\cdot}$ on ROS production. Cells were incubated with sodium nitroprusside at the concentrations indicated for 12 hours. OLYMPUS BX-60 Florescence microscopy (X10 objective) was used.

In order to quantify NO. induced reactive oxygen species (ROS) production, the generation of ROS was measured by means of fluorometer. After treatment, cells were incubated with the fluorescence probe, DCFH-DA for 30 minutes. Then, cells were washed with PBS twice and read on Fluorometer with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

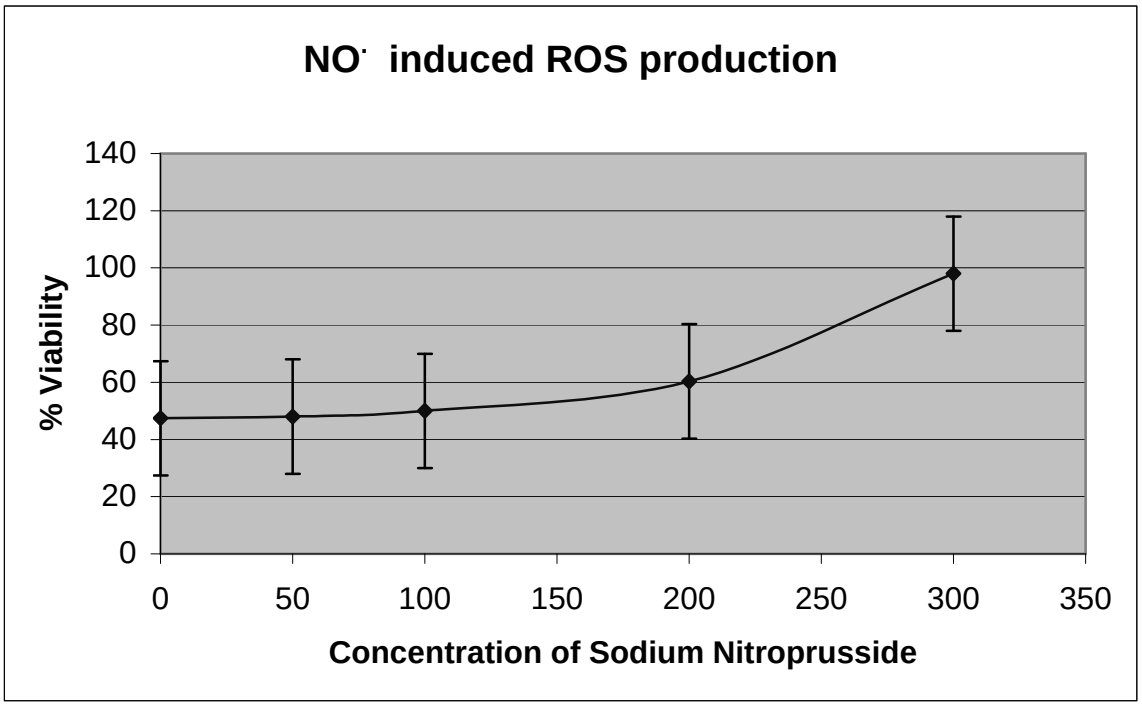

Figure 5.5: NO. induced ROS production. Cells were incubated with indicated concentrations of sodium nitroprusside for 12 hours. The values are mean (± S.D) of two experiments in duplicate.

5.1.3 Apoptotic Effect of $NO^{\cdot}$

Hoescht 33342 (HO) is one of the fluorescence dyes used for cell viability. HO is known to access into the apoptotic nuclei and bind to poly (d (A-T)) tracts. This dye makes it possible to label the cells from early to late apoptotic stage [4]. After the cells were treated with the agents at the concentration indicated, the cells were incubated with HO for 20 min following the required treatments. Arrows indicate the apoptotic cells which have more condensed and bright nucleus.

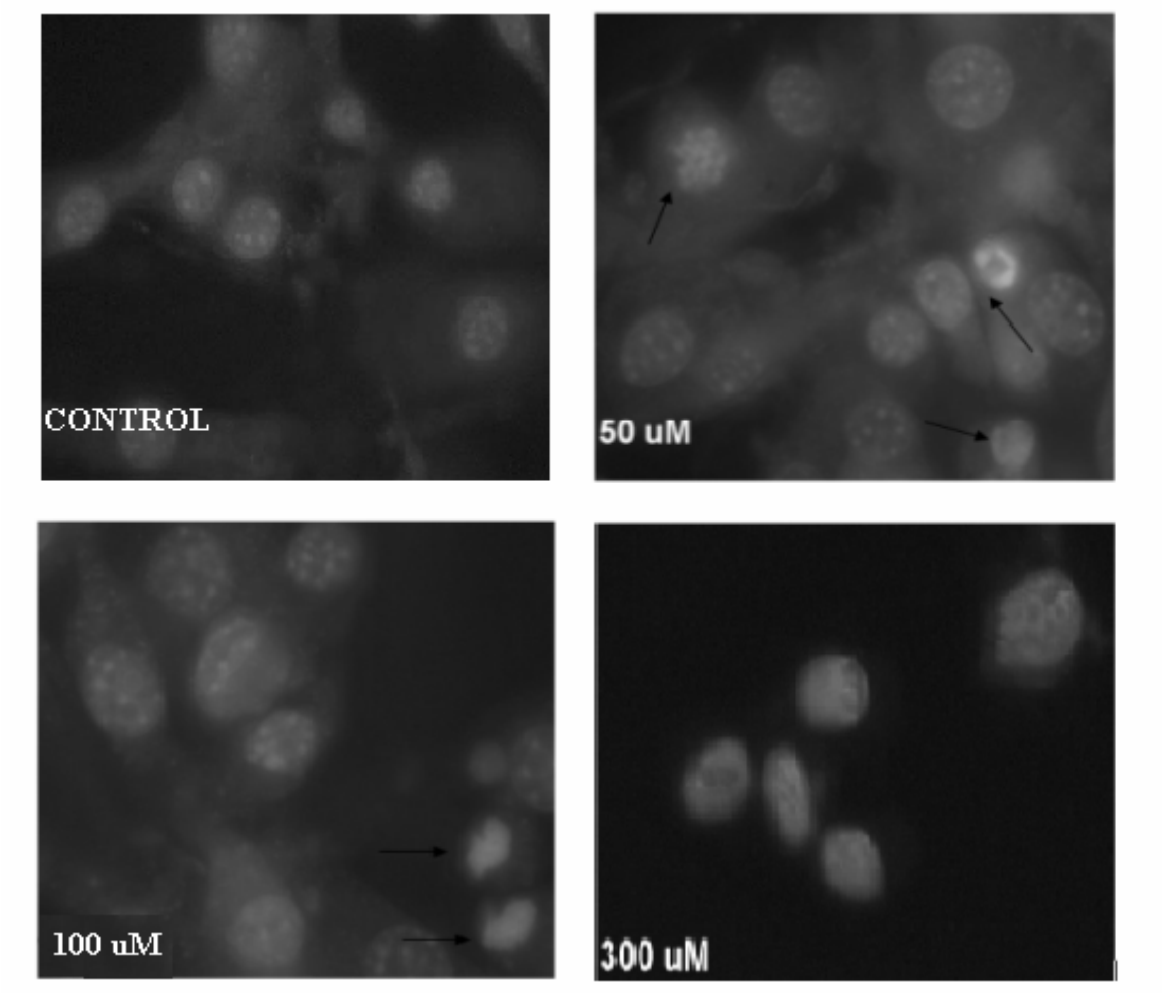

Figure 5.6: Apoptotic effect of $NO^{\cdot}$. Cells were treated with sodium nitroprusside for 12 hours. OLYMPUS BX-60 Florescence microscopy (X100 objective) was used.

Acridine Orange (AO) and Propidium Iodide (PI) are two fluorescence dyes used for cell viability analysis. AO can enter living cells and binds to normal nuclei. PI is able to enter late apoptotic and necrotic cells, then intercalate nucleic acids every 4-5 bp [4]. The usage of HO, AO and PI as a combination is called as differential staining. This method makes it possible to visualize the healthy, apoptotic and necrotic cells at the same time.

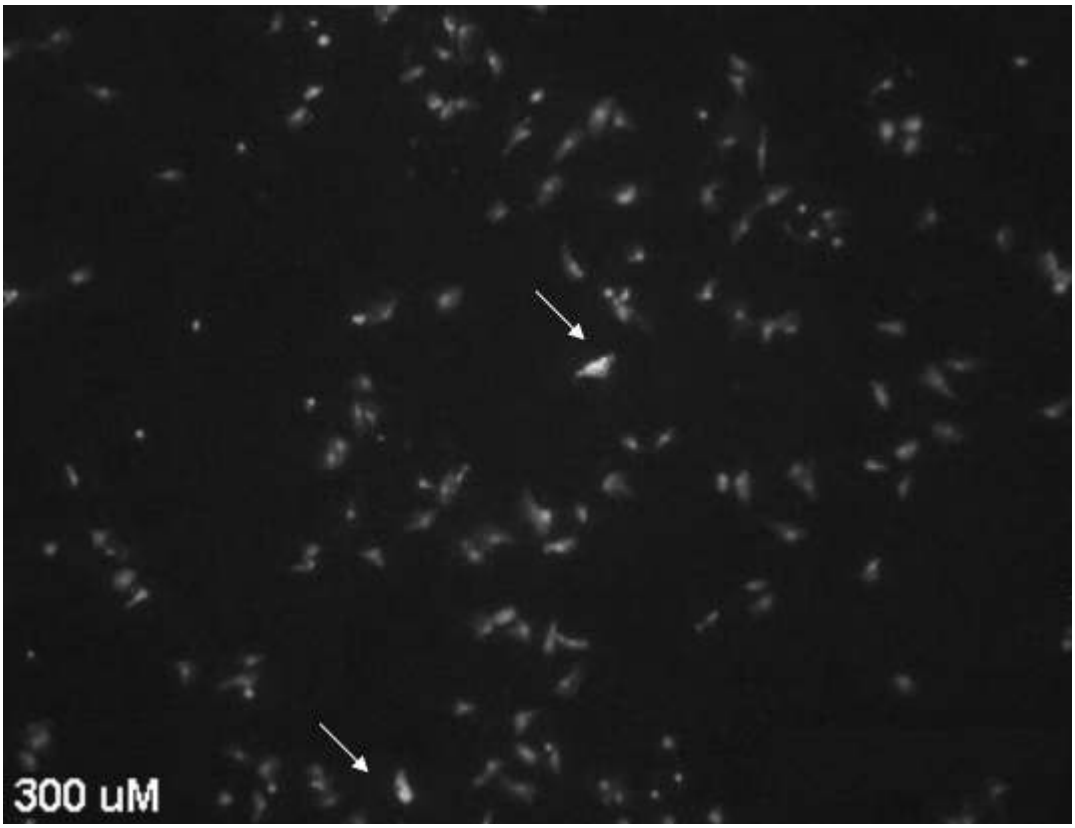

Figure 5.7: Differential staining of healthy, necrotic and apoptotic cells. After treatment with sodium nitroprusside at the indicated concentration for 12 hours, the cells were incubated with HO, AO and PI in this order. OLYMPUS BX-60 Florescence microscopy (X10 objective) was used. Arrows show the late apoptotic/necrotic cells.

Experiments were repeated at least three times and the photograph above is the best representation of the three.

5.1.4. Effect of $NO^{\cdot}$ on DNA Fragmentation

Genomic DNA has been isolated in order to detect apoptosis at the DNA level. DNA was isolated from the cells treated with indicated concentrations of sodium nitroprusside. There is no DNA fragmentation observed.

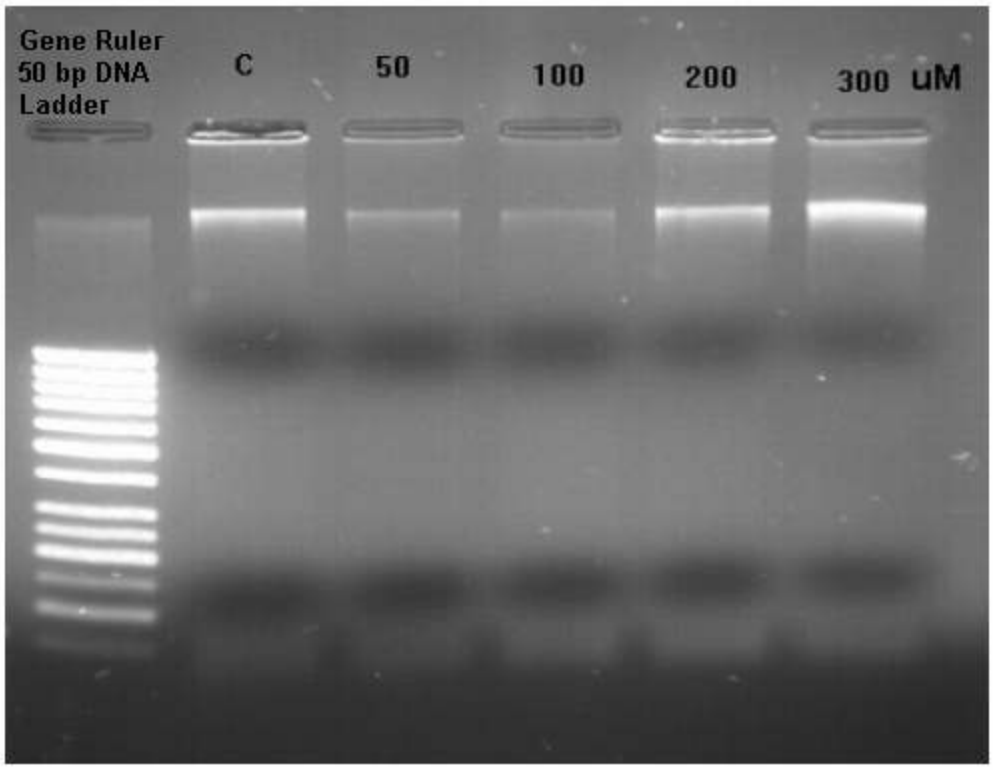

Figure 5.8: Effect of different concentrations of $NO^{\cdot}$ on DNA Fragmentation. Genomic DNA was isolated and analysed by running 1.5 % gel electrophoresis at 90 V for 1hour.

Cells were exposed to 300 μM of sodium nitroprusside for longer periods such as 24, 48, 72 hours. DNA was isolated for detection of Apoptosis and DNA was observed to be intact.

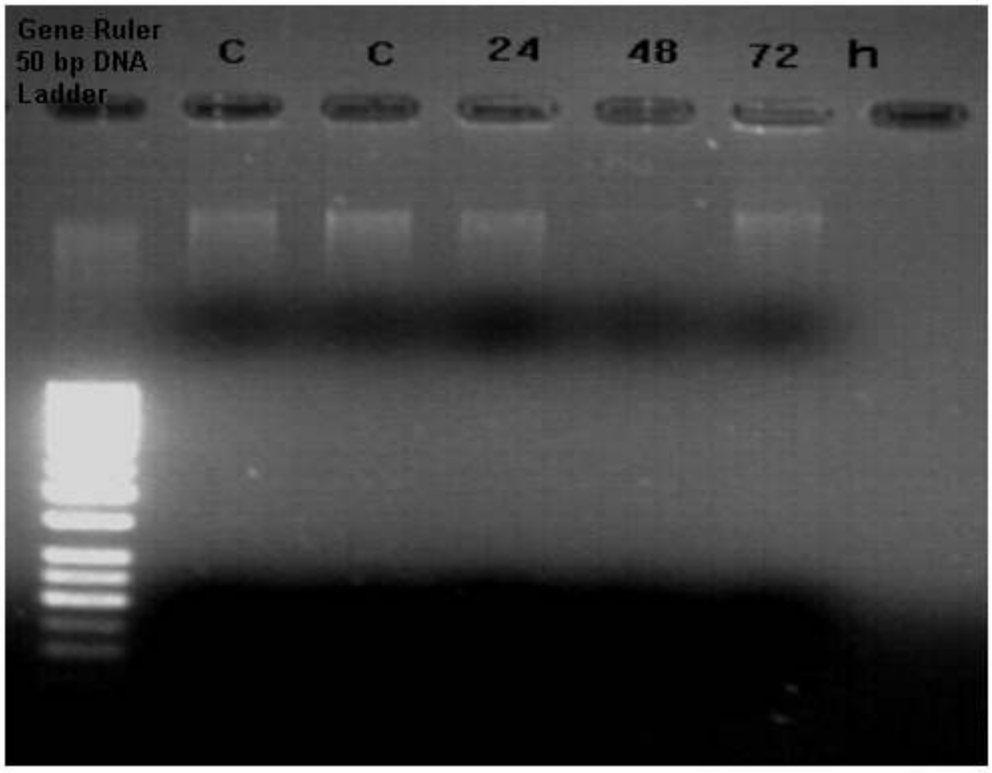

Figure 5.9: Effect of time on DNA Fragmentation. Genomic DNA was isolated and analyzed by running 1.5 % gel electrophoresis at 90 V for 1hour.

5.2. Effect of Peroxynitrite ($ONOO^-$) on 3T3 Fibroblast Cell Line

5.2.1. Effect of $ONOO^-$ on Cell Viability

Cells were incubated with 50, 100, 200, 300 μM $ONOO^-$ for 2 hours and cell viability was evaluated by trypan blue dye exclusion method. Non-viable cells were seen blue under light microscopy and viable cells were seen white since they exclude trypan blue dye.

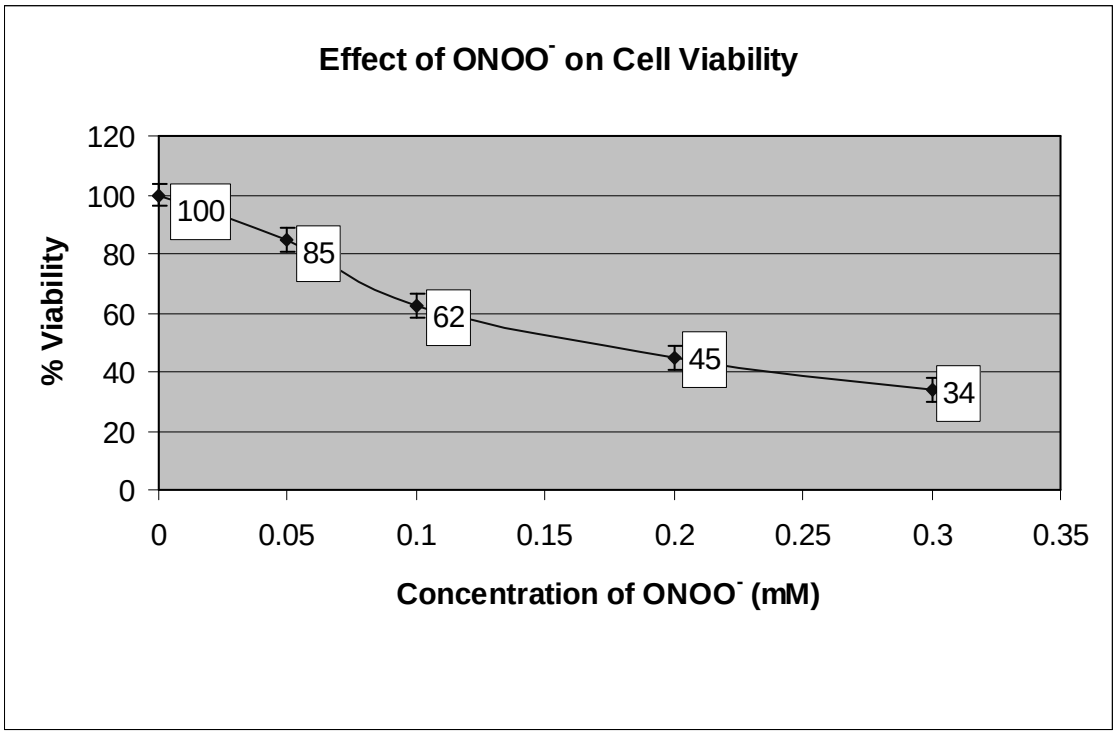

Figure 5.10: Effect of $ONOO^-$ on cell viability. 3T3 Fibroblast cells were treated with $ONOO^-$ at the concentrations indicated for 2 hours. Values are means (± S.D.) of three determinations.

5.2.2 Effect of $ONOO^-$ on ROS Production

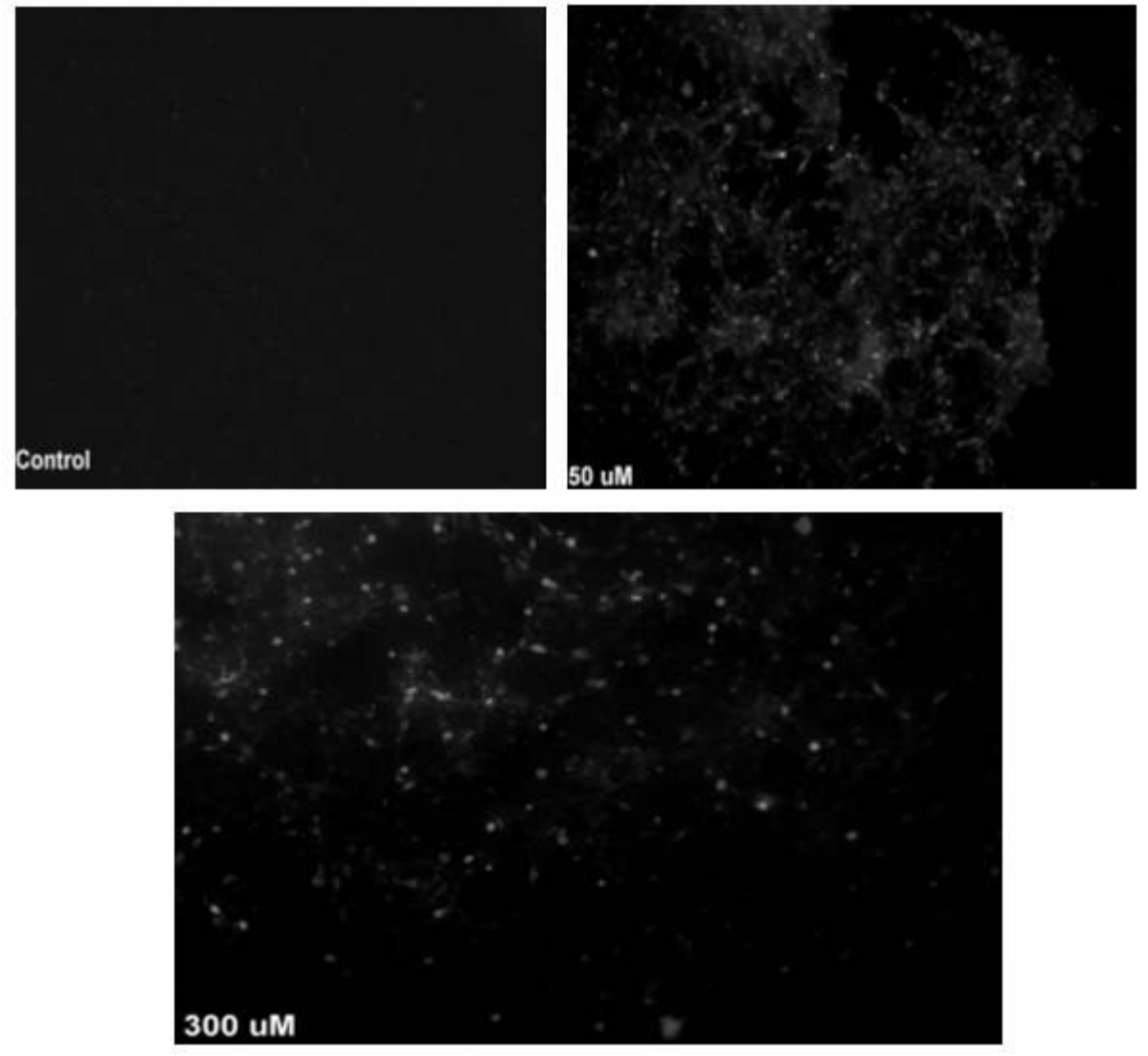

Figure 5.11: Effect of $ONOO^-$ on ROS production. . Cells were incubated with $ONOO^-$ at the concentrations indicated for 2 hours. OLYMPUS BX-60 Florescence microscopy (X10 objective) was used.

5.2.3. Apoptotic Effect of $ONOO^-$

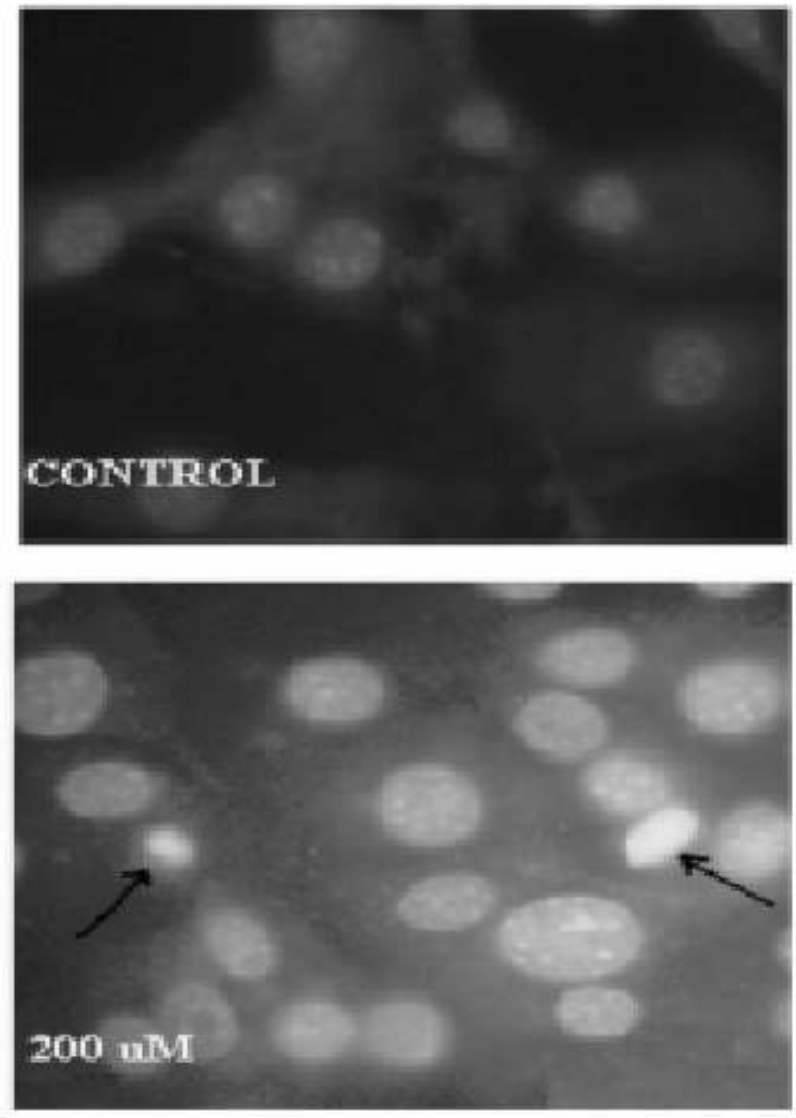

Figure 5.12: Apoptotic effect of $ONOO^-$. Cells were treated with $ONOO^-$ at the concentration indicated for 2 hours. OLYMPUS BX-60 Florescence microscopy (X100 objective) was used.

5.2.4. Effect of $ONOO^-$ on DNA Fragmentation

DNA was isolated from the cells treated with indicated concentrations of $ONOO^-$ synthesized for 12 hours. DNA Fragmentation was not detected.

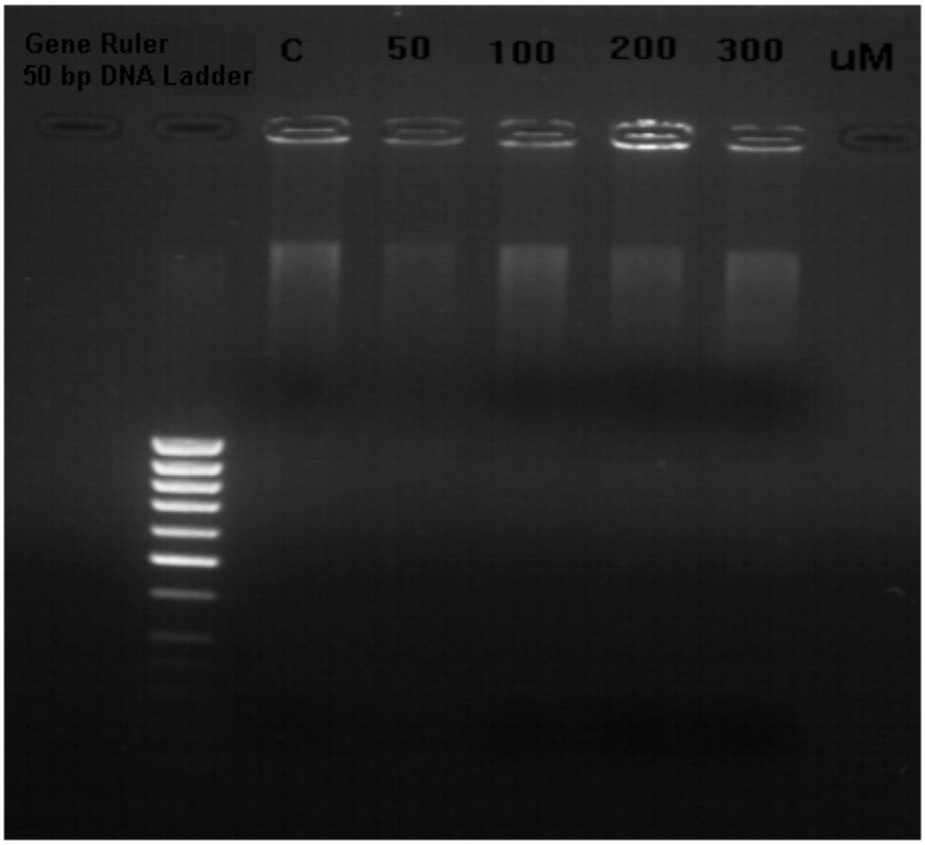

Figure 5.13: Effect of different concentrations of $ONOO^-$ on DNA Fragmentation. Genomic DNA was isolated and analyzed by running 1.5 % gel electrophoresis at 90 V for 1hour.

5.3. Effect of Catechin on $ONOO^-$ and $NO^\cdot$ Induced Cytotoxicity

5.3.1. Chemical Model System

Catechin has been known to have the ability to scavenge ROS [21]. Scavenging effect of Catechin on $ONOO^-$ was investigated in chemical model system. 0.5 mM of Catechin has left to react with 3 mM of $ONOO^-$ for 10 min and the end product was analyzed by FT-IR. In order to compare the end product with reactants, also reactants were detected by FT-IR.

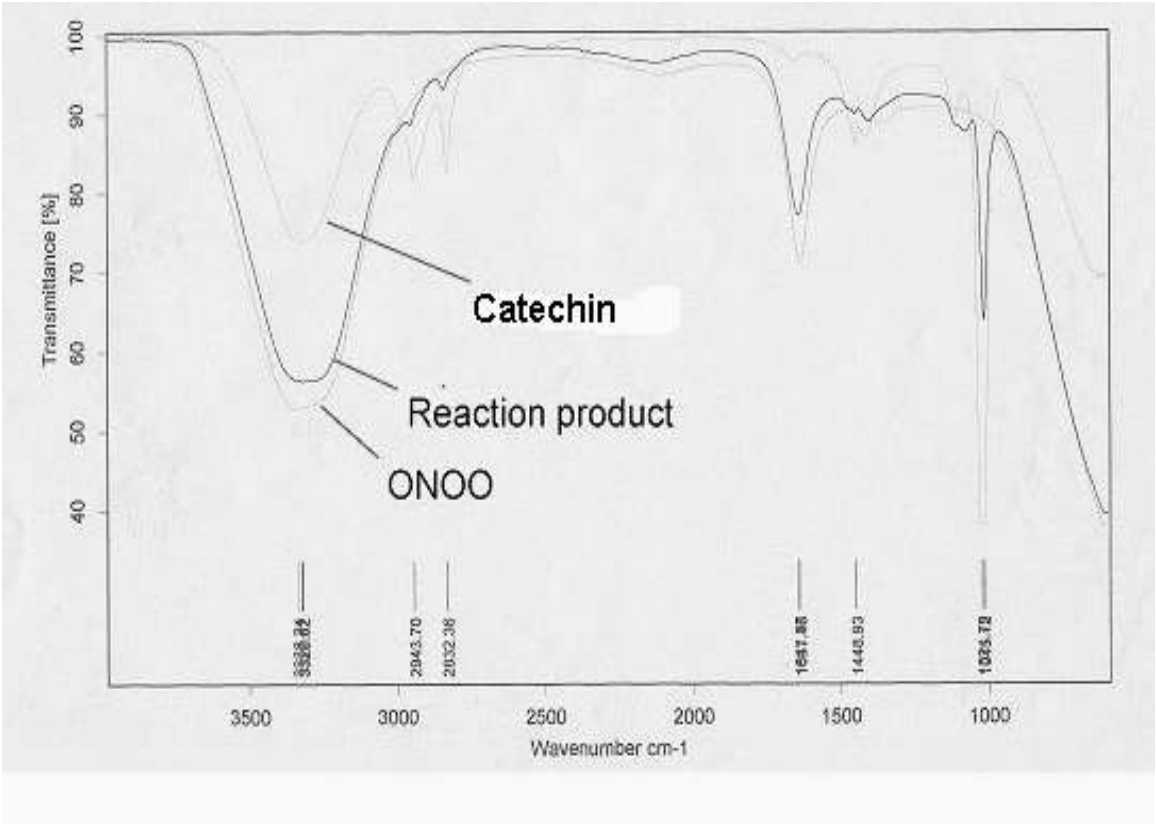

Figure 5.14: Scavenging effect of Catechin on $ONOO^-$. The end product of the reaction between Catechin and $ONOO^-$ was analyzed by FT-IR .

The end product of the reaction between $ONOO^-$ and Catechin was freeze-dried to remove excess water and analyzed in FT-IR.

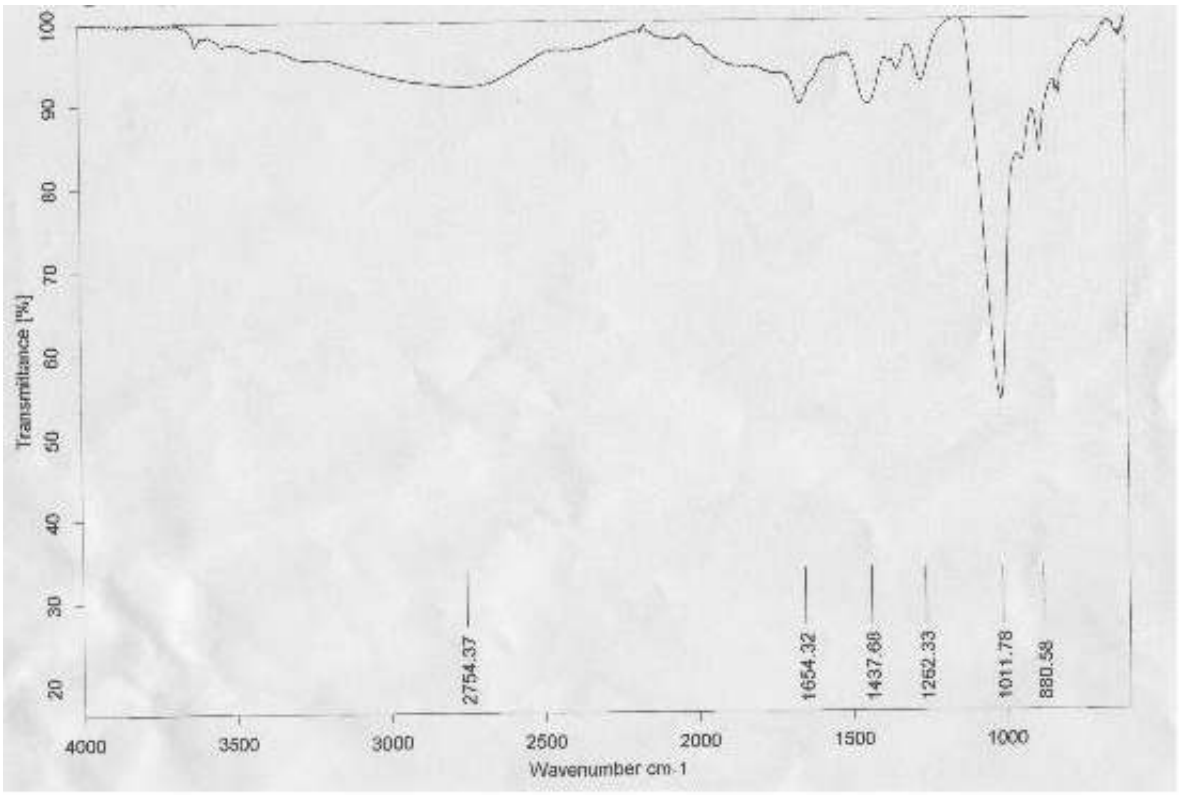

Figure 5.15: Scavenging effect of Catechin on $ONOO^-$. The freeze-dried end product was analyzed by FT-IR.

The freeze-dried form of the end product was analyzed by means of H-NMR and ^{13}C-NMR to obtain further information about its structure.

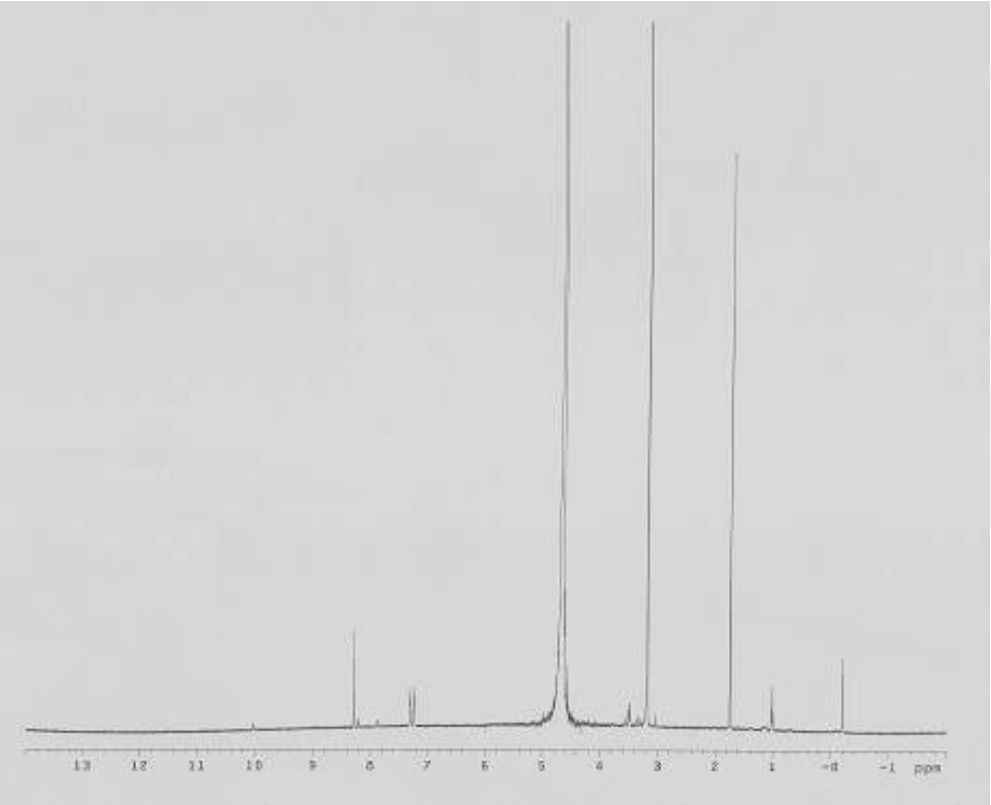

Figure 5.16: H-NMR analysis of the end product. Freeze-dried end product was analyzed by means of H-NMR.

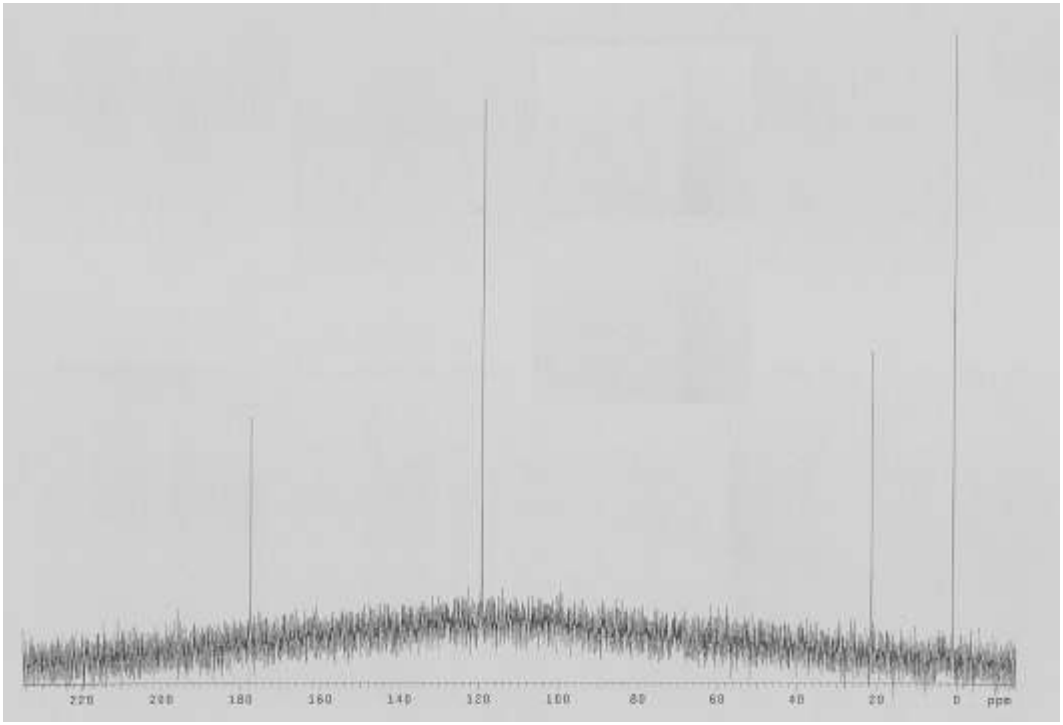

Figure 5.17: ^{13}C-NMR analysis of the end product. Freeze-dried end product was analyzed by means of ^{13}C -NMR.

5.3.2. Cellular Studies

The effect of Catechin on 3T3 Fibroblast cell line was investigated. Cells were treated with 5 and 10 μM of Catechin for 2 hours and cell viability was checked by MTT Assay.

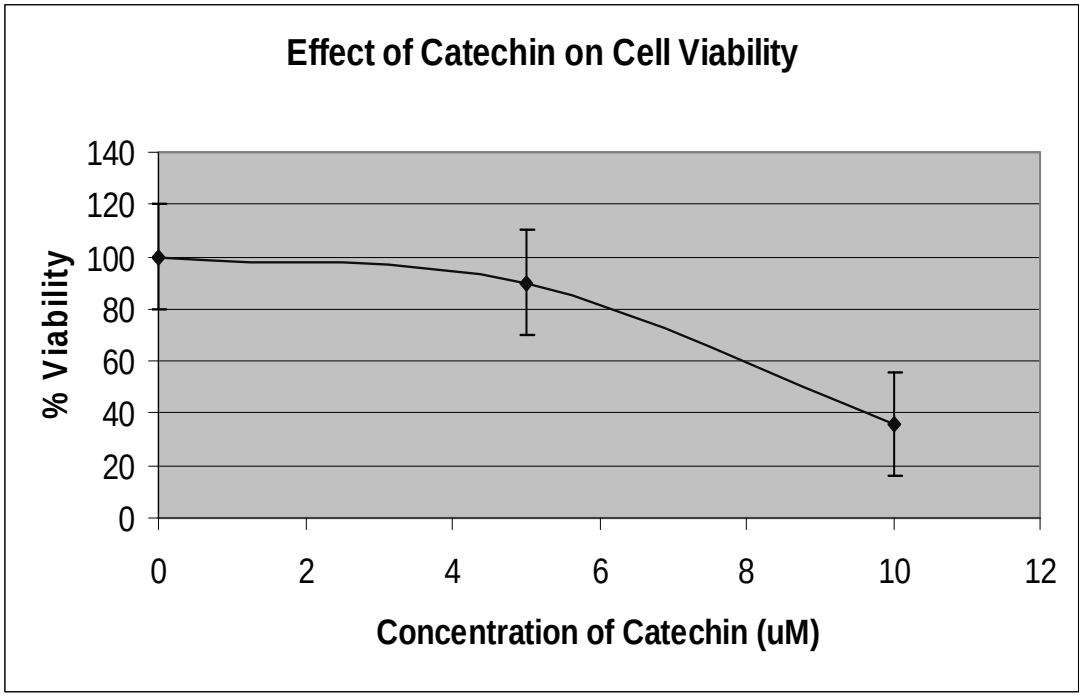

Figure 5.18: Effect of Catechin on 3T3 Fibroblast cell line. Values are means (± S.D.) of two experiments.

5.3.2.1. Effect of Catechin on $ONOO^-$ Induced Cytotoxicity

In order to determine the effect of Catechin on $ONOO^-$ induced cytotoxicity, cells were pre-incubated with Catechin for 2 hours, then treated with $ONOO^-$ for 2 hours. Cell viability was checked by Trypan Blue dye exclusion method.

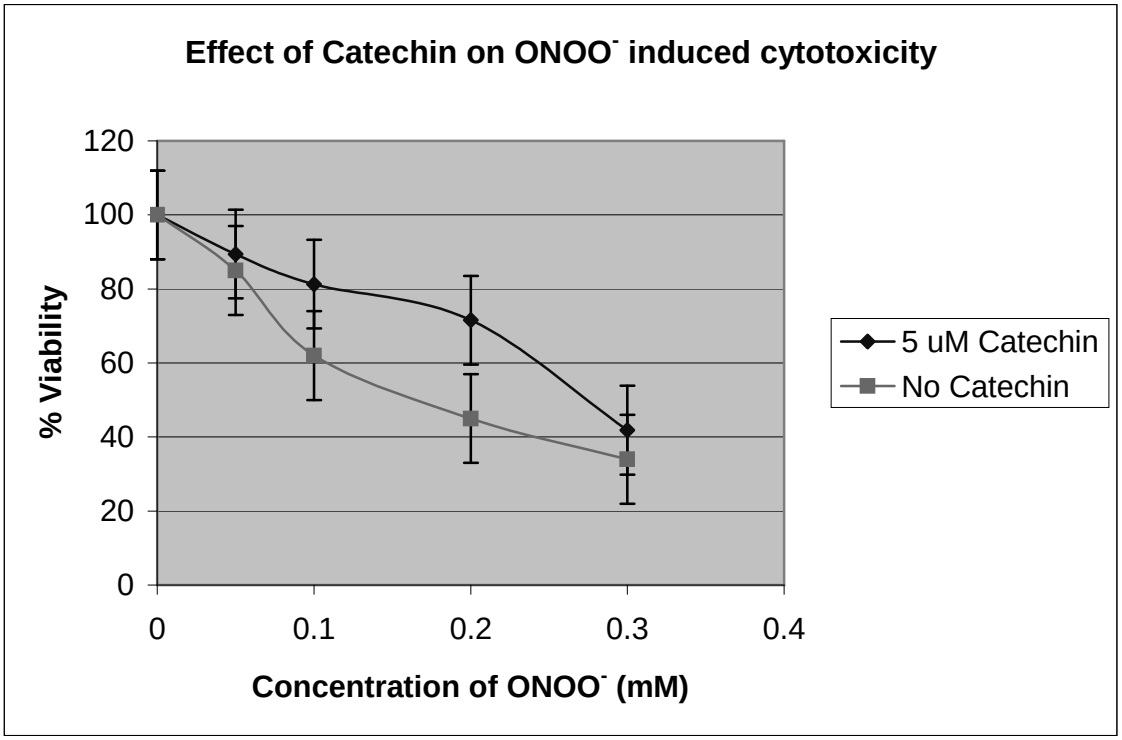

Figure 5.19: Effect of Catechin on $ONOO^-$ induced cytotoxicity. Values are means (± S.D.) of two determinations.

5.3.2.2. Effect of Catechin on $ONOO^-$ Induced ROS Production

In order to detect the effect of Catechin on $ONOO^-$ induced ROS production cells were pre-incubated with 5 μM Catechin for 2 hours, then treated with $ONOO^-$ at the indicated concentrations for 2 hours.

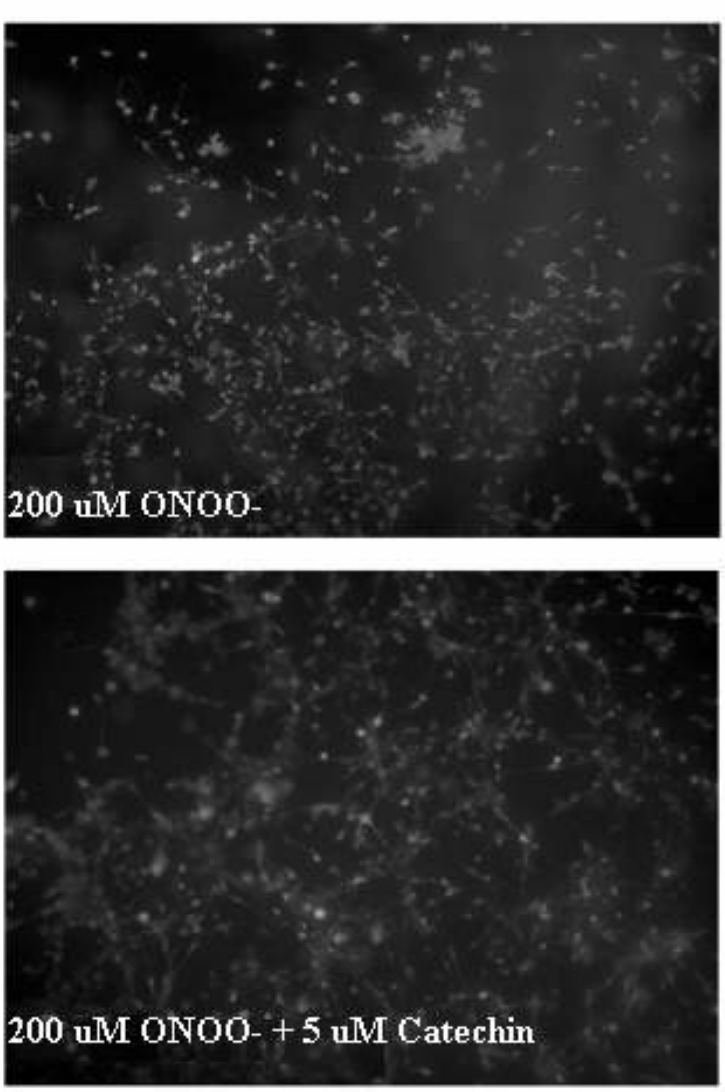

Figure 5.20 Effect of Catechin on $ONOO^-$ induced ROS production. OLYMPUS BX-60 Florescence microscopy (X10 objective) was used.

5.3.2.3. Effect of Catechin on NO· Induced Cytotoxicity

The effect of Catechin on NO· induced cytotoxicity was investigated. Cells were pre-incubated with 5 and 10 μM of Catechin for 2 hours, then treated with indicated concentrations of sodium nitroprusside for 12 hours. Cell viability was checked by MTT Assay.

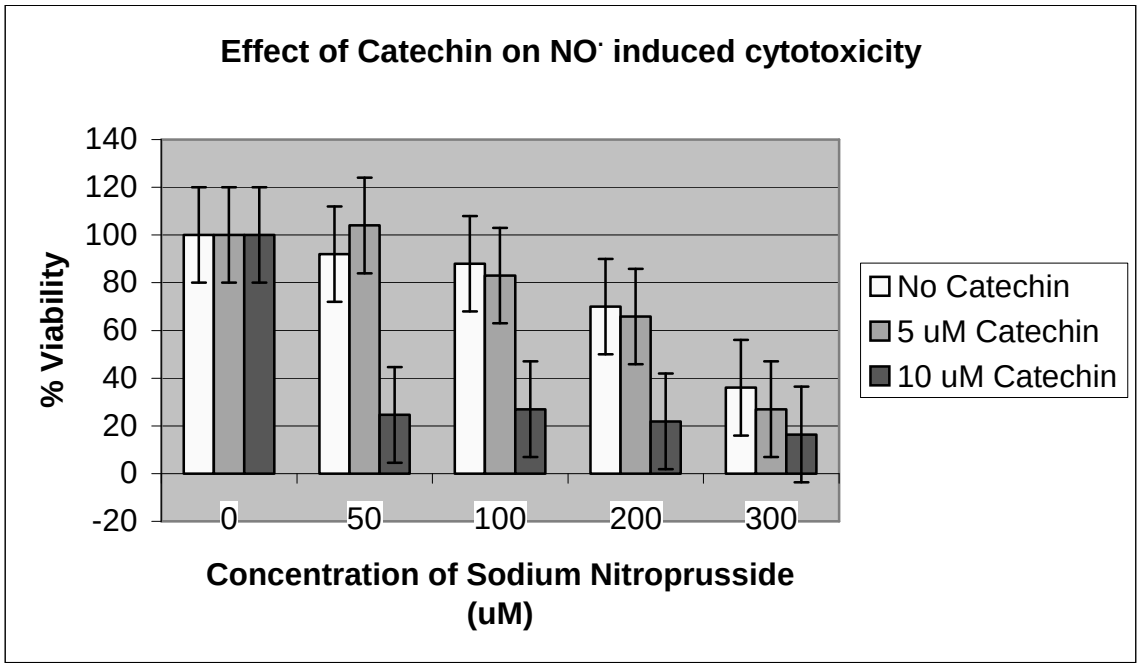

Figure 5.21: Effect of Catechin on NO· induced cytotoxicity. Decrease in absorbance indicates the cytotoxic effect of the agents. The values are mean (± S.D) of two experiments in duplicate.

Cell were pre-incubated with Catechin for 2 hours then incubated with $NO^{\cdot}$ and $ONOO^{-}$ for 12 and 2 hours respectively. Cell viability was checked by Trypan Blue dye exclusion method and MTT Assay.

	50 µM	**+5 µM Catechin**	**100 µM**	**+5 µM Catechin**	**200µM**	**+5 µM Catechin**	**300µM**	**+5 µM Catechin**
$NO^{\cdot}$	**92**	**104**	**88**	**83**	**70**	**65.8**	**36**	**27**
$ONOO^{-}$	**85**	**89.4**	**62**	**81.3**	**45**	**71.5**	**34**	**41**

Table 5.1: % cell viability in the presence of Catechin, $NO^{\cdot}$ and $ONOO^{-}$

CHAPTER 6

DISCUSSION

6.1. Nitric Oxide ($NO^{\cdot}$) Induced Oxidative Stress

After its discovery, $NO^{\cdot}$ has been one of the highly studied biological molecules since it is not only a diatomic free radical but also a molecule involved in various regulatory functions such as neurotransmission, vasodilation and immune defense [17]. $NO^{\cdot}$ was chosen to be the molecule of the year by Science in 1992 and Furcgott et al. won the Nobel Prize for their discoveries concerning "$NO^{\cdot}$ as a signaling molecule in cardiovascular system". Beside its role as a messenger in the cell, it was shown that high concentrations of $NO^{\cdot}$ (> 1μM) is indicated to have cytotoxic effects via formation of ROS and RNS leading to DNA damage, cell death or mutagenesis implicated in the pathogenesis of several diseases such as atherosclerosis, cardiovascular diseases and cancer [15]. Studies about $NO^{\cdot}$ induced cytotoxicity in different cell types have shown that this is a complex process involving multiple pathways such as blockage of DNA synthesis, damage to mitochondria, occurrence in DNA strand breakages, inhibition of key enzymes in the cell [17].

Although $NO^{\cdot}$ is one of the most studied biological molecule on several cell types, little is known about the effects of $NO^{\cdot}$ on 3T3 Fibroblast cell line. In previous studies reporting the relationship between the NOS and $NO^{\cdot}$ formation in macrophages, it has been suggested that local $NO^{\cdot}$ concentrations near and inside the cells maybe as high as 10 μM for prolonged periods of times [15]. In another study, it was shown that treatment with 0.5 mM and 1mM of GSNO, which is a $NO^{\cdot}$ releasing factor, for 12 and 24 hours caused a significant toxicity in Calu-1 lung endothelial cells and induced apoptosis. These two high

concentrations were chosen since the level of $NO^{\cdot}$ in lungs due to cigarette smoke reaches to 500 ppm, which is impossible to exist in vessels [10]. In another study, cytotoxic effect of sodium nitroprusside has been investigated in smooth muscle cells and it was shown that in two different phenotypes of smooth muscle cells sodium nitroprusside at high concentrations (0.1-1 mM) decreases the cell viability depending on the phenotype [33].

In this study the possible cytotoxic and morphological effects of $NO^{\cdot}$ was investigated in which sodium nitroprusside was used as $NO^{\cdot}$ donor in the cells. The concentration range of sodium nitroprusside was chosen between 50-300 μM based on the previous studies reported. In preliminary studies, Trypan Blue dye exclusion method was used, but these results are later confirmed by more sensitive spectrophotometric method where $NO^{\cdot}$ was also shown to be cytotoxic. Trypan blue dye exclusion method showed that $NO^{\cdot}$ at the concentrations between 50-300 μM decreased the cell viability to % 33.33 (Figure 5.2). Also 33 % loss in cell viability was detected by spectrophotometric MTT Assay (Figure 5.3).

$NO^{\cdot}$ induced ROS production was investigated in 3T3 fibroblast cell line by the fluorescence probe DCFH-DA and flourescent microscopy results showed that $NO^{\cdot}$ induces ROS production at concentrations between 50-300 μM dose-dependently (Figure 5.4). At concentrations higher than 100 μM, $NO^{\cdot}$ causes morphological changes and cellular detachment when incubated for 12 hours (Figure 5.4).

Fluorometric measurements of ROS production showed that 2.6 %, 12.9 % and 50.6 % increase in ROS production at the concentrations of 50, 100, 200 and 300 μM respectively (Figure 5.5).

$NO^{\cdot}$ induced cell death was investigated in different cell types such as macrophages, thymocytes, chondrocytes, neurons, endothelial cells various tumor cells. First evidences of $NO^{\cdot}$ induced apoptosis was provided by microscopic examinations of chromatin condensation and DNA fragmentation. However, all studies have not discriminated between apoptotic and necrotic cell death and also all examinations have not demonstrated

whether endogenously generated $NO^{\cdot}$ would suffice to initiate cell death [16]. In previous studies it was suggested that $NO^{\cdot}$-induced formation of ROS leads to cell death [15].

We have shown precisely that $NO^{\cdot}$ induces intracellular ROS production as demonstrated by imaging studies as well as flourimetric studies and to further investigate whether this event triggers apoptosis or not a fluorescence probe, Hoeschst 33342 (HO) labeling the cells from early to late apoptosis, has been used. A number of apoptotic cells were visualized among sodium nitroprusside treated cells by this method (Figure 5.6). Furthermore, cells were differentially stained by Acridine Orange (AO), Propidium Iodide (PI) and Hoeschst 33342 (HO). AO is the indicator of the viable cells and PI shows the late apoptotic and necrotic cells. Our fluorescent microscopy results demonstrate a certain number of late apoptotic and necrotic cells (Figure 5.7).

In order to support imaging data with molecular data, genomic DNA was isolated and analyzed for DNA fragmentation. Cells were treated with sodium nitroprusside at the concentrations between 50-300 μM for 12 hours, isolated DNA was gel electrophorised against a marker and DNA fragmentation was not observed (Figure 5.8). Furthermore, cells were incubated with the highest concentration of $NO^{\cdot}$ for longer periods such as 24, 48 72 hours and DNA was still intact (Figure 5.9).

6.2. Peroxynitrite ($ONOO^-$) Induced Oxidative Stress

$NO^{\cdot}$ reacts quickly with superoxide radical ($O_2^{\cdot -}$) to give $ONOO^-$ which is a primary pathway of $NO^{\cdot}$ metabolism and the effect of $ONOO^-$ on 3T3 Fibroblast cell line should also be investigated to better understand $NO^{\cdot}$ induced oxidative stress.

$ONOO^-$ in the cell decomposes rapidly to give hydroxyl radical and nitrogen dioxide, both of which are strong oxides inducing oxidative stress in several cell types leading to oxidation and nitration of proteins, initiation of lipid peroxidation and DNA breakages [10].

$ONOO^-$ was synthesized as described in Chapter 4.7 and then added to the cells

directly at the concentrations between 50-300 μM. $ONOO^-$ was shown to be cytotoxic in 3T3 Fibroblast cell line as causing a decrease in cell viability to % 34 at the concentration of 300 μM after 2 hour treatment (Figure 5.10).

Previous studies showed that $ONOO^-$ promotes ROS and RNS formation via oxidation, nitration and reacting with other compounds such as CO_2 in different cell types [5]. $ONOO^-$ induced ROS production was detected by a fluorescence probe, DCFH-DA and it was observed that $ONOO^-$ at the concentrations of 50-300 μM stimulates ROS production in 3T3 Fibroblast cell line in a dose-dependently (Figure 5.11).

$ONOO^-$ is known to damage DNA or any cellular compound by nitration or oxidation. Previous studies have been shown that treatment of $ONOO^-$ with an equivalent dose of NO gives much more damage than $NO^\cdot$ to the cell [17]. $ONOO^-$ has been shown to cause both apoptosis and necrosis in various cell types such as entrocytes, endothelials and neurons [11]. $ONOO^-$ induced cell death in 3T3 fibroblast cells has been investigated firstly by Hoeschst 33342 (HO) dye staining. Flourescent microscopy results demonstrate that after 2-hour treatment of $ONOO^-$ at the concentrations between 50-300 μM induced apoptosis in the cells (Figure 5.12).

In molecular studies, genomic DNA was isolated from cells treated with $ONOO^-$ and DNA was gel electrophorised and again intact DNA was observed. (Figure 5.13). Previous studies have been indicated that necrosis occurs as a result of catastrophic toxicity and the parameters of necrosis are passive cell swelling, injury in cytoplasmic organelles, membrane lysis and activation of PARP [10]. To further investigate $ONOO^-$ induced cell death these parameters should be considered in depth.

6.3 Effect of Catechin on $ONOO^-$ and $NO^\cdot$ induced cytotoxicity

Catechin, a potential antioxidant compound has been shown to prevent oxidative stress. As it has hydroxyl groups in its structure it is capable of donating electrons and this feature makes it a scavenger of free radicals or chelator of metal ions [29]. Catechin is a member of flavonoids in which there are more than 4000 members known in this family [30]. The antioxidant effects of flavonoids have been investigated both in vivo and in vitro. However, there is not much study about the preventive effect of Catechin on ONOO- induced cytotoxicty. In chemical model system Catechin reacted with $ONOO^-$ in the presence of pH 7.4 buffer and the end product was analyzed by two different methods; FT-IR and Nuclear Magnetic Resonance (NMR).

In FT-IR result, Catechin gives a peak at about 3300 cm^{-1} indicating hydroxyl group on water. The peaks between 2800-2900 cm^{-1} showing C-H aliphatic groups and a peak at 1100 cm^{-1} indicating C-O strecth. The peak at 1647 cm^{-1} indicates the aromatic benzene group on Catechin. $ONOO^-$ does not give all these peaks, as there is no carbon bonding on its structure except hydroxyl group on water. The end product gives the same hydroxyl group peak as the mixture is in water. Besides this, the end product gives very slight peaks between 2800-2900 cm^{-1} indicating aliphatic groups, which do not seem to be present. The peak at 1647 cm^{-1}, which is less intense than Catechin has, indicates aromatic groups and the peak at 1100 cm^{-1} shows C-O bonding on the end product (Figure 5.14).

In order to remove excess water, the end product was freeze-dried and analyzed. The peak at 1654 cm^{-1} indicates aromatic C strech and the peak at 1011 cm^{-1} shows C-O stretching. Besides this, there are two peaks detected at 1437 and 1262 cm^{-1}, which could not be defined (Figure 5.15) It is certain that the end product has aromatic groups and C-O, but further LC-MS studies are required to determine the structure of the end product.

In H-NMR analysis, the resonance at 8.3 δ indicates aromatic protons. The resonance at about 4.6 δ may indicate benzyl acetate and the resonance at 3.2 δ indicates the presence of hydroxyl group. Also the resonance at 2.1 δ belongs to methyl groups (Figure 5.16). In ^{13}C-NMR analysis, the resonance detected at 178 δ may belong to carboxylic group, although no carboxylic group detected on FT-IR analysis. Also there is a resonance at 120 δ indicating aromatic carbon and another resonance at 22 δ, which may indicate aliphatic groups (Figure 5.17).

FT-IR and NMR analysis have given preliminary information about the structure of the end product. These analyses indicate that the product has aliphatic group(s), aromatic group(s) and hydroxyl group on it. Considering the structure of the reactants, a nitro group expected, however this group has not been in these preliminary studies. One possible explanation could be that the reaction product is expected to be a radical, which adds further difficulty to the detection by conventional methods. Our analytical studies indicate that the structural difference between the reactants and the product, further studies are required to determine the structure of the product more precisely.

In cellular studies; cells were treated with 5 and 10 μM of Catechin for 2 hours and the cell viability has been checked by MTT assay. Results show that Catechin causing 10 % and 64 % viability loss at concentration 5 and 10 μM respectively (Figure 5.18).

It was observed that pre-treatment with 5 μM of Catechin for 2 hours caused an increase in cell viability with respect to the viability of $ONOO^-$ treated cells (Figure 5.19). Catechin is found to be most effective on the cells incubated with 200 μM of $ONOO^-$ with 26 % viability increase and least effective with 4.4 % viability increase on 50 μM $ONOO^-$ treated cells (Table 5.1).

Furthermore, the effect of Catechin on $ONOO^-$ induced ROS production has been investigated by DCFH-DA staining. After pre-treatment with Catechin for 2 hours, cells were incubated with 200 μM of $ONOO^-$ for 1 hour. A slight difference has been observed between the cells pre-incubated with Catechin and the control indicating that Catechin does not protect cells from $ONOO^-$ induced ROS production (Figure 5.20).

Cells were pre-incubated with 5 and 10 μM of Catechin for 2 hours and treated with $NO^\cdot$ for 12 hours. The results showed that 5 μM of Catechin causes 12 % protection on 50 μM of $NO^\cdot$ induced toxicity, but this preventive effect could not be observed at higher concentrations of $NO^\cdot$ Besides, pre-treatment with 10 μM of Catechin causes viability loss with respect to cells incubated with $NO^\cdot$ (Figure 5.21).

Two parameters affecting the preventive effect of antioxidants are the concentrations of antioxidant and oxidant. In previous studies, it was shown that compounds acting as antioxidant at low concentrations may become oxidizing agents at high concentrations such as phenolic compound, quercetin [30]. The optimum conditions determined for preventive effect of Catechin according to our results were 5μM of Catechin for 50 μM of $NO^\cdot$ and 200 μM of $ONOO^-$ (Table 5.1).

CHAPTER 7

CONCLUSIONS

In the present study, the oxidative and cytotoxic effects of two biologically important molecules, $NO^{\cdot}$ and $ONOO^{-}$ have been studied on 3T3 fibroblast cell line.

The major outcomes of this study can be summarized as;

- $NO^{\cdot}$ and $ONOO^{-}$ induce cytotoxicity and intracellular ROS production lieading to cell death in 3T3 fibroblast cell line at concentrations of 50-300 μM incubated for 2, 12 hours respectively.
- Although apoptotic cells were visualized by differential staining, $NO^{\cdot}$ and $ONOO^{-}$ do not lead to DNA fragmentation indicating that the cytotoxic effect of these oxidants is due to another mechanism other than apoptosis.
- A potential antioxidant molecule, Catechin was found to be cytotoxic dose-dependently. Moreover, Catechin potetiates $NO^{\cdot}$ induced cytotoxicity when used at the concentrations higher than 100 μM.

In view of our findings some points need to be clarified in order to better understand the underlying mechanisms of $NO^{\cdot}$ and $ONOO^{-}$ induced oxidative stress cytotoxicity.

- The molecular mechanism of $NO^{\cdot}$ and $ONOO^{-}$ induced cell death.
- Mechanism of the reaction between Catechin and $ONOO^{-}$.
- Mechanism of Catechin cytotoxicity as well as its potentiation on $NO^{\cdot}$ induced effect.

By understanding the underlying molecular mechanism for $NO^{\cdot}$ and $ONOO^{-}$ induced cytotoxicity and ROS production triggering cell death, better therapeutic strategies can be designed.

CHAPTER 8

REFERENCES

[1] Forman H. J. Oxidative Stress and Signal Transduction International Thomson Publishing, 1997.

[2] Koppenol,W. H.; ‘’The basic chemistry of nitrogen monoxide and $ONOO^-$’’, Free Radical Biology & Medicine, Vol. 25, Nos. 4/5, pp. 385-391, 1998.

[3] Squadrito, G.L.; Pryor, W. A.; ‘’Oxidative chemistry of $NO^·$: The roles of superoxide, $ONOO^-$, and carbon dioxide’’ , Free Radical Biology & Medicine, Vol. 25, Nos. 4/5, pp. 392-403, 1998.

[4] Foglieni, C.; Meoni, C.; Davalli, A., M.; ‘’ Fluorescent dyes for cell viability: an application on prefixed conditions’’, Histochemistry and Cell Biology

[5] Radi, R.; Cassina, A.; Hodara, R.; Hodara, R.; ‘’ $ONOO^-$ reactions and formation mitochondria’’, Free Radical Biology & Medicine, Vol. 25, No. 33, pp. 1451-1464, 2001.

[6] Gonzalez-Zulueta, M.; Ensz, L. M.; Mukhina, G.; ‘’Manganese superoxide dismutase protects nNOS neurons from NMDA and $NO^·$-mediated neurotoxicity’’, Journal of Neuroscience, Vol.18, pp. 2040-2055, 1998.

[7] Tsuda, T.; Kato, Kato, Y.; Osawa, T.; ‘’Mechanism for the $ONOO^-$ scavenging activity by anthocyanins’’, FEBS, Vol.484, pp. 207-210, 2000

[8] Virág, L.; Szabó, É.; Gergely, P.; ‘’$ONOO^-$-induced cytotoxicity: mechanism and opportunities for intervention’’, Toxicology Letters, Vol. 140-141, pp 113-124, 2003.

[9] Lin, K.; Xue, J.; Sun, F. F.; ‘’ Reactive oxygen species participate in $ONOO^-$ induced Apoptosis in HL-60 cells’’, Biochemical and Biophysical Research Communications, Vol.230, pp.115-119, 1997.

[10] Wang, Y.; Ho, Y.; Pan M.; ‘’Mechanisms of cell death induced by $NO^\cdot$ and peroxy nitrite in Calu-1 cells’’, Environmental Toxicology and Pharmacology, Vol.6, pp.35-44, 1998.

[11] Virág, L.; Szabó, C.; ‘’ BCL-2 protects $ONOO^-$ treated thymocytes from poly(ADP-Ribose) synthase (PARS)-independent apoptotic but not from PARS-mediated necrotic cell death’’, Free Radical Biology & Medicine, Vol.29, No.8, pp 704-713, 2000.

[12] Klotz, L.; Schroeder, P.; Sies,H.; ‘’Peroxynitride signaling: Receptor tyrosine kinases and activation of stress-responsive pathways’’, Free Radical Biology & Medicine, Vol.33, No.6, pp.737-743, 2002.

[13] Minetti, M.; Mallozzi, C.; Di Stasi A. M. M. ;‘’$ONOO^-$ activates kinases of the SRC family and upregulates tyrosine phosphorylation signaling’’, Free Radical Biology & Medicine, Vol.33, No.6, pp. 744-754,2002.

[14] Potoka, D. A.; Upperman, J. S.; Nadler, E. P.; Wang, C. T.; ‘’ NF-KB inhibition enhances $ONOO^-$-induced entrocyte apoptosis’’, Journal of Surgical Research, Vol.106, Issue 1, July 2002.

[15] Wink, D. A.; Mithchell J. B.; '' Chemical biology of nitric oxide: insight into regulatory, cytotoxic, and cytoprotective mechanisms of $NO^{\cdot}$'', Free Radical Biology & Medicine, Vol. 25, Nos.4/5, pp. 434-456, 1998.

[16] Brüne, B.; Knethen, A.; Sandau, K. B.; ''$NO^{\cdot}$ and its role in apoptosis'', Euoropean of Pharmacology, Vol. 351, 261-272,1998.

[17] Burney, S.; Caulfield, J. L. ; Niles, J. C.; ''The chemistry of DNA damage from $NO^{\cdot}$ and $ONOO^{-}$'', Mutation Research, Vol. 424, pp. 37-49, 1999.

[18] Janero, D.; '' $NO^{\cdot}$ -related pharmaceuticals: Contemporary approaches to therapeutic no modulation'', Free Radical Biology & Medicine, Vol.28, No.10, pp 1495-1506, 2000.

[19] Halliwell, B.; Gutteridge, John M.C.; 'Free Radicals in Biology and Medicine', Oxford University Press, 1999.

[20] Bapat, S.; Verkleij, A.; Post J. A.; '' $ONOO^{-}$ activates mitogen-activated protein kinase (MAPK) via a MEK-independent pathway: a role for protein kinase'', FEBS Letters, Vol.499, pp.21-26, 2001.

[21] Schroeter, H.; Boyd, C.; Spencer, J. P. E.; ''MAPK signaling in neurodegeneration: influences of flavonoids and $NO^{\cdot}$'', Neurobiology of Aging, Vol. 23, pp. 861-880,2002.

[22] Mondoro, T. H.; Shafer, B. C.; '' $ONOO^{-}$-induced tyrosine nitration and phosphorylation in human platelets'', Free Radical Biology & Medicine, Vol.22, No.6, pp. 1055-1063, 1997.

[23] MacMillan-Crow, L. A.; Greendofer, J. S.; Vickers, S. M.; ''Tyrosine nitration of c-SRC tyrosine kinase in human pancreatic ductal adenocarcinoma'', Archives of Biochemistry and Biophysics, Vol. 377, No. 2, pp.

[24] Chang, L.; Karin, M.; ‘’ Mammalian MAP kinase signalling cascades’’, Nature, Vol. 410, pp. 37-40,2001

[25] Deak, M.; Clifton, A.D.; Lucocq, L.M.; Alessi D.R.; ‘’Mitogen and stress-activated protein kinase-1 (MSK 1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB’’, EMBO J., Vol. 17, No.15, pp. 4426-4441, 1998.

[26] Shaffer, H.F.; Weber, M.J.; ‘’Mitogen-activated protein kinase: specific messages from ubiquitous messengers’’, Molecular Cell Biology, Vol. 19, pp. 2435-2444, 1999.

[27] So, H.S.; Park, R.K., Kim M.S.; ‘$NO^{\cdot}$ inhibits c-Jun N terminal kinase 2 (JNK2) via S-nitrosylation, ‘’Biochem. Biophys Res Commun, Vol. 247, No. 3, pp, 809-813, 1998.

[28] Soleas, G.J.; Grass, L.; Josephy, P.D.; Goldberg, D.M.; ‘’ A comparison of the anticarcinogenic properties of four red wine polyphenols’’, Clinival Biochemistry, Vol.35, pp, 119-124, 2002.

[29] Ratna, W.N.; Simonelli, J.A.; ‘’ The action of dietary phytochemicals quercetin, catechin, resveratrol and naringenin on estrogen-mediated gene expression’’, Life Sciences, Vol. 70, pp. 1557-1589, 2002.

[30] Galati, G.; Sebzevari, O.; Wilson, J.X.; ‘Prooxidant activity and cellular effects of the phenoxyl radicals dietary flavonoids and other polyphenolics’’, Toxicology, Vol. 177, pp. 91-104, 2002.

[31] Ishige, K.; Schubert, D.; Sagara, Y.; ‘’ Flavonoids Protect Neuronal cells from oxidative stress by three distinct mechanisms’’, Free Radical Biology & Medicine, Vol. 30, No. 5, pp. 433-446, 2001.

[32] Meβmer, U.K., Ankarcrona, M., Nicotera, P.; ‘’ p53 expression in $NO^{\cdot}$-induced apoptosis’’, FEBS Letter, Vol. 355, pp.23-26,1994.

[33] Lau, H.K.F.; ‘’ Cytotoxicity of $NO^{\cdot}$ donors in smooth muscle cells is dependent on phenotype, and mainly due to apoptosis’’, Atherosclerosis, Vol. 166, pp. 223-232, 2003.

[34] Meβmer, U.K., Brüne, B.; ‘’$NO^{\cdot}$ induced apoptosis: p53-dependent and p53-independent signalling pathways’’, Biochemistry Journals, Vol. 319, pp. 299-305, 1996.

[35] Verstraeten, S.V.; Keen, C.L., Schmitz, H.H.; ‘’ Flavan-3-ols and procyanidins protect liposomes against lipid oxidation and distruption of the bilayer structure’’, Free Radical Biology & Medicine, Vol.22, No.6, pp. 1055-1063, 1997.

CHAPTER 8

REFERENCES

[1] Forman H. J. Oxidative Stress and Signal Transduction International Thomson Publishing, 1997.

[2] Koppenol,W. H.; ‘’The basic chemistry of nitrogen monoxide and $ONOO^-$’’, Free Radical Biology & Medicine, Vol. 25, Nos. 4/5, pp. 385-391, 1998.

[3] Squadrito, G.L.; Pryor, W. A.; ‘’Oxidative chemistry of $NO^·$: The roles of superoxide, $ONOO^-$, and carbon dioxide’’ , Free Radical Biology & Medicine, Vol. 25, Nos. 4/5, pp. 392-403, 1998.

[4] Foglieni, C.; Meoni, C.; Davalli, A., M.; ‘’ Fluorescent dyes for cell viability: an application on prefixed conditions’’, Histochemistry and Cell Biology

[5] Radi, R.; Cassina, A.; Hodara, R.; Hodara, R.; ‘’ $ONOO^-$ reactions and formation mitochondria’’, Free Radical Biology & Medicine, Vol. 25, No. 33, pp. 1451-1464, 2001.

[6] Gonzalez-Zulueta, M.; Ensz, L. M.; Mukhina, G.; ‘’Manganese superoxide dismutase protects nNOS neurons from NMDA and $NO^·$-mediated neurotoxicity’’, Journal of Neuroscience, Vol.18, pp. 2040-2055, 1998.

[7] Tsuda, T.; Kato, Kato, Y.; Osawa, T.; ‘’Mechanism for the $ONOO^-$ scavenging activity by anthocyanins’’, FEBS, Vol.484, pp. 207-210, 2000

[8] Virág, L.; Szabó, É.; Gergely, P.; ‘’$ONOO^-$-induced cytotoxicity: mechanism and opportunities for intervention’’, Toxicology Letters, Vol. 140-141, pp 113-124, 2003.

[9] Lin, K.; Xue, J.; Sun, F. F.; ‘’ Reactive oxygen species participate in $ONOO^-$ induced Apoptosis in HL-60 cells’’, Biochemical and Biophysical Research Communications, Vol.230, pp.115-119, 1997.

[10] Wang, Y.; Ho, Y.; Pan M.; ‘’Mechanisms of cell death induced by $NO^\cdot$ and peroxy nitrite in Calu-1 cells’’, Environmental Toxicology and Pharmacology, Vol.6, pp.35-44, 1998.

[11] Virág, L.; Szabó, C.; ‘’ BCL-2 protects $ONOO^-$ treated thymocytes from poly(ADP-Ribose) synthase (PARS)-independent apoptotic but not from PARS-mediated necrotic cell death’’, Free Radical Biology & Medicine, Vol.29, No.8, pp 704-713, 2000.

[12] Klotz, L.; Schroeder, P.; Sies,H.; ‘’Peroxynitride signaling: Receptor tyrosine kinases and activation of stress-responsive pathways’’, Free Radical Biology & Medicine, Vol.33, No.6, pp.737-743, 2002.

[13] Minetti, M.; Mallozzi, C.; Di Stasi A. M. M. ;‘’$ONOO^-$ activates kinases of the SRC family and upregulates tyrosine phosphorylation signaling’’, Free Radical Biology & Medicine, Vol.33, No.6, pp. 744-754,2002.

[14] Potoka, D. A.; Upperman, J. S.; Nadler, E. P.; Wang, C. T.; ‘’ NF-KB inhibition enhances $ONOO^-$-induced entrocyte apoptosis’’, Journal of Surgical Research, Vol.106, Issue 1, July 2002.

[15] Wink, D. A.; Mithchell J. B.; '' Chemical biology of nitric oxide: insight into regulatory, cytotoxic, and cytoprotective mechanisms of $NO^{\cdot}$'', Free Radical Biology & Medicine, Vol. 25, Nos.4/5, pp. 434-456, 1998.

[16] Brüne, B.; Knethen, A.; Sandau, K. B.; ''$NO^{\cdot}$ and its role in apoptosis'', Euoropean of Pharmacology, Vol. 351, 261-272,1998.

[17] Burney, S.; Caulfield, J. L. ; Niles, J. C.; ''The chemistry of DNA damage from $NO^{\cdot}$ and $ONOO^{-}$'', Mutation Research, Vol. 424, pp. 37-49, 1999.

[18] Janero, D.; '' $NO^{\cdot}$ -related pharmaceuticals: Contemporary approaches to therapeutic no modulation'', Free Radical Biology & Medicine, Vol.28, No.10, pp 1495-1506, 2000.

[19] Halliwell, B.; Gutteridge, John M.C.; 'Free Radicals in Biology and Medicine', Oxford University Press, 1999.

[20] Bapat, S.; Verkleij, A.; Post J. A.; '' $ONOO^{-}$ activates mitogen-activated protein kinase (MAPK) via a MEK-independent pathway: a role for protein kinase'', FEBS Letters, Vol.499, pp.21-26, 2001.

[21] Schroeter, H.; Boyd, C.; Spencer, J. P. E.; ''MAPK signaling in neurodegeneration: influences of flavonoids and $NO^{\cdot}$'', Neurobiology of Aging, Vol. 23, pp. 861-880,2002.

[22] Mondoro, T. H.; Shafer, B. C.; '' $ONOO^{-}$-induced tyrosine nitration and phosphorylation in human platelets'', Free Radical Biology & Medicine, Vol.22, No.6, pp. 1055-1063, 1997.

[23] MacMillan-Crow, L. A.; Greendofer, J. S.; Vickers, S. M.; ''Tyrosine nitration of c-SRC tyrosine kinase in human pancreatic ductal adenocarcinoma'', Archives of Biochemistry and Biophysics, Vol. 377, No. 2, pp.

[24] Chang, L.; Karin, M.; ‘’ Mammalian MAP kinase signalling cascades’’, Nature, Vol. 410, pp. 37-40,2001

[25] Deak, M.; Clifton, A.D.; Lucocq, L.M.; Alessi D.R.; ‘’Mitogen and stress-activated protein kinase-1 (MSK 1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB’’, EMBO J., Vol. 17, No.15, pp. 4426-4441, 1998.

[26] Shaffer, H.F.; Weber, M.J.; ‘’Mitogen-activated protein kinase: specific messages from ubiquitous messengers’’, Molecular Cell Biology, Vol. 19, pp. 2435-2444, 1999.

[27] So, H.S.; Park, R.K., Kim M.S.; ‘$NO^{\cdot}$ inhibits c-Jun N terminal kinase 2 (JNK2) via S-nitrosylation, ‘’Biochem. Biophys Res Commun, Vol. 247, No. 3, pp, 809-813, 1998.

[28] Soleas, G.J.; Grass, L.; Josephy, P.D.; Goldberg, D.M.; ‘’ A comparison of the anticarcinogenic properties of four red wine polyphenols’’, Clinival Biochemistry, Vol.35, pp, 119-124, 2002.

[29] Ratna, W.N.; Simonelli, J.A.; ‘’ The action of dietary phytochemicals quercetin, catechin, resveratrol and naringenin on estrogen-mediated gene expression’’, Life Sciences, Vol. 70, pp. 1557-1589, 2002.

[30] Galati, G.; Sebzevari, O.; Wilson, J.X.; ‘Prooxidant activity and cellular effects of the phenoxyl radicals dietary flavonoids and other polyphenolics’’, Toxicology, Vol. 177, pp. 91-104, 2002.

[31] Ishige, K.; Schubert, D.; Sagara, Y.; ‘’ Flavonoids Protect Neuronal cells from oxidative stress by three distinct mechanisms’’, Free Radical Biology & Medicine, Vol. 30, No. 5, pp. 433-446, 2001.

[32] Meβmer, U.K., Ankarcrona, M., Nicotera, P.; ‘’ p53 expression in $NO^{\cdot}$-induced apoptosis’’, FEBS Letter, Vol. 355, pp.23-26,1994.

[33] Lau, H.K.F.; ‘’ Cytotoxicity of $NO^{\cdot}$ donors in smooth muscle cells is dependent on phenotype, and mainly due to apoptosis’’, Atherosclerosis, Vol. 166, pp. 223-232, 2003.

[34] Meβmer, U.K., Brüne, B.; ‘’$NO^{\cdot}$ induced apoptosis: p53-dependent and p53-independent signalling pathways’’, Biochemistry Journals, Vol. 319, pp. 299-305, 1996.

[35] Verstraeten, S.V.; Keen, C.L., Schmitz, H.H.; ‘’ Flavan-3-ols and procyanidins protect liposomes against lipid oxidation and distruption of the bilayer structure’’, Free Radical Biology & Medicine, Vol.22, No.6, pp. 1055-1063, 1997.