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Role of mitochondria in neuronal cell death induced by oxidative stress: Neuroprotection by coenzyme Q(10).

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ROLE OF MITOCHONDRIA IN NEURONAL CELL DEATH

INDUCED BY OXIDATIVE STRESS; NEUROPROTECTION

BY COENZYME Q_{10}.

By Mallika Somayajulu

A Thesis Submitted to the Faculty of Graduate Studies and Research through the

Department of Chemistry and Biochemistry in Partial

Fulfillment of the Requirements for

The Degree of Master of Science at

The University of Windsor

Windsor, Ontario, Canada

2004

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ABSTRACT

ROLE OF MITOCHONDRIA IN NEURONAL CELL DEATH INDUCED BY OXIDATIVE STRESS; NEUROPROTECTION BY COENZYME Q_{10}

In past research, oxidative stress has been implicated in aging and age related disorders. Cell death caused by oxidative stress has been shown to play an important role in neuro-degenerative diseases. We used differentiated Human Neuroblastoma (SH-SY5Y) and Teratocarcinoma (NT2N) cells as models to study the mechanism of cell death induced by oxidative stress. We observed that differentiated NT2N AND SH-SY5Y cells underwent apoptosis following oxidative stress induced by direct hydrogen peroxide treatment. Morphological, apoptotic features including nuclear condensation and membrane blebbing and biochemical changes including DNA fragmentation and caspase and proteasome activation were evident following oxidative stress. We further investigated the production of Reactive oxygen species and mitochondrial dysfunction in the cell under oxidative stress. There was an increase in total ROS produced by the cells after H_2O_2 treatment. Furthermore, there was a decrease in the mitochondrial membrane potential and an increase in mitochondrial ROS generation.

CoQ_{10} has been shown to be a potent anti-oxidant and an important component of the mitochondrial respiratory chain. In order to study the effects of CoQ_{10} on neuronal oxidative stress, we pre-treated differentiated NT2 and SH-SY5Y cells with CoQ_{10} for 24 hours prior to H_2O_2 treatment. Our results indeed indicated that pre-treatment with CoQ_{10} inhibited total ROS production and

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reduced caspase-3 activity and proteasome activity considerably. Moreover, CoQ_{10} maintained a mitochondrial membrane potential even following oxidative stress and reduced the amount of ROS produced by mitochondria. My study suggests that water soluble CoQ_{10} not only acts as an anti-oxidant but also stabilizes the mitochondrial membrane during oxidative stress. We found that CoQ_{10} also offered neuroprotection when neuronal cells were subjected to oxidative stress induced using environmental toxin paraquat. Taken together, the results of this study suggested that water soluble CoQ_{10} can prevent mitochondrial collapse and block neuronal apoptosis. Thus CoQ_{10} has a great potential for chemotherapeutic intervention in neurodegenerative diseases.

Dedicated to my parents

ACKNOWLEDGEMENTS

I wish to express my deep gratitude towards my supervisor, Dr. Siyaram Pandey, for accepting me as his student and giving me a chance to study here at the University of Windsor. I would also like to thank him for all his guidance throughout the past two years, for all the good times that we have had and for all the help and support he gave me while I was new here and learning to adjust to a new city and culture.

My sincere thanks to Dr. Lee and Dr. Zielinski for being on my committee, for all the valuable suggestions, advice, encouragement, and support.

I am very thankful to Dr. Mutus, Dr. Lee and Dr. Ananvoranich for allowing me to use some of their instrument facilities for this work. I am very grateful to Jafar, who helped me learn many of the techniques and also for sharing his knowledge and experience. I am also very thankful to Sarah for all her help and support. I would also like to thank Christopher, Danielle, Natasha, Daisy and Aubri. Thanks to Yit, for all her help and support. I am also thankful to Paul for all his help and suggestions. I would also like to thank Monique, Arun, Shane, Ju, Dana, Eck, Jiyun, Anupama, Ritu, Ahmed and Tom for their kindness and assistance.

I would take this opportunity to thank my friends Christina, Inga, and Fatema for all the care, encouragement, help and support especially when I miss home and get cranky. I am also very thankful to all the people in the chemistry

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and biochemistry graduate office for all their help and the University of Windsor for the financial support throughout the past two years.

Finally, I want to thank my family for their constant encouragement and support.

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LIST OF ABBREVIATIONS

AMC	amino methyl coumarin
ANT	adenosine nucleotide transporter
araC	cytosine arabinoside
BSA	bovine serum albumin
CARD	caspase recruitment domain
CAT	catalase
CK	creatine kinase
DCFDA	2', 7'- dichlorofluorescein diacetate
DD	death domain
DMEM	Dulbeco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribo nucleic acid
DTNB	5, 5'-dithio-bis (2-nitrobenzoic acid)
EDTA	ethylenediaminetetra-acetic acid disodium salt
EGTA	ethylene glycol-bis (β-amino methyl ether) N, N, N', N' tetra acetic acid
FAK	focal adhesion kinase
FBS	fetal bovine serum
FrdU	fluorodeoxyuridine
GPx	glutathione peroxidase
GSH	reduced glutathione
H_2O_2	hydrogen peroxide
hBDNF	human brain derived neurotrophic factor
HK	hexokinase
HRP	horse radish peroxidase
ICAD	inhibitor of caspase activated DNase
LMP Agarose	low melting point agarose
MDA	Malonaldehyde
MMP	mitochondrial membrane permeability
MPP^+	N methyl, 4-phenyl pyridinium
mRNA	messenger RNA
NO	nitric acid
NT2	human teratocarcinoma
NT2A	differented human astrocytes
NT2N	differentiated human neurons
O_2^{*-}	superoxide anion radical
PBS	phosphate buffer saline
PHPA	para-phenyl acetic acid
PTP	permeability transition pore
ROS	reactive oxygen species
SH-SY5Y	human neuroblastoma
SOD	superoxide dismutase
TNF	tumour necrosis factor

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Urd	uridine
VDAC	voltage dependent anionic channel
$\Delta\Psi_m$	mitochondrial membrane potential

INTRODUCTION

Excessive loss of neuronal cells due to programmed cell death has been reported in several neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, ischemia and amyotrophic lateral sclerosis (Beal 1999, Behl 1999, Fiskum *et al.*, 1999, Piantadosi *et al.*, 1996, Coyle *et al.*, 1993, Siesjo et al., 1993, Traystman *et al.*, 1991). In most of these diseases, oxidative stress has been implicated to cause apoptosis. Neuronal cells are very sensitive to free radicals partly due to the high rate of oxygen consumption. Neurons are post mitotic tissues and hence once lost, cannot be re generated. A severe loss in neurons could lead to impaired brain function and affect memory and cognition.

1.1 Apoptosis

Cell death plays a significant role not only in the development of multi-cellular organisms, but also in maintaining tissue homeostasis. Kerr et al. proposed that there are two distinct cell death pathways that cells may undergo: one pathological and the other physiological. They recognized these two distinct morphological forms of cell death and coined the terms 'necrosis' for the accidental cell death and 'apoptosis' for the programmed cell death (Kerr *et al.*, 1972). Necrotic cell death is an uncontrolled process, which may be induced by factors such as physical damage, injury, toxic agents, extreme heat and pressure. Some of the prominent morphological features of necrosis include an increase in cell volume, causing cells to rupture and releasing cellular contents such as enzymes into the intercellular milieu (Gores *et al.*, 1990).

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This can further cause tissue damage by affecting neighbouring cells or by attracting proinflammatory cells to the affected area and causing necrotic cells to be engulfed (Haslett 1992). The biochemical characteristics of necrosis are random degradation of DNA and random proteolysis of proteins.

Apoptosis differs from necrosis in that it is a highly orchestrated form of cell death (**Figure 1**). Physiological stimuli such as lack of growth factor and hormones, or oxidative stress DNA damaging agents (such as ultra-violet radiation), chemotherapeutic drugs may induce the apoptotic cell death pathway. Cells have a withered and shrunken appearance and they do not adhere to the neighbouring cells. Nuclear condensation is simultaneously followed by membrane blebbing and formation of apoptotic bodies occurs. Neighbouring cells then phagocytose these apoptotic cells. As the plasma membrane remains intact when the cell dies, there is no inflammation. DNA degradation of cells undergoing apoptosis also tends to be very systematic. In addition, there is a rapid alteration in the organization of phospholipids in most cell types leading to the exposure of phosphatidyl serine on the cell surface.

Apoptosis plays an important role in embryo development and in the adult animal during tissue turnover or at the end of an immune response (Ashkenazi et al., 1998). It also acts as a safe guard against growth of cells carrying mutations and which are potentially capable of becoming cancerous. The significance of apoptosis in maintaining the normal functioning of the body is demonstrated when it malfunctions. If there is decreased apoptosis, it results in autoimmune diseases and cancers, causing cells to grow uncontrollably. On the other hand,

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Figure 1. The two different forms of cell death: Apoptosis and Necrosis

NECROSIS   APOPTOSIS
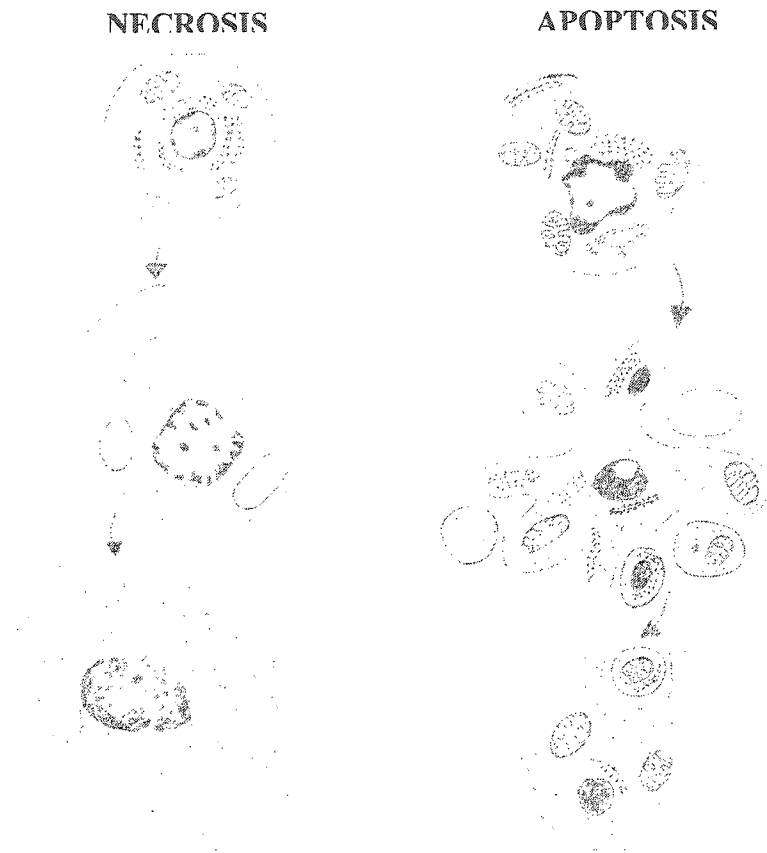
Cell death can be of two types: apoptosis and necrosis. Apoptosis is marked by nuclear condensation, cell shrinkage, membrane blebbing and formation of apoptotic bodies which are engulfed by neighbouring cells. Necrosis is accompanied by random DNA degradation and an increase in cell volume ultimately causing the cell to burst, spilling its contents into the inter-cellular milieu.

excessive cell death may result in stroke damage and neurodegenerative diseases and AIDS.

1.2. The biochemical mechanism of apoptosis

Two pathways causing apoptosis have been well established. The extrinsic pathway employs the death ligand induced activation of death receptors resulting in the activation of initiator caspases (Ashkenazi *et al.*, 1998). In the intrinsic pathway, apoptotic signals affect the mitochondria such that apoptogenic factors are released into the cytoplasm.

1.2.1. The extrinsic pathway

Death receptors are cell surface receptors belonging to the tumour necrosis factor receptor (TNF) family. Death receptors contain a homologous cytoplasmic sequence called the death domain (DD) (Tartaglia *et al.*, 1997). These DD are responsible for recruiting adapter molecules, which in turn recruit caspases to the receptor complex (**Figure 2**). In most cases, caspase-8 is recruited. Once activated, caspase-8 activates effector caspase, caspase-3. Caspase-8 can also cleave Bid, a pro-apoptotic protein, which translocates to the mitochondria and affects the mitochondrial membrane potential by having pronounced effects on the other pro-apoptotic proteins.

1.2.2. The intrinsic pathway

This pathway is also known as the mitochondrial pathway. The role of mitochondria in the activation of caspases is implicated in various studies (Zou *et al.*, 1999, Li *et al.*, 1997, Zou *et al.*, 1997). Different types of stress such as oxidative stress, DNA damage, etc. can result in the leaking of cytochrome c

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Figure 2. The extrinsic pathway in apoptosis
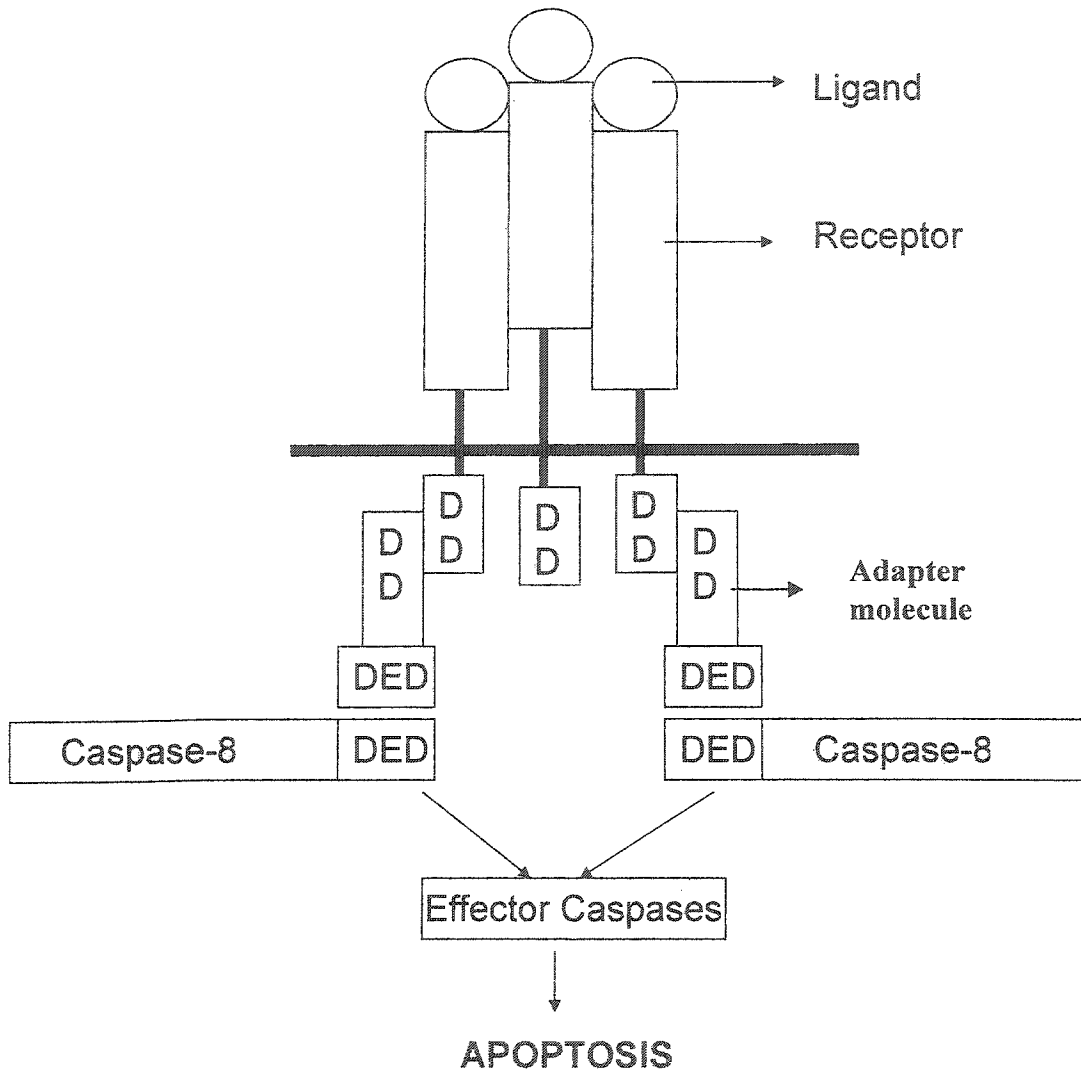
The binding to the ligands to the death receptors causes the recruitment of adapter molecules via death domains (DD). These adapters in turn recruit initiator caspases such as caspase-8 via death effector domains (DED). Upon activation of caspase-8, effector caspases like caspase-3 are activated. Activation of effector caspases ultimately leads to apoptosis.

from the inner membrane space of the mitochondria into the cytoplasm (Kroemer *et al.*, 1997). Cytochrome c binds to Apaf-1 in the presence of ATP causing a conformational change in Apaf-1 allowing it to bind to procaspase-9 via the caspase recruitment domains (CARD) present in both the molecules. Aggregation of procaspase-9 leads to auto-proteolysis and activation of downstream caspases like caspases-3 (**Figure 3**). The role of mitochondria in apoptosis is described in detail in the later section.

1.3. Proteases involved in apoptosis

There exists a special machinery to execute apoptotic cell death. The central component of this machinery is a proteolytic system called caspases. Recent studies have shown the activation of another enzyme pathway called the ubiquitin-proteasome pathway that mediates protein degradation is activated during apoptosis.

Caspases are a very important component of the apoptotic pathway. Enzymes belong to a family of cysteine specific proteases (Thornberry *et al.*, 1998). These enzymes use cysteine as the nucleophilic group for substrate cleavage and cleave peptide bonds on the carboxyl side of aspartic acid residue (Earnshaw *et al.*, 1999, Nicholson 1999, Cohen 1997, Villa *et al.*, 1997). At least 14 caspases have been identified in mammals. They are expressed as zymogens and require proteolytic processing between domains to form the mature protein. Upon initiation of an apoptotic signal, multiple caspases are activated via caspase cascades in the cell (Macfarlane *et al.*, 1997, Casciola-

Figure 3. The intrinsic pathway in apoptosis
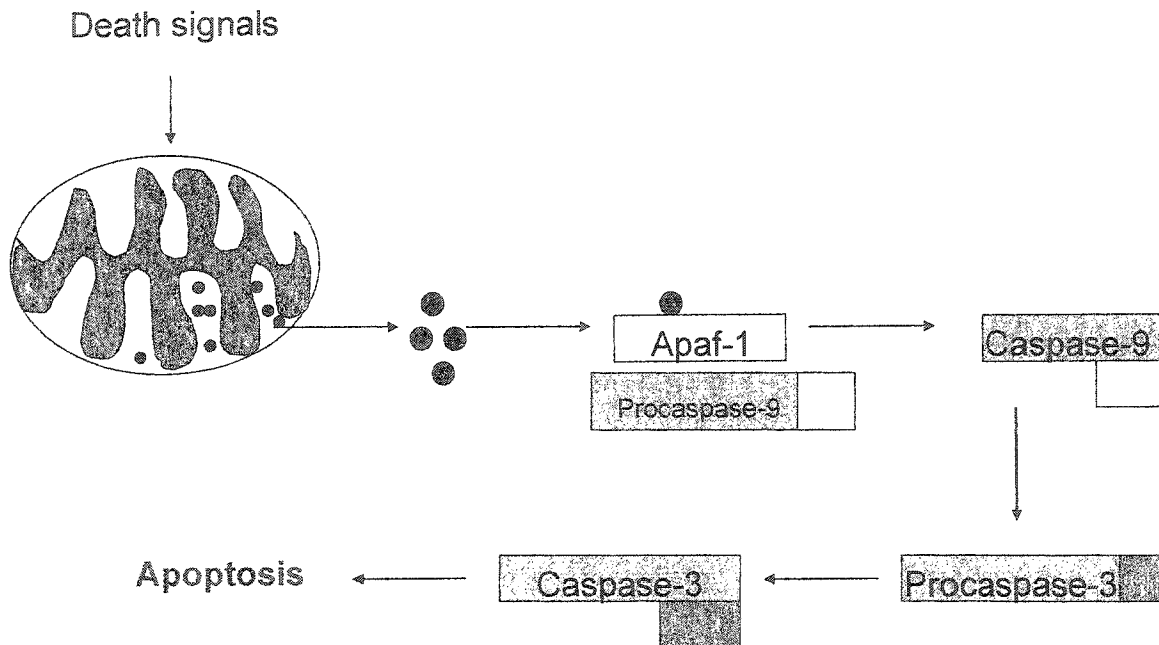
Release of apoptogenic factors such as cytochrome c from the mitochondria leads to the activation of initiator caspases. Apaf-1 undergoes a conformational change upon cytochrome c binding and binds to procaspase-9, and activates it. Caspase-9 then causes the activation of effector caspases such as caspase-3. Caspase-3 inactivates important proteins ultimately leading to the apoptosis.

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Rosen *et al.*, 1996, Casciola-Rosen *et al.*, 1994). Caspases can be divided into two categories depending on their order of activation. The caspases that are activated first in the cascade are called initiator caspases such as caspase-8. Upon activation, these initiator caspases activate the effector caspases like caspase-3 that execute the apoptotic program.

One of the major roles of caspases is to inactivate the proteins that protect cells from apoptosis like the cleavage of the inhibitor of caspase activated DNase (ICAD) (Enari *et al.*, 1998, Liu *et al.*, 1997). ICAD is an inhibitor of a nuclease responsible for DNA degradation called Caspase activated DNase (CAD). Cleavage of ICAD results in the activation of CAD, which enters the nucleus and degrades the DNA. Some of the other functions of caspases include disassembly of cell structures like destruction of nuclear lamina (Takahashi *et al.*, 1996, Orth *et al.*, 1996), reorganizing proteins such as gelosin (Kothakota *et al.*, 1997), focal adhesion kinase (FAK) (Wen *et al.*, 1997), etc. which are involved in cytoskeleton leading to deregulation of their activity, inactivation of proteins involved in DNA repair and replication and mRNA splicing (Cryns *et al.*, 1998, Enari *et al.*, 1998).

Recent studies have shown protein degradation by the ubiquitin-proteasome pathway during apoptosis. The proteasome pathway is involved in the general degradation of damaged, non-functional and unwanted proteins in the cells (Rodgers *et al.*, 2002). Activation of proteasome has been observed during serum deprivation-induced apoptosis (Pandey *et al.*, 2003).

1.4. Role of mitochondria in apoptosis

Mitochondria play a very important role in apoptosis (Desagher *et al.*, 2000 Green *et al.*, 1998). Mitochondria control apoptosis at various levels, including maintenance of ATP production (Leist *et al.*, 1997), mitochondrial membrane potential and mitochondrial membrane permeability for the release of apoptogenic factors from the inter membrane space of the mitochondria into the cytosol (Kroemer *et al.*, 2000, Vayssière *et al.*, 1994, Zoratti *et al.*, 1995). During apoptosis, the changes in the mitochondrial membrane potential have been associated with the opening of the mitochondrial permeability transition pore (MPTP) (Halestrap *et al.*, 2000, Lemasters *et al.*, 1998).

The permeability transition pore is a large multi protein complex that spans the mitochondrial membranes, creating a channel between the cytosol and the mitochondrial matrix (Harris *et al.*, 2000, Zoratti *et al.*, 1995). The components of this channel include a voltage dependent anionic channel (VDAC), adenosine nucleotide transporter (ANT), the benzodiazapine receptor, hexokinase (HK), creatine kinase (CK) and cyclophilin D (Zoratti *et al.*, 1995) (**Figure 4**). The opening of the permeability transition pore (PTP) depends on the mitochondrial membrane potential and the binding of different molecules. Marked increase in the intra mitochondrial Ca^{+2} levels, increased oxidative radical levels, partial failure of respiratory complexes, either individually or together can produce a fall in mitochondrial membrane potential ($\Delta\Psi_m$) (Richter 1993).

The opening of the PTP dissipates remaining proton gradient across the mitochondrial membrane and further reduces $\Delta\Psi_m$. Several factors have been

Figure 4. Components of the permeability transition pore
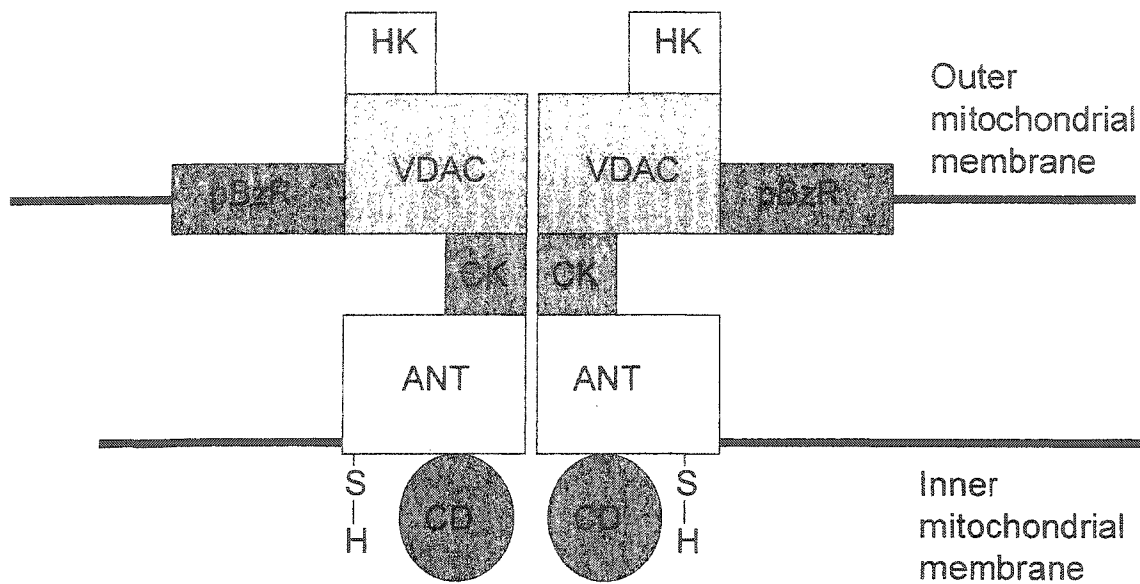
The permeability transition pore is a channel between the cytosol and the mitochondrial matrix. The components of this channel include a voltage dependent anionic channel (VDAC), adenosine nucleotide transporter (ANT), the benzodiazapine receptor, hexokinase (HK), creatine kinase (CK) and cyclophilin D. The opening of the pore causes a loss in the potential and allows the release of apoptogenic substances from the mitochondria into the cytoplasm.

shown to influence the opening or the closure of PTP. Pore opening is favoured

by events which include cross linking of protein thiols by oxidative agents, high

levels of intracellular Ca^{+2}, release of glutathione from the mitochondria (Hirsch *et*

al., 1997) and increased ROS levels in the mitochondrial matrix (Chernyak *et al.*,

1996). However, binding of Bcl-2 to the PTP favours PTP closure.

1.5. The Bcl-2 family: activators and inhibitors of apoptosis

The Bcl-2 family of proteins plays an important role in regulating apoptosis. This

family of proteins can be divided into two functional groups: the anti-apoptotic

(Bcl-2, Bcl-xl, Bcl-w) and pro-apoptotic group (Bax, Bid, Bak, Bim). One of the

key features of this family is that the members share sequence homology (Hunter

et al., 1996, Chittenden *et al.*, 1995, Yin *et al.*, 1994). Upon apoptotic stimuli,

many pro-apoptotic proteins tanslocate from the cytoplasm to the mitochondria

(Gross *et al.*, 1999, Huang *et al.*, 1998), undergo a conformational change and

oligomerize within the mitochondrial membranes (Leist *et al.*, 2001, Gross *et al.*,

1999, Goping *et al.*, 1998). The pro and anti-apoptotic proteins can interact with

each other to form homodimers and heterodimers. The anti-apoptotic members

of the Bcl-2 family can inhibit the translocation and homodimerization of pro-

apoptotic Bcl-2 family members. For example Bcl-2 forms heterodimers with Bax,

preventing the formation of Bax: Bax homodimers thereby interfering with the

ability of Bax to increase MMP (Benedict *et al.*, 2000). The anti-apoptotic

members of the Bcl-2 family shut off the apoptotic signal cascade upstream of

effector caspase activation by preventing the release of cytochrome c from the

mitochondria (Boulakia *et al.*, 1996, Chinnaiyan *et al.*, 1996, Shimizu *et al.*, A, 1996, Shimizu *et al.*, B, 1996).

1.6. Oxidative stress

Oxidative stress refers to the undue oxidation of biomolecules leading to cellular damage, and it is carried out by reactive oxygen species (ROS). Various neurodegenerative disorders and syndromes are associated with oxidative stress (Behl 2002).

ROS include a number of reactive molecules derived from oxygen and are generated during normal metabolic processes and in response to various stimuli (Fleury *et al.*, 2002, Frodovich 1978). Mitochondria are the major source of ROS in the cell. Levels of ROS generated during 'leaky' mitochondrial respiration are not negligible: the amount of H_2O_2 and other ROS produced by brain mitochondria can reach 5% of O_2 metabolized (Arnaiz *et al.*, 1999, Chance *et al.*, 1979).The mitochondrial electron transport chain produces ROS at Complex-I and Complex- III.

Much o f the d ata a re in a greement w ith t he h ypothesis t hat i ncrease i n ROS is a consequence of the impairment of mitochondrial respiratory chain (Fleury *et al.*, 2002). Stepwise reduction of molecular oxygen occurs via one electron transfer leading to the formation of ROS like superoxide and hydrogen peroxide (see below). H_2O_2 is not a free radical but can penetrate cell membranes making it very toxic to the cell. It acts as an intracellular signaling molecule (Rhee 1999, Sunderesan *et al.*, 1995). H_2O_2 can give rise to an OH *

radical which is probably capable of causing more damage than any other ROS (Betteridge 2000, Halliwell 1987). The superoxide radical can also react with nitric oxide (NO) to form peroxynitrite, which is highly reactive and cytotoxic.

$$O_2 \xrightarrow{e^-} O_2^{\cdot -} \xrightarrow[2H^+]{e^-} H_2O_2 \xrightarrow{e^-} {}^\cdot OH + OH^- \xrightarrow[2H^+]{e^-} 2H_2O$$
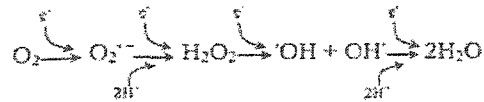
ROS can react with different biomolecules like proteins, DNA and lipids. *In vitro* studies have revealed that ROS can react with several amino acid residues leading to less active enzymes and malfunction of proteins (Butterfield *et al.*, 1998, Steinberg 1997). ROS can cause modifications in DNA leading to mutagenesis. Studies have shown a high prevalence of cancer in individuals exposed to oxidative stress (Marnett 2000, Mates *et al.*, 1999). ROS has also been shown to target mitochondrial DNA (Shoji *et al.*, 1995). In many cases apoptosis is mediated by ROS (Kamata *et al.*, 1999). Recent studies have indicated that ROS could be responsible for decrease in Bcl-xl mRNA (Carmody *et al.*, 2000). Moreover ROS can also lead to the activation of cell death mediators (Burdon 1996, Burdon 1995) and also cause lipid peroxidation (Butterfield *et al.*, 1998, Steinberg 1997).

1.7. Oxidative stress by neurotoxins

Paraquat (1, 1'- dimethyl-4, 4' bipyridinium) is a non selective herbicide widely used for broadleaf weed control (Suntres 2002) has been found to selectively kill nigrostriatal dopaminergic neurons in animal models. Paraquat is

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very toxic and can be lethal to both animals and humans upon acute exposure (Onyon *et al.*, 1987). The structure of paraquat resembles closely to the structure of N Methyl, 4-phenyl pyridinium (MPP$^+$), which is a dopaminergic neurotoxin (Shimizu *et al.*, 2001). Due to the similarity in structure with MPP$^+$ (**Figure 5**), there may be a possible role of paraquat exposure in the development of neurodegenerative disease (Corasoniti *et al.*, 1998). Moreover, studies have revealed a strong co relation between the amount of paraquat used and Parkinson's disease (Liou *et al.*, 1997, Liou *et al.*, 1996, Morano *et al.*, 1994). Though research has shown that paraquat can cause oxidative stress, the mechanism by which paraquat induces cell death is still not clearly understood. Further studies are essential in order to determine the mechanism of paraquat induced cell death.

1.8. Anti-oxidants

Oxidative metabolism in brain tissue as in all cells occurs in mitochondria. During oxidative phosphorylation, a finite amount of O_2 is left partially reduced as the superoxide anion (O_2^{*-}). ROS emanating from within the cell can threaten homeostasis if they are not appropriately resolved. Local levels of ROS at any given time will depend not only on the rate of generation, but also on the efficacy of the antioxidant network. Neuroprotective antioxidants are considered a promising approach to slowing the progression and limiting the extent of neuronal cell loss in neurodegenerative disorders.

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Figure 5. Structural similarities between paraquat and MPP$^+$

Paraquat

MPP$^+$
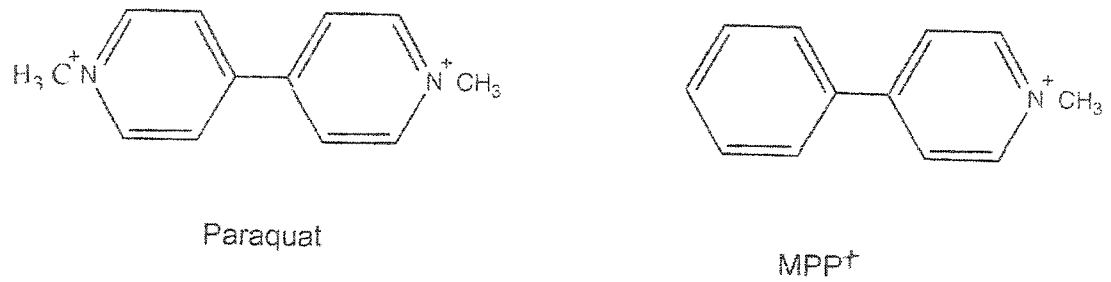
The structure of paraquat resembles that of N methyl, 4-phenyl pyridinium (MPP$^+$). MPP$^+$ is a dopaminergic neurotoxin. Paraquat exposure has been linked to Parkinson's disease.

Under normal physiological conditions, free radicals are countered by various antioxidant defenses; enzymatic and non-enzymatic. Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) are among the first line of defense against oxygen toxicity. SOD converts O_2^*- to H_2O_2, which is further converted to H_2O with the help of GPx and CAT. SOD inhibits

*OH production. It is considered to be the first line of defense against O_2 toxicity (Ray et al., 2002).

The non-enzymatic antioxidants like glutathione (GSH) are essential for the cellular detoxification of reactive oxygen species in brain cells. A compromised GSH system in the brain has been connected with the oxidative stress (Dringen et al., 2003). As an antioxidant, GSH metabolizes ROS and peroxides primarily by serving as a cofactor for GSH-dependent enzymes such as GSH peroxidase. In addition, antioxidants such as the lipophilic free radical scavenger α-tocopherol (vitamin E), or the hydrophilic compound ascorbate (vitamin C), the two most prominent antioxidants of their class, can directly interact with ROS at the molecular level (Finkel et al., 2000, Halliwell 1996, Sies 1993). Studies in perfused rat liver (Valls et al., 1994) and isolated rat hepatocytes (Beyer et al., 1996) have shown that exogenously added CoQ_{10} can act as an antioxidant.

1.8.1. CoQ_{10}

CoQ_{10} or ubiquinone (2,3 di methoxy-5methyl 6 multi-prenyl-1, 4, benzoquinone) exerts its main natural function in mitochondria as a part of the

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electron transport chain, but is also present in low concentrations in plasma and in cell membranes where it functions as an antioxidant by preventing lipid peroxidation (Ernster *et al.*, 1995). It is a highly lipophilic compound which carries electrons from complex-I and complex-II to complex-III. CoQ_{10} can accept one electron and get converted to an intermediate semi-ubiquinone which then can accept one electron to get converted to its reduced form called ubiquinol (**Figure 6**). However, due to its insoluble nature, to study the mechanism by which CoQ_{10} offers protection is difficult. Recently, a water soluble formulation of CoQ_{10} was prepared by using a patented protocol, at the National Research Council, Ottawa, by Dr. Marianna Sikorska and Dr. Henryk Borowy-Borowski. The water soluble formulation is readily taken up by cells in culture thereby enabling further studies to exemplify its anti-oxidant capabilities.

1.9. Cell lines

It is difficult to study the biochemical mechanisms of neurodegenerative diseases due to the lack of availability of human neurons because neurons are post-mitotic tissues. Human teratocarcinoma (NT2N) and Human Neuroblastoma (SH-SY5Y) cells have been used as neuronal models to study neuronal functions.

1.9.1. Human teratocarcinoma cells

NT2 cells can be differentiated into neuronal phenotypes by exposing them with retinoic acid. Upon the exposure to RA, the use of differential adhesion matrices

Figure 6. The different forms of CoQ$_{10}$

Ubiquinone (Q)
(fully oxidized)

$H^+ + e$

Semiquinone radical
(QH$^\cdot$)

$H^+ + e$

Ubiquinol (QH$_2$)
(fully reduced)
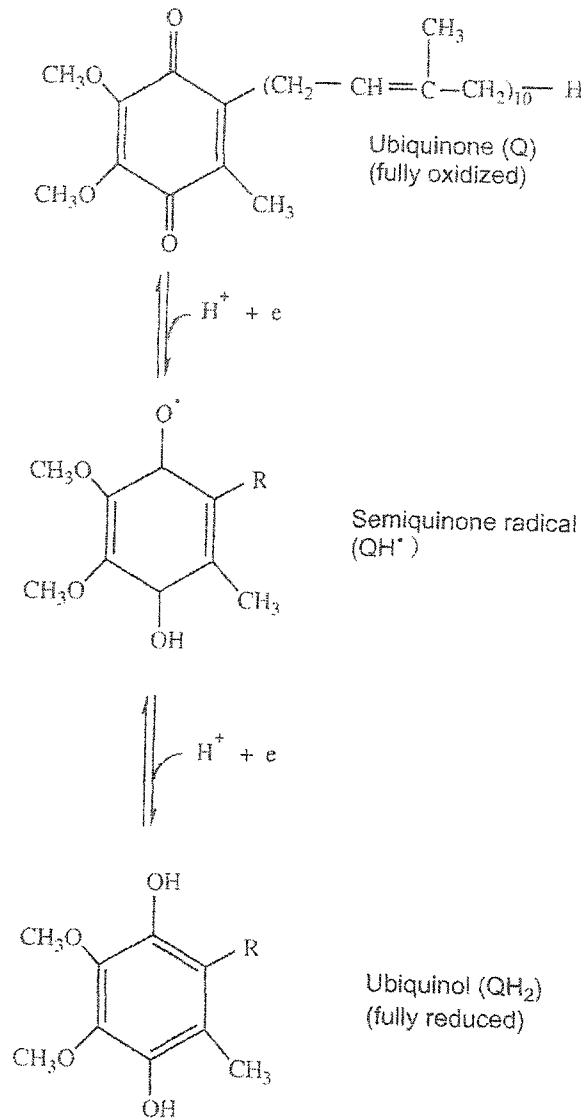
Oxidized CoQ$_{10}$ or ubiquinone can accept one electron and form an intermediate semiubiquinone radical. This radical can then accept one more electron to form the reduced form also known as ubiquinol.

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and mitotic inhibitors, the cells develop morphological and cytoskeletal characteristics of postmitotic central nervous system neurons. They resemble human primary neurons and like them, elaborate processes that differentiate into dendrites (Pleasure et al., 1992, Pleasure et al., 1993). Commitment of NT2 cells to a stable neuronal phenotype is irreversible.

1.9.2. Human neuroblastoma cells (SH-SY5Y)

SH-SY5Y cells can be differentiated into human neuron like cells by treating them with retinoic acid and are dependent on brain derived neurotrophic factor (BDNF).This treatment with RA and BDNF causes expression of neuron specific markers like neurofilaments and withdrawal from cell cycle differentiation (Encinas et al., 2000). Differentiation of neuroblasts occurs once they are arrested in the G_0 phase of the cell cycle (Encinas et al., 2000). Removal of BDNF makes the cells to re-enter S phase, leading to apoptosis due to unscheduled entry into the S phase (Encinas et al., 2000).

Objectives

1. To differentiate NT2 and SH-SY5Y cells into neuronal cells in order to use them as a model to study oxidative stress induced neuronal cell death.

2. To investigate if direct oxidative stress causes apoptotic cell death.

3. To examine if water soluble CoQ_{10} can offer neuroprotection against oxidative stress and prevent apoptosis.

4. To study the mechanism of neuroprotection by CoQ_{10}:

   1. As an antioxidant

   2. Restoring mitochondrial function.

5. To study the effect of environmental toxin: paraquat, an herbicide on neuronal cells and determine if water soluble CoQ_{10} can offer protection.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemical and supplies

2.1.1. Cell Lines

Human Neuroblastoma (SH-SY5Y) cells were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA. The Human Teratocarcinoma (NT2) cells were purchased from Stratagene cloning systems, La Jolla, CA, USA.

2.1.2. Media

DMEM F12, L-Glutamine and Gentamycin were purchased from Gibco BRL, VWR, Mississauga, ON, Canada. Fetal bovine serum was purchased from Winsent Inc, Quebec, Canada. Matrigel matrix was purchased from Becton-Dickinson, CA, USA. Recombinant h BDNF was p urchased from Almone Labs, Israel. Uridine, fluorodeoxyuridine, cytosine arabinoside, retinoic acid and collagen were purchased from Sigma Chemical Company, Mississauga, ON, Canada.

2.1.3. Coenzyme Q_{10}

Water-soluble CoQ_{10} was formulated in the National Research Council (NRC), Ottawa, Canada by a patented protocol developed by Dr. Marianna Sikorska and Dr. Henryk Borowy-Borowski (US Patent No. 6,045, 826).

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2.1.4. Other chemicals

Most of the chemicals including ATP, BSA, CHAPS, DTT, DTNB, EDTA, EGTA, GSH, GSH Reductase, HEPES, hydrogen peroxide, HRP, luciferin-luciferase, low melting point agarose, HEPES, MDA, $MgCl_2$, NADPH, Na_2EDTA, parahydroxyphenylaceticacid, paraquat, succinate, thiobarbituric acid, trichloroacetic acid and trypsin were purchased from Sigma Chemical Company, Mississauga, ON, Canada. Triton X-100 was obtained from Gibco BRL, VWR, Mississauga, ON, Canada. DMSO, NaOH, NaCl, $NaHCO_3$ and sucrose were purchased from BDH Inc., Toronto, Canada. Glycine and agarose were purchased from EM Sciences, NJ, USA. DCFDA, DEVD-AMC Hoechst, LLVY-AMC and mitotracker Red CM-H_2XRos were obtained from Molecular Probes, Eugene, OR, USA. Protein assay reagent was purchased from Bio Rad, Ontario, Canada.

2.2. Apparatus and instrumentation

Fluorescent and phase contrast pictures were taken using a fluorescent microscope (Leica DM IRB, Germany). Fluorescence measurements were conducted in multiwell plate reader with the help of Spectra Max Gemini XS (Molecular Devices, Sunnyvale, California). Absorbance was measured by a UV-Visible Spectrophotometer (Agilent Technologies). Absorbances in 96 well micro-titer plates were measured using a Bio-tek ELx 808ru Ultra Microplate Reader.

Cell culture was conducted under sterile conditions in the class-II type A/B3 Biosafety cabinet (Nuaire), and all cultures were maintained in a CO_2

incubator containing a HEPA filter (Thermo Forma). Centrifugation was done using low speed centrifuge (Jouan) and DESAGA (Sarstedt-Gruppe). Other general laboratory equipment used included the following:

A pH Meter (VWR, Model 8100) with buffer solutions from VWR, an Adventurer ^{TM} balance (OHAUS), Vortex Jr. Mixer from Scientific Industries Inc, 1296-002 DELFIA ^{R} plate shaker from Wallac, a heat block (Gibco BRL, VWR, Canada) Rocking platform model 200 from VWR, a Corning stirrer from Fisher Scientific (Toronto, Ontario), a Dounce homogenizer from Kontes Glass Company (NJ, USA), freezer vials (VWR) and Eppendorf pipettes (Fisher Scientific).

2.3. Cell Culture

2.3.1. Human Teratocarcinoma Cells

Cells were grown and differentiated using a slight modification of the manufacturer's protocol. NT2 cells were grown in D-MEM/F-12 growth medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 2 mM L-glutamine and 20 µg/mL gentamycin at 37°C at 5% CO_2. The cells were sub cultured by splitting 1:5 twice a week. For differentiation, cells were seeded in a $25m^2$ flask and treated with normal growth medium containing 10 µM retinoic acid twice weekly for 5 weeks. The cells were then sub cultured 1:3 and replated in complete D-MEM/F-12 media for one day. Next day, the cells were put in serum free media for 5 minutes and differentiated neuronal cells were mechanically dislodged by gently striking the flasks. Then the cells were replated in 60x15mm petri-plates,

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which were pre-coated with Matrigel Matrix and maintained in normal growth media containing mitotic inhibitors (1 μM cytosine arabinoside (araC), 10 μM fluorodeoxyuridine (FrdU) and 10 μM uridine (Urd)) for at least 3 weeks. Over the weeks, half of the medium was changed twice a week. Differentiated neurons (NT2N) were obtained 3-4 weeks after treatment with mitotic inhibitors. A mixed culture containing both neurons and astrocytes (NT2N/A) was obtained during some experiments.

2.3.2. Human Neuroblastoma Cells

SH-SY5Y cells were grown in Ham's F12 medium with 2 mM L-glutamine that was modified to contain 1.5 g/L sodium carbonate, 10% Fetal Bovine Serum and 10 μg/mL gentamycin at 37°C at 5% CO_2. Differentiation of these cells was carried out using a previously desribed method (Encinas *et al.*, 2000). Cells were plated in 60x15mm petri-plates, which were pre-coated with 0.05% collagen. On the day after plating the cells, *All-trans* Retinoic acid was added to a final concentration of 10 μM in F12 medium containing 15% Fetal Bovine Serum for 5 days. After 5 days in the presence of RA, the cells were washed twice with F12 medium and incubated with 50 ng/mL brain derived neurotrophic factor (BDNF) in Serum Free Media (F12 medium without FBS) for 7 days.

2.4. Experimental treatments

2.4.1. Oxidative Stress

2.4.1.1. H_2O_2 Treatment

Differentiated NT2N and SH-SY5Y cells were either pre-treated with 30 μg/mL Coenzyme Q_{10} (CoQ$_{10}$) for 24 hours prior to treatment or directly subjected to oxidative stress by incubation with 100 μM H_2O_2 for 1 hour at 37°C. Then the media was replaced with fresh complete media without H_2O_2, with or without 30 μg/mL CoQ$_{10}$ and experiments were carried out at various time intervals as indicated in the figures.

2.4.1.2. Paraquat Treatment

Differentiated SH-SY5Y cells were either pre-treated with 30 μg/mL water-soluble Coenzyme Q_{10} (CoQ$_{10}$) for 24 hours or directly subjected to paraquat treatment by incubation with 100 μM paraquat in complete media for 48 hours at 37°C.

2.5. Cellular Staining and Microscopy

In order to study the morphology, NT2N and SH-SY5Y cells were grown, differentiated and treated as mentioned above. After 6 hours of treating both NT2 and SH-SY5Y cells with H_2O_2, and 48 hours of treating SH-SY5Y cells with paraquat, the cells were stained with Hoechst 33342 (10 μM final concentration) for 10 minutes. The media containing excess Hoechst dye was removed by aspiration and replaced with 2 mL of 1X PBS. The cells were then examined under a fluorescent microscope, and phase contrast and fluorescence pictures were taken. These pictures were processed using Adobe Photoshop 7.0 software.

2.6. Comet Assay

The comet assay was performed using a slight modification of a previously described method (Gajendran et al., 2000). NT2N cells were treated with hydrogen peroxide and SH-SY5Y cells were treated with paraquat as mentioned above. After 3 hours (for H_2O_2) and 48 hours (for paraquat) of treatment respectively, the cells were incubated with 0.1% trypsin for about 5 minutes. The cells were harvested by mechanical dislodging. 10 µL of cells (about 10,000 cells) from the control and treated samples were mixed with 80 µL of warm low-melting point agarose (LMP) (0.75%, 37°C) in a microfuge tube and spread on a glass slide pre-coated with 200 µL of 0.1% agarose in such a way that half of the gel was on the coarse surface and the other half on the smooth, transparent surface (agarose gel tends to slide away from the smooth surface during processing). The slides were incubated at 4°C for about 15 minutes. Slides were then immersed immediately in a tray containing a freshly prepared cold lysis buffer (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% Triton X-100 and 10% DMSO). The slides were incubated in the dark for 1 hour at 4°C. Slides were then washed in freshly prepared alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH>13). The DNA was electrophoresed at 300 mA (0.8V/cm), washed twice in a neutralizing buffer (0.4 M Tris, pH 7.5) and stained with Hoechst 33342 (10 µM). The cells were photographed under a fluorescent microscope (Zeiss, Axiovert 200), and phase contrast and fluorescence pictures were processed using Adobe Photoshop 7.0 software.

2.7. Total Cell Lysate

The cells were harvested by mechanical dislodging using a rubber policeman. Cells were then centrifuged at 500xg for 5 minutes. The supernatant was removed and the pellet was then washed twice with 1X PBS pH 7.4 and centrifuged again at 500xg for 5 minutes. The supernatant was removed and the pellet was resuspended in 0.4 mL of extraction buffer (25 mM HEPES pH 7.25, 5 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X 100). The re suspension in the buffer was transferred into a cell homogenizer. Cells were homogenized with 20 strokes to produce the total cell lysate.

2.8. Protein Estimation

The concentration of proteins present in the total cell lysate sample was estimated using the protocol from BioRad Laboratories. The protein estimation was carried out by taking 10 µL aliquots of each total cell lysate sample, 790 µL of water and 200 µL of BioRad protein assay reagent to a total volume of 1 mL in plastic cuvettes. The mixtures were vortexed and allowed to stand for 10 minutes at room temperature. The absorbance was then taken using a UV-Visible Spectrophotometer and analyzed at 595 nm. The standard curve was prepared by using various amounts of a standard protein solution and recording the absorbance in identical condition.

2.9. Proteasome and Caspase-3 Activity

Proteasome and caspase-3 assays were performed using a previously published method (Naderi et al, 2003). Total NT2N and SH-SY5Y cell lysates were obtained as mentioned earlier. The proteasome assay was performed in a Sarstedt 96 well plate. 20 µL of total cell lysate was incubated with 50 µL LLVY-AMC fluorogenic peptide substrate in a 1X LLVYase buffer (0.1 M Tris-HCl, pH 8.0, 5 mM MgCl₂) for 1 hour at 37°C. The total volume of the reaction mixture was made to 100 µL with distilled water. After incubation for 1 hour, fluorescence was measured at an excitation and emission wavelengths of 370 nm and 440 nm respectively, using the Spectra Max Gemini XS. For the caspase-3 assay, 20 µL of total cell lysate was incubated with 200 µL DEVD-AMC fluorogenic peptide substrate in a 1X DEVDase buffer (0.1 M HEPES, pH 7.4, 2 mM DTT, 0.1% CHAPS, 1% Sucrose) for 1 hour at 37°C. After incubation for 1 hour, fluorescence was measured in a 96 well micro-titer plate at an excitation and emission wavelengths of 400 nm and 505 nm respectively, using the Spectra Max Gemini XS. The Caspase-3 and proteasome activity were expressed per microgram of protein. Protein concentration was measured using the Bio Rad protein assay reagent, and bovine serum albumin was used as a standard. Microsoft Excel version 6.0 software was used for data representation and statistical analysis.

2.10. Reduced Glutathione (GSH) Measurements

GSH assay was performed using a previously published method (Baker et al., 2003). NT2 cells were grown, differentiated and treated with 100 µM H₂O₂. GSH levels were measured in cells 24 hours after H₂O₂ treatment. The total cell

extract was obtained as mentioned above. 50 μL of the total cell lysate was incubated with 100□L of reaction mixture containing 1 mM NADPH, 100 mM Na_2HPO_4, 100 units GSH reductase and 1 mM DTNB for 5 minutes. The absorbance was measured at 412nm in a 96 well micro-titer plate using Bio-tek ELx 808ru Ultra Micro plate Reader. GSH levels were measured by running internal standards of GSH. Results were expressed per microgram of protein. Protein concentration was measured using the Bio Rad protein assay reagent and bovine serum albumin was used as a standard as mentioned above. Microsoft Excel version 6.0 software was used for data representation and statistical analysis.

2.11. Lipid Peroxidation Assay

Lipid peroxidation was performed using a previously described method (Cereser *et al.*, 2001). SH-SY5Y cells were grown, differentiated and treated for 24 hours with H_2O_2 as mentioned earlier. The total cell lysate was obtained as mentioned earlier and then centrifuged at 500xg for 5 minutes and the supernatant was removed. The pellets were re suspended in 0.25 mL of extraction buffer. 100 μL of cell membrane sample was incubated with 1ml of thiobarbituric acid on a 100°C heatblock for 15 minutes. After cooling, absorbance at 535 nm was measured using a spectrophotometer. Lipid peroxidation levels were determined running internal standards of Malonaldehyde (MDA). Results were calculated per microgram of protein. Protein concentration was measured using the Bio Rad protein assay reagent with bovine serum albumin as a standard (mentioned

earlier). Microsoft Excel 6.0 software was used for data representation and statistical analysis.

2.12. Measurement of Total ROS

Production of total ROS was measured after 6 hours and 24 hours of H_2O_2 treatment in NT2N cells, while total ROS production was monitored after 48 hours of paraquat treatment in SH-SY5Y cells. The membrane permeable dye 2', 7' - dichlorofluorescein diacetate (DCFDA) was used to detect ROS production using a modification of a previously published procedure (Siraki et al., 2002). Briefly, at various time points after treatment, the cells were incubated with DCFDA to a final concentration of 10 μM for 10 minutes at 37°C. Cells were harvested by mechanical dislodging using a rubber policeman and centrifuged at 500xg for 5 minutes. The pellet was washed and re suspended in PBS, and fluorescence (excitation 500 nm and emission at 520 nm) was measured using a Spectra Max Gemini XS multi-well plate fluorescence reader (Molecular Device, Sunnyvale, California). Protein concentration was measured using the Bio Rad protein assay r eagent a nd r esults w ere s hown p er m icrogram o f protein using bovine serum albumin as a standard. Microsoft Excel version 6.0 software was used for data representation and statistical analysis.

2.13. Measurement of ROS Production from Isolated Mitochondria

SH-SY5Y cells were grown, differentiated and treated for 24 hours with H_2O_2 as mentioned above and intact mitochondria were isolated using a previously

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published method (Li *et al.*, 2003). Cells were harvested by mechanical dislodging using a rubber policeman and centrifuged at 500xg for 5 minutes. They were washed twice with ice cold PBS and centrifuged at 250xg, the cells were re suspended in isolation buffer containing 1 mM EDTA, 10 mM HEPES (pH 7.4) and 250mM sucrose. Cells were homogenized by a Dounce homogenizer with 20 strokes. The disrupted cells were centrifuged for 5 minutes at 600xg at 4°C. The supernatant was collected and centrifuged at 15,000xg at 4°C for 5 minutes and the resulting pellet was mitochondria. The pellet was re-suspended in buffer without EDTA. Mitochondrial suspensions were kept on ice and all the experiments were performed within 5 hours.

Mitochondrial h ydrogen p eroxide w as m easured u sing p -hydroxy phenyl acetic acid (PHPA) by an increase in the fluorescence due to oxidized p-hydroxyphenylacetate fluorescence by horseradish peroxidase as previously described (Li *et al.*, 2003). 0.25 mg of mitochondrial suspension was added to a 100µl reaction mixture containing 0.25 M Sucrose, 1 mM $MgCl_2$, 10 mM HEPES 0.5 mg/mL PHPA and 0.4 units of HRP per well. 100 mM succinate was added and the contents were mixed. After 30 minutes of incubation, the fluorescence of oxidized PHPA (excitation 320 nm, emission 400 nm) was measured in a 96 well micro-titer plate using the Spectra Max Gemini XS. Mitochondrial hydrogen peroxide production was determined by interpolation from the standard curve generated by reagent hydrogen peroxide. Total mitochondrial protein was estimated using BioRad with bovine serum albumin as a standard and the results

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were expressed per microgram of protein. Microsoft Excel version 6.0 software was used for data representation and statistical analysis.

2.14. Mitochondrial Membrane Potential

NT2 and SH-SY5Y cells were grown and treated with H_2O_2 as mentioned above. Mitochondrial membrane potential was measured using Mitotracker CM-XH_2-Ros dye. The cells were treated with 0.5 μM of the dye and incubated for 15 minutes. Media containing the dye was removed and replaced by 3 mL 1X PBS. The cells were observed under a fluorescent microscope, and phase contrast and fluorescence pictures were processed using Adobe Photoshop 7.0 software.

2.15. ATP Determination

ATP was measured from isolated mitochondria. SH-SY5Y cells were grown and treated as mentioned above and intact mitochondria were isolated as mentioned previously (Li et al., 2003). Isolated mitochondria were re-suspended in a reaction mixture containing 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM HEPES and 1 mM EDTA. This suspension was briefly sonicated and centrifuged at 5000xg for 5 minutes. 100 μL of this supernatant was incubated with 100 μL luciferin-luciferase (5 mg/mL) and bioluminescence was measured using the Spectra Max Gemini XS. Mitochondrial ATP levels were determined by running internal standards. Total mitochondrial protein was estimated using Bio Rad method with bovine serum albumin as a standard and the results were expressed as ATP amount per microgram of protein. Microsoft Excel version 6.0 software was used for data representation and statistical analysis.

CHAPTER 3
RESULTS

3.1.1. Differentiation of NT2 and SH-SY5Y Cells

Human Teratocarcinoma cells and Human Neuroblastoma cells were used to study oxidative stress caused by hydrogen peroxide treatment. Previous studies have revealed that upon differentiation these cells behave similar to the neurons of the Central Nervous System (CNS) (Sandhu et al., 2003, Guillemain et al., 2000). Undifferentiated NT2 cells (**Figure 7A**) were treated with retinoic acid (RA) for 5 weeks. Cells appeared to form a dense, multilayer culture in which it is impossible to visualize any structure using phase-contrast microscopy (**Figure 7B**). After evenly replating the cells in media containing mitotic inhibitors, the cells came together to form cellular aggregates within 2 weeks. Moreover an extensive network of neurites developed and eventually formed clusters, which were connected to one another by large bundles of processes (**Figure 7C**).

Undifferentiated SH-SY5Y cells (**Figure 7D**) were treated with RA for 5 days during which some cells differentiated to a more neuronal phenotype by extending neuritic processes. Then the cells were incubated with hBDNF for 5 days. After the first day, some cells displayed neuritic processes and eventually formed aggregates (**Figure 7E**).

3.1.2. Oxidative Stress Caused by Hydrogen Peroxide Induces Apoptosis in Differentiated Neuronal Cells

Cells were grown and differentiated as described in Materials and Methods. The differentiated neuronal cells were exposed briefly to 100 μM H_2O_2 (1 hour) in complete media, and then they were washed and incubated at 37°C in complete

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Figure 7.   Morphology of NT2 cells during differentiation

A	B	C
Undifferentiated	RA treatment	Mitotic inhibitors
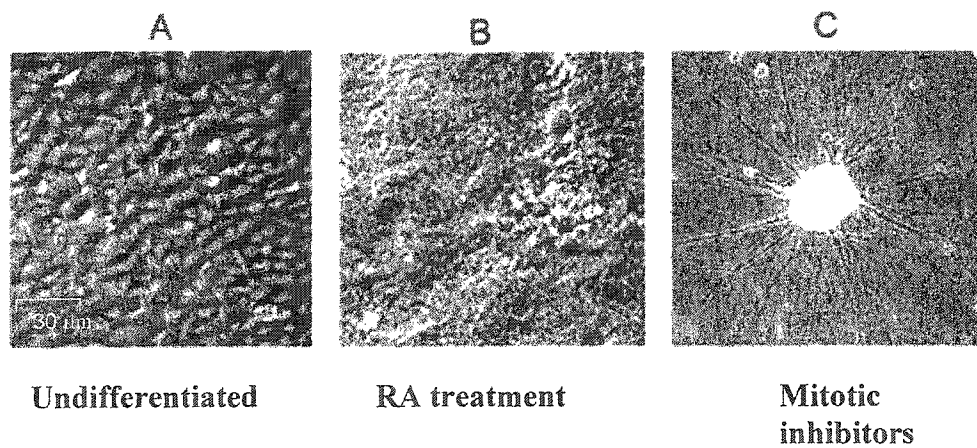
Undifferentiated NT2 cells (A) were treated with Retinoic acid (RA) for 5 weeks in order to introduce differentiation. After 5 weeks the cells were incubated with media containing mitotic inhibitors for about 3 weeks. Phase contrast pictures were taken at the end of RA treatment (B) and after 3 weeks of incubation with mitotic inhibitors (C) at 400X using a Leica DM IRB microscope.

Figure 7.   Morphology of SH-SY5Y cells during differentiation

Undifferentiated   After RA and BDNF
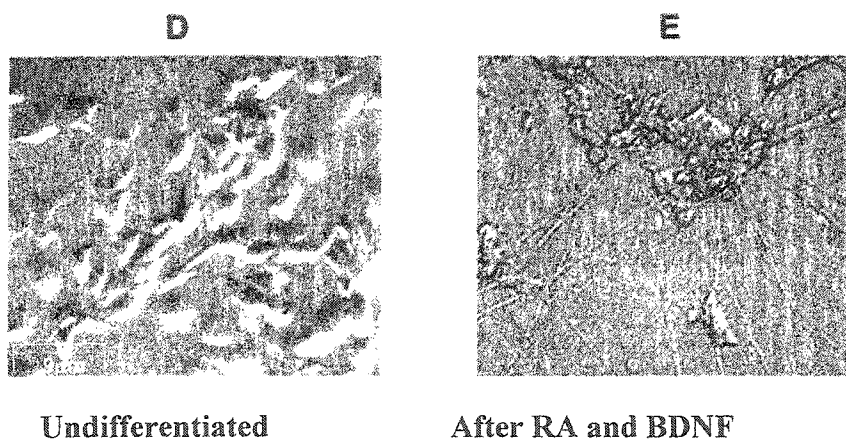
Undifferentiated SH-SY5Y cells (D) were treated with RA for 5 days and with
BDNF for 7 days. After 7 days of hBDNF treatment (E), phase contrast pictures
were taken at 400X using a Leica DM IRB microscope.

media. Various characteristics including cellular morphology and biochemical changes were monitored at various time intervals. Results (**Figure 8B**) indicated changes in morphology such as beading in the neuronal processes 6 hours after hydrogen peroxide treatment. The neurites appeared weakened, and the cells eventually lifted off from the substratum 24 hours after treatment (**Figure 8 E**). Figures **8 A & 8 D** show the morphology of differentiated neuronal cells without H_2O_2 treatment.

Nuclear condensation and DNA fragmentation are major characteristic features of apoptosis. Control and treated neuronal cells were stained with cell-permeable Hoechst dye to assess the nuclear morphology. The results shown in figure **9B & 9E** showed nuclear condensation in treated cells (**Figure 9A & 9D** represents the control cells), indicating that oxidative stress induced by H_2O_2 treatment caused apoptosis in the neuronal cells. When the numbers of apoptotic nuclei were counted for the treated and untreated cells, an increase of about 35% (in the case of NT2N cells) and 25% (in the case of SH-SY5Y cells) was observed after treatment when compared to control (**Figure 10**). In order to assess the DNA degradation during apoptosis of neurons following oxidative stress, a comet assay was performed on the treated and control NT2N cells. The cells treated with hydrogen peroxide showed comet images indicating DNA damage (**Figure 11B**), while the control cells did not show any comet images (**Figure 11A**). These results indicated that the differentiated neuronal cells undergo apoptosis displaying all the characteristic apoptotic features following hydrogen peroxide treatment.

Figure 8. Morphology of NT2N cell clusters following hydrogen peroxide treatment

| A | B | C |
| Control | H_2O_2 - CoQ_{10} | H_2O_2 + CoQ_{10} |
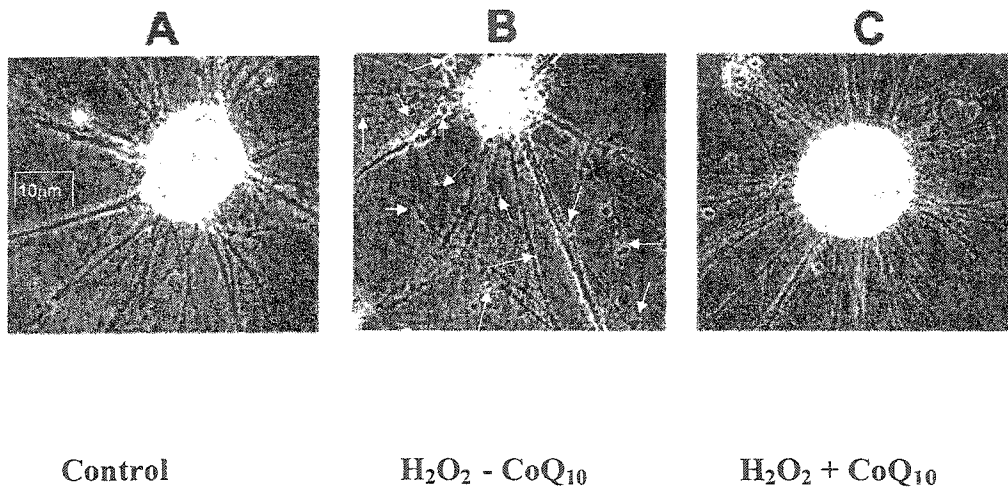
Differentiated NT2 were treated with 100 μM hydrogen peroxide in presence (C) and absence (B) of CoQ_{10} as described in the Materials and Methods. Figure A represents control cells which were not treated with hydrogen peroxide. After 6 hours of treatment, phase contrast pictures of NT2N were taken at magnifications of 400 X, using a Leica DM IRB microscope. Arrows in B indicate beading in the neuronal processes.

Figure 8. Morphology of SH-SY5Y cells following hydrogen peroxide treatment

D	E	F
Control	H_2O_2 - CoQ_{10}	H_2O_2 + CoQ_{10}
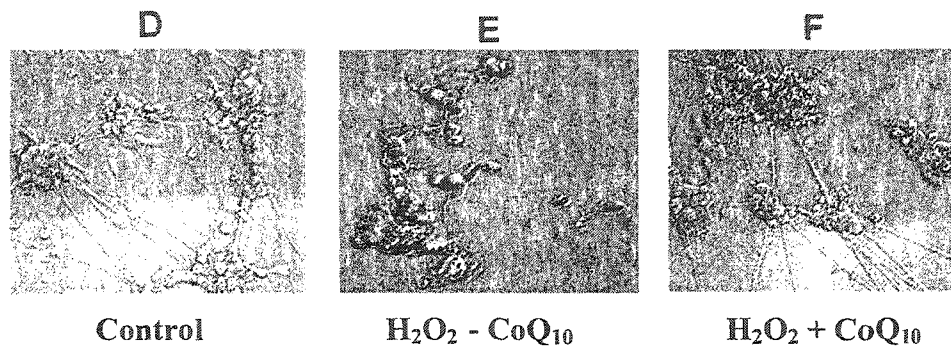
Differentiated SH-SY5Y cells were treated with 100 μM hydrogen peroxide in presence (F) and absence (E) of CoQ_{10} as described in the Materials and Methods section. Figure D represents control neuronal cells which were not treated with hydrogen peroxide. Phase contrast pictures for SH-SY5Y cells were taken 24 hours after treatment at a magnification of 400X.

Figure 9. Nuclear morphology of NT2N cells after hydrogen peroxide treatment

A	B	C
Control	H_2O_2 - CoQ_{10}	H_2O_2 + CoQ_{10}
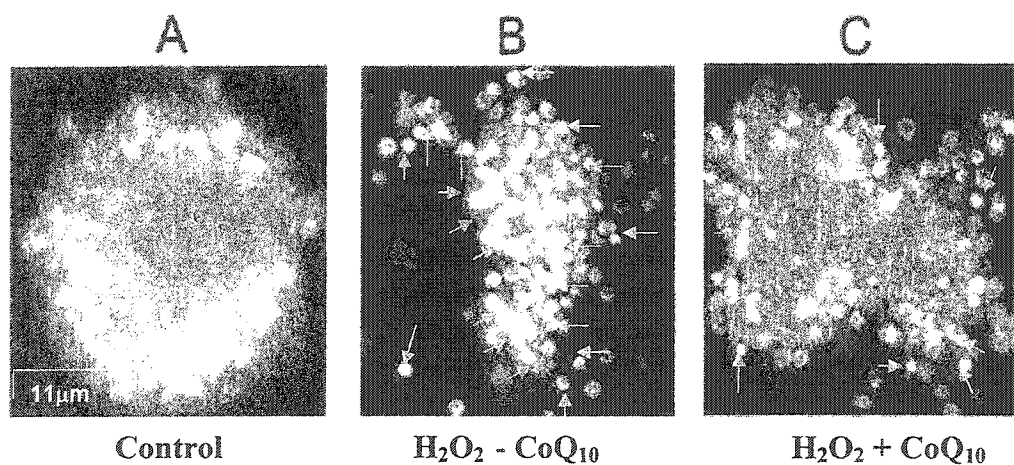
After treating NT2N cells for 6 hours with hydrogen peroxide in the presence and absence of CoQ_{10}, Hoechst 33342 dye was added to the cells to study nuclear morphology. Nuclear condensation is clearly observed in cells treated with H_2O_2, in the absence of CoQ_{10}. The arrows indicate bright, condensed and rounded nuclei (B). Fluorescent pictures were taken at magnification of 400X using Leica DM IRB microscope. Figure C indicates cells treated with H_2O_2 in the presence of CoQ_{10}, while figure A represents control cells.

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Figure 9. Nuclear morphology of SH-SY5Y cells after hydrogen peroxide treatment

D	E	F
Control	H_2O_2 - CoQ_{10}	H_2O_2 + CoQ_{10}
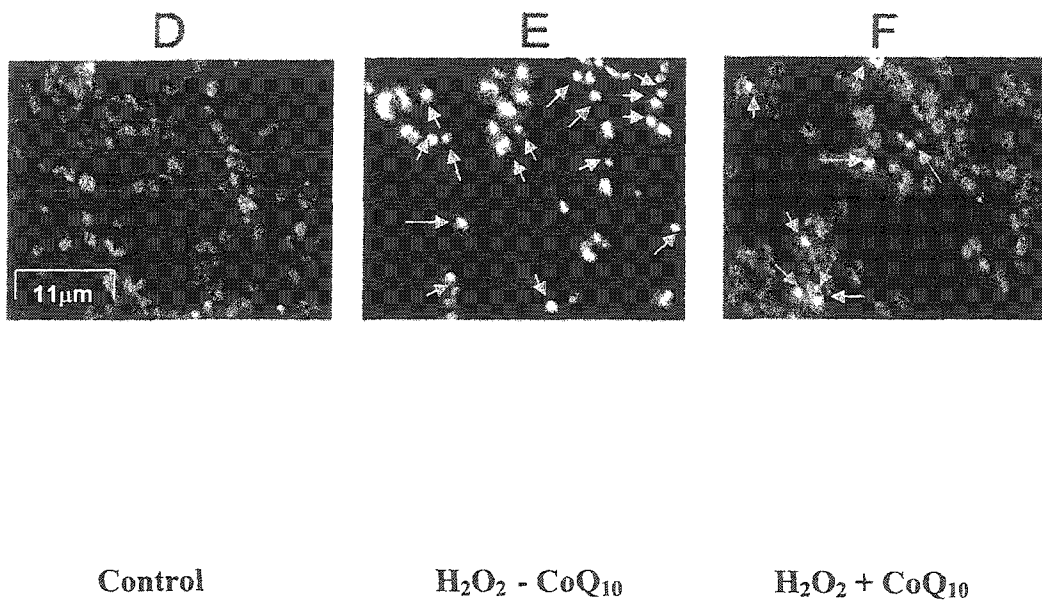
After treating SH-SY5Y cells for 24 hours, Hoechst 33342 dye was added to the cells to study nuclear morphology. Nuclear condensation is clearly observed in cells treated with H_2O_2, in the absence of CoQ_{10}. Arrows indicate bright, condensed and rounded nuclei (E). Fluorescent pictures were taken at magnification of 400X using Leica DM IRB microscope. Cells that were pre-treated with CoQ_{10} prior to H_2O_2 treatment are represented in figure F. Figure D represents control cells which were not treated with hydrogen peroxide.

Figure 10. CoQ$_{10}$ rescues cells from undergoing apoptosis after hydrogen peroxide treatment
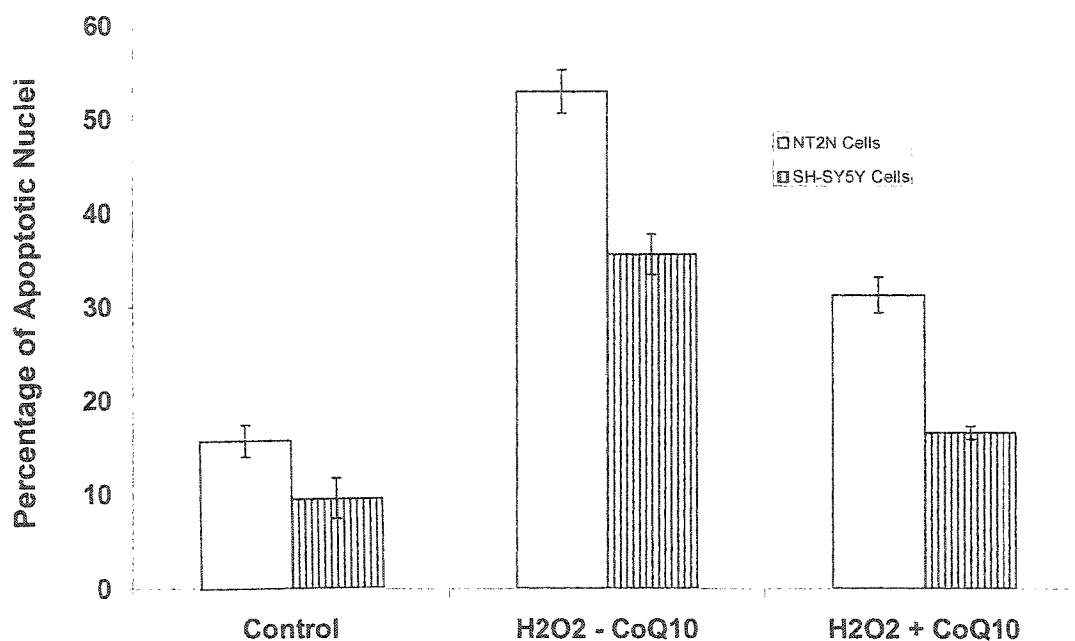
SH-SY5Y and NT2N cells were treated with 100 μM H$_2$O$_2$ for 1 hour in absence and presence of CoQ$_{10}$. After 6 hours, they were stained with Hoechst 33324 and apoptotic nuclei were counted using Leica DM IRB microscope. Standard errors were calculated using data from three sets of separate experiments. Microsoft excel version 6.0 was used for data representation and statistical analysis.

Figure 11. DNA fragmentation during apoptosis induced by hydrogen peroxide treatment

A   **B**   **C**

Control H_2O_2 - CoQ_{10} H_2O_2 + CoQ_{10}
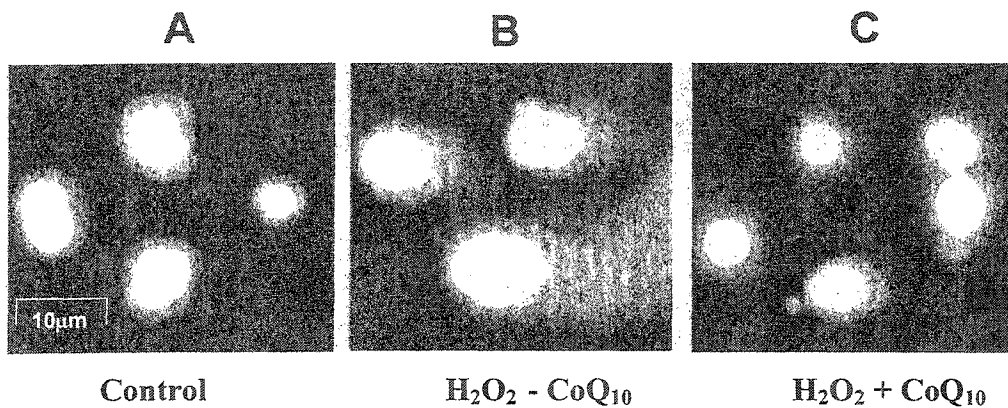
Differentiated NT2 cells were treated with 100 µM H_2O_2 in the absence (B) and presence (C) of CoQ_{10}. A comet assay was conducted 3 hours after treatment. Figure A represents the control cells. Pictures of cells were taken under fluorescent light after staining with Hoechst 33342 dye, at a magnification of 400 X, using a Leica DM IRB microscope. DNA degradation is shown by comet-like images.

3.1.3. Generation of ROS by NT2N Cells following Oxidative Stress

Several studies have shown that H_2O_2 treatment causes oxidative stress (Chang *et al.*, 2003, Ratan *et al.*, 1994, Enokido *et al.*, 1990). In order to determine whether the brief exposure to H_2O_2 caused oxidative stress on Human Teratocarcinoma (NT2N) cells, the production of reactive oxygen species was monitored during the early and late phases of apoptosis using a redox sensitive dye 2', 7'– dichlorofluorescein diacetate (DCFDA). Results shown in figure 4 indicated a considerable increase in generation of ROS at intervals of 6 hours and 24 hours after hydrogen peroxide treatment. This indicated that H_2O_2 treatment led to oxidative stress in differentiated neuronal cells (**Figure 12 A & 12 B**).

3.1.4. Neuroprotective Effect of Water Soluble Coenzyme Q_{10} on Neuronal Cells after Hydrogen Peroxide Treatment.

CoQ_{10} is not only an important component of the mitochondrial electron transport chain but is also a potent anti-oxidant. To evaluate its role in protection against oxidative stress induced by hydrogen peroxide, a water-soluble formulation containing both oxidized and reduced forms of CoQ_{10} was used (Borowy-Borowski H, US Patent No. 6,045, 826). Cells were pre-treated with CoQ_{10} 24 hours prior to inducing oxidative stress by hydrogen peroxide treatment as described in materials and methods. We observed that CoQ_{10} pre-treated cells were resistant to apoptosis following treatment. The percentage of apoptotic nuclei were less in the presence of CoQ_{10} (**Figure 9C & 9F**) as compared to

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Figure 12.A. Production of total reactive oxygen species in NT2N cells after hydrogen peroxide treatment
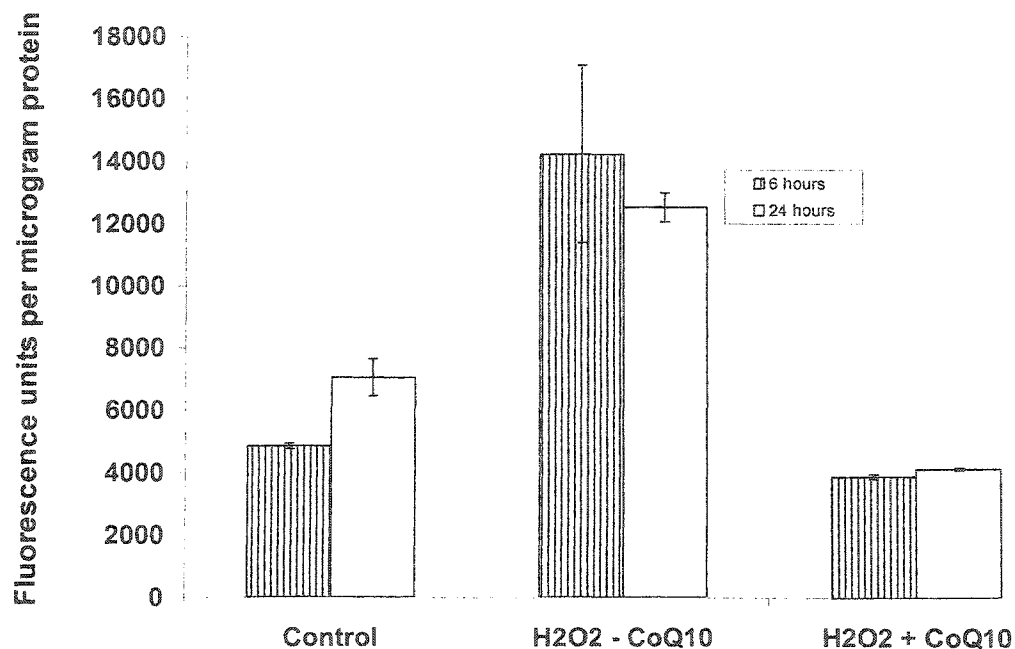
NT2N cells were treated with 100 μM H_2O_2 for 1 hour in absence or presence of CoQ_{10}, after which cells were washed with fresh media and incubated at 37°C. ROS was measured following 6 hours and 24 hours of treatment using 2', 7'-dihydrofluorescein diacetate (DCFDA) Microsoft excel version 6.0 software was used to represent data and calculate standard deviation. Standard errors were calculated using data from three sets of separate experiments.

Figure 12.B. Production of total reactive oxygen species in differentiated SH-SY5Y cells after hydrogen peroxide treatment.
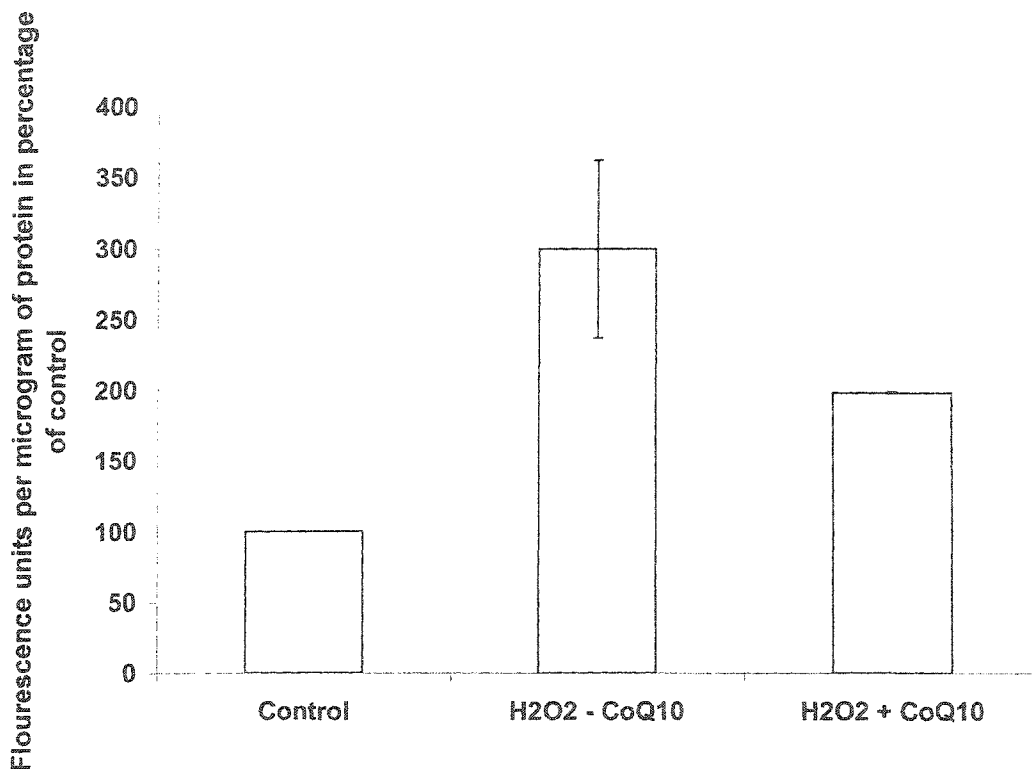
SH-SY5Y cells were treated with 100 µM H_2O_2 for 1 hour in absence or presence of CoQ_{10}, after which cells were washed with fresh media and incubated at 37°C. ROS was measured following 24 hours of treatment using 2', 7'-dihydrofluorescein diacetate (DCFDA) Microsoft excel version 6.0 software was used to represent data and calculate standard deviation. Standard errors were calculated using data from three sets of separate

those treated in the absence of CoQ_{10} (**Figure 9B, 9E**). The cells appeared to have healthier neuronal processes as compared to the cells treated with peroxide in the absence of CoQ_{10}, and apoptotic features like nuclear condensation and DNA fragmentation were considerably inhibited in CoQ_{10} pre-treated cells (**Figure 8C & 8F**) and the neuronal processes showed less beading. The percentage of cells undergoing apoptosis after hydrogen peroxide treatment was significantly reduced in CoQ_{10} pre-treated cells (**Figure 10**). Similarly, DNA degradation was inhibited by CoQ_{10} pre-treatment (**Figure 11C**).

In order to determine whether or not CoQ_{10} reduced the amount of ROS generated, NT2N cells were treated as mentioned earlier, and ROS levels were monitored 6 hours and 24 hours after treatment. Results indicated that CoQ_{10} pre-treatment was able to bring down the level of ROS at all time points after treatment. (**Figure 12A**). Similar results were seen when ROS measurements were made after SHSY-5Y cells were treated for 24 hours (**Figure 12 B**).

3.1.5. Oxidative Stress Causes an Increase in Lipid Peroxidation which is decreased in the Presence of CoQ_{10}.

An increase in lipid peroxidation production is a characteristic feature of cellular oxidative stress. Malonaldehyde (MDA), a bi-product of lipid peroxidation (LPO), is involved in DNA adduct formations, which can be responsible for carcinogenesis (Ray *et al.*, 2002).

In order to determine whether H_2O_2 treatment increased lipid peroxidation in SH-SY5Y cells, a lipid peroxidation assay was conducted. Levels of MDA were

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assessed in order to study lipid peroxidation. After 24 hours of H_2O_2 treatment, cells showed a considerable increase in lipid peroxidation production compared to control cells or cells pre-treated with CoQ_{10} (**Figure 13**). These results demonstrate that treatment with H_2O_2 induced oxidative stress on SH-SY5Y cells, resulting in an increased measurement of lipid peroxidation which was inhibited by CoQ_{10}.

3.1.6. CoQ_{10} Pre-treatment Results in Maintaining GSH Levels following Oxidative Stress

Reduced GSH has an anti-oxidative effect against oxidative stress. In cells under oxidative stress, reduced glutathione (GSH) levels are decreased. In order to determine if GSH levels change following H_2O_2 stress. NT2N cells were treated with H_2O_2 in the presence and absence of CoQ_{10}. After 24 hours of treatment, the GSH levels were monitored by using DTNB as described in Materials and Methods. Results indicated a decrease in the GSH levels after oxidative stress. However, in the cells pre-treated with CoQ_{10}, the cellular levels of GSH were higher (**Figure 14**). These results show that CoQ_{10} pre-treatment helped cells maintain the GSH levels during oxidative stress.

Figure 13. Lipid peroxidation in differentiated SH-SY5Y cells after hydrogen peroxide treatment
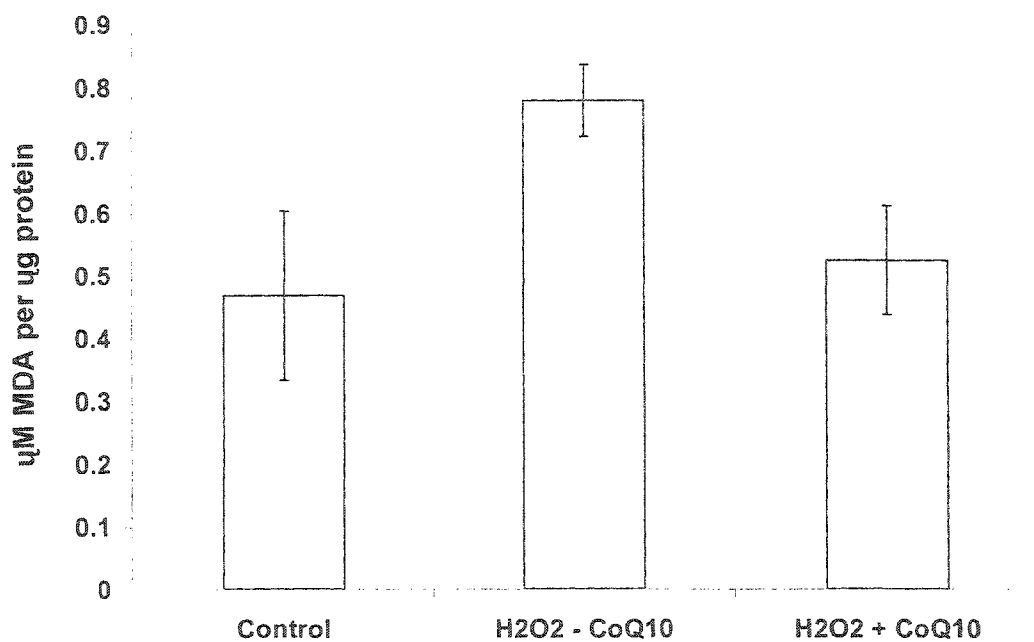
Differentiated SHSY-5Y cells were exposed to 24 hours of H_2O_2 treatment in the presence and absence of CoQ_{10}, followed by a lipid peroxidation assay. Malonaldehyde was measured using thiobarbituric acid as mentioned in Materials and Methods. Microsoft Excel Version 6.0 software was used to represent data and calculate standard deviation. Results were calculated using internal standards of Malonaldehyde and expressed per microgram of protein. Standard errors were calculated using data from three separate sets of experiments.

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Figure 14.   GSH levels in NT2N cells after hydrogen peroxide treatment
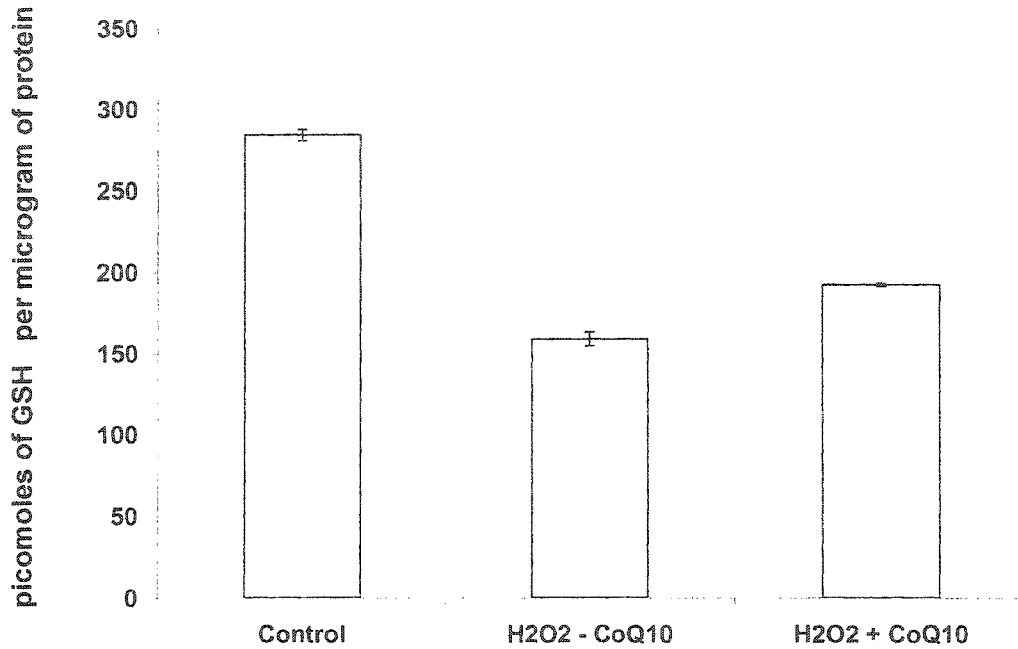
After 24 hours of H_2O_2 treatment, NT2N cells were subjected to a GSH assay as mentioned in Materials and Methods. Microsoft Excel Version 6.0 software was used to represent data and calculate standard deviation.   Results were calculated per microgram of protein and standard errors were calculated using data from three separate sets of experiments. ($p < 0.05$)

3.1.7. CoQ$_{10}$ reduces the activity of caspase-3 and proteasome

Proteases play a very essential role in apoptotic cell death. They are activated during apoptosis in many systems. Caspase-3 is a cysteine protease, generally thought to be involved in the execution phase of apoptosis. Similarly, proteasome proteases have also shown to be involved in apoptosis. We investigated if these proteases are activated in the cells challenged with oxidative stress.

Caspase-3 activity was measured 24 hours after treating NT2N cells with H$_2$O$_2$. There was a significant increase in caspase-3 activity following oxidative stress as compared to the control. Cells pre-treated with CoQ$_{10}$ showed lowered caspase-3 activity as compared to oxidatively stressed cells that were not pre-treated with CoQ$_{10}$ (**Figure 15A**). Proteasome activation was observed 24 hours after oxidative stress was induced in NT2N cells (**Figure 15B**). The results indicated an increase in activity of proteases upon hydrogen peroxide treatment, in the absence of CoQ$_{10}$. But CoQ$_{10}$ pre-treatment reduced the proteasome activity after oxidative stress.

3.1.8. Oxidative stress causes mitochondrial dysfunction and increased hydrogen peroxide generation from mitochondria; inhibition by Coenzyme Q$_{10}$.

Mitochondrial dysfunction leads to the increase in the production of ROS. To investigate whether hydrogen peroxide treatment caused mitochondrial dysfunction, we monitored the production of ROS from the mitochondria. It has

Figure 15. A. Caspase-3 activity following hydrogen peroxide treatment
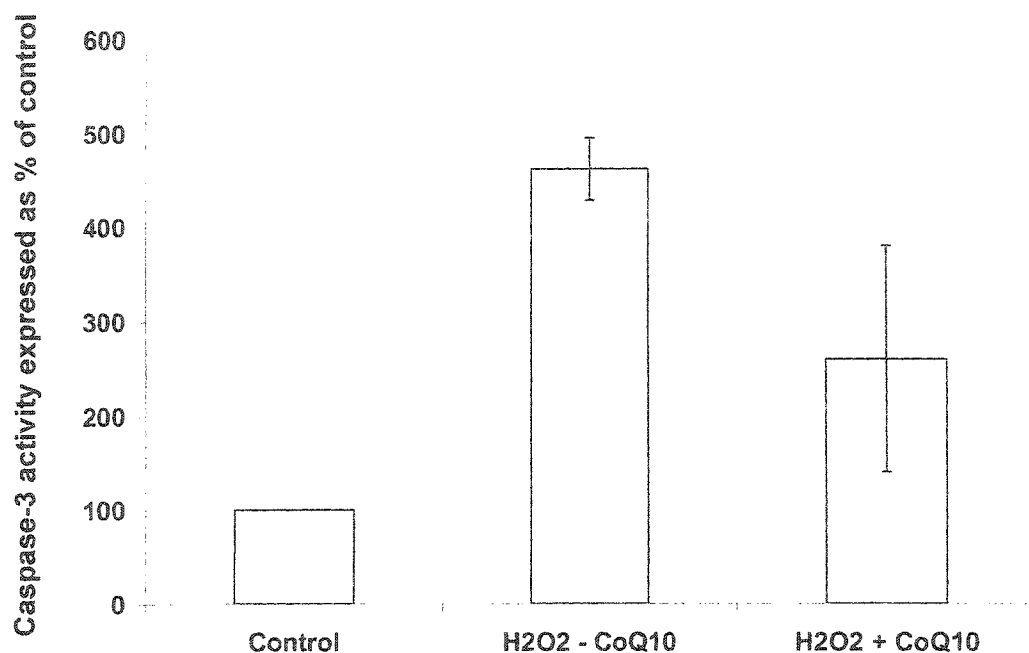
Caspase-3 assay was performed employing cell extracts from control and treated NT2N cells 24 hours after hydrogen peroxide treatment in the absence and presence of CoQ_{10}. Activity of Caspase-3 was expressed per µg protein. Note that the caspase-3 activity in the extract from control cells is taken as 100%. Standard errors were calculated using data from three sets of separate experiments. Microsoft excel version 6.0 software was used to represent data and calculate standard deviation.

Figure 15. B.   Proteosome activity after hydrogen peroxide treatment
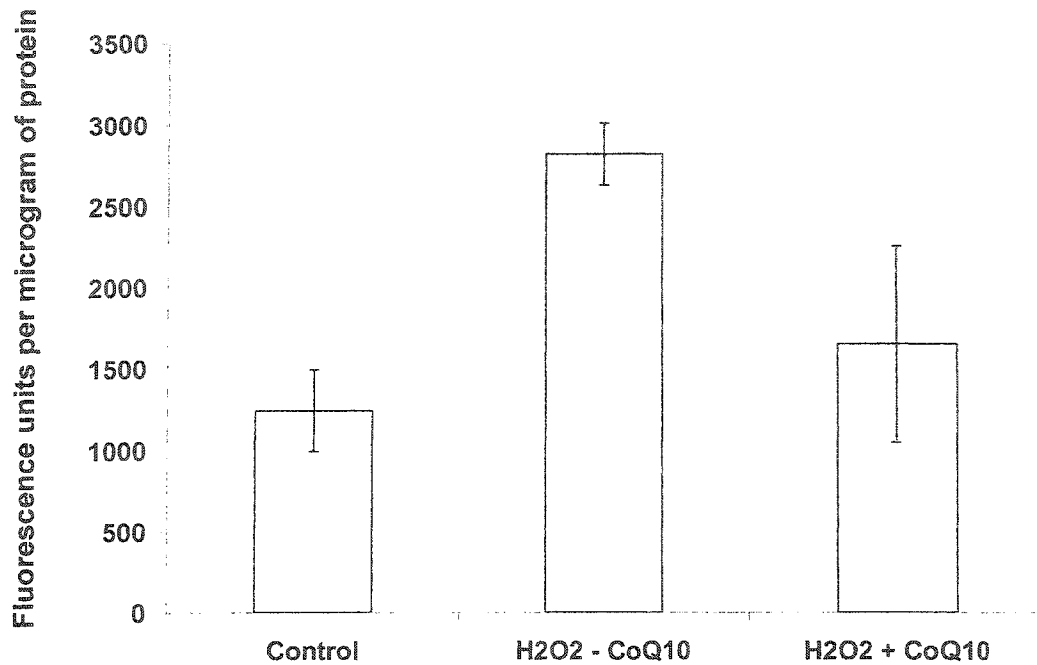
Proteasome assay was performed employing cell extracts from control and treated N T2N c ells a fter 2 4 h ours a s m entioned e arlier a nd expressed p er μ g protein. Standard errors were calculated using data from three sets of separate experiments. Microsoft excel version 6.0 software was used to represent data and calculate standard deviation. (p< 0.05)

been shown previously that H_2O_2 produced in the mitochondria can be measured spectrofluorometrically by using the PHPA method (Li *et al.*, 2003).

We monitored H_2O_2 production from mitochondria isolated from control and treated SH-SY5Y cells 24 hours after inducing oxidative stress as described in Materials and Methods. Results obtained indicated that mitochondria isolated from treated cells generated more H_2O_2 as compared to untreated cells. Furthermore, we observed that the increase in the production of ROS from the mitochondria after inducing oxidative stress was inhibited by CoQ_{10} pre-treatment (**Figure 16**).

3.1.9. CoQ_{10} prevents the collapse of mitochondrial membrane potential

It has been established that cells are capable of CoQ_{10} internalization when CoQ_{10} is added to tissue culture media in an aqueous solution. Increase in CoQ_{10} content in the mitochondria as well as in membranes has been reported (Sandhu *et al.*, 2003). Since CoQ_{10} is present in the mitochondrial membrane, it may stabilize the mitochondrial membrane and prevent it from collapsing under oxidative stress. In order to evaluate this possibility, mitochondrial membrane potential was studied using Mitotracker Red dye. Mitotracker Red is a membrane permeable dye and fluoresces brightly once it enters the mitochondria. If the mitochondrial membrane potential is decreased then the fluorescence

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Figure 16. Production of mitochondrial reactive oxygen species after hydrogen peroxide treatment
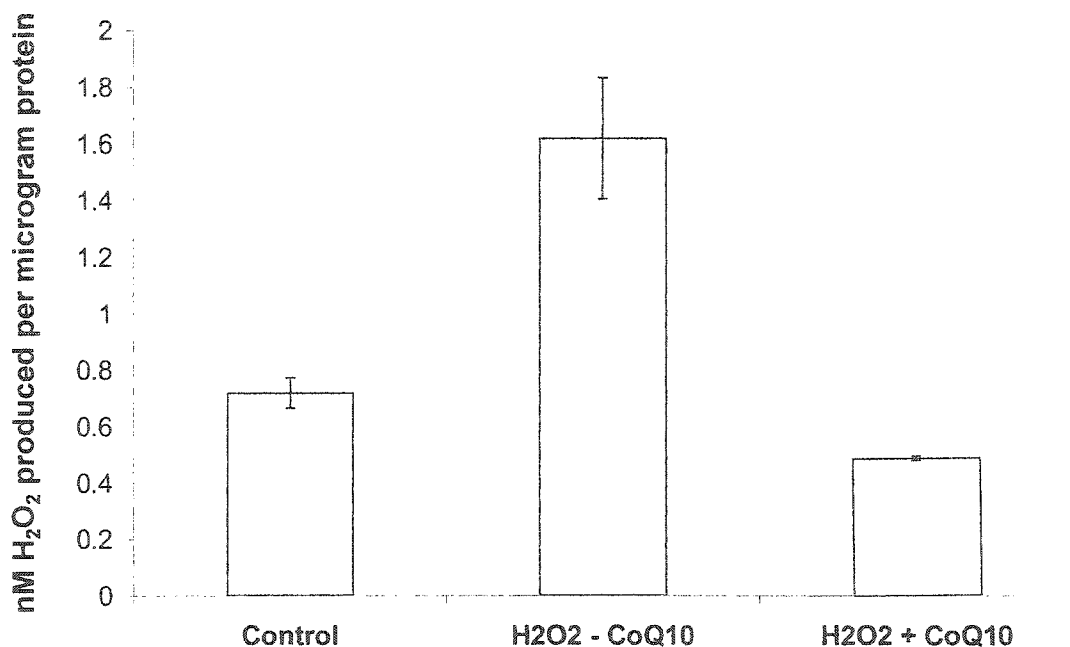
Mitochondria were isolated from SH-SY5Y cells after 24 hours of H_2O_2 treatment in presence and absence of CoQ_{10}. Hydrogen peroxide generated by the mitochondria was measured using PHPA as mentioned in Materials and Methods. Hydrogen peroxide production was expressed per µg of total mitochondrial protein. Microsoft excel version 6.0 software was used to represent data and calculate standard deviation. Standard errors were calculated using data from three sets of separate experiments.

is diffused. SH-SY5Y cells were stained with Mitotracker Red 24 hours after treatment, as described previously. As shown in figure **17.B**, a decrease in the mitochondrial membrane potential after treatment was evident. But CoQ_{10} prevented the mitochondrial membrane potential collapse after induction of oxidative stress (**Figure 17C**), suggesting that CoQ_{10} stabilizes the mitochondrial membrane. Control cells are seen in figure **17A**. Similar results were obtained using NT2 astrocytes (**Figures 17D, 17E & 17F**).

3.1.10. CoQ_{10} restores ATP production in cells under oxidative stress

Neurons depend on oxidative phosphorylation in the mitochondria in order to generate ATP. To determine the ATP content following oxidative stress, SH-SY5Y cells were grown, differentiated and treated with H_2O_2 as described in materials and methods. Mitochondria were isolated from SH-SY5Y cells 24 hours after treatment and luciferin-luciferase assay was used to measure ATP production. Results showed that there was a decrease in the ATP levels following oxidative stress. However, CoQ_{10} pretreatment prevented the loss in ATP production following oxidative stress significantly (**Figure 18**). Each of the last three experiments- inhibition of ROS production from isolated mitochondria, restoration of mitochondrial membrane potential and ATP production from isolated mitochondria suggested that CoQ_{10} functions at the mitochondrial level, to protect neuronal cell death due to oxidative stress.

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Figure 17. Mitochondrial membrane potential in differentiated SH-SY5Y cells following hydrogen peroxide treatment
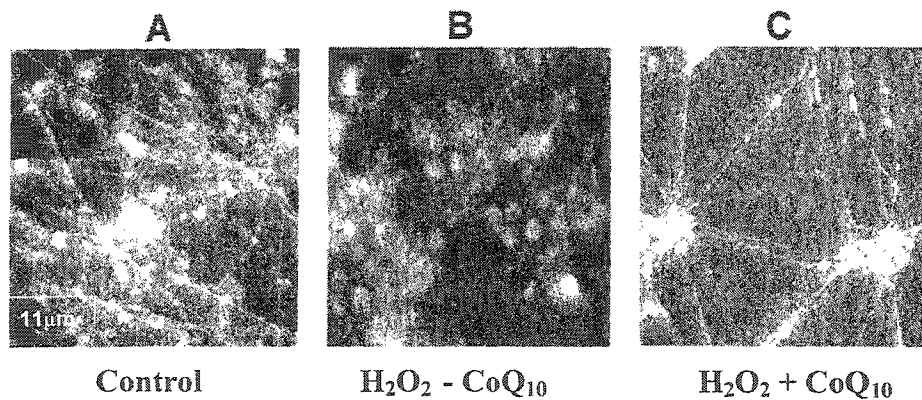
Control $H_2O_2 - CoQ_{10}$ $H_2O_2 + CoQ_{10}$

SH-SY5Ycells were treated with H_2O_2 for 1 hour in absence or presence of CoQ_{10}, after which cells were washed with fresh media and incubated at 37°C for 24 hours and then stained with Mitotracker Red dye as mentioned in materials and methods. Pictures were taken at a magnification of 400 X using Leica DM IRB microscope. Figure A represents control cells while B represents H_2O_2 treated cells in the absence of CoQ_{10} while C represents cells treated in the presence of CoQ_{10}.

Figure 17. Mitochondrial membrane potential in differentiated NT2

astrocytes following hydrogen peroxide treatment

D	E	F
Control	H_2O_2 - CoQ_{10}	H_2O_2 + CoQ_{10}
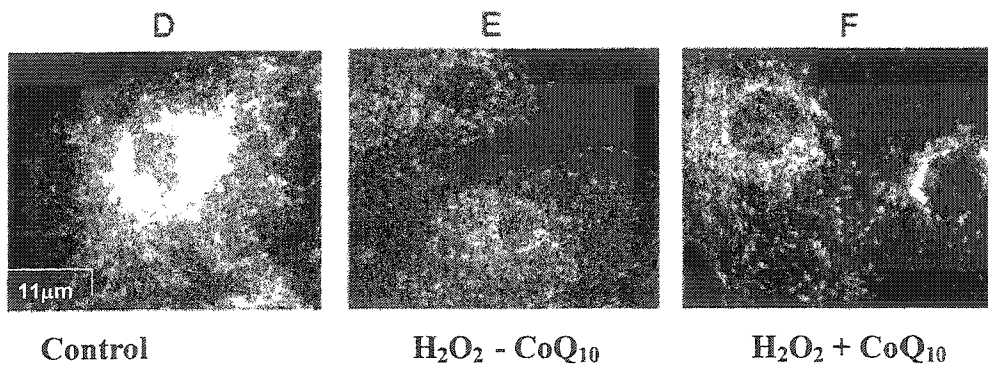
NT2 astrocytes were treated with H_2O_2 for 1 hour in absence or presence of CoQ_{10}, after which cells were washed with fresh media and incubated at 37°C for 24 hours and then stained with Mitotracker Red dye as mentioned in Materials and Methods. Pictures were taken at a magnification of 400 X using Leica DM IRB microscope. Figure D represents untreated cells while E represents H_2O_2 treated cells in the absence of CoQ_{10} while F represents cells treated in the presence of CoQ_{10}.

Figure 18.   Mitochondrial ATP production in SH-SY5Y cells after hydrogen

peroxide treatment
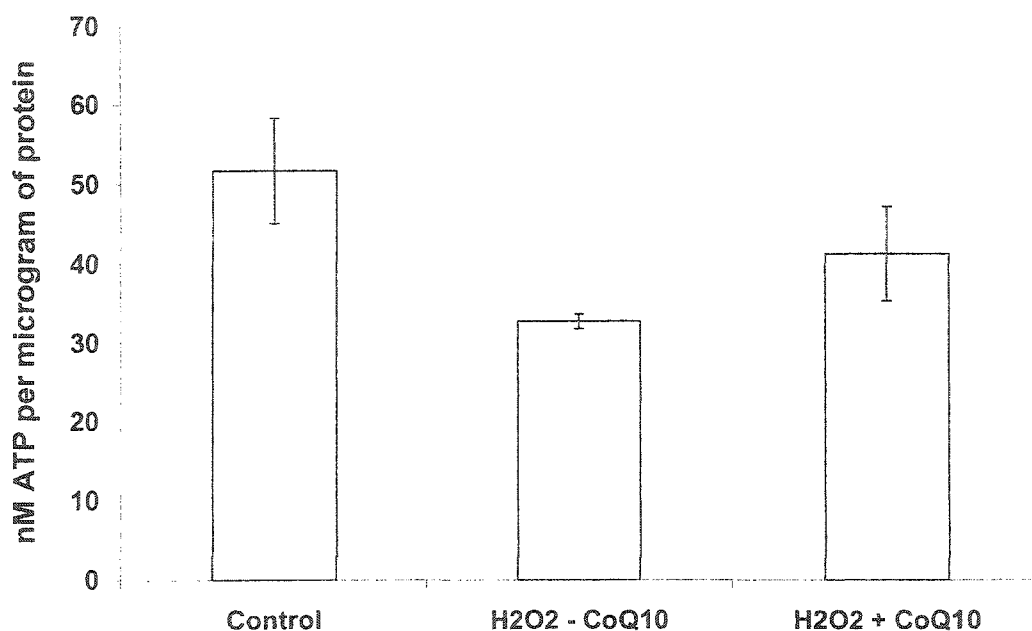
SH-SY5Y cells were treated with H_2O_2 for 24 hours in the presence and absence of CoQ_{10} and mitochondria were isolated. Luciferin – Luciferase was used to measure ATP production. An internal ATP standard was used and ATP production was expressed per μg of total mitochondrial protein. Microsoft excel version 6.0 software was used to represent data and calculate standard deviation. Standard errors were calculated using data from three sets of separate experiments. ($p < 0.05$)

3.2.1. Paraquat induces Oxidative Stress that can induce apoptosis in differentiated neuronal SH-SY5Y cells

Paraquat is a herbicide that has been shown to be related to development of Parkinson's disease. The structure of paraquat resembles that of MPP^+ and cell death induced by paraquat has been reported.

In order to study the effects of paraquat on neuronal cells, SH-SY5Y cells were grown, differentiated and treated as previously described. Cellular morphology was observed after 48 hours of paraquat treatment. Morphological changes were evident, including beading on neurites and rounding up of the cells, after 48 hours of paraquat treatment (**Figure 19B**). Control cells did not have evident morphological changes as indicated by figure **19A**. Nuclear condensation, a characteristic feature of apoptosis was clearly observed after treatment as shown in figure **20B**, whereas the nuclei of control cells did not show nuclear condensation (**Figure 20A**). DNA degradation is also a characteristic feature of apoptotic cell death. A comet assay was employed to examine DNA degradation changes after treatment with paraquat. The cells treated with paraquat showed comet-like images (**Figure 21B**), indicating DNA damage, whereas control cells did not (**Figure 21A**). All these results to strongly indicate that SHSY-5Y cells undergo apoptosis after paraquat treatment induced oxidative stress.

Figure 19. Morphology of SH-SY5Y cells after paraquat treatment
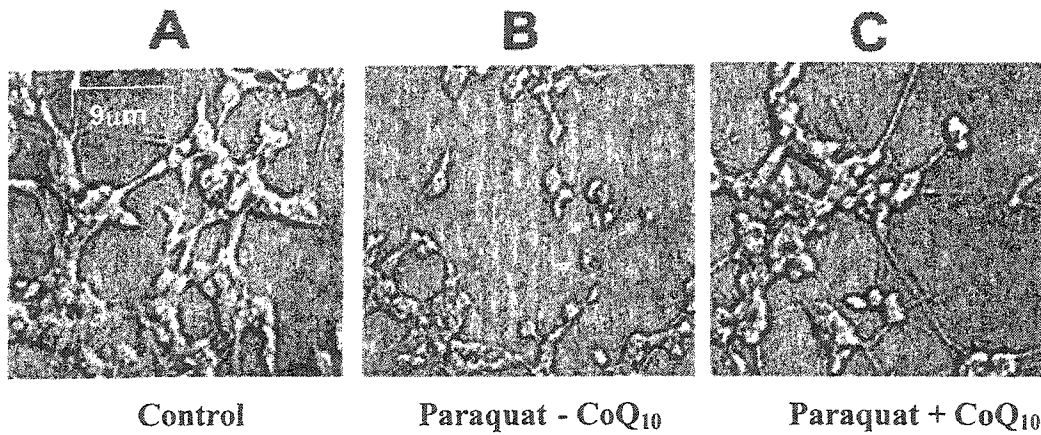
Control   Paraquat - CoQ$_{10}$ Paraquat + CoQ$_{10}$

Differentiated SH-SY5Y cells were treated with 100 μM paraquat in presence (C) and absence (B) of CoQ$_{10}$ as described in Materials and Methods. Figure A depicts control neuronal cells which were not treated with paraquat. After 48 hours of treatment, pictures were taken at 400X magnification using a Leica DM IRB microscope. Arrows indicate rounding up of cells.

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Figure 20. Nuclear morphology of SH-SY5Y cells after paraquat treatment
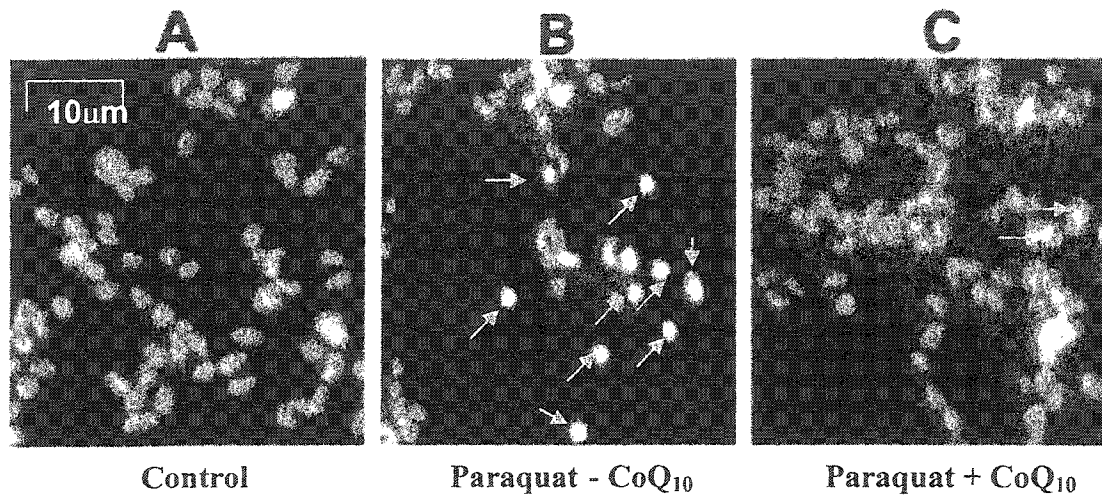
As described in methods, differentiated SH-SY5Y cells were treated with 100 µM paraquat in presence (C) and absence (B) of CoQ_{10} as mentioned in Materials and Methods. Figure A depicts control neuronal cells which were not treated with paraquat. After 48 hours of treatment, Hoechst 33342 dye was applied to the cells to examine nuclear morphology. Pictures were taken at 400X magnification using a Leica DM IRB microscope. Nuclear condensation is evident in cells with bright, condensed and rounded nuclei and indicated by arrows.

Figure 21. DNA degradation after paraquat treatment
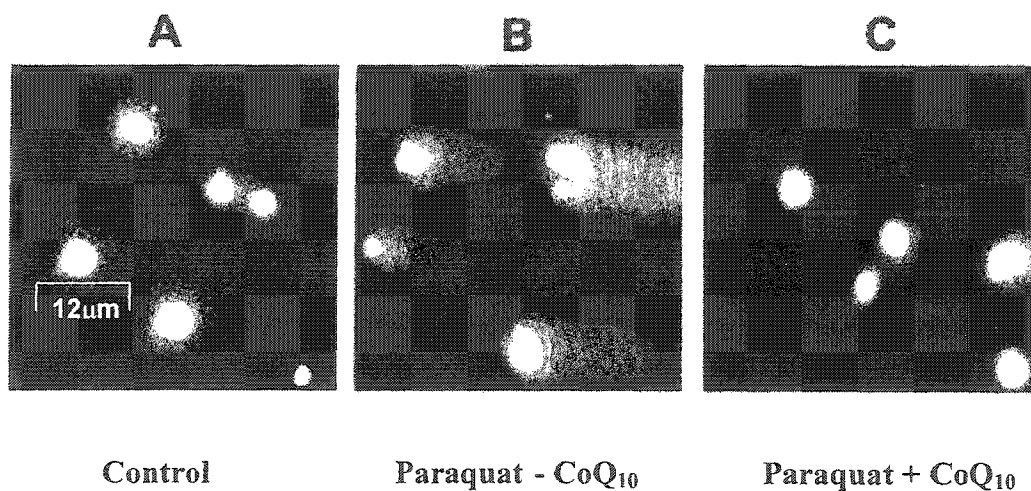
A	B	C
Control	Paraquat - CoQ$_{10}$	Paraquat + CoQ$_{10}$

Differentiated SH-SY5Y cells were treated with 100 µM paraquat in presence (C) and absence (B) of CoQ$_{10}$ as previously described. Figure A depicts neuronal cells which were not treated with paraquat. After 48 hours of treatment, a comet assay was conducted, as described in Materials and Methods. Pictures were taken at 400X magnification using a Leica DM IRB microscope. DNA degradation is indicated by comet-like images due to migration of damaged DNA during electrophoresis.

3.2.2. Pre-treatment with water-soluble CoQ$_{10}$ protects cells from paraquat-induced apoptosis

SH-SY5Y cells were grown, differentiated and treated as described in the materials and methods section, followed by observation of the cellular morphology. After 24 hours of pre-treatment with CoQ$_{10}$ and 48 hours of paraquat treatment, SH-SY5Y cells did not show any apoptotic morphology (**Figure 19C**). Similarly the nuclear morphology (**Figure 20C**) indicated that the nuclear chromatin condensation was inhibited in cells pre-treated with CoQ$_{10}$. Pre-treatment with CoQ$_{10}$ also resulted in fewer number of comet-like images (**Figure 21C**) as cells treated with paraquat alone, indicating less DNA damage. All these results taken together suggest that CoQ$_{10}$ protects SH-SY5Y cells from undergoing apoptosis.

3.2.3. Paraquat induces oxidative stress in differentiated SH-SY5Y cells and CoQ$_{10}$ offers protection

In order to determine whether paraquat treatment caused oxidative stress on SH-SY5Y cells, the production of ROS was measured. ROS measurements were collected after treatment of SH-SY5Y cells as previously described. After 48 hours of paraquat treatment, cells showed a considerable raise in ROS production compared to control cells or cells pre-treated with CoQ$_{10}$ as indicated by figure **22**. Results have been expressed as percentage of control.

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Figure 22. Generation of reactive oxygen species after paraquat treatment
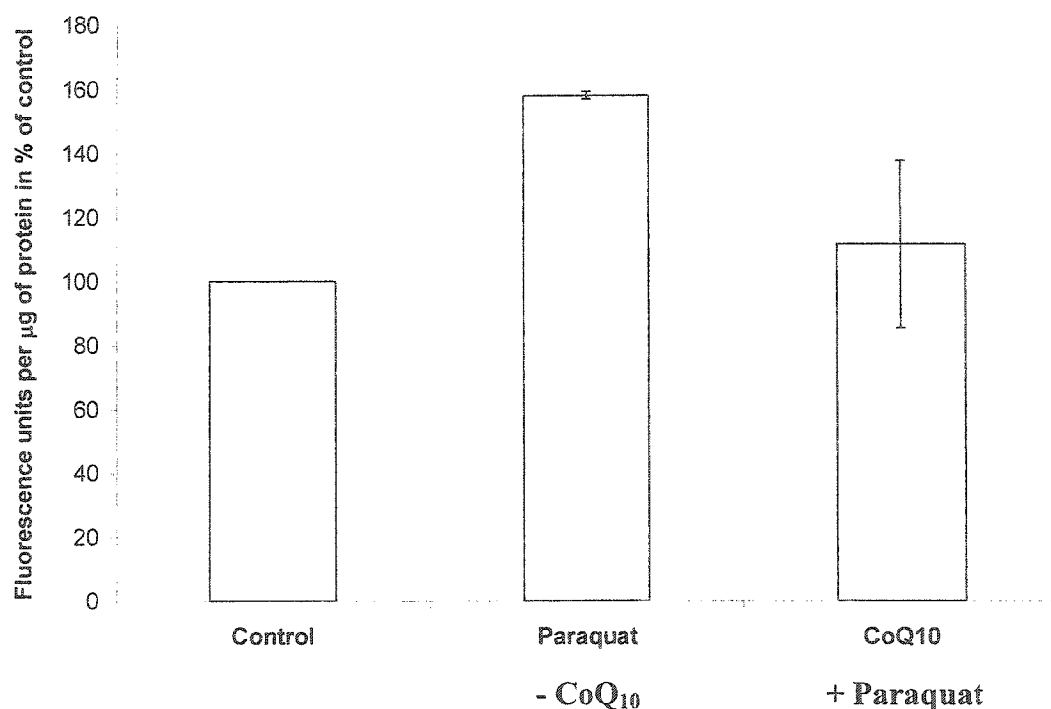
After 48 hours of paraquat treatment with differentiated SHSY-5Y cells in the absence and presence of CoQ_{10}, DCFDA was used to measure total cell ROS generation, as described in Materials and Methods. Microsoft Excel Version 6.0 software was used to represent data and calculate standard deviation. Results were calculated per microgram of protein and expressed in percentage of control. Standard errors were calculated using data from three separate sets of experiments ($p < 0.05$).

CHAPTER 4

DISCUSSION

Oxidative stress induced apoptosis has been implicated with several neurodegenerative diseases. In the present study, we have investigated the role of oxidative stress on neuronal cell death and neuro-protection by water soluble CoQ_{10} following H_2O_2 and paraquat induced oxidative stress.

4.1. NT2 and SH-SY5Y cells as neuronal models

Neurons are post-mitotic tissues. It is difficult to study the biochemical mechanisms of neurodegenerative diseases due to the lack of availability of human neurons. Human teratocarcinoma (NT2N) and Human Neuroblastoma (SH-SY5Y) cells have been used as neuronal models to study neuronal functions. Previous studies have shown that NT2 cells can be differentiated into neurons and astrocytes, and the commitment of these cells to a stable phenotype is irreversible (Sandhu et al., 2003, Guillemain et al., 2000). NT2-N cells have also been shown to survive and integrate within the host brain after transplantation and help in function recovery in animal models of stroke, Parkinson's and Huntington's disease (Sandhu et al., 2003, Hartley et al., 1999, Borlongan et al., 1998). SH-SY5Y cells can be differentiated into human neuron like cells, but the survival of these cells is dependent on BDNF and removal of BDNF leads to apoptosis in these cells. Differentiated cells express neuronal markers (Leypoldt et al., 2002, Encinas et al., 2000). Therefore the results

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obtained using the choice of these two these cell lines for the study of neuronal cell death would be more relevant to human neurodegenerative diseases.

4.2. Induction of apoptosis by external oxidative stress

It has been shown that oxidative stress such as H_2O_2 can induce apoptosis in cells and cultured neurons (Chang *et al.*, 2003, Ratan *et al.*, 1994, Enokido *et al.*, 1990). We subjected the differentiated neuronal cells to direct oxidative stress by a brief H_2O_2 treatment. We have demonstrated that differentiated neuronal cells are very sensitive and undergo apoptosis following direct oxidative stress by H_2O_2 treatment, as clearly indicated by cellular morphology, nuclear condensation and DNA fragmentation-the characteristics features of apoptosis. It has been observed that undifferentiated NT2 or SH-SY5Y cells are not sensitive to oxidative stress induced by similar concentration of hydrogen peroxide. Although 100 $\square$M H_2O_2 is not a physiological concentration during any disease, but for *in vitro* models, a brief exposure to100 μM H_2O_2 causes oxidative stress.

Our results showed that indeed there was increase in ROS production after H_2O_2 treatment, a decrease in GSH level and increased lipid peroxidation. A decreased GSH levels and an affiliated increase in ROS during apoptosis have been reported in previous studies (Tan *et al.*, 1998). Moreover, lipid peroxidation is also a direct measure of oxidative stress. Our data showed an increase in lipid peroxidation upon treatment with H_2O_2. All these results taken together suggest that cells were under oxidative stress which in turn led to apoptotic cell death.

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4.3. Oxidative stress causes mitochondrial dysfunction

Mitochondria have been shown to play a key role in the apoptotic process. Mitochondrial control of apoptosis can be described at two important levels; one is the maintenance of ATP production. The other is mitochondrial membrane potential and mitochondrial membrane permeability for the release of certain apoptogenic factors such as cytochrome C into the cytoplasm. Cytochrome C can bind to pro-caspase-9 and Apaf-1 to form the apoptosome, further causing the activation caspase-3. Treatment with H_2O_2 has been shown to cause mitochondrial dysfunction (Leducq et al., 2003, Tsutsumi et al., 2002). Disruption of the mitochondrial respiratory chain can not only result in over production of ROS leading to oxidative stress, but also activation of apoptotic mediators (Bortner et al., 2002). Mitochondria actively generate ROS such as super oxide anions and hydrogen peroxide (Kannan et al., 2000). When we monitored the ROS production from mitochondria isolated from differentiated NT2N and SH-SY5Y cells after inducing oxidative stress, an increase in ROS production by after treatment occurred. Each of these experiments confirmed that oxidative stress was induced by hydrogen peroxide treatment. Increased mitochondrial ROS and caspase-3 activation also suggested mitochondrial dysfunction, following oxidative stress.

4.4. Neuroprotection offered by CoQ_{10} prevents mitochondrial dysfunction and rescues cells from oxidative stress

Coenzyme Q_{10} is hydrophobic and localized in the inner mitochondrial membrane. Previous studies have used oil formulations of CoQ_{10}. Since CoQ_{10} is very hydrophobic, cells cannot take it up easily. Recently water soluble CoQ_{10} containing both the oxidized and reduced forms was formulated (Borowy-Borovski, US Patent No. 6,045, 826). Water-soluble formulations of CoQ_{10} are readily taken up by the cells, when added to tissue culture media, making it possible to study the mechanism by which CoQ_{10} offers protection against oxidative stress. An increase in CoQ_{10} content in the mitochondrial membranes and cell membranes has been observed in cells pre-treated with CoQ_{10} (Sandhu et al., 2003). Our data have shown that water soluble CoQ_{10} can provide protection by functioning as an antioxidant against oxidative stress indicated by high ROS levels, elevated lipid peroxidation and decreased levels of GSH.

Coenzyme Q_{10} carries electrons from Complex-I and Complex-II to Complex-III. Since it is a component of the electron transport chain, we were interested to see whether an extra amount of CoQ_{10} would help restore mitochondrial functions during oxidative stress. Our study has indicated CoQ_{10} protection at the mitochondrial level. Firstly, in the cells pre-treated with CoQ_{10} before H_2O_2 treatment, the mitochondrial ROS was significantly low when compared to those isolated from the cells that had not been pre-treated with CoQ_{10} before H_2O_2 treatment. Furthermore, we observed that mitochondria maintained the membrane potential in presence of CoQ_{10} (figures 17C, 17F).

These observations strongly suggest that CoQ_{10} can act not only as an anti-oxidant but also to stabilize the mitochondrial membrane during oxidative stress. Another interesting observation was that mitochondria in cells treated with hydrogen peroxide in the presence of CoQ_{10} remained functional and maintained ATP production at almost a normal level, led us to believe that CoQ_{10} may have enhanced the electron transport from complex-I and complex-II to complex-III. We have demonstrated CoQ_{10} protects cells at all the different levels in the apoptotic pathway both morphologically and biochemically.

Our study demonstrates that CoQ_{10} can prevent mitochondrial dysfunction during oxidative stress. Results in our lab have also shown that Bax induced ROS generation from mitochondria can be prevented by CoQ_{10} (Pandey et al., unpublished data).

Recent studies have focused on designing new therapeutics for protection against neurodegeneration. These include the investigation of the role of microglial activation as a target for neuro-protection (Dommergues et al., 2003), testing neuro-protective agents such as docosahexaenoic acid, nimodipine and citicoline (Hogyes et al., 2003, Sobrado et al., 2003) and using anti-oxidants like estradiol, (Marin et al., 2003) melatonin (Erol et al., 2003) and vitamin E (Garcia-Estrada et al., 2003). However, Vitamin E cannot prevent mitochondrial depolarization, cytochrome c release and caspase-9 activation induced by apoptotic stimuli (Papucci et al., 2003). It is not clearly known how hydrogen peroxide causes mitochondrial dysfunction. One of the hypotheses is that it may

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cause distinct changes including lipid peroxidation and these changes may trigger a cascade.

4.5. Mechanism of CoQ$_{10}$ neuroprotection

Recent reports have suggested that the permeability transition pore (PTP) in the mitochondria harbors a ubiquinone binding site and is regulated by complex-I of the mitochondrial respiratory chain (Papucci *et al.*, 2003, Walter *et al.*, 2002, Petronilli et al., 2001, Walter L *et al.*, 2000, Fontaine E *et al.*, 1999, Fontaine E *et al.*, 1998). Our next steps would include considering whether PTP are involved in the pathway by using PTP inhibitors and also to see how CoQ$_{10}$ interferes at PTP level. It will also be important to see if there is any competition between Bax and CoQ$_{10}$ at the PTP binding sites and whether oxidatively modified protein can cause the opening of the PTP.

4.6. Neuroprotection by CoQ$_{10}$ against paraquat toxicity

Paraquat, a non-selective herbicide which is still used in some countries, has been found to selectively kill nigrostriatal dopaminergic neurons in animal models (McCormack *et al.*, 2002). Several investigations have revealed that environmental toxins, including paraquat, are responsible for the development of sporadic age-related Parkinsonism (Di Monte, 2002). A strong association between paraquat exposure and risk of Parkinson's disease has been reported in farmers in Taiwan where paraquat is frequently sprayed in rice fields (Liou *et al.*, 1997). Paraquat induced cell death has been reported in lung epithelial cells and

rat PC12 cells (Capelletti *et al.*, 1998, Fabisiak *et al.*, 1998). One of the problems with previous animal models of paraquat-induced Parkinsonism has been its direct toxicity to the lung and liver before the behavioral symptoms of the disease appear (Onyon *et al.*, 1987). It has been postulated that paraquat is structurally similar to MPP^+, the active metabolite of MTPT, therefore the mechanism of paraquat neurotoxicity has been assumed to be similar (Shimizu *et al.*, 2001). MPP^+ has been shown to cause cell death in a nigral dopaminergic cell line due to increased oxidative stress (Chun *et al.*, 2001). Previous reports have shown that paraquat mediates its genotoxic effect, partly via its capacity to generate ROS (Suntres 2002). Our results are in agreement with these reports. We have observed an increase in the total ROS production as well as mitochondrial ROS production confirming that paraquat can generate ROS.

Although vitamin E confers protection against paraquat-induced injuries in vitamin E-deficient animals, normal animals receive little benefit from additional pharmacologic supplementation with vitamin E. In a study investigating the presence of lipid peroxidation as a potential marker of sub acute toxic reaction, it was shown that vitamin E supplementation to humans (100–900 mg per day) was ineffective in protecting against paraquat poisoning and did not affect the levels of lipid peroxidation (Yasaka *et al.*, 1986).

In conclusion, our results clearly indicate that CoQ_{10} offers neuroprotection at all the stages in the apoptotic pathway against oxidative stress induced by hydrogen peroxide and paraquat. Therefore, water-soluble CoQ_{10} is a potential

therapeutic agent for the treatment of neurodegenerative diseases should be further explored.

FUTURE PROSPECTIVES

How external oxidative stress causes mitochondrial dysfunction is still not clearly understood. Further investigation is required in order to establish the mechanism by which external oxidative stress causes the loss of mitochondrial function.

It would also be interesting to find out which proteins are modified by ROS. Proteomics can aid in finding the proteins modified during oxidative stress. Oxy blot analysis using mass spectrometry can be used to identify proteins that are oxidatively modified.

Water soluble CoQ_{10} has shown to prevent isolated mitochondria from oxidative stress. However, the mechanism by which CoQ_{10} offers neuro protection by preventing mitochondrial damage is still not clear. The mechanism by which CoQ_{10} prevents mitochondrial dysfunction will be extremely interesting. Whether CoQ_{10} can bind to PTP and thereby affect the opening and closing the pore will be a very important step in understanding the mechanism of CoQ_{10} neuro protection.

It would also be fascinating to study the differences in the mechanism of protection offered by CoQ_{10} and other anti-oxidants like Vitamin E.

All the experiments mentioned here have been performed at the cellular level. It is very essential to see how CoQ_{10} functions *in vivo* brain level. Preliminary results in our lab showed that normal rats fed with water soluble

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CoQ_{10} showed a systematic reduction in oxidative stress in the brain and liver as shown by higher GSH levels and lower levels of lipid peroxidation as compared to rats that were not fed with CoQ_{10}. However, further investigation is required to study the properties of water soluble CoQ_{10} as a neuro-protective agent. Moreover it will be very interesting to see the effect of oxidative stress and inhibition by water soluble CoQ_{10} on behaviour of these animals.

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