Pesticides and Pesticide Mixtures Induce Neurotoxicity: Potentiation of Apoptosis and Oxidative Stress

by
Zhenquan Jia

Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University in
partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
in
Biomedical and Veterinary Sciences

Department of Biomedical Sciences and Pathobiology
Virginia-Maryland Regional College of Veterinary Medicine

Approved:

_____
Hara P. Misra, BVSc & AH, MS, Ph.D., Committee Chair

_____ _____
Chris Reilly, Ph.D., Committee member Bradley G. Klein, Ph.D., Committee Member

_____ _____
Korinn Saker, DVM, Ph.D., Committee Member Eugene Gregory, Ph.D., Committee Member

July 7, 2006
Blacksburg, Virginia

Keywords: endosulfan, zineb, pesticide mixtures, neurotoxicity, apoptosis, necrosis, oxidative stress, antioxidants

Pesticides and Pesticide Mixtures Induce Neurotoxicity: Potentiation of Apoptosis and Oxidative Stress

by

Zhenquan Jia

Abstract

Several epidemiological studies have suggested a role for environmental chemicals in the etiology of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. Endosulfan (an organochlorine) and zineb (zinc-ethylene-bis-dithiocarbamate) are used as pesticides on a variety of crops worldwide and pose potential health risks to humans and animals. Both endosulfan and zineb are known to affect nervous system. Because the dopaminergic system continues to develop postnatally, we hypothesized that developmental exposure to endosulfan or zineb alone or in combination would result in alteration of nigrostrial neurotransmitters and would render the nigrostrial dopamine system more susceptible to chemical challenge later in life. The objectives of this study were (1) to determine the effects of endosulfan and zineb individually and in combination on dopaminergic or cholinergic pathways *in vivo*, (2) to investigate the effects of exposure to endosulfan, zineb and their mixtures administered in early life (during brain development) on subsequent exposure to these pesticides on the dopaminergic and cholinergic systems, *in vivo*, (3) to investigate the mechanism(s) of induction of neuronal cell death caused by these pesticides using human neuroblastoma SH-SY5Y cells in culture, (4) to define the role of oxidative stress in pesticide-induced neuronal cell death *in vitro.* Male C57Bl/6 mice of 7-9 months old exposed to zineb (50 and 100 mg/kg), endosulfan (1.55, 3.1 and 6.2 mg/kg) and their mixtures every other day over a 2-week period exhibited higher levels of dopamine accumulation in the striatum. Both pesticide-treated groups displayed significantly lower norepinephrine levels in the striatum ($\rho \leq 0.05$) than the controls. The developmental exposure to zineb, endosulfan and their combination enhanced the vulnerability to subsequent neurotoxic challenges occurring later in life. Thus, C57BL/6 mice exposed to zineb, endosulfan and their mixtures as juveniles (postnatal days 5 to 19) and re-exposed at 8 months of age showed a significant depletion of striatal dopamine, to 22%, 16%, and 35% of control, respectively. Acetylcholinesterase activity in the cerebral cortex was found to be significantly increased in all pesticide treated groups. Mice given mixtures of

pesticides also showed significantly increased levels of normal and aggregated alpha-synuclein, a hallmark of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. The results of these studies indicate that exposure to these pesticides as neonates and re-exposure as adults could result in neurochemical changes that did not reveal at adulthood when the exposure was at juvenile age only.

We further investigated the mechanism(s) of activation of pesticide-induced neuronal cell death *in vitro*. The characteristic of cell death in SH-SY5Y human neuroblastoma cells was examined. These cells are known to retain catecholaminergic phenotype. Cells were exposed to endosulfan, zineb and mixtures of two pesticides, in concentrations ranging from 50 μM to 400 μM. These exposures caused both apoptotic and necrotic cell death in SH-SY5Y cells as evaluated by lactate dehydrogenase release, 7-aminoactinomycin-D and Annexin-V/PI assays. Exposure to mixtures of the pesticides enhanced both the early apoptosis and late apoptosis/necrosis compared to either chemical alone. Visual evaluation using DNA ladder assay and fluorescence Annexin V/PI assay confirmed the contribution of both apoptotic and necrotic events. Furthermore, endosulfan and zineb alone and in combination altered the caspase-3 activity indicating that both pesticides exposure exert their apoptotic effect via the caspase-3 pathway. Because there has been increasing evidence of the role of reactive oxygen species (ROS) and oxidative stress in pesticide-induced neuronal cell death (apoptosis and necrosis), the levels of ROS and antioxidant enzymes were examined. Cells treated with pesticides were found to enhance the generation of superoxide anion and hydrogen peroxide both in a dose- and time-dependent manner. Mixture of pesticides significantly enhanced the production of these reactive oxygen species compared to cells exposed to individual pesticide. Cells treated with pesticides showed a decrease in superoxide dismutase, glutathione peroxidase, and catalase levels. These pesticides also induced lipid peroxides (thiobarbituric acid reactive products) formation in SH-SY5Y cells. Furthermore, cells exposed to these pesticides were found to have increased in the expression of NFkappaB activity in the nucleus. These data support the hypothesis that oxidative stress was induced in neuronal cells by exposing to these pesticides *in vitro*.

Taken together, the results of this study support the above hypothesis and suggest that the cytotoxicity of endosulfan and zineb and their combinations may, at least in part, be associated with the generation of ROS. Furthermore, mice exposed at early age and re-exposed at adulthood become more susceptible to alteration of neurotransmitter levels compared to mice exposed to these pesticides only as juveniles. These findings could add to the growing body of knowledge on the mechanism of pesticide-induced dopaminergic neuronal cell death and could hold tremendous implication for the future understanding of the possible involvement of environmental risk factors in the pathogenesis of Parkinson's disease.

Acknowledgements

I am grateful to all those who helped me complete this dissertation. First, I would like to thank my committee for their guidance and support throughout my project. Also, I am indebted to my advisor Dr. Hara P Misra for his constant support and guidance during my graduate program; he made me believe in myself. In addition, I gratefully acknowledge Dr. Chris Reilly for his suggestions and great help on my future career development and his brilliant guidance and comments on my research, allowing me to use graph software, cell culture incubator, and any other facility in his lab. I would further like to thank Dr. Bradley G. Klein for all his guidance and suggestions in the neuroscience aspects of this dissertation, particularly the chance to learn the striatum isolation techniques in his lab from Ms. Celia Dodd, whose expertise in this technique was valuable in this project. I would like to gratefully acknowledge Dr. Korinn Saker for her insight and dedication to my project and allowing me to use Oxyscan equipment in her lab. I would also like to acknowledge Dr. Eugene Gregory for his continuous support for my research and for his interest and willingness to serve as my committee members.

I also deeply thank Dr. Marion Ehrich for offering valuable suggestions and time in reviewing the two manuscripts (*in vivo* parts) of this dissertation. I admire her enthusiasm for science; she remains a worthy role model for my future endeavors. Dr. Geraldine Magnin and Ms. Barbara Wise deserve recognition for their help and expertise in HPLC equipment, Ms. Kristel Fuhrman for providing support with the acetylcholinesterase assay, Ms. Joan Kalnitsky for her help and expertise in flow cytometry, Mr. Jimmy Martin for sharing his expertise in Oxyscan equipment, Mr. Dan Ward for his assistance with statistics, and Ms. Kristin Knight for her training me with cell culture.

I would also like to thank my friends in CMMID. Special thanks to Dr. Xisheng Wang, Dr. Fangfang Huang, and Dr. Sheela Ramamoorthy for their help, friendship, advice, and many valuable discussions that facilitated my research. I also appreciate Dr. Selen Olgun for her friendship and encouragement, especially when I was the only person in the lab. I extend many thanks to Ms. Carolina Aguilar, Ms. Nicole McKeown, Dr. Jinghong Kou, Dr. Chao Shang, Dr. Juhong Liu, Vimala Vemireddi, Dr. Gliceria Pimentel-Smith, Mr. Denis Guenette, Dr. Padma Billam and Mr.

Mohamed Seleem for their friendship and help in the lab. Many thanks, also, to Dr. Dongmin Liu, Hongwei Si and Xiaolun Sun for their warm friendship while we lived in Blacksburg.

I would also like to extend my special thanks to everybody at the animal facility and IDU for taking care of the animals.

I would also like to thank the Pratt Fellowship, VCOM, VMRCVM, Dr. Ludeman A Eng, Dr. John Lee, and Dr. Roger J Avery for their financial support without which I would not have been able to complete my studies.

Finally, I would like to thank my family for their continued moral support. I am especially thankful to my wife, Yanling Li and daughter, Wenyue Jia for their company, patience, and long hours of waiting during my studies.

Declaration of work performed

I, Zhenquan Jia declare that I performed all the work herein, except for the portions of HPLC, flow cytometric and statistical analysis.

Table of Contents

Table of Figures:

Table of Table:

List of abbreviation

7AAD- 7-aminoactinomycin-D

AChE – acetylcholinesterase

AcTCh – acetylthiocholine

AD – Alzheimer's disease

ANOVA – analysis of variance

Caspases – cysteinyl-aspartate-specific proteinases

CAT – catalase

CMMID – Center for Molecular Medicine and Infectious disease

COMT – catechol-O-methyltransferase

DA– dopamine

DAT – dopamine transporter

DCF – 2',7'-dichlorofluorescein

DCF-DA – 2',7'-dichlorofluoroscin diacetate

DDC – N-N'-diethyl-dithiocarbamate

DMSO – dimethylsulfoxide

DOPAC – dihydroxyphenylacetic acid

DTNB – dithio-bis-nitrobenzoic acid

EBDC – ethylene bisdithiocarbamate

EMSA – electromobility shift assay

EN – endosulfan

EPA – Environmental Protection Agency

GABA – gamma-aminobutyric acid

GLM – general linear model

GP – globus pallidus

GPe – globus pallidus external

GPi – globus pallidus interna

GPX – glutathione Peroxidase

GR – glutathione Reductase

GSH – reduced glutathione

GSSG – oxidized glutathione

H_2O_2 – hydrogen peroxide

HBSS – Hanks' Balanced Salt Solution

HE – hydroethidine

Hrs – hours

HPLC – high performance liquid chromatography

HRP – horseradish peroxidase

HVA – homovanillic acid

i.p – intraperitoneal

JV – juveniles

LBD – lewy body dementias

LC_{25} – lethal dose that kills 25% of the population

LD_{50} – lethal dose that kills 50% of the population

LDH – lactate dehydrogenase

MAO– monoamine oxidase

MAO-B – monoamino oxidase type B

MDA – malondialdehyde

Mg/kg – milligrams/kilograms

Min – minutes

$MPDP^{+}$ – methyl-4-phenyl-1, 2-dihydropyridinium ion

MPP^{+} – 1-methyl-4-phenylpyridinium

MPTP-1-methyl-4-phenyl-1, 2,3,6-tethrahydropyridine

NADH – nicotinamide adenine dinucleotide

NADPH– nicotinamide adenine dinucleotide phosphate-reduced form

NE – norepinephrine

$O_2^{-\bullet}$ – superoxide anion

$O_2^{\uparrow\downarrow}$ – singlet oxygen

$OH^{\bullet}$ – hydroxyl radical

Paraquat – N,N′-dimethyl-4,4′-bipyridylium

PBS – phosphate buffered saline

PD – Parkinson's disease

PHF's – paired helical filaments

PI- propidium iodide

pNA – p-nitroaniline

PS – phosphatidyl serine

PVDF – polyvinyldiene difluoride

SDS – sodium lauryl sulfate

SEM – standard error of the mean

SNc – substantia nigra pars compacta

SN – substantia nigra

SNr – substantia nigra pars reticulata

SOD – superoxide dismutase

STN – subthalamic nucleus

TBA – thiobarbituric acid

TBARS – thiobarbituric acid reactive substances

TBS – tris buffered saline

TH - tyrosine hydroxylase

ZB – zineb

CHAPTER 1 Introduction

1.1. Statement of hypothesis

The hypothesis was that exposure to endosulfan or zineb alone, or in combination would disrupt the dopaminergic and/or cholinergic pathways; that exposure to these chemicals during the critical period of brain development would render the nigrostriatal dopamine system more susceptible to subsequent chemical challenge later in life; and that concurrent exposure to both of these pesticides would exert effects greater than each alone because combination would enhance the generation of reactive oxygen species (ROS), which may exert proapoptotic effects on neuronal cells. The results of the proposed study would provide possible relevance in the pathogenesis of neurodegenerative diseases, often related to the exposure of pesticides.

1.2. Study Rationale:

To test the above hypothesis, studies were designed to investigate the role of endosulfan, zineb, singly and in mixtures for their capability to induce neurochemical processes that could contribute to neurodegeneration. The studies were also designed to help to understand ROS and oxidative stress as possible mechanisms by which concurrent exposure to theses pesticides could cause neuronal cell death (apoptosis and necrosis) in an *in vivo* system. This research was initiated to better understand the toxic effects of multiple pesticides exposures and could make a potential contribution toward the identification of environmental toxicants as risk factors in the etiology of neurodegenerative diseases.

Although the etiology of Parkinson's disease (PD) and Alzheimer's disease (AD) is not known, most studies strongly suggest that environmental factors, particularly pesticide exposure, may play an important role in the pathogenesis of these disorders. Discovery of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) as a neurotoxicant and a model of Parkinson's disease led to studies on increased risk of PD following exposure to other

chemical substances (Betarbet *et al.*, 2000). Experiments in animals exposed to certain pesticides such as paraquat and rotenone suggest that these pesticides could contribute to neurodegenerative diseases such as PD and AD (Manning-Bog *et al.*, 2002; Thiruchelvam *et al.*, 2002). Many dithiocarbamate fungicides such as maneb, and mancozeb have been reported to cause neurodegeneration and demylination of rat neurons (Ferraz *et al.*, 1988; Seaton *et al.*, 1997; McGrew *et al.*, 2000; Zhang *et al.*, 2003). In addition, maneb appears to possess potent dopaminergic activity and is well known to enhance the toxic effects of MPTP in mice (Takahashi *et al.*, 1989; Thiruchelvam *et al.*, 2000a; Thiruchelvam *et al.*, 2002; Thiruchelvam *et al.*, 2003). Although zineb (a zinc- substituted dithiocarbamate) is structurally similar to maneb (a manganese-substituted dithiocarbamate), studies on the action of zineb on the dopamine system have not been studied in details in the literature, thus, further investigation is needed. Endosulfan, a commonly used organochlorine cyclodiene pesticide, is commonly used to control a number of insects on food crops and appears to have major effects on the central nervous system in human and animal species (Dikshith *et al.*, 1984; Paul *et al.*, 1994; Balasubramaniam *et al.*, 1995; Boereboom *et al.*, 1998). Since both zineb and endosulfan are widely used to protect a variety of crops against insects, human exposure to these chemicals, either concurrently or sequentially, is virtually unavoidable due to contamination of air, water, ground and food. No studies have been done with combination of the organochlorine endosulfan and the dithiocarbamate zineb. Mixtures could trigger some effects that single chemical exposure does not. Therefore, we proposed to examine the effect of the pesticides, zineb and endosulfan, individually or in combination to neurochemical processes that could contribute to neurodegeneration.

The potential for adverse effects of pesticide exposures to infants and children is of significant concern (Kimmel and Makris, 2001; Dietert *et al.*, 2002). Children are reported to absorb higher amounts of pesticides per body weight than adults (O.T.A., 1990). The parameters of fetal, infant, and childhood growth may be predictors of disease in later life (Osmond and Barker, 2000). Furthermore, the susceptibility of the developing nervous system to degeneration following exposure to environmental chemicals is well recognized (de la Fuente-Fernandez and Calne, 2002; Thiruchelvam *et al.*, 2002). Under

this hypothesis, early exposure of pesticides to infants and children could later affect the brain, whose immature development could make these children more susceptible to neurotoxicity later in life. In fact, the study showed that developmental exposure followed by adult rechallenge unmasked silent toxicity, which was apparently produced by maneb treatment during development (Thiruchelvam et al., 2002). No similar studies have been done with endosulfan and zineb. This dissertation includes studies to test the hypothesis that exposure to endosulfan and zineb during the critical period of postnatal development could result in neuronal dysfunction and enhance the impact if rechallenge as adults.

Accumulation of alpha-synuclein in Lewy bodies and neurites is a pathological hallmark of PD. The pathway (s) of alpha- synuclein in neuropathology leading to dopaminergic neurons death is not clearly understood. It has been suggested that dopamine-induced oxidative stress and free radical generation could introduce mutation in alpha- synuclein leading to destruction of dopaminergic neurons (Lotharius and Brundin, 2002). The impaired dopamine neurotransmitter storage arising from mutations in alpha- synuclein could lead to reduced vesicular storage of dopamine and increased dopamine release into synapse. The breakdown of this labile dopamine neurotransmitter in the cytoplasm could, in turn, promote oxidative stress and metabolic dysfunction, which in turn, lead to the death of dopamine containing neurons. There have been studies with pesticides such as paraquat that demonstrated its ability to cause up-regulation and aggregation of α-synuclein, which in turn lead to a variety of neurodegenerative disorders, including PD and AD (Manning-Bog *et al* 2002). No similar studies have been done with organochlorine endosulfan and dithiocarbamate zineb. Hence, we proposed to study the effects of endosulfan and/or zineb on the expression of alpha-synuclein in mouse brain in order to elucidate the molecular pathogenesis of neurodegeneration caused by the pesticides.

Overproduction of reactive oxygen species (ROS) has been implicated as a mechanism contributing to the toxicity of several organochlorine pesticides (Latchoumycandane and Mathur, 2002; Sohn *et al.*, 2004) and dithiocarbamate pesticides (Burkitt *et al.*, 1998;

Thiruchelvam *et al.*, 2005). For example, ROS are proposed to be responsible for endosulfan-induced toxicity in Jurkat- T cells (Kannan and Jain, 2003). Oxidative stress may occur when ROS are produced faster than they can be removed by cellular defense mechanisms. To respond to acute and chronic increases in oxidative stress, cells have adaptive mechanisms to increase antioxidant defenses (Zhou *et al.*, 2001). Antioxidant enzymes including isoforms of superoxide dismutase (SOD), catalase, glutathione reductase (GR) and glutathione peroxidase (GPX) have been found to be inducible in different cells in response to oxidative stress (Harris, 1992). Oxidative stress has been implicated in the pathogenesis of neurodegenerative diseases, such as PD and AD (Jenner, 1991; Jenner *et al.*, 1992; Gotz *et al.*, 1994; Owen *et al.*, 1996; Christen, 2000; Rottkamp *et al.*, 2000; Zhang *et al.*, 2000; Pratico *et al.*, 2002; Zafrilla *et al.*, 2006).

Furthermore, chemical exposure of various kinds can result in apoptotic cell death. Apoptotic cell death is also an important developmental process and occurs during both pre- and postnatal brain development. Exposure to toxicants during these critical periods may perturb this process by altering the systems that regulate apoptotic signals, resulting in undesirable increases in apoptosis and consequent decreases in cell numbers. Excessive free radical or ROS generation has been implicated as initiators of apoptosis (McConkey *et al*. 1994; Corcoran *et al*. 1994). In most cases, cellular defenses can overcome oxidative insults. However, sustained or overwhelming free radical generation may ultimately result in cell death or dysfunction. One such species, hydrogen peroxide, has been shown to induce both apoptotic and necrotic cell d8ath (Lennon *et al.*, 1991; Escargueil-Blanc *et al.*, 1994). It was also shown that higher concentrations of hydrogen peroxide rapidly kills cells with no evidence of apoptosis; moderate concentrations of hydrogen peroxide induces ladder-like DNA cleavage and was associated with morphologic evidence of apoptosis (Gardner *et al.*, 1997). In neuronal cells, H_2O_2 was also shown to induce cell death with necrotic and/or apoptotic characteristics depending on the H_2O_2 concentration (Cole and Perez-Polo, 2002; Valencia and Moran, 2004). It has been hypothesized that endosulfan could induce apoptosis/necrosis via ROS generation. This is supported by evidence that endosulfan induces apoptosis at lower

concentrations and necrosis at higher concentrations in a human leukemic T-cell line (Kannan *et al.*, 2000).

Moreover, oxidative stress has been shown to differentiate certain families of genes in cells (Curutti and Trump, 1991). Reactive oxidants can induce the transcription of several genes such as c-fos, c-jun, c-myc and b-actin (Puri *et al*., 1995). It has been widely accepted that NFkappaB is the first eukaryotic transcription factor that is specifically activated upon oxidative stress (Schreck *et al.*, 1991), and plays a key role in the regulation of numerous genes involved in pathogen responses and cellular defense mechanisms during the programmed cell death (Guerrini *et al.*, 1995). It is also known that the pyrrolidine dithiocarbamates are potent inhibitors of the oxidative stress-induced activation of the transcription factor NFkappaB (Schreck *et al.*, 1992b; Meyer *et al.*, 1993; Schenk *et al.*, 1994). Therefore, in order to relate their possible relevance in the pathogenesis of neurodegenerative diseases, this dissertation included examination of apoptosis, necrosis, ROS formation and activation of NFkappaB in SH-SY5Y neuroblastoma cells following exposure to endosulfan and zineb, *in vitro.*

It is accepted that humans are exposed to a large number of chemicals through various routes of exposure either concurrently or sequentially (Simmons, 1995). Until recently, about 95% of all chemical studies were performed only on individual chemicals. So, there is paucity of data about the effects of various chemicals in mixtures. Research on chemical mixtures has increased due to an initiative set forth by the USEPA and NIEHS to promote a broader understanding of chemicals in mixtures and the mechanisms associated with multiple exposures (NCERQA 1998). This research was initiated to improve understanding of the neurotoxic effects of the mixture of endosulfan and zineb exposure in C57BL/6 mice and on SH-SY5Y cells in culture.

1.3. Specific Aims:

In order to test the above hypotheses, experiments were designed with following specific aims:

Aim 1: To determine the effect of endosulfan, zineb and their mixtures on the dopaminergic and cholinergic systems, in vivo.

Seven to nine-month-old male C57Bl/6 mice were exposed to pesticides. Monoamine levels in both the striatum and cerebral cortex were measured by HPLC and acetylcholinesterase activity (AChE) in the cerebral cortex was measured by spectrophotometric analysis.

Aim 2: To investigate the effects of exposure to endosulfan, zineb and their mixtures administered in early life on the dopaminergic system and cholinergic system following subsequent exposure to these chemicals at adulthood.

The C57BL/6 mice were exposed to each of the pesticides or their mixtures as juveniles (5-19 days) and re-challenged as adults at 8 months age. As also noted above, the level of dopamine and its metabolites in the striatum were measured by HPLC and acetylcholinesterase activity in the cerebral cortex was measured by spectrophotometry. As alpha-synuclein has been reported to be involved in the development of Parkinson's disease, the level of this protein in cerebral cortex was also measured using Western blot analysis.

Aim 3: To assess the cytotoxicity of endosulfan and zineb, alone and in combination, on the SH-SY5Y neuroblastoma cell line and investigate the characteristics of cell death (apoptosis and necrosis), in vitro.

Cytotoxicity was evaluated using lactate dehydrogenase release. A dose-dependent response was obtained by exposure to individual pesticides. For mixture studies, concentrations of pesticides (100 μM each) were chosen based on LC_{25} (lethal

concentration) that would result in minimal cell death. The apoptotic and necrotic effects of pesticides on SH-SY5Y cells were evaluated by flow cytometric analysis using 7-AAD and FITC-Annexin-V dyes. Fragmentation of DNA (a hallmark of apoptosis) was examined by agarose/ethidium bromide gel electrophoresis. The morphological characteristics of the cells were observed by phase-contrast microscopy. Apoptotic and necrotic cells were further identified by visualization under a fluorescence microscope in combination with Annexin-V-FITC-propidium iodide staining.

Aim 4: To examine the role of oxidative stress, caspase-3 and NFkappaB in pesticide-induced cytotoxicity in vitro.

Intracellular H_2O_2 production and superoxide anion ($O_2^{-\bullet}$) production in cells following pesticide exposure were monitored using DCF-DA and hydroethidium spectrofluorometric assays, respectively. Superoxide dismutase, glutathione peroxidase and catalase were also measured by spectrophotometric analysis. Lipid peroxides were measured spectrophotometrically as thiobarbituric acid reactive products (TBARS). Caspase-3 was measured by spectrofluorometric assay. The activation of NFkappaB transcription factor was monitored using an Enzyme Linked Immunosorbent Assay (ELISA) procedure.

CHAPTER 2 Literature review

2.1. Parkinson's disease:

Parkinson's disease is a chronic, progressive neurodegenerative movement disorder. Tremors, rigidity, slow movement (bradykinesia), poor balance, and difficulty walking (called parkinsonian gait) are characteristic primary symptoms of Parkinson's disease (Sandyk *et al.*, 1988; Leenders and Oertel, 2001). Parkinson's disease has a prevalence of approximately 0.5 to 1 percent among persons 65 to 69 years of age, rising to 1 to 3 percent among persons 80 years of age and older (Aschner, 2000; Shastry, 2001). Parkinson's disease occurs when dopaminergic cells, or neurons, in an area of the brain known as the substantia nigra die or become impaired. These neurons produce an important brain chemical known as dopamine, a chemical messenger responsible for transmitting signals between the substantia nigra and the next "relay station" of the brain, the corpus striatum, to produce smooth, purposeful muscle activity. The loss of dopamine causes the nerve cells of the striatum to fire out of control, leaving patients unable to direct or control their movements in a normal manner (Barbeau, 1967; Barbeau, 1968; Rinne, 1982). Previous studies have shown Parkinson's patients lose 80 percent or more of dopamine-producing cells in the substantia nigra (Rondot *et al.*, 1986; Hornykiewicz, 1989; Fearnley and Lees, 1991).

2.2. Basal ganglia:

The basal ganglia have been implicated in a wide variety of motor functions, including the planning, initiation and execution of movements, the performance of learned movements and sequencing of movements (Marsden, 1982). Understanding of the normal circuitry and physiological function of the basal ganglia is essential to gain a clear understanding of the dysfunction leading to Parkinson's disease and related movement disorders.

2.2.1. Basal ganglia anatomy:

The basal ganglion consists of four main structures: the striatum (caudate nucleus, putamen and nucleus accumbens), the pallidum (external and internal segments of the globus pallidus and ventral pallidum), the subthalamic nucleus (STN) and the substantia nigra (pars compacta and pars reticulata). The caudate and putamen together are called the striatum, which is the target of the cortical input to the basal ganglia. The globus pallidus has two segments separated by a medullary lamina, the internal segment of the globus pallidus (GPi) and the external segment of the globus pallidus (GPe). The globus pallidus internal is an output structure of the basal ganglia. The substantia nigra has two parts: the ventral part substantia nigra pars reticulata (SNr) and the dorsal part substantia nigra pars compacta (SNc) that overlaps on the SNr (Lee, 1987; Marsden and Obeso, 1994; Graybiel, 1996; Blandini *et al.*, 2000).

2.2.2. Circuitry:

A basic model of basal ganglia circuitry that has been useful in understanding the symptoms of Parkinson's disease is based on two major loops that process cortical information and feed the processed information back to the cortex. The direct and indirect pathways of the basal ganglia act as a fine-tuning mechanism in movement control (Alexander *et al.*, 1986; Segawa, 2000). In the motor circuit, areas of the motor cortex project in a somatotopic pattern to the posterolateral putamen. There they synapse through excitatory glutamatergic neurons onto the medium spiny striatal neurons. These striatal neurons use gamma-aminobutyric acid (GABA) as their primary neurotransmitter and either substance P (SP) or enkephalin as co-transmitters (Figure 2.1a). They are organized into two pathways: the 'direct' and the 'indirect' pathway (Figure 2.1a). The direct pathway connects the striatum to the internal segment of the GPi and SNr. The GPi and SNr are the output nuclei of the basal ganglia (GPi/SNr) and project to the brainstem and the thalamus (Figure 2.1a). The influence of the GPi and SNr on the thalamus is inhibitory, whereas the thalamic projection to the cortex is excitatory. The indirect pathway also connects the striatum to the output nuclei of the basal ganglia but these fibers first pass through synaptic connections in the GPe and then STN. Output from the STN to the GPi/SNr is excitatory (Figure 2.1a). At the level of the output structures of the basal ganglia, these two pathways oppose each other in controlling the excitatory output

of the VA/VL complex to the motor and premotor cortices (Romo and Schultz, 1992; Jueptner and Krukenberg, 2001; Exner *et al.*, 2002).

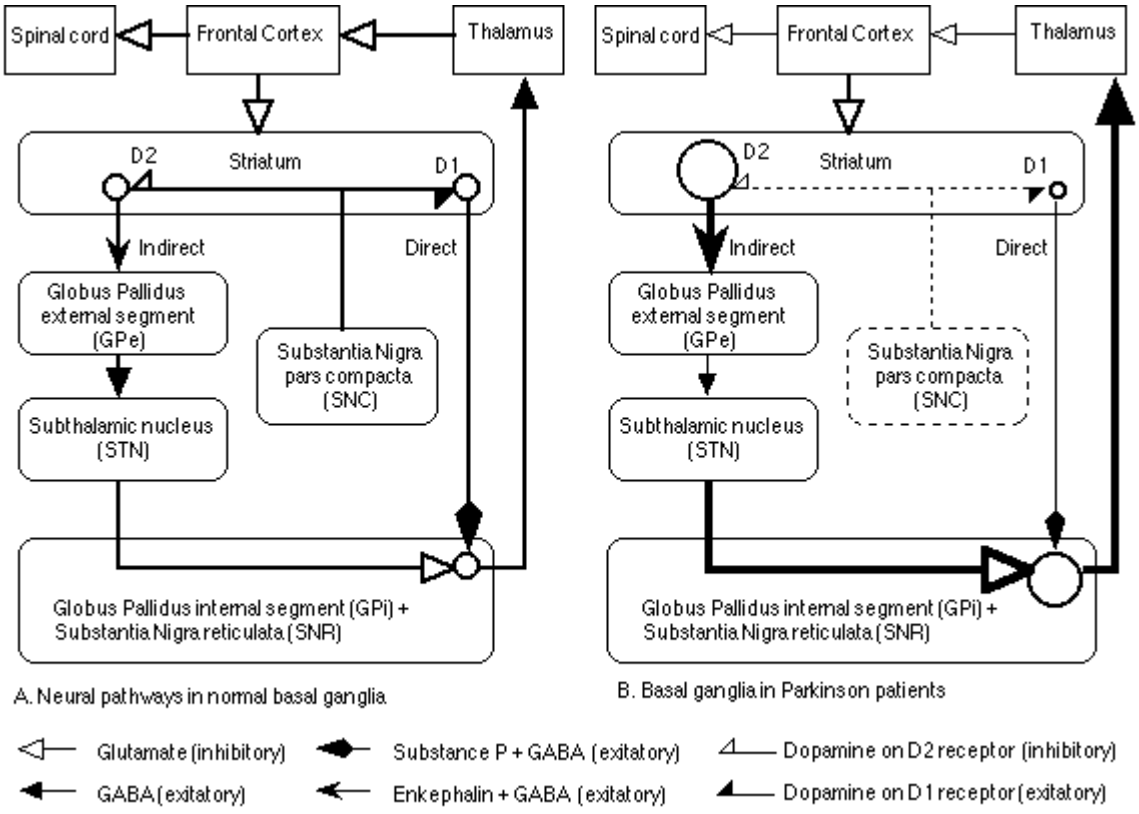


Figure 2.1: Basal ganglia circuitry in normal conditions and Parkinson's disease.
The thickness of the arrows represents the strength of the signal.
http://tcw2.ppsw.rug.nl/~vdbosch/pd.html

2.2.3. The role of dopamine containing neurons in basal ganglia function:

Dopamine-containing neurons play a significant role in regulating the flow of information through the basal ganglia. The dopamine-containing neurons can be divided into three groups: nigrostriatal, mesocortical and tuberohypophysial. The balance of transmission through the direct and indirect pathways is tightly regulated by a major modulator projection from dopaminergic neurons in the SNc. Dopaminergic containing neurons of the substantia nigra communicate with neurons of the basal ganglia by

liberating the neurotransmitter dopamine. This interaction at the biochemical level is responsible for the fine-tuning of an organism's movements. Nearly 80% of all dopamine in the brain is found in the corpus striatum. The dopamine input to the striatum regulates the direct and indirect pathways differentially due to the presence of different postsynaptic dopamine receptors on the two populations of medium spiny neurons. D1 receptors are primarily expressed on medium spiny neurons that project directly to SNr, while D2 receptors are primarily expressed on the medium spiny neurons of the striatum that protect to the GPe in the indirect pathway (Gerfen *et al.*, 1990; Lester *et al.*, 1993; Gerfen *et al.*, 1995; Ince *et al.*, 1997). Dopamine promotes motor behavior by its action on both the direct and indirect pathway. Dopamine excites striatonigral neurons of the direct pathway expressing D1 receptors. Dopamine inhibits striatopallidal neurons of the indirect pathway expressing D2 receptor. Because of the differential expression of dopamine receptors, the release of dopamine in the striatum has a net excitatory effect on the direct pathway, and an inhibitory influence on the indirect pathway (Figure 2.1).

2.2.4 The pathological changes in dopamine containing neurons in Parkinson's disease and the functional effect on degeneration:

Parkinson's disease is a neurological disorder that affects movement control. The primary pathological change giving rise to the motor symptoms of Parkinson's disease is the selective death of dopaminergic neurons in the SNc (Barbeau, 1968; Rinne, 1982). Degeneration of the dopamine nigrostriatal pathway is also accompanied with projection from the substantia nigra of the midbrain to the striatum of the forebrain. This pathway contains 90% of the dopamine within the brain. As a result, the amount of dopamine available for neurotransmission in the corpus striatum is lowered. Parkinsonian signs appear when dopaminergic neurons death exceeds a critical threshold of 70-80% of the nigrostrital nurons (Leenders and Oertel, 2001). Degeneration of dopamine neurons of SNc in Parkinson's disease results in a change of the basal ganglia circuitry that regulates the initiation of voluntary movement. The loss of this important modulator input results in a decrease in activity through the direct pathway and an increase in activity through the indirect pathway (Penney and Young, 1983; Albin *et al.*, 1989; Albin *et al.*, 1995). Specifically, loss of dopaminergic neurons in the SNc reduces the normal inhibition by

the nigrostriatal pathway on GABA-Enkephalin neurons, which increases their activity, and thus over inhibits the GPe (Figure 2.1b). The inhibitory tone of Gpe on STN is reduced and the STN increases its activity well over the normal to excite the GPi/SNr (Figure 2.1b) This increases the GABAergic outputs from the internal segment of the globus pallidus and the pars reticulata of the substantia nigra to the thalamus (Crossman, 1987; DeLong, 1990). Such activity causes increased inhibition of the thalamic ventral nuclei. Because these nuclei are responsible for the activation of cortical areas involved in the initiation of movements, the ultimate effect of dopamine deficiency is paucity of movement (Piccirilli *et al.*, 1984; Scherman *et al.*, 1989), which accounts for most of the symptoms of Parkinson's disease. Furthermore, any alteration of the normal dopaminergic input to the striatum should have serious consequences for hyperactivity of the STN and overexcitation of its efferent targets can now be considered to be the pathophysiological hallmark of the parkinsonian state. L-DOPA (used for the treatment of Parkinson's disease) increases in dopaminergic transmission, thereby restoring the critical dopaminergic modulation of basal ganglia by an increase in activity through the direct pathway and a corresponding decrease in activity through the indirect pathway (Schmidt, 1995; Suri *et al.*, 1997; Merello and Cammarota, 2000).

2.3. Alzheimer's disease:

Alzheimer's disease is an irreversible, progressive disorder in which brain cells (neurons) deteriorate, resulting in the loss of cognitive functions, primarily memory, judgment and reasoning, movement coordination, and pattern recognition. In advanced stages of this disease, all memory and mental functioning may be lost. The two most significant physical findings in the cells of brains affected by Alzheimer's disease are neuritic plaques and neurofibrillary tangles (Masters and Beyreuther, 1990; Goedert *et al.*, 1991; Brion, 1998; Brion, 1999). The neurofibrillary tangles are also called paired helical filaments (PHF's) of Alzheimer's disease type. PHF's are found in the cerebral cortex, hippocampus, locus coeruleus and dorsal raphe of Alzheimer's disease patients. The topographically widespread and cytologically heterogeneous populations of neurons affected in the Alzheimer's disease brain are associated with a complex array of

neurotransmitter deficits. The first transmitter related alteration to be defined was a marked decline in the activities of choline acetyltransferase and acetylcholinesterase, indicating dysfunction and loss of basal forebrain cholinergic neurons and their cortical projections. Acetylcholine is necessary for cognitive function (Enz *et al.*, 1993; Shen, 1994; Taylor, 1998; Talesa, 2001). The activity of acetylcholinetransferase in the cortex and the hippocampus is 70-90% lower in Alzheimer's disease patients than in aged normal controls (Mann and Yates, 1982). This disease is believed to be caused by deterioration of more than a single neurotransmitter system because many neurons releasing monoamine or neuropeptide transmitters also become morphologically abnormal and undergo attrition (Wilcock *et al.*, 1982; Bird *et al.*, 1983; Nagai *et al.*, 1983; Foster *et al.*, 1986; Boissiere *et al.*, 1997; Kim *et al.*, 2004).

2.4. The similarities and the link (Alpha-synuclein) between Parkinson's disease and Alzheimer's disease:

Although Parkinson's Disease and Alzheimer's disease affect different cell types within the CNS, the two diseases also often coexist (Ebmeier *et al.*, 1988; Forno, 1992). In one study 23% of people with Alzheimer's also met the criteria for Parkinson's disease (Cooper, 1991). In basal ganglia, dopamine regulates striatal cholinergic function through D1 and D2 receptors expressed by cholinergic interneurons (Dawson *et al.*, 1990; Asanuma *et al.*, 1992; Abercrombie and DeBoer, 1997; McGinty, 1999). The stimulation of D1 receptor increases acetylcholine release while the stimulation of D2 decreases acetylcholine release. A balance between acetylcholine and dopamine has been recognized as necessary for normal striatal function, thereby suggesting that acetylcholine usually opposes the effects of dopamine (Pradhan, 1974; Denaro *et al.*, 1984; Calabresi *et al.*, 1989; Westerink *et al.*, 1990). For this reason, anything that affects dopamine will also affect acetylcholine and this will have a repercussion in other major neurodegenerative disease such as Alzheimer's disease.

The presence of aggregates of alpha-synuclein in brain is one of the emerging factors linking neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease.

Alpha-synuclein has been identified as a major component of Lewy bodies. The presence of Lewy bodies is a feature of neurodegenerative disease such as Alzheimer's disease and Parkinson's disease. Alpha synuclein (from the locus PARK1) is a 140 amino acid protein, which is highly conserved among vertebrates and belongs to a family of four closely related members, comprising alpha, beta and gamma synuclein (Clayton and George, 1998; Clayton and George, 1999; George, 2002). Alpha-synuclein is a soluble unfolded protein, but it can aggregate to form insoluble amyloid fibrils, particularly in its mutated form (Spillantini *et al.*, 1996; Spillantini *et al.*, 1997). Mutations in the alpha-synuclein gene may make it easier for the protein to be in the random coil state so that aggregation is more likely to occur (Weinreb *et al.*, 1996; Clayton and George, 1999; Di Rosa *et al.*, 2003; Kaplan *et al.*, 2003; Klucken *et al.*, 2003). Aggregated insoluble alpha-synuclein may be toxic and cause neurodegeneration. Full-length alpha-synuclein normally occurs in a natively unfolded state, but at high concentrations, it can form oligomers of beta pleated sheets, called protofibrils, that can seed in a nucleation-dependent manner to form the amyloid fibrils present in Lewy bodies. The normal physiological role of alpha-synuclein is not clear, but it could modulate dopamine storage, synthesis and uptake by regulating synaptic vesicle recycling in nerve terminal. Studies using a newly characterized human mesencephalic cell line indicate that alpha-synuclein, may be important for dopamine storage (Lotharius *et al.*, 2002). In addition to regulating dopamine storage, alpha-synuclein could modulate intracellular dopamine handling through interactions with proteins that regulate dopamine synthesis and uptake. For instance, direct binding of alpha-synuclein to the dopamine transporter via its C-terminal domain seems to enhance extracellular dopamine uptake by increasing the number of functional transporters at the cell surface (Lee *et al.*, 2001). Furthermore, *in vitro* studies suggest that alpha-synuclein can directly bind to and inhibit tyrosine hydroxylase (TH), the first enzyme in the pathway of synthesis of dopamine from tyrosine (Perez *et al.*, 2002; Baptista *et al.*, 2003; Gillette and Bloomquist, 2003).

The biochemical mechanism of action of alpha-synuclein in neuropathology leading to dopaminergic neurons death is not clearly understood. Expression of mutant or wild type forms of alpha-synuclein in transgenic mice produced adult-onset loss of dopaminergic

neurons, filamentous intraneuronal inclusions containing alpha-synuclein and locomotor dysfunction in transgenic fruit flies (Feany and Bender, 2000). One hypothesis for the ultimate cause of the destruction of dopaminergic neurons by mutant or overexpressed alpha-synuclein suggests that the damage may results from pathway of dopamine-induced oxidative stress and over production of oxygen free radicals (Souza *et al.*, 2000; Shavali *et al.*, 2006). Free radical generation may play a pivotal role in dopaminergic neuron death. As mentioned above, alpha-synuclein may regulate vesicle recycling at the nerve terminal. Altered function of alpha-synuclein brought about by mutations or over express could result in a shortage of monoaminergic vesicles, which would, in turn, lead to a gradual accumulation of dopamine in the cytoplasm. Those high levels of dopamine in the cytoplasm are easily oxidized by catabolism. The catabolism of dopamine itself, via both enzymatic deamination and auto-oxidation, is reputed to generate toxic superoxide and hydroxyl radicals, which may in turn trigger a self-amplifying cell-destruction cycle. In normal degradative pathway of dopamine by monoamine oxidase, one molecule of H_2O_2 can cause lipid peroxidation and/or can be further metabolized to other reactive oxidative species. This oxidative stress primarily occurs at nigrostriatal terminals, where most of this neurotransmitter is synthesized and stored. In turn, the onset of the dopamine containing neurons death could be triggered (Lotharius *et al.*, 2002).

2.5. Environmental risk factor and PD disease:

2.5.1. MPTP:

Although the cause of Parkinson's disease is not known, most studies strongly suggest that environmental factors, particularly pesticides exposure, may play an important role in the pathogenesis of this disorder. The environmental factors hypothesis emerged in the Parkinson's disease field following the discovery of the Parkinsonian toxin MPTP (1-methyl-4-phenyl-1, 2,3,6- tetrahydropyridine), an analog of the opioid meperidine used by drug abusers (Langston *et al.*, 1983). Primates treated with MPTP exhibit behavioral, biochemical and neuropathological deficits which are very similar to those observed in human parkinsonian patients (Burns *et al.*, 1983; Chiueh *et al.*, 1984). The impairment of dopaminergic neurotransmission is characterized by a reduction in the concentrations of dopamine and of its metabolite (3,4, -dihydroxyphenylacetic acid and homovanillic acid),

the diminished activity of TH, and a decrease in the density of dopamine recepotors (Chiueh *et al.*, 1984; Jarvis and Wagner, 1985; Sirinathsinghji *et al.*, 1986; Sundstrom *et al.*, 1990; Russ *et al.*, 1991; Schmidt and Ferger, 2001; Perry *et al.*, 2004). MPTP-induced Parkinsonism in laboratory animals, widely regarded as the best animal model of Parkinson disease, has yielded clues to some of the cause of idiopathic Parkinson's disease. As soon as it was recognized that MPTP was the neurotoxicant, a number of laboratories began to investigate its mechanism of action. Although MPTP can cross the blood-brain barrier, MPTP itself is not toxic, but has to be oxidized to the active toxin MPP^+ by two-step biotransformation process (Da Prada and Kettler, 1986; Chang and Ramirez, 1987; Yang *et al.*, 1988). MPTP is first oxidized by MAO-B to 1-methyl-4-phenyl-1, 2-dihydropyridinium ion ($MPDP^+$). In a second step, $MPDP^+$ is spontaneously oxidized to MPP^+. Many studies support the hypothesis that MPP^+ is the primary toxic metabolite of MPTP (Mytilineou *et al.*, 1985; D'Amato *et al.*, 1986; Singer *et al.*, 1988). MPP^+ is toxic to neuronal cells in tissue culture and produces long term dopamine depletion after intraventricular injection in rodents. MPP^+ is transformed and accumulated in the periphery as well as in the central nervous system. However, MPP^+ formed in the periphery does not contribute to brain toxicity, since it cannot readily pass the blood brain barrier because of its charged configuration (Dostert and Strolin Benedetti, 1988). It is now generally agreed that glia cells release MPP^+ into the extracellular space by an active mechanism using the extraneuronal monoamine transporter which was studied in detail by Ruuss and coworkers (Russ *et al.*, 1996). Subsequently, MPP^+ can be specifically taken into dopaminergic nerve terminals via plasma membrane dopamine transporter. Recently it was demonstrated that mice with a deficiency of the dopamine transporter are resistant against MPTP toxicity (Gainetdinov *et al.*, 1997; Takahashi *et al.*, 1997; Hogan *et al.*, 2000; Kilbourn *et al.*, 2000; Kurosaki *et al.*, 2003). Over expression of dopamine transporter may result in enhanced MPTP neurotoxicity (Gainetdinov *et al.*, 1997; Bezard *et al.*, 1999; Eberling *et al.*, 1999; Kurosaki *et al.*, 2003).

2.5.2. Pesticides:

Pesticides are used extensively throughout the world. Each year about 5 billion pounds of pesticides are used worldwide (Smith, 2001). Such widespread use results in pervasive human exposure. Numerous studies have suggested exposure to pesticide has a negative impact on public health (Rhile *et al.*, 1996; Hampton and Orrenius, 1998; Tebourbi *et al.*, 1998; Thiruchelvam *et al.*, 2000b; Camacho *et al.*, 2001; Thiruchelvam *et al.*, 2002b; Thiruchelvam *et al.*, 2004). Chronic use and human exposure to certain pesticides have been linked to neurodegenerative disease such as Parkinson's and Alzheimer's disease. Several classes of pesticides, particularly insecticides including organophosphorous compounds, carbamates, pyrethroids and organochlorines have been implicated in increased risk of Parkinson disease. The clinical and pathological syndromes associated with both the familial PD cases and those caused by environmental agents are remarkably similar.

The herbicide paraquat (N,N′-dimethyl-4,4′-bipyridylium), an environmental agricultural chemical, has been repeatedly posed as a risk factor for Parkinsonian-syndrome in humans. The injections of paraquat directly into brain can change dopamine levels and behavior of animals and produce neuronal loss (Calo *et al.*, 1990; Bagetta *et al.*, 1992; Liou *et al.*, 1996) (Brooks *et al.*, 1999). Furthermore, the exposure of mice to paraquat leads to a significant increase in brain levels of alpha-synuclein and is accompanied by the accumulation of alpha-synuclein-containing lesions within neurons of the SN pars compacta (Manning-Bog *et al.*, 2002). Dithiocarbamate-based fungicides have also been found to impose dopaminergic neurotoxicity. The dithiocarbamate fungicides, such as maneb and mancozeb are known to possess potent dopaminergic activity and enhance the toxic effects of MPTP in mice (Takahashi *et al.*, 1989). The exposure of mice to maneb produced selective nigrostriatal dopamine system neurotoxicity, including loss of striatal dopamine and loss of cell bodies of dopamine neurons in the substantia nigra pars compacta (Thiruchelvam *et al.*, 2002b). Exposure to rotenone, the naturally occurring and commonly used insecticide, has been associated with increased incidence of PD. The rotenone model appears to be an accurate model in that systemic mitochondrial complex I inhibition results in specific, progressive and chronic degeneration of the nigrostriatal pathway similar to that observed in human Parkinson' disease (Tai and Truong, 2002;

Gao *et al.*, 2003; Greenamyre *et al.*, 2003; Perier *et al.*, 2003; Sherer *et al.*, 2003; Trojanowski, 2003; Alam and Schmidt, 2004). It also reproduces the neuronal inclusions and oxidative stress damage seen in Parkinson's disease (Testa *et al.*, 2005). Organochlorine pesticides have potential dopaminergic neurotoxic effects. The neurotoxic and dopamine-depleting effects of the organochlorine dieldrin were initially observed in intoxicated ducks, doves, and rats (Sharma *et al.*, 1976; Wagner and Greene, 1978; Heinz *et al.*, 1980). The organochlorine heptachlor has been associated with nigrostriatal damage and alteration of the dopamine transporter, contributing towards these diseases (Miller *et al.*, 1999). In addition to the above-mentioned pesticides, case reports have associated development of Parkinsonism with exposure to organophosphorus and pyrethroid insecticides (Muller-Vahl *et al.*, 1999; Karen *et al.*, 2001). Other reviews have, however, suggested that there is insufficient evidence to correlate PD with pesticide exposure (IOM, 2003).

Until recently, about 95% of all chemical studies were performed only on individual chemicals (Groten et al. 1999). It is accepted that humans are exposed to a large number of chemicals through various routes of exposure either concurrently or sequentially (Simmons, 1995). These exposures are often highly correlated, particularly within functional or chemical groups, making it difficult to identify effects of particular agents. Often when chemicals are in mixture, one compound will intensify the effect of other compounds by altering biotransformation processes, such as modulating certain metabolic enzymes or interrupting cellular receptors (Groten et al. 1999). Recent research on chemical mixtures has increased due to an initiative set forth by the USEPA and NIEHS to promote a broader understanding of chemicals in mixtures and the mechanisms associated with multiple exposures (NCERQA 1998). The studies on chemical mixtures are few in regard to the nervous system. More research on chemical mixtures is needed to gain a better understanding of multiple chemical toxicity.

2.5.2.1. Endosulfan:

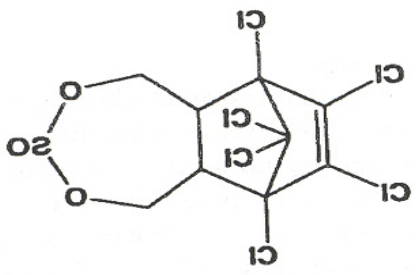


Figure 2.2: Chemical structure of endosulfan

Endosulfan (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzadioxathiepin 3-oxide) is a member of the cyclodiene group of organochlorine pesticides used worldwide in agriculture (Figure 2.2). It is commonly used to control a number of insects on food crops such as grains, tea, fruits, and vegetables and on nonfood crops such as tobacco and cotton. It is made up of a mixture of two isomers, alpha and beta isomers with approximately 7:3 ratio. Approximately 1.4 - 2.2 million pounds of active ingredient are used annually in the U.S (EPA, 1972). Although endosulfan is no longer produced in the United States, it is registered for use on 60 crops and is a major environmental concern in southern Florida (Baughman, 1986). Endosulfan is fairly resistant to photo-degradation, persistent in soil and tends to be uniformly distributed through all parts of the environment. Its half-life ranges from few months to years in soil and 1-6 months in water depending on pH and other climatic conditions. The general public may be exposed to endosulfan simply by ingesting contaminated food, inhaling contaminated air, drinking a beverage made from endosulfan contaminated water, or by skin contact.

In humans with endosulfan poisoning, overstimulation of the central nervous system is the major characteristic (Boyd and Dobos, 1969; Boyd *et al.*, 1970; Aleksandrowicz, 1979; Blanco-Coronado *et al.*, 1992; Gilbert and Mack, 1995; Boereboom *et al.*, 1998; Chugh *et al.*, 1998). Cyclodiene pesticides such as endosulfan antagonize the action of the neurotransmitter gamma-aminobutyric acid (GABA), which induces the uptake of chloride ions by neurons. Binding of GABA to its receptor opens chloride-selective ion

channels leading to influx of chloride into neurons through electrochemical gradient, resulting in hyperpolarization of the membrane and inhibition of cell firing. Studies in animals have shown changes in neurotransmitter levels and alterations in neurobehavioral processes after exposure to endosulfan (Lakshmana and Raju, 1994; Paul *et al.*, 1995). The most prominent signs of acute overexposure to endosulfan in both humans and animals are hyperactivity, tremors, decreased respiration, dyspnea, salivation, and tonic-clonic convulsions. Possible mechanisms of endosulfan-induced neurotoxicity include alteration of neurotransmitter levels in brain areas by affecting synthesis, degradation, rates of release and re-uptake, and interference with the binding of those neurotransmitter to their receptors (Gupta, 1976; Agrawal *et al.*, 1983; Zaidi *et al.*, 1985; Abalis *et al.*, 1986; Cole and Casida, 1986; Ansari *et al.*, 1987; Lakshmana and Raju, 1994; Paul *et al.*, 1994; Pomes *et al.*, 1994; Paul *et al.*, 1995; Rosa *et al.*, 1996). Changes in neurotransmitter levels (specifically serotonin, GABA, and dopamine) in the brain were believed to be partly responsible for the neurotoxicity of endosulfan in rats after observing hyperactivity, tremors, and convulsions following a single intraperitoneal injection of 40 mg/kg of endosulfan (Ansari *et al.*, 1987). Changes in the concentrations of dopamine, noradrenaline, and serotonin in various brain areas of endosulfan-treated rats were also reported (Lakshmana and Raju, 1994). The cyclodiene pesticides antagonize the action of the neurotransmitter GABA, which induces the uptake of chloride ions by neurons. GABA receptor is in involved in endosulfan-induced neurotoxicity (Abalis *et al.*, 1986; Cole and Casida, 1986; Ozoe and Matsumura, 1986). Endosulfan was also reported to affect serotonic system, significant increase in frontal cortical ^{3}H-serotonin binding that in turn induces neurotoxicity observed in rats following multiple exposure endosulfan (Agrawal *et al.*, 1983; Zaidi *et al.*, 1985).

2.5.2.2. Zineb:

Zineb (Zinc-ethylene-bis-dithiocarbamate) belongs to ethylene bis-dithiocarbamate (EBDC), a class of fungicides used worldwide in agriculture (Figure 2.3). EBDC are used on a wide range of crops world wide including potatoes, cereals, apples, pears, and leafy vegetables due to the reported low acute toxicity and scarce persistence in the

environment((Maroni *et al.*, 2000). They control many fungal diseases such as blight, leaf spot, rust, downy mildew and scab.

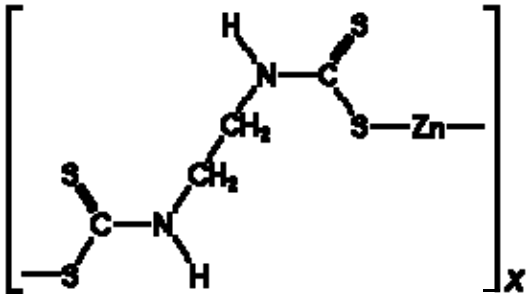


Figure 2.3: Structure of zineb

The major metal derivatives of ethylene bis-dithiocarbamate widely used in agriculture are maneb (manganese-ethylene-bis-dithiocarbamate), zineb, mancozeb (manganese-zinc- ethylene-bis-dithiocarbamate), metiram (zineb-ethylene-thiuram-disulphide adduct) and propineb (polymeric zinc propylenebisdithiocarbamate). For examples, 3.0 million pounds of maneb and 8.3 million pound of mancozeb are applied to annually in the United States (www.usgs.gov). Exposure to these compounds can be via ingestion, inhalation, or dermal pathways. Dithiocarbamate fungicides such as maneb possess potent dopaminergic activity and are well known to enhance the toxic effects of MPTP in mice (Takahashi *et al.*, 1989). Exposures of maneb to mice produced selective nigrostriatal dopamine system neurotoxicity, including loss of striatal dopamine and of cell bodies of dopamine neurons in the substantia nigra pars compacta (Thiruchelvam *et al.*, 2000a; Thiruchelvam *et al.*, 2000b). Combination of maneb and paraquat targets nigrostriatal dopamine systems and induces locomotor impairment. This suggest that these combinations may be considered as potential environmental risk factors for Parkinsonism (Thiruchelvam *et al.*, 2000a; Thiruchelvam *et al.*, 2000b). The symptoms of a mild, generalized bradykinesia and rigidity, postural tremor in the right limbs, mild tremor of the lips, mild slowness of gait with reduced swinging of the arms, seborrhea,

mild hypomimia, and slurred speech was found in human exposure with maneb (Meco *et al.*, 1994). Exposure to zineb and mancozeb at 50 and 10 μM for 24 hours significantly reduced specific dopamine and GABA uptakes in mesencephalic-striatal coculture (Soleo *et al.*, 1996).

Much less is known about the mechanisms of zineb neurotoxicity. Based on the neurochemical and behavioral changes and its apparently selective disruption of the nigrostriatal system, just like maneb, zineb is also assumed to be able to cross the blood–brain barrier (Thiruchelvam et al. 2002). Zinc–EBDC, being relatively stable in vitro, could potentially degenerate to zinc and EBDC in vivo, and both of these compounds are potentially neurotoxic. Several studies suggest zinc might be involved in several neurological dysfunctions (Dineley *et al.*, 2003; Hipkiss, 2005; Mocchegiani *et al.*, 2005). Zinc interacts with the proteins, beta-amyloid and its precursor protein, which are believed to be involved in the causation of degenerative processes in the brain, particularly in Alzheimer's disease (Constantinidis, 1990; Bush *et al.*, 1993; Fitzgerald, 1995; Cuajungco and Lees, 1997; Curtain *et al.*, 2001; Cuajungco and Faget, 2003). On the other hand, the EBDC component has been suggested to be primarily responsible for the neurotoxicity on neuronal system relevant to the pathophysiology of Parkinsonism (Soleo *et al.*, 1996). This conclusion follows from the finding that both mancozeb (Mn–Zinc–EBDC) and zineb (Zinc–EBDC) produce neurotoxicity in cell cultures (Soleo *et al.*, 1996). Other studies also support the suggestion that EBDC can enhance MPTP-induced neurotoxicity (McGrew *et al.*, 2000). Oxidative stress is a key aspect of dithiocarbamate pesticide-induced neurotoxicity. Several dithiocarbamate pesticides have been reported to increase ROS formation in neuronal cells (Tariq *et al.*, 2000; Barlow *et al.*, 2005).Oxidative damage to protein (inhibit mitochondrial function) was associated with Mn-EBDC induced neurotoxicity (Zhang *et al.*, 2003).

2.6. Reactive Oxygen Species and Oxidative Stress:

Free radicals are chemical species which have unpaired electrons. Usually, free radicals are not stable and in general reactive and attack other molecules unless there are some structural factors to stabilize their unpaired electrons. There are many forms of free radical species, such as oxygen-centered, carbon-centered, nitrogen centered. Any free radical involving oxygen can be referred to as reactive oxygen species (ROS). ROS include free radical such as superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($O_2^{\uparrow\downarrow}$), and the partially reduced form of oxygen, hydrogen peroxide (H_2O_2). The one-electron reduction product of oxygen is the superoxide radical. If two electrons are transferred, the product is hydrogen peroxide. Certain chelates of ferrous iron and cuprous copper are capable of transferring a third electron to hydrogen peroxide, causing lysis of the O-O bond generating the hydroxyl free radical, $OH^{\bullet}$, one of the most potent oxidants known.

$O_2 + e^- \rightarrow O_2^-$ ……... (i)

$O_2^- + e^- + 2H^+ \rightarrow H_2O_2$ ……. (ii)

$Fe^{2+}/Cu^+ + H_2O_2 \rightarrow Fe^{3+}/Cu^{2+} + OH\cdot + OH^-$ ……..(III)

Superoxide anion lacks the ability to penetrate lipid membranes and is therefore enclosed in the compartment where it was produced. So, it is not highly reactive. Hydrogen peroxide is not a free radical but is nonetheless highly important much because of its ability to penetrate biological membranes. Hydrogen peroxide is therefore toxic to cells and a cause of further free radical generation, particularly when reacting with reduced transition metals to form hydroxyl radicals. Hydrogen peroxide is one of the most powerful oxidizers know, stronger than chlorine, chlorine dioxide. Hydroxyl radical is extremely reactive and very short lived. Highly reactive hydroxyl radical can cause damage to DNA and other biological molecules (Muindi *et al.*, 1984; Jackson *et al.*, 1987; Muller and Gurster, 1993).

To control the level of free radicals, the aerobic organism has utilized several anti-oxidative mechanisms including enzymatic and nonenzymatic antioxidants (Storey,

1996). The antioxidant enzymes include superoxide dismutase (SOD), catalase, glutathione reductase (GR) and glutathione peroxidase (GPX). SOD catalyses the dismutation of superoxide to H_2O_2. Mammalian cells contain 3 forms of SOD, Mn-SOD, Cu/Zn SOD, and the extracellular SOD. The manganese-containing enzyme is most abundant in mitochondria, while the zinc/copper forms predominant in cytoplasm (Fridovich, 1995). Catalase is major antioxidant enzyme that catalyzes the decomposition of H_2O_2 to H_2O, sharing this function with GPX. Both enzymes detoxify oxygen reactive radicals by catalyzing the formation of H_2O_2 derived from superoxide and hence finish the detoxification reaction started by SOD. GR catalyzes the reduction of oxidized glutathione (GSSG) to glutathione (GSH), so it is essential for the glutathione redox cycle that maintains adequate levels of reduced cellular GSH. Non-enzyme low molecular weight antioxidant compounds include vitamins C and E, as well as glutathione, β-carotene and uric acid. Vitamin C is widely recognized for its antioxidant function in extracellular fluid' and it has been shown to neutralize $O_2^{-\bullet}$ and $^{\bullet}OH$ (Schneider *et al.*, 2005). Vitamin E is an important intracellular antioxidant bound predominately in the membrane due to its highly lipophilic properties. Vitamin E provides antioxidant action in and near lipid membranes by converting $O_2^{-\bullet}$, $^{\bullet}OH$, to less reactive forms (*Bagchi et al.*, 1997). Vitamin A and β-carotene are also lipophilic and scavenge $O_2^{-\bullet}$ or react with peroxyl radicals. Uric acid is hydrophilic and scavenges $O_2^{-\bullet}$, $^{\bullet}OH$, and peroxyl radicals. GSH is the primary low-molecular-weight thiol in the cytoplasm and is a major reserve for cysteine. GSH in conjunction with the reductant NADPH can reduce lipid peroxides, superoxide anion, hydroxyl radical and hydrogen peroxide.

Oxidative stress may occur when ROS are produced faster than they can be removed by cellular defense mechanisms. The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, proteins, and DNA. Lipid peroxidation is the most common and dangerous type of free radical-induced cellular oxidation. It is a consequence of interaction of free radical and lipid to form lipid radicals, lipid hydroperoxide and peroxide intermediates, which

subsequently leads to autocatalytic radical chain reactions, resulting in membrane damage.

Oxidative stress plays an important role in the pathogenesis of neurodegenerative diseases, such as PD and AD (Jenner, 1991; Jenner *et al.*, 1992; Gotz *et al.*, 1994; Owen *et al.*, 1996; Christen, 2000; Rottkamp *et al.*, 2000; Zhang *et al.*, 2000; Pratico *et al.*, 2002; Zafrilla *et al.*, 2006). Neurodegenerative disease has been also related to free radicals generation, which plays a pivotal role in neuronal death. The decreased level of reduced glutathione in substantia nigra in Parkinson's disease could be the result of neuronal loss (Perry *et al.*, 1982; Sofic *et al.*, 1992; Spencer *et al.*, 1995; Sechi *et al.*, 1996). The activity of mitochondrial superoxide dismutase in the substantia nigra in Parkinson's disease was increased (Saggu *et al.*, 1989). Excess free radical could increase the risk of lipid peroxidation that plays a role in producing the Parkinson-like syndrome (Dexter *et al.*, 1986; Pall *et al.*, 1986; Smith *et al.*, 1987; Kilinc *et al.*, 1988; Dexter *et al.*, 1989; Agil *et al.*, 2006).

Oxidative damage to tissues caused by ROS has been proposed to be vital for the induction of a variety of chemical-induced toxic manifestations. The mechanism of action of MPTP has been suggested to be mediated by a series of free radical reaction and oxidative stress. The ultimate cause of the destruction of dopaminergic neurons by MPTP suggests that the damage may result from intraneuronal generation of superoxides and other cytotoxic free radicals (Chiueh *et al.*, 1992; Zang and Misra, 1992a; Zang and Misra, 1992b; Adams *et al.*, 1993; Chiueh *et al.*, 1993; Chiueh and Rauhala, 1998; Obata and Yamanaka, 2001). MPP^+ is concentrated in mitochondria and impairs mitochondrial respiration (Heikkila *et al.*, 1984) by inhibiting complex I of the electron transfer complex (Mizuno *et al.*, 1987; Mizuno *et al.*, 1988; Arora *et al.*, 1990; Kotake and Ohta, 2003) and consequently causes the death of neurons. Blockade of mitochondrial respiration leads to an acute deficit in ATP formation and increases production of ROS, especially of superoxide (Sinha *et al.*, 1986; Hasegawa *et al.*, 1990; Walker *et al.*, 1991; Ramsay and Singer, 1992). MPP^+ has been shown to release toxic Fe^{+2}, which in turn may react with hydrogen peroxide to generate hydroxyl radicals via the Fenton reaction

(Mohanakumar *et al.*, 1994; Double *et al.*, 1998). Transgenic mice which overexpress the superoxide detoxifying enzyme SOD show a significant protection against MPTP toxicity (Andrews *et al.*, 1996; Sanchez-Ramos *et al.*, 1997). Dopaminergic cell death by MPTP caused by oxidative stress is followed by lipid peroxidation (Mizuno *et al.*, 1987; Mizuno *et al.*, 1988). Mmany pesticides are capable of inducing oxidative stress by overwhelming or modulating cellular drug metabolizing system (Dikshith, 1991; Bachowski *et al.*, 1998; Bagchi *et al.*, 2000; Gauthier *et al.*, 2001). Several different studies indicate that within different cells or in organ systems various classes of pesticides including organophosphates, bipyridyl herbicides and organochlorines increase free racial that leads to oxidative stress (Cicchetti and Argentin, 2003; Kovacic, 2003; Abdollahi *et al.*, 2004). These pesticides exposures can result in the alteration of secondary messengers, such as free radicals or other ROS, and these alterations have been linked to the induction of apoptosis in cells.

2.7. Apoptotic and necrotic cell death:

Cell death is part of the normal development and maturation cycle, and is a component of many response patterns of living cells to xenobiotic agents such as pesticides exposure (Carlson *et al.*, 2000; Kitazawa *et al.*, 2001; Kitazawa *et al.*, 2004; McCarthy *et al.*, 2004). Cell death can be partitioned into distinct cell death pathways, apoptosis and necrosis. Apoptosis is a very important phenomenon for the survival of an organism. In order to maintain a constant size, multicellular organisms have to equalize the rates of cell production and cell death. Controlled, normal physiological death of unwanted cells occurs via apoptosis. Necrosis appears to be the result of acute cellular dysfunction in response to severe stress conditions or after exposure to toxic agents. There are many observable morphological and biochemical differences between apoptosis and necrosis. Apoptosis is a complex process characterized by mitochondrial depolarization, alterations in phospholipid asymmetry, chromatin condensation, nuclear fragmentation, membrane blebbing, cell shrinkage and the formation of membrane bound vesicles termed apoptotic bodies (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Buja *et al.*, 1993; Farber, 1994; Columbano, 1995; Trump *et al.*, 1997; Maruyama, 1998). The most important feature of apoptotic cell

death is that it eliminates the dying cells without eliciting an inflammatory response. Necrosis, in contrast, is characterized morphologically by cytoplasmic and mitochondrial swelling, plasma membrane rupturing and release of the cellular contents into the extracellular space (Buja *et al.*, 1993; Majno and Joris, 1995; Zhivotovsky, 2004). Due to the ultimate breakdown of the plasma membrane that leads to release of cytoplasmic contents into the extracellular fluid, necrotic cell death is often associated with inflammatory response that can cause further tissue damage by affecting neighboring cells (Haslett, 1992).

Caspase (cysteinyl-aspartate-specific proteinases) activation plays a central role in the execution of apoptosis (Cohen *et al.*, 2004; Sun *et al.*, 2004; Cohen, 2005). Caspases are intracellular cysteine proteases related to CED-3, the death gene of the nematode C. elegans. Fourteen caspases have been identified so far, all of which contain an active site thiol group necessary for activity (Thornberry and Lazebnik, 1998). Based on their homology in amino acid sequences, caspases are divided into three subgroups. The first subgroup includes caspase 2, 8, 9 and 10. This subgroup is involved primarily in the apoptosis activator. The second subgroup including caspase 3, 6 and 7 is primarily involved in executing apoptosis. The third subgroup, including caspase 4, 5, 11, 12, 13, and 14, is involved primarily in the control of inflammation and is not thought to be involved in the process of apoptosis.

Neurodegenerative disease often occurs via apoptosis (Tatton and Olanow, 1999; Honig and Rosenberg, 2000; Rosenstock *et al.*, 2004; Waldmeier and Tatton, 2004; Clayton *et al.*, 2005; Novikova *et al.*, 2006). Caspase-3 has been implicated as a key mediator of apoptosis. A downstream caspase activated by both caspase-9 and caspase-8, caspase-3-like proteases were the first caspases linked to apoptosis. Caspase-3 also appears to play an important role in developmentally regulated cell death in the brain (de Bilbao *et al.*, 1999; Shimohama *et al.*, 2001; Yakovlev *et al.*, 2001). Activation of caspase-3 is a necessary step for neuronal apoptosis after exposure to neurotoxins such as MPP^+ (Fall and Bennett, 1999). In addition, the activation of caspase-3 has been found to be related to Parkinson's disease and Alzheimer's disease (Stadelmann *et al.*, 1999; Hartmann *et al.*,

2000; Jellinger, 2000; Jordan *et al.*, 2000; Rideout and Stefanis, 2001). This suggests that caspase-3 may act as a common downstream effector of cell death in many neurodegenerative disorders.

Several classes of pesticides, including the insecticides that are organophosphorous compounds carbamates, pyrethroids and organochlorines have been shown to induce apoptosis (Ahmadi *et al.*, 2003; Masoud *et al.*, 2003; Olgun *et al.*, 2004; Bloom *et al.*, 2006; Calviello *et al.*, 2006). Most, if not all, chemicals seem to be capable of producing apoptosis at low doses and necrosis at higher doses (Wyllie, 1987; Lennon *et al.*, 1991; Raffray and Cohen, 1991).

Examination of the type of cell death caused by endosulfan, zineb, or by their mixtures may help improve our understanding of cellular events that may lead to cell death and could make a potential contribution for the risk assessment in the etiology of neurodegenerative diseases.

2.8. Literature Cited:

Abalis, I. M., Eldefrawi, M. E., and Eldefrawi, A. T. (1986). Effects of insecticides on GABA-induced chloride influx into rat brain microsacs. *J Toxicol Environ Health* **18**, 13-23.

Abdollahi, M., Ranjbar, A., Shadnia, S., Nikfar, S., and Rezaiee, A. (2004). Pesticides and oxidative stress: a review. *Med Sci Monit* **10**, RA141-147.

Abercrombie, E. D., and DeBoer, P. (1997). Substantia nigra D1 receptors and stimulation of striatal cholinergic interneurons by dopamine: a proposed circuit mechanism. *J Neurosci* **17**, 8498-8505.

Adams, J. D., Jr., Klaidman, L. K., and Leung, A. C. (1993). MPP+ and MPDP+ induced oxygen radical formation with mitochondrial enzymes. *Free Radic Biol Med* **15**, 181-186.

Agil, A., Duran, R., Barrero, F., Morales, B., Arauzo, M., Alba, F., Miranda, M. T., Prieto, I., Ramirez, M., and Vives, F. (2006). Plasma lipid peroxidation in sporadic Parkinson's disease. Role of the L-dopa. *J Neurol Sci* **240**, 31-36.

Agrawal, A. K., Anand, M., Zaidi, N. F., and Seth, P. K. (1983). Involvement of serotonergic receptors in endosulfan neurotoxicity. *Biochem Pharmacol* **32**, 3591-3593.

Ahmadi, F. A., Linseman, D. A., Grammatopoulos, T. N., Jones, S. M., Bouchard, R. J., Freed, C. R., Heidenreich, K. A., and Zawada, W. M. (2003). The pesticide rotenone induces caspase-3-mediated apoptosis in ventral mesencephalic dopaminergic neurons. *J Neurochem* **87**, 914-921.

Alam, M., and Schmidt, W. J. (2004). Mitochondrial complex I inhibition depletes plasma testosterone in the rotenone model of Parkinson's disease. *Physiol Behav* **83**, 395-400.

Albin, R. L., Young, A. B., and Penney, J. B. (1989). The functional anatomy of basal ganglia disorders. *Trends Neurosci* **12**, 366-375.

Albin, R. L., Young, A. B., and Penney, J. B. (1995). The functional anatomy of disorders of the basal ganglia. *Trends Neurosci* **18**, 63-64.

Aleksandrowicz, D. R. (1979). Endosulfan poisoning and chronic brain syndrome. *Arch Toxicol* **43**, 65-68.

Alexander, G. E., DeLong, M. R., and Strick, P. L. (1986). Parallel organization of functionally segregated circuits linking basal ganglia and cortex. *Annu Rev Neurosci* **9**, 357-381.

Andrews, A. M., Ladenheim, B., Epstein, C. J., Cadet, J. L., and Murphy, D. L. (1996). Transgenic mice with high levels of superoxide dismutase activity are protected from the neurotoxic effects of 2'-NH2-MPTP on serotonergic and noradrenergic nerve terminals. *Mol Pharmacol* **50**, 1511-1519.

Ansari, R. A., Husain, K., and Gupta, P. K. (1987). Endosulfan toxicity influence on biogenic amines of rat brain. *J Environ Biol* **8**, 229-236.

Arora, P. K., Riachi, N. J., Fiedler, G. C., Singh, M. P., Abdallah, F., Harik, S. I., and Sayre, L. M. (1990). Structure-neurotoxicity trends of analogues of 1-methyl-4-phenylpyridinium (MPP+), the cytotoxic metabolite of the dopaminergic neurotoxin MPTP. *Life Sci* **46**, 379-390.

Asanuma, M., Ogawa, N., Haba, K., Hirata, H., and Mori, A. (1992). Effects of chronic catecholamine depletions on muscarinic M1-receptor and its mRNA in rat brain. *J Neurol Sci* **110**, 205-214.

Aschner, M. (2000). Manganese: brain transport and emerging research needs. *Environ Health Perspect* **108 Suppl 3**, 429-432.

Bachowski, S., Xu, Y., Stevenson, D. E., Walborg, E. F., Jr., and Klaunig, J. E. (1998). Role of oxidative stress in the selective toxicity of dieldrin in the mouse liver. *Toxicol Appl Pharmacol* **150**, 301-309.

Bagchi, D., Balmoori, J., Bagchi, M., Ye, X., Williams, C. B., and Stohs, S. J. (2000). Role of p53 tumor suppressor gene in the toxicity of TCDD, endrin, naphthalene, and chromium (VI) in liver and brain tissues of mice. *Free Radic Biol Med* **28**, 895-903.

Bagetta, G., Corasaniti, M. T., Iannone, M., Nistico, G., and Stephenson, J. D. (1992). Production of limbic motor seizures and brain damage by systemic and intracerebral injections of paraquat in rats. *Pharmacol Toxicol* **71**, 443-448.

Balasubramaniam, E., Paul, V., and Kazi, M. (1995). Cyclodiene insecticides-induced changes in the central depressive effect of chlorpromazine in rats. *Indian J Physiol Pharmacol* **39**, 231-236.

Baptista, M. J., O'Farrell, C., Daya, S., Ahmad, R., Miller, D. W., Hardy, J., Farrer, M. J., and Cookson, M. R. (2003). Co-ordinate transcriptional regulation of dopamine synthesis genes by alpha-synuclein in human neuroblastoma cell lines. *J Neurochem* **85**, 957-968.

Barbeau, A. (1967). The "pink spot", 3,4-dimethoxyphenylethylamine and dopamine. Relationship to Parkinson's disease and to schizophrenia. *Rev Can Biol* **26**, 55-79.

Barbeau, A. (1968). Dopamine and dopamine metabolites in Parkinson's disease-a review. *Proc Aust Assoc Neurol* **5**, 95-100.

Barlow, B. K., Lee, D. W., Cory-Slechta, D. A., and Opanashuk, L. A. (2005). Modulation of antioxidant defense systems by the environmental pesticide maneb in dopaminergic cells. *Neurotoxicology* **26**, 63-75.

Baughman, D. S. (1986). The acute toxicity of inseticides associated with agricultural runoff to the grass shimp Palaemonetes Pugio. , pp. 1-70. University of Carolina, Columbia.

Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., and Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* **3**, 1301-1306.

Bezard, E., Gross, C. E., Fournier, M. C., Dovero, S., Bloch, B., and Jaber, M. (1999). Absence of MPTP-induced neuronal death in mice lacking the dopamine transporter. *Exp Neurol* **155**, 268-273.

Bird, T. D., Stranahan, S., Sumi, S. M., and Raskind, M. (1983). Alzheimer's disease: choline acetyltransferase activity in brain tissue from clinical and pathological subgroups. *Ann Neurol* **14**, 284-293.

Blanco-Coronado, J. L., Repetto, M., Ginestal, R. J., Vicente, J. R., Yelamos, F., and Lardelli, A. (1992). Acute intoxication by endosulfan. *J Toxicol Clin Toxicol* **30**, 575-583.

Blandini, F., Nappi, G., Tassorelli, C., and Martignoni, E. (2000). Functional changes of the basal ganglia circuitry in Parkinson's disease. *Prog Neurobiol* **62**, 63-88.

Bloom, S. E., Lemley, A. T., and Muscarella, D. E. (2006). Potentiation of apoptosis by heat stress plus pesticide exposure in stress resistant human B-lymphoma cells and its attenuation through interaction with follicular dendritic cells: role for c-Jun N-terminal kinase signaling. *Toxicol Sci* **89**, 214-223.

Boereboom, F. T., van Dijk, A., van Zoonen, P., and Meulenbelt, J. (1998). Nonaccidental endosulfan intoxication: a case report with toxicokinetic calculations and tissue concentrations. *J Toxicol Clin Toxicol* **36**, 345-352.

Boissiere, F., Faucheux, B., Agid, Y., and Hirsch, E. C. (1997). Choline acetyltransferase mRNA expression in the striatal neurons of patients with Alzheimer's disease. *Neurosci Lett* **225**, 169-172.

Boyd, E. M., and Dobos, I. (1969). Protein deficiency and tolerated oral doses of endosulfan. *Arch Int Pharmacodyn Ther* **178**, 152-165.

Boyd, E. M., Dobos, I., and Krijnen, C. J. (1970). Endosulfan toxicity and dietary protein. *Arch Environ Health* **21**, 15-19.

Brion, J. P. (1998). Neurofibrillary tangles and Alzheimer's disease. *Eur Neurol* **40**, 130-140.

Brion, J. P. (1999). [Neurofibrillary tangles and early modification of the neuronal cytoskeleton in Alzheimer's disease and in experimental models]. *Bull Mem Acad R Med Belg* **154**, 287-294.

Brooks, A. I., Chadwick, C. A., Gelbard, H. A., Cory-Slechta, D. A., and Federoff, H. J. (1999). Paraquat elicited neurobehavioral syndrome caused by dopaminergic neuron loss. *Brain Res* **823**, 1-10.

Buja, L. M., Eigenbrodt, M. L., and Eigenbrodt, E. H. (1993). Apoptosis and necrosis. Basic types and mechanisms of cell death. *Arch Pathol Lab Med* **117**, 1208-1214.

Burkitt, M. J., Bishop, H. S., Milne, L., Tsang, S. Y., Provan, G. J., Nobel, C. S., Orrenius, S., and Slater, A. F. (1998). Dithiocarbamate toxicity toward thymocytes involves their copper-catalyzed conversion to thiuram disulfides, which oxidize glutathione in a redox cycle without the release of reactive oxygen species. *Arch Biochem Biophys* **353**, 73-84.

Burns, R. S., Chiueh, C. C., Markey, S. P., Ebert, M. H., Jacobowitz, D. M., and Kopin, I. J. (1983). A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc Natl Acad Sci U S A* **80**, 4546-4550.

Bush, A. I., Multhaup, G., Moir, R. D., Williamson, T. G., Small, D. H., Rumble, B., Pollwein, P., Beyreuther, K., and Masters, C. L. (1993). A novel zinc(II) binding site modulates the function of the beta A4 amyloid protein precursor of Alzheimer's disease. *J Biol Chem* **268**, 16109-16112.

Calabresi, P., Stefani, A., Mercuri, N. B., and Bernardi, G. (1989). Acetylcholine-dopamine balance in striatum: is it still a target for antiparkinsonian therapy? *Exs* **57**, 315-321.

Calo, M., Iannone, M., Passafaro, M., and Nistico, G. (1990). Selective vulnerability of hippocampal CA3 neurones after microinfusion of paraquat into the rat substantia nigra or into the ventral tegmental area. *J Comp Pathol* **103**, 73-78.

Calviello, G., Piccioni, E., Boninsegna, A., Tedesco, B., Maggiano, N., Serini, S., Wolf, F. I., and Palozza, P. (2006). DNA damage and apoptosis induction by the

pesticide Mancozeb in rat cells: involvement of the oxidative mechanism. *Toxicol Appl Pharmacol* **211**, 87-96.

Camacho, I. A., Hassuneh, M. R., Nagarkatti, M., and Nagarkatti, P. S. (2001). Enhanced activation-induced cell death as a mechanism of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity in peripheral T cells. *Toxicology* **165**, 51-63.

Carlson, K., Jortner, B. S., and Ehrich, M. (2000). Organophosphorus compound-induced apoptosis in SH-SY5Y human neuroblastoma cells. *Toxicol Appl Pharmacol* **168**, 102-113.

Chang, G. D., and Ramirez, V. D. (1987). Effects on dopamine metabolism of MPTP and MPP+ infused through a push-pull cannula into the caudate nucleus of awake adult male rats. *Brain Res* **424**, 49-57.

Chiueh, C. C., Huang, S. J., and Murphy, D. L. (1992). Enhanced hydroxyl radical generation by 2'-methyl analog of MPTP: suppression by clorgyline and deprenyl. *Synapse* **11**, 346-348.

Chiueh, C. C., Markey, S. P., Burns, R. S., Johannessen, J. N., Jacobowitz, D. M., and Kopin, I. J. (1984). Neurochemical and behavioral effects of 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) in rat, guinea pig, and monkey. *Psychopharmacol Bull* **20**, 548-553.

Chiueh, C. C., Miyake, H., and Peng, M. T. (1993). Role of dopamine autoxidation, hydroxyl radical generation, and calcium overload in underlying mechanisms involved in MPTP-induced parkinsonism. *Adv Neurol* **60**, 251-258.

Chiueh, C. C., and Rauhala, P. (1998). Free radicals and MPTP-induced selective destruction of substantia nigra compacta neurons. *Adv Pharmacol* **42**, 796-800.

Christen, Y. (2000). Oxidative stress and Alzheimer disease. *Am J Clin Nutr* **71**, 621S-629S.

Chugh, S. N., Dhawan, R., Agrawal, N., and Mahajan, S. K. (1998). Endosulfan poisoning in Northern India: a report of 18 cases. *Int J Clin Pharmacol Ther* **36**, 474-477.

Cicchetti, R., and Argentin, G. (2003). The role of oxidative stress in the in vitro induction of micronuclei by pesticides in mouse lung fibroblasts. *Mutagenesis* **18**, 127-132.

Clayton, D. F., and George, J. M. (1998). The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends Neurosci* **21**, 249-254.

Clayton, D. F., and George, J. M. (1999). Synucleins in synaptic plasticity and neurodegenerative disorders. *J Neurosci Res* **58**, 120-129.

Clayton, R., Clark, J. B., and Sharpe, M. (2005). Cytochrome c release from rat brain mitochondria is proportional to the mitochondrial functional deficit: implications for apoptosis and neurodegenerative disease. *J Neurochem* **92**, 840-849.

Cohen, G. M. (2005). Caspase inactivation of the proteasome. *Cell Death Differ* **12**, 1218.

Cohen, Z., Wilson, J., Ritter, L., and McDonagh, P. (2004). Caspase inhibition decreases both platelet phosphatidylserine exposure and aggregation: caspase inhibition of platelets. *Thromb Res* **113**, 387-393.

Cole, K. K., and Perez-Polo, J. R. (2002). Poly(ADP-ribose) polymerase inhibition prevents both apoptotic-like delayed neuronal death and necrosis after H(2)O(2) injury. *J Neurochem* **82**, 19-29.

Cole, L. M., and Casida, J. E. (1986). Polychlorocycloalkane insecticide-induced convulsions in mice in relation to disruption of the GABA-regulated chloride ionophore. *Life Sci* **39**, 1855-1862.

Columbano, A. (1995). Cell death: current difficulties in discriminating apoptosis from necrosis in the context of pathological processes in vivo. *J Cell Biochem* **58**, 181-190.

Constantinidis, J. (1990). [Alzheimer's disease and the zinc theory]. *Encephale* **16**, 231-239.

Cooper, B. (1991). The epidemiology of primary degenerative dementia and related neurological disorders. *Eur Arch Psychiatry Clin Neurosci* **240**, 223-233.

Crossman, A. R. (1987). Primate models of dyskinesia: the experimental approach to the study of basal ganglia-related involuntary movement disorders. *Neuroscience* **21**, 1-40.

Cuajungco, M. P., and Faget, K. Y. (2003). Zinc takes the center stage: its paradoxical role in Alzheimer's disease. *Brain Res Brain Res Rev* **41**, 44-56.

Cuajungco, M. P., and Lees, G. J. (1997). Zinc and Alzheimer's disease: is there a direct link? *Brain Res Brain Res Rev* **23**, 219-236.

Curtain, C. C., Ali, F., Volitakis, I., Cherny, R. A., Norton, R. S., Beyreuther, K., Barrow, C. J., Masters, C. L., Bush, A. I., and Barnham, K. J. (2001). Alzheimer's disease amyloid-beta binds copper and zinc to generate an allosterically ordered membrane-penetrating structure containing superoxide dismutase-like subunits. *J Biol Chem* **276**, 20466-20473.

D'Amato, R. J., Lipman, Z. P., and Snyder, S. H. (1986). Selectivity of the parkinsonian neurotoxin MPTP: toxic metabolite MPP+ binds to neuromelanin. *Science* **231**, 987-989.

Da Prada, M., and Kettler, R. (1986). Uptake, metabolism and subcellular localization of MPTP and MPP+ in blood platelets. *Clin Neuropharmacol* **9 Suppl 4**, 347-349.

Dawson, V. L., Dawson, T. M., and Wamsley, J. K. (1990). Muscarinic and dopaminergic receptor subtypes on striatal cholinergic interneurons. *Brain Res Bull* **25**, 903-912.

de Bilbao, F., Guarin, E., Nef, P., Vallet, P., Giannakopoulos, P., and Dubois-Dauphin, M. (1999). Postnatal distribution of cpp32/caspase 3 mRNA in the mouse central nervous system: an in situ hybridization study. *J Comp Neurol* **409**, 339-357.

de la Fuente-Fernandez, R., and Calne, D. B. (2002). Evidence for environmental causation of Parkinson's disease. *Parkinsonism Relat Disord* **8**, 235-241.

DeLong, M. R. (1990). Primate models of movement disorders of basal ganglia origin. *Trends Neurosci* **13**, 281-285.

Denaro, A., Tamminga, C. A., Ruggieri, S., Chase, T. N., and Agnoli, A. (1984). State of DA/ACh balance after chronic treatment with haloperidol and with THIP in rats. *Adv Neurol* **40**, 141-147.

Dexter, D., Carter, C., Agid, F., Agid, Y., Lees, A. J., Jenner, P., and Marsden, C. D. (1986). Lipid peroxidation as cause of nigral cell death in Parkinson's disease. *Lancet* **2**, 639-640.

Dexter, D. T., Carter, C. J., Wells, F. R., Javoy-Agid, F., Agid, Y., Lees, A., Jenner, P., and Marsden, C. D. (1989). Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. *J Neurochem* **52**, 381-389.

Di Rosa, G., Puzzo, D., Sant'Angelo, A., Trinchese, F., and Arancio, O. (2003). Alpha-synuclein: between synaptic function and dysfunction. *Histol Histopathol* **18**, 1257-1266.

Dietert, R. R., Lee, J. E., and Bunn, T. L. (2002). Developmental immunotoxicology: emerging issues. *Hum Exp Toxicol* **21**, 479-485.

Dikshith, T. S., Raizada, R. B., Srivastava, M. K., and Kaphalia, B. S. (1984). Response of rats to repeated oral administration of endosulfan. *Ind Health* **22**, 295-304.

Dikshith, T. S. S. (1991). Pesticides. In *Pesticides of Toxicology in Animals* (T. S. S. Dikshith, Ed.), pp. 1-41. CRC Press, Boca Raton, FL.

Dineley, K. E., Votyakova, T. V., and Reynolds, I. J. (2003). Zinc inhibition of cellular energy production: implications for mitochondria and neurodegeneration. *J Neurochem* **85**, 563-570.

Dostert, P., and Strolin Benedetti, M. (1988). [The bases of MPTP neurotoxicity]. *Encephale* **14**, 399-412.

Double, K. L., Maywald, M., Schmittel, M., Riederer, P., and Gerlach, M. (1998). In vitro studies of ferritin iron release and neurotoxicity. *J Neurochem* **70**, 2492-2499.

Eberling, J. L., Bankiewicz, K. S., Pivirotto, P., Bringas, J., Chen, K., Nowotnik, D. P., Steiner, J. P., Budinger, T. F., and Jagust, W. J. (1999). Dopamine transporter loss and clinical changes in MPTP-lesioned primates. *Brain Res* **832**, 184-187.

Ebmeier, K. P., Besson, J. A., Cherryman, G., and Smith, F. (1988). [Parkinson's disease and Alzheimer's disease--similarities in magnetic resonance imaging parameters and their possible explanation]. *Z Gerontol* **21**, 142-145.

Enz, A., Amstutz, R., Boddeke, H., Gmelin, G., and Malanowski, J. (1993). Brain selective inhibition of acetylcholinesterase: a novel approach to therapy for Alzheimer's disease. *Prog Brain Res* **98**, 431-438.

EPA (1972). The pollution potential in pesticide manufacturing. Pesticide Study Series 5. U.S. Environmental Protection Agency, Office of Water Products.

Escargueil-Blanc, I., Salvayre, R., and Negre-Salvayre, A. (1994). Necrosis and apoptosis induced by oxidized low density lipoproteins occur through two calcium-dependent pathways in lymphoblastoid cells. *Faseb J* **8**, 1075-1080.

Exner, C., Koschack, J., and Irle, E. (2002). The differential role of premotor frontal cortex and basal ganglia in motor sequence learning: evidence from focal basal ganglia lesions. *Learn Mem* **9**, 376-386.

Fall, C. P., and Bennett, J. P., Jr. (1999). Characterization and time course of MPP+ -induced apoptosis in human SH-SY5Y neuroblastoma cells. *J Neurosci Res* **55**, 620-628.

Farber, E. (1994). Programmed cell death: necrosis versus apoptosis. *Mod Pathol* **7**, 605-609.

Feany, M. B., and Bender, W. W. (2000). A Drosophila model of Parkinson's disease. *Nature* **404**, 394-398.

Fearnley, J. M., and Lees, A. J. (1991). Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain* **114 (Pt 5)**, 2283-2301.

Ferraz, H. B., Bertolucci, P. H., Pereira, J. S., Lima, J. G., and Andrade, L. A. (1988). Chronic exposure to the fungicide maneb may produce symptoms and signs of CNS manganese intoxication. *Neurology* **38**, 550-553.

Fitzgerald, D. J. (1995). Zinc and Alzheimer's disease. *Science* **268**, 1920; author reply 1921-1923.

Forno, L. S. (1992). Neuropathologic features of Parkinson's, Huntington's, and Alzheimer's diseases. *Ann N Y Acad Sci* **648**, 6-16.

Foster, N. L., Tamminga, C. A., O'Donohue, T. L., Tanimoto, K., Bird, E. D., and Chase, T. N. (1986). Brain choline acetyltransferase activity and neuropeptide Y concentrations in Alzheimer's disease. *Neurosci Lett* **63**, 71-75.

Fridovich, I. (1995). Superoxide radical and superoxide dismutases. *Annu Rev Biochem* **64**, 97-112.

Gainetdinov, R. R., Fumagalli, F., Jones, S. R., and Caron, M. G. (1997). Dopamine transporter is required for in vivo MPTP neurotoxicity: evidence from mice lacking the transporter. *J Neurochem* **69**, 1322-1325.

Gao, H. M., Hong, J. S., Zhang, W., and Liu, B. (2003). Synergistic dopaminergic neurotoxicity of the pesticide rotenone and inflammogen lipopolysaccharide: relevance to the etiology of Parkinson's disease. *J Neurosci* **23**, 1228-1236.

Gardner, A. M., Xu, F. H., Fady, C., Jacoby, F. J., Duffey, D. C., Tu, Y., and Lichtenstein, A. (1997). Apoptotic vs. nonapoptotic cytotoxicity induced by hydrogen peroxide. *Free Radic Biol Med* **22**, 73-83.

Gauthier, M., Roberge, C. J., Pelletier, M., Tessier, P. A., and Girard, D. (2001). Activation of human neutrophils by technical toxaphene. *Clin Immunol* **98**, 46-53.

George, J. M. (2002). The synucleins. *Genome Biol* **3**, REVIEWS3002.

Gerfen, C. R., Engber, T. M., Mahan, L. C., Susel, Z., Chase, T. N., Monsma, F. J., Jr., and Sibley, D. R. (1990). D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* **250**, 1429-1432.

Gerfen, C. R., Keefe, K. A., and Gauda, E. B. (1995). D1 and D2 dopamine receptor function in the striatum: coactivation of D1- and D2-dopamine receptors on separate populations of neurons results in potentiated immediate early gene response in D1-containing neurons. *J Neurosci* **15**, 8167-8176.

Gilbert, M. E., and Mack, C. M. (1995). Seizure thresholds in kindled animals are reduced by the pesticides lindane and endosulfan. *Neurotoxicol Teratol* **17**, 143-150.

Gillette, J. S., and Bloomquist, J. R. (2003). Differential up-regulation of striatal dopamine transporter and alpha-synuclein by the pyrethroid insecticide permethrin. *Toxicol Appl Pharmacol* **192**, 287-293.

Goedert, M., Sisodia, S. S., and Price, D. L. (1991). Neurofibrillary tangles and beta-amyloid deposits in Alzheimer's disease. *Curr Opin Neurobiol* **1**, 441-447.

Gotz, M. E., Kunig, G., Riederer, P., and Youdim, M. B. (1994). Oxidative stress: free radical production in neural degeneration. *Pharmacol Ther* **63**, 37-122.

Graybiel, A. M. (1996). Basal ganglia: new therapeutic approaches to Parkinson's disease. *Curr Biol* **6**, 368-371.

Greenamyre, J. T., Betarbet, R., and Sherer, T. B. (2003). The rotenone model of Parkinson's disease: genes, environment and mitochondria. *Parkinsonism Relat Disord* **9 Suppl 2**, S59-64.

Guerrini, L., Blasi, F., and Denis-Donini, S. (1995). Synaptic activation of NF-kappa B by glutamate in cerebellar granule neurons in vitro. *Proc Natl Acad Sci U S A* **92**, 9077-9081.

Gupta, P. K. (1976). Endosulfan-induced neurotoxicity in rats and mice. *Bull Environ Contam Toxicol* **15**, 708-713.

Hampton, M. B., and Orrenius, S. (1998). Redox regulation of apoptotic cell death. *Biofactors* **8**, 1-5.

Harris, E. D. (1992). Regulation of antioxidant enzymes. *Faseb J* **6**, 2675-2683.

Hartmann, A., Hunot, S., Michel, P. P., Muriel, M. P., Vyas, S., Faucheux, B. A., Mouatt-Prigent, A., Turmel, H., Srinivasan, A., Ruberg, M., Evan, G. I., Agid, Y., and Hirsch, E. C. (2000). Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. *Proc Natl Acad Sci U S A* **97**, 2875-2880.

Hasegawa, E., Takeshige, K., Oishi, T., Murai, Y., and Minakami, S. (1990). 1-Methyl-4-phenylpyridinium (MPP+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. *Biochem Biophys Res Commun* **170**, 1049-1055.

Haslett, C. (1992). Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. *Clin Sci (Lond)* **83**, 639-648.

Heikkila, R. E., Manzino, L., Cabbat, F. S., and Duvoisin, R. C. (1984). Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine by monoamine oxidase inhibitors. *Nature* **311**, 467-469.

Heinz, G. H., Hill, E. F., and Contrera, J. F. (1980). Dopamine and norepinephrine depletion in ring doves fed DDE, dieldrin, and Aroclor 1254. *Toxicol Appl Pharmacol* **53**, 75-82.

Hipkiss, A. R. (2005). Could carnosine suppress zinc-mediated proteasome inhibition and neurodegeneration? Therapeutic potential of a non-toxic but non-patentable dipeptide. *Biogerontology* **6**, 147-149.

Hogan, K. A., Staal, R. G., and Sonsalla, P. K. (2000). Analysis of VMAT2 binding after methamphetamine or MPTP treatment: disparity between homogenates and vesicle preparations. *J Neurochem* **74**, 2217-2220.

Honig, L. S., and Rosenberg, R. N. (2000). Apoptosis and neurologic disease. *Am J Med* **108**, 317-330.

Hornykiewicz, O. (1989). Ageing and neurotoxins as causative factors in idiopathic Parkinson's disease--a critical analysis of the neurochemical evidence. *Prog Neuropsychopharmacol Biol Psychiatry* **13**, 319-328.

Ince, E., Ciliax, B. J., and Levey, A. I. (1997). Differential expression of D1 and D2 dopamine and m4 muscarinic acetylcholine receptor proteins in identified striatonigral neurons. *Synapse* **27**, 357-366.

IOM (2003). In *Institute of Medicine (IOM), Gulf War and Health: Volume 2, Insecticides and Solvents* pp. 412-418. The National Academics Press, Washington, DC.

Jackson, J. H., Schraufstatter, I. U., Hyslop, P. A., Vosbeck, K., Sauerheber, R., Weitzman, S. A., and Cochrane, C. G. (1987). Role of hydroxyl radical in DNA damage. *Trans Assoc Am Physicians* **100**, 147-157.

Jarvis, M. F., and Wagner, G. C. (1985). Neurochemical and functional consequences following 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) and methamphetamine. *Life Sci* **36**, 249-254.

Jellinger, K. A. (2000). Cell death mechanisms in Parkinson's disease. *J Neural Transm* **107**, 1-29.

Jenner, P. (1991). Oxidative stress as a cause of Parkinson's disease. *Acta Neurol Scand Suppl* **136**, 6-15.

Jenner, P., Dexter, D. T., Sian, J., Schapira, A. H., and Marsden, C. D. (1992). Oxidative stress as a cause of nigral cell death in Parkinson's disease and incidental Lewy body disease. The Royal Kings and Queens Parkinson's Disease Research Group. *Ann Neurol* **32 Suppl**, S82-87.

Jordan, J., Galindo, M. F., Cena, V., and Gonzalez-Garcia, C. (2000). [Cysteine proteinase and neurodegeneration]. *Rev Neurol* **31**, 333-340.

Jueptner, M., and Krukenberg, M. (2001). Motor system: cortex, basal ganglia, and cerebellum. *Neuroimaging Clin N Am* **11**, 203-219, viii.

Kannan, K., and Jain, S. K. (2003). Oxygen radical generation and endosulfan toxicity in Jurkat T-cells. *Mol Cell Biochem* **247**, 1-7.

Kaplan, B., Ratner, V., and Haas, E. (2003). Alpha-synuclein: its biological function and role in neurodegenerative diseases. *J Mol Neurosci* **20**, 83-92.

Karen, D. J., Li, W., Harp, P. R., Gillette, J. S., and Bloomquis, J. R. (2001). Striatal dopaminergic pathways as a target for the insecticides permethrin and chlorpyrifos. *Neurotoxicology* **22**, 811-817.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-257.

Kilbourn, M. R., Kuszpit, K., and Sherman, P. (2000). Rapid and differential losses of in vivo dopamine transporter (DAT) and vesicular monoamine transporter (VMAT2) radioligand binding in MPTP-treated mice. *Synapse* **35**, 250-255.

Kilinc, A., Yalcin, A. S., Yalcin, D., Taga, Y., and Emerk, K. (1988). Increased erythrocyte susceptibility to lipid peroxidation in human Parkinson's disease. *Neurosci Lett* **87**, 307-310.

Kim, K. W., Suh, Y. J., Park, W. Y., Jhoo, J. H., Lee, D. Y., Youn, J. C., Lee, K. H., Seo, J. S., and Woo, J. I. (2004). Choline acetyltransferase G +4 A polymorphism confers a risk for Alzheimer's disease in concert with Apolipoprotein E epsilon4. *Neurosci Lett* **366**, 182-186.

Kimmel, C. A., and Makris, S. L. (2001). Recent developments in regulatory requirements for developmental toxicology. *Toxicol Lett* **120**, 73-82.

Kitazawa, M., Anantharam, V., Kanthasamy, A., and Kanthasamy, A. G. (2004). Dieldrin promotes proteolytic cleavage of poly(ADP-ribose) polymerase and apoptosis in dopaminergic cells: protective effect of mitochondrial anti-apoptotic protein Bcl-2. *Neurotoxicology* **25**, 589-598.

Kitazawa, M., Anantharam, V., and Kanthasamy, A. G. (2001). Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptopic cell death in dopaminergic cells. *Free Radic Biol Med* **31**, 1473-1485.

Klucken, J., McLean, P. J., Gomez-Tortosa, E., Ingelsson, M., and Hyman, B. T. (2003). Neuritic alterations and neural system dysfunction in Alzheimer's disease and dementia with Lewy bodies. *Neurochem Res* **28**, 1683-1691.

Kotake, Y., and Ohta, S. (2003). MPP+ analogs acting on mitochondria and inducing neuro-degeneration. *Curr Med Chem* **10**, 2507-2516.

Kovacic, P. (2003). Mechanism of organophosphates (nerve gases and pesticides) and antidotes: electron transfer and oxidative stress. *Curr Med Chem* **10**, 2705-2709.

Kurosaki, R., Muramatsu, Y., Watanabe, H., Michimata, M., Matsubara, M., Imai, Y., and Araki, T. (2003). Role of dopamine transporter against MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) neurotoxicity in mice. *Metab Brain Dis* **18**, 139-146.

Lakshmana, M. K., and Raju, T. R. (1994). Endosulfan induces small but significant changes in the levels of noradrenaline, dopamine and serotonin in the developing rat brain and deficits in the operant learning performance. *Toxicology* **91**, 139-150.

Langston, J. W., Ballard, P., Tetrud, J. W., and Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**, 979-980.

Latchoumycandane, C., and Mathur, P. P. (2002). Induction of oxidative stress in the rat testis after short-term exposure to the organochlorine pesticide methoxychlor. *Arch Toxicol* **76**, 692-698.

Lee, F. J., Liu, F., Pristupa, Z. B., and Niznik, H. B. (2001). Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. *Faseb J* **15**, 916-926.

Lee, R. G. (1987). Physiology of the basal ganglia and pathophysiology of Parkinson's disease. *Can J Neurol Sci* **14**, 373-380.

Leenders, K. L., and Oertel, W. H. (2001). Parkinson's disease: clinical signs and symptoms, neural mechanisms, positron emission tomography, and therapeutic interventions. *Neural Plast* **8**, 99-110.

Lennon, S. V., Martin, S. J., and Cotter, T. G. (1991). Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Prolif* **24**, 203-214.

Lester, J., Fink, S., Aronin, N., and DiFiglia, M. (1993). Colocalization of D1 and D2 dopamine receptor mRNAs in striatal neurons. *Brain Res* **621**, 106-110.

Liou, H. H., Chen, R. C., Tsai, Y. F., Chen, W. P., Chang, Y. C., and Tsai, M. C. (1996). Effects of paraquat on the substantia nigra of the wistar rats: neurochemical, histological, and behavioral studies. *Toxicol Appl Pharmacol* **137**, 34-41.

Lotharius, J., Barg, S., Wiekop, P., Lundberg, C., Raymon, H. K., and Brundin, P. (2002). Effect of mutant alpha-synuclein on dopamine homeostasis in a new human mesencephalic cell line. *J Biol Chem* **277**, 38884-38894.

Lotharius, J., and Brundin, P. (2002). Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. *Nat Rev Neurosci* **3**, 932-942.

Majno, G., and Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* **146**, 3-15.

Mann, D. M., and Yates, P. O. (1982). Is the loss of cerebral cortical choline acetyl transferase activity in Alzheimer's disease due to degeneration of ascending cholinergic nerve cells. *J Neurol Neurosurg Psychiatry* **45**, 936.

Manning-Bog, A. B., McCormack, A. L., Li, J., Uversky, V. N., Fink, A. L., and Di Monte, D. A. (2002). The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. *J Biol Chem* **277**, 1641-1644.

Maroni, M., Colosio, C., Ferioli, A., and Fait, A. (2000). Biological Monitoring of Pesticide Exposure: a review. Introduction. *Toxicology* **143**, 1-118.

Marsden, C. D. (1982). The mysterious motor function of the basal ganglia: the Robert Wartenberg Lecture. *Neurology* **32**, 514-539.

Marsden, C. D., and Obeso, J. A. (1994). The functions of the basal ganglia and the paradox of stereotaxic surgery in Parkinson's disease. *Brain* **117 (Pt 4)**, 877-897.

Maruyama, S. (1998). [Necrosis and apoptosis]. *Masui* **47 Suppl**, S77-80.

Masoud, L., Vijayasarathy, C., Fernandez-Cabezudo, M., Petroianu, G., and Saleh, A. M. (2003). Effect of malathion on apoptosis of murine L929 fibroblasts: a possible mechanism for toxicity in low dose exposure. *Toxicology* **185**, 89-102.

Masters, C. L., and Beyreuther, K. (1990). Protein abnormalities in neurofibrillary tangles: their relation to the extracellular amyloid deposits of the A4 protein in Alzheimer's disease. *Adv Neurol* **51**, 151-161.

McCarthy, S., Somayajulu, M., Sikorska, M., Borowy-Borowski, H., and Pandey, S. (2004). Paraquat induces oxidative stress and neuronal cell death; neuroprotection by water-soluble Coenzyme Q10. *Toxicol Appl Pharmacol* **201**, 21-31.

McGinty, J. F. (1999). Regulation of neurotransmitter interactions in the ventral striatum. *Ann N Y Acad Sci* **877**, 129-139.

McGrew, D. M., Irwin, I., and Langston, J. W. (2000). Ethylenebisdithiocarbamate enhances MPTP-induced striatal dopamine depletion in mice. *Neurotoxicology* **21**, 309-312.

Meco, G., Bonifati, V., Vanacore, N., and Fabrizio, E. (1994). Parkinsonism after chronic exposure to the fungicide maneb (manganese ethylene-bis-dithiocarbamate). *Scand J Work Environ Health* **20**, 301-305.

Merello, M., and Cammarota, A. (2000). [Functional anatomy of the basal ganglia]. *Rev Neurol* **30**, 1055-1060.

Meyer, M., Schreck, R., and Baeuerle, P. A. (1993). H2O2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *Embo J* **12**, 2005-2015.

Miller, G. W., Kirby, M. L., Levey, A. I., and Bloomquist, J. R. (1999). Heptachlor alters expression and function of dopamine transporters. *Neurotoxicology* **20**, 631-637.

Mizuno, Y., Sone, N., Suzuki, K., and Saitoh, T. (1988). Studies on the toxicity of 1-methyl-4-phenylpyridinium ion (MPP+) against mitochondria of mouse brain. *J Neurol Sci* **86**, 97-110.

Mizuno, Y., Suzuki, K., Sone, N., and Saitoh, T. (1987). Inhibition of ATP synthesis by 1-methyl-4-phenylpyridinium ion (MPP+) in isolated mitochondria from mouse brains. *Neurosci Lett* **81**, 204-208.

Mocchegiani, E., Bertoni-Freddari, C., Marcellini, F., and Malavolta, M. (2005). Brain, aging and neurodegeneration: role of zinc ion availability. *Prog Neurobiol* **75**, 367-390.

Mohanakumar, K. P., de Bartolomeis, A., Wu, R. M., Yeh, K. J., Sternberger, L. M., Peng, S. Y., Murphy, D. L., and Chiueh, C. C. (1994). Ferrous-citrate complex

and nigral degeneration: evidence for free-radical formation and lipid peroxidation. *Ann N Y Acad Sci* **738**, 392-399.

Muindi, J. R., Sinha, B. K., Gianni, L., and Myers, C. E. (1984). Hydroxyl radical production and DNA damage induced by anthracycline-iron complex. *FEBS Lett* **172**, 226-230.

Muller-Vahl, K. R., Kolbe, H., and Dengler, R. (1999). Transient severe parkinsonism after acute organophosphate poisoning. *J Neurol Neurosurg Psychiatry* **66**, 253-254.

Muller, K., and Gurster, D. (1993). Hydroxyl radical damage to DNA sugar and model membranes induced by anthralin (dithranol). *Biochem Pharmacol* **46**, 1695-1704.

Mytilineou, C., Cohen, G., and Heikkila, R. E. (1985). 1-Methyl-4-phenylpyridine (MPP+) is toxic to mesencephalic dopamine neurons in culture. *Neurosci Lett* **57**, 19-24.

Nagai, T., McGeer, P. L., Peng, J. H., McGeer, E. G., and Dolman, C. E. (1983). Choline acetyltransferase immunohistochemistry in brains of Alzheimer's disease patients and controls. *Neurosci Lett* **36**, 195-199.

Novikova, L., Garris, B. L., Garris, D. R., and Lau, Y. S. (2006). Early signs of neuronal apoptosis in the substantia nigra pars compacta of the progressive neurodegenerative mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/probenecid model of Parkinson's disease. *Neuroscience*.

O.T.A. (1990). *United States. Congress. Office of Technology Assessment. Neurotoxicity : identifying and controlling poisons of the nervous system : summary : new developments in neuroscience*. Congress of the U.S. For sale by the Supt. of Docs., U.S. G.P.O., Washington DC.

Obata, T., and Yamanaka, Y. (2001). [Parkinsonism induced by MPTP and free radical generation]. *Nippon Yakurigaku Zasshi* **117**, 105-110.

Olgun, S., Gogal, R. M., Jr., Adeshina, F., Choudhury, H., and Misra, H. P. (2004). Pesticide mixtures potentiate the toxicity in murine thymocytes. *Toxicology* **196**, 181-195.

Osmond, C., and Barker, D. J. (2000). Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women. *Environ Health Perspect* **108 Suppl 3**, 545-553.

Owen, A. D., Schapira, A. H., Jenner, P., and Marsden, C. D. (1996). Oxidative stress and Parkinson's disease. *Ann N Y Acad Sci* **786**, 217-223.

Ozoe, Y., and Matsumura, F. (1986). Structural requirements for bridged bicyclic compounds acting on
picrotoxinin receptor. *J Agric Food Chem* **34**, 126-134.

Pall, H. S., Williams, A. C., Blake, D. R., Winyard, P., and Lunec, J. (1986). Lipid peroxidation and Parkinson's disease. *Lancet* **2**, 870-871.

Paul, V., Balasubramaniam, E., Jayakumar, A. R., and Kazi, M. (1995). A sex-related difference in the neurobehavioral and hepatic effects following chronic endosulfan treatment in rats. *Eur J Pharmacol* **293**, 355-360.

Paul, V., Balasubramaniam, E., and Kazi, M. (1994). The neurobehavioural toxicity of endosulfan in rats: a serotonergic involvement in learning impairment. *Eur J Pharmacol* **270**, 1-7.

Penney, J. B., Jr., and Young, A. B. (1983). Speculations on the functional anatomy of basal ganglia disorders. *Annu Rev Neurosci* **6**, 73-94.
Perez, R. G., Waymire, J. C., Lin, E., Liu, J. J., Guo, F., and Zigmond, M. J. (2002). A role for alpha-synuclein in the regulation of dopamine biosynthesis. *J Neurosci* **22**, 3090-3099.
Perier, C., Bove, J., Vila, M., and Przedborski, S. (2003). The rotenone model of Parkinson's disease. *Trends Neurosci* **26**, 345-346.
Perry, J. C., Da Cunha, C., Anselmo-Franci, J., Andreatini, R., Miyoshi, E., Tufik, S., and Vital, M. A. (2004). Behavioural and neurochemical effects of phosphatidylserine in MPTP lesion of the substantia nigra of rats. *Eur J Pharmacol* **484**, 225-233.
Perry, T. L., Godin, D. V., and Hansen, S. (1982). Parkinson's disease: a disorder due to nigral glutathione deficiency? *Neurosci Lett* **33**, 305-310.
Piccirilli, M., Piccinin, G. L., and Agostini, L. (1984). Dopamine deficiency in Parkinson's disease. *Neurology* **34**, 265-266.
Pomes, A., Rodriguez-Farre, E., and Sunol, C. (1994). Disruption of GABA-dependent chloride flux by cyclodienes and hexachlorocyclohexanes in primary cultures of cortical neurons. *J Pharmacol Exp Ther* **271**, 1616-1623.
Pradhan, S. N. (1974). Proceedings: Balance between acetylcholine, serotonin, norepinephrine, and dopamine in self-stimulation. *Psychopharmacol Bull* **10**, 47-48.
Pratico, D., Clark, C. M., Liun, F., Rokach, J., Lee, V. Y., and Trojanowski, J. Q. (2002). Increase of brain oxidative stress in mild cognitive impairment: a possible predictor of Alzheimer disease. *Arch Neurol* **59**, 972-976.
Raffray, M., and Cohen, G. M. (1991). Bis(tri-n-butyltin)oxide induces programmed cell death (apoptosis) in immature rat thymocytes. *Arch Toxicol* **65**, 135-139.
Ramsay, R. R., and Singer, T. P. (1992). Relation of superoxide generation and lipid peroxidation to the inhibition of NADH-Q oxidoreductase by rotenone, piericidin A, and MPP+. *Biochem Biophys Res Commun* **189**, 47-52.
Rhile, M. J., Nagarkatti, M., and Nagarkatti, P. S. (1996). Role of Fas apoptosis and MHC genes in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity of T cells. *Toxicology* **110**, 153-167.
Rideout, H. J., and Stefanis, L. (2001). Caspase inhibition: a potential therapeutic strategy in neurological diseases. *Histol Histopathol* **16**, 895-908.
Rinne, U. K. (1982). Parkinson's disease as a model for changes in dopamine receptor dynamics with aging. *Gerontology* **28 Suppl 1**, 35-52.
Romo, R., and Schultz, W. (1992). Role of primate basal ganglia and frontal cortex in the internal generation of movements. III. Neuronal activity in the supplementary motor area. *Exp Brain Res* **91**, 396-407.
Rondot, P., Bianco, C., and de Recondo, J. (1986). Ageing and Parkinson's disease. *Gerontology* **32 Suppl 1**, 102-105.
Rosa, R., Rodriguez-Farre, E., and Sanfeliu, C. (1996). Cytotoxicity of hexachlorocyclohexane isomers and cyclodienes in primary cultures of cerebellar granule cells. *J Pharmacol Exp Ther* **278**, 163-169.
Rosenstock, T. R., Carvalho, A. C., Jurkiewicz, A., Frussa-Filho, R., and Smaili, S. S. (2004). Mitochondrial calcium, oxidative stress and apoptosis in a

neurodegenerative disease model induced by 3-nitropropionic acid. *J Neurochem* **88**, 1220-1228.

Rottkamp, C. A., Nunomura, A., Raina, A. K., Sayre, L. M., Perry, G., and Smith, M. A. (2000). Oxidative stress, antioxidants, and Alzheimer disease. *Alzheimer Dis Assoc Disord* **14 Suppl 1**, S62-66.

Russ, H., Mihatsch, W., Gerlach, M., Riederer, P., and Przuntek, H. (1991). Neurochemical and behavioural features induced by chronic low dose treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the common marmoset: implications for Parkinson's disease? *Neurosci Lett* **123**, 115-118.

Russ, H., Staust, K., Martel, F., Gliese, M., and Schomig, E. (1996). The extraneuronal transporter for monoamine transmitters exists in cells derived from human central nervous system glia. *Eur J Neurosci* **8**, 1256-1264.

Saggu, H., Cooksey, J., Dexter, D., Wells, F. R., Lees, A., Jenner, P., and Marsden, C. D. (1989). A selective increase in particulate superoxide dismutase activity in parkinsonian substantia nigra. *J Neurochem* **53**, 692-697.

Sanchez-Ramos, J. R., Song, S., Facca, A., Basit, A., and Epstein, C. J. (1997). Transgenic murine dopaminergic neurons expressing human Cu/Zn superoxide dismutase exhibit increased density in culture, but no resistance to methylphenylpyridinium-induced degeneration. *J Neurochem* **68**, 58-67.

Sandyk, R., Bamford, C. R., and Iacono, R. P. (1988). Pain and sensory symptoms in Parkinson's disease. *Int J Neurosci* **39**, 15-25.

Schenk, H., Klein, M., Erdbrugger, W., Droge, W., and Schulze-Osthoff, K. (1994). Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-kappa B and AP-1. *Proc Natl Acad Sci U S A* **91**, 1672-1676.

Scherman, D., Desnos, C., Darchen, F., Pollak, P., Javoy-Agid, F., and Agid, Y. (1989). Striatal dopamine deficiency in Parkinson's disease: role of aging. *Ann Neurol* **26**, 551-557.

Schmidt, N., and Ferger, B. (2001). Neurochemical findings in the MPTP model of Parkinson's disease. *J Neural Transm* **108**, 1263-1282.

Schmidt, W. J. (1995). Balance of transmitter activities in the basal ganglia loops. *J Neural Transm Suppl* **46**, 67-76.

Schreck, R., Meier, B., Mannel, D. N., Droge, W., and Baeuerle, P. A. (1992). Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. *J Exp Med* **175**, 1181-1194.

Schreck, R., Rieber, P., and Baeuerle, P. A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *Embo J* **10**, 2247-2258.

Seaton, T. A., Cooper, J. M., and Schapira, A. H. (1997). Free radical scavengers protect dopaminergic cell lines from apoptosis induced by complex I inhibitors. *Brain Res* **777**, 110-118.

Sechi, G., Deledda, M. G., Bua, G., Satta, W. M., Deiana, G. A., Pes, G. M., and Rosati, G. (1996). Reduced intravenous glutathione in the treatment of early Parkinson's disease. *Prog Neuropsychopharmacol Biol Psychiatry* **20**, 1159-1170.

Segawa, M. (2000). Development of the nigrostriatal dopamine neuron and the pathways in the basal ganglia. *Brain Dev* **22 Suppl 1**, S1-4.

Sharma, R. P., Winn, D. S., and Low, J. B. (1976). Toxic, neurochemical and behavioral effects of dieldrin exposure in mallard ducks. *Arch Environ Contam Toxicol* **5**, 43-53.

Shastry, B. S. (2001). Parkinson disease: etiology, pathogenesis and future of gene therapy. *Neurosci Res* **41**, 5-12.

Shavali, S., Combs, C. K., and Ebadi, M. (2006). Reactive macrophages increase oxidative stress and alpha-synuclein nitration during death of dopaminergic neuronal cells in co-culture: relevance to Parkinson's disease. *Neurochem Res* **31**, 85-94.

Shen, Z. X. (1994). Acetylcholinesterase provides deeper insights into Alzheimer's disease. *Med Hypotheses* **43**, 21-30.

Sherer, T. B., Betarbet, R., Kim, J. H., and Greenamyre, J. T. (2003). Selective microglial activation in the rat rotenone model of Parkinson's disease. *Neurosci Lett* **341**, 87-90.

Shimohama, S., Tanino, H., and Fujimoto, S. (2001). Differential expression of rat brain caspase family proteins during development and aging. *Biochem Biophys Res Commun* **289**, 1063-1066.

Simmons, J. E. (1995). Chemical mixtures: challenge for toxicology and risk assessment. *Toxicology* **105**, 111-119.

Singer, T. P., Ramsay, R. R., McKeown, K., Trevor, A., and Castagnoli, N. E., Jr. (1988). Mechanism of the neurotoxicity of 1-methyl-4-phenylpyridinium (MPP+), the toxic bioactivation product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Toxicology* **49**, 17-23.

Sinha, B. K., Singh, Y., and Krishna, G. (1986). Formation of superoxide and hydroxyl radicals from 1-methyl-4-phenylpyridinium ion (MPP+): reductive activation by NADPH cytochrome P-450 reductase. *Biochem Biophys Res Commun* **135**, 583-588.

Sirinathsinghji, D. J., Whittington, P. E., and Audsley, A. R. (1986). Neurochemical changes in the substantiae nigrae and caudate nuclei following acute unilateral intranigral infusions of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Brain Res* **399**, 339-345.

Smith, C. (2001). Pesticide exports from U.S. ports, 1997-2000. *Int J Occup Environ Health* **7**, 266-274.

Smith, M. T., Sandy, M. S., and Di Monte, D. (1987). Free radicals, lipid peroxidation, and Parkinson's disease. *Lancet* **1**, 38.

Sofic, E., Lange, K. W., Jellinger, K., and Riederer, P. (1992). Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. *Neurosci Lett* **142**, 128-130.

Sohn, H. Y., Kwon, C. S., Kwon, G. S., Lee, J. B., and Kim, E. (2004). Induction of oxidative stress by endosulfan and protective effect of lipid-soluble antioxidants against endosulfan-induced oxidative damage. *Toxicol Lett* **151**, 357-365.

Soleo, L., Defazio, G., Scarselli, R., Zefferino, R., Livrea, P., and Foa, V. (1996). Toxicity of fungicides containing ethylene-bis-dithiocarbamate in serumless dissociated mesencephalic-striatal primary coculture. *Arch Toxicol* **70**, 678-682.

Souza, J. M., Giasson, B. I., Chen, Q., Lee, V. M., and Ischiropoulos, H. (2000). Dityrosine cross-linking promotes formation of stable alpha -synuclein polymers.

Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J Biol Chem* **275**, 18344-18349.
Spencer, J. P., Jenner, P., and Halliwell, B. (1995). Superoxide-dependent depletion of reduced glutathione by L-DOPA and dopamine. Relevance to Parkinson's disease. *Neuroreport* **6**, 1480-1484.
Spillantini, M. G., Crowther, R. A., and Goedert, M. (1996). Comparison of the neurofibrillary pathology in Alzheimer's disease and familial presenile dementia with tangles. *Acta Neuropathol (Berl)* **92**, 42-48.
Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997). Alpha-synuclein in Lewy bodies. *Nature* **388**, 839-840.
Stadelmann, C., Deckwerth, T. L., Srinivasan, A., Bancher, C., Bruck, W., Jellinger, K., and Lassmann, H. (1999). Activation of caspase-3 in single neurons and autophagic granules of granulovacuolar degeneration in Alzheimer's disease. Evidence for apoptotic cell death. *Am J Pathol* **155**, 1459-1466.
Storey, K. B. (1996). Oxidative stress: animal adaptations in nature. *Braz J Med Biol Res* **29**, 1715-1733.
Sun, X. M., Butterworth, M., MacFarlane, M., Dubiel, W., Ciechanover, A., and Cohen, G. M. (2004). Caspase activation inhibits proteasome function during apoptosis. *Mol Cell* **14**, 81-93.
Sundstrom, E., Fredriksson, A., and Archer, T. (1990). Chronic neurochemical and behavioral changes in MPTP-lesioned C57BL/6 mice: a model for Parkinson's disease. *Brain Res* **528**, 181-188.
Suri, R. E., Albani, C., and Glattfelder, A. H. (1997). A dynamic model of motor basal ganglia functions. *Biol Cybern* **76**, 451-458.
Tai, K. K., and Truong, D. D. (2002). Activation of adenosine triphosphate-sensitive potassium channels confers protection against rotenone-induced cell death: therapeutic implications for Parkinson's disease. *J Neurosci Res* **69**, 559-566.
Takahashi, N., Miner, L. L., Sora, I., Ujike, H., Revay, R. S., Kostic, V., Jackson-Lewis, V., Przedborski, S., and Uhl, G. R. (1997). VMAT2 knockout mice: heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. *Proc Natl Acad Sci U S A* **94**, 9938-9943.
Takahashi, R. N., Rogerio, R., and Zanin, M. (1989). Maneb enhances MPTP neurotoxicity in mice. *Res Commun Chem Pathol Pharmacol* **66**, 167-170.
Talesa, V. N. (2001). Acetylcholinesterase in Alzheimer's disease. *Mech Ageing Dev* **122**, 1961-1969.
Tariq, M., Arshaduddin, M., Biary, N., Al Deeb, S., and Al Moutaery, K. (2000). Diethyldithiocarbamate (DEDC) impairs neuronal recovery following sciatic nerve injury in rats. *Restor Neurol Neurosci* **17**, 135-141.
Tatton, W. G., and Olanow, C. W. (1999). Apoptosis in neurodegenerative diseases: the role of mitochondria. *Biochim Biophys Acta* **1410**, 195-213.
Taylor, P. (1998). Development of acetylcholinesterase inhibitors in the therapy of Alzheimer's disease. *Neurology* **51**, S30-35; discussion S65-37.
Tebourbi, O., Rhouma, K. B., and Sakly, M. (1998). DDT induces apoptosis in rat thymocytes. *Bull Environ Contam Toxicol* **61**, 216-223.

Testa, C. M., Sherer, T. B., and Greenamyre, J. T. (2005). Rotenone induces oxidative stress and dopaminergic neuron damage in organotypic substantia nigra cultures. *Brain Res Mol Brain Res* **134**, 109-118.

Thiruchelvam, M., Brockel, B. J., Richfield, E. K., Baggs, R. B., and Cory-Slechta, D. A. (2000a). Potentiated and preferential effects of combined paraquat and maneb on nigrostriatal dopamine systems: environmental risk factors for Parkinson's disease? *Brain Res* **873**, 225-234.

Thiruchelvam, M., McCormack, A., Richfield, E. K., Baggs, R. B., Tank, A. W., Di Monte, D. A., and Cory-Slechta, D. A. (2003). Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. *Eur J Neurosci* **18**, 589-600.

Thiruchelvam, M., Prokopenko, O., Cory-Slechta, D. A., Richfield, E. K., Buckley, B., and Mirochnitchenko, O. (2005). Overexpression of superoxide dismutase or glutathione peroxidase protects against the paraquat + maneb-induced Parkinson disease phenotype. *J Biol Chem* **280**, 22530-22539.

Thiruchelvam, M., Richfield, E. K., Baggs, R. B., Tank, A. W., and Cory-Slechta, D. A. (2000b). The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. *J Neurosci* **20**, 9207-9214.

Thiruchelvam, M., Richfield, E. K., Goodman, B. M., Baggs, R. B., and Cory-Slechta, D. A. (2002). Developmental exposure to the pesticides paraquat and maneb and the Parkinson's disease phenotype. *Neurotoxicology* **23**, 621-633.

Thiruchelvam, M., Richfield, E. K., Goodman, B. M., Baggs, R. B., and Cory-Slechta, D. A. (2002b). Developmental exposure to the pesticides paraquat and maneb and the Parkinson's disease phenotype. *Neurotoxicology* **23**, 621-633.

Thiruchelvam, M. J., Powers, J. M., Cory-Slechta, D. A., and Richfield, E. K. (2004). Risk factors for dopaminergic neuron loss in human alpha-synuclein transgenic mice. *Eur J Neurosci* **19**, 845-854.

Thornberry, N. A., and Lazebnik, Y. (1998). Caspases: enemies within. *Science* **281**, 1312-1316.

Trojanowski, J. Q. (2003). Rotenone neurotoxicity: a new window on environmental causes of Parkinson's disease and related brain amyloidoses. *Exp Neurol* **179**, 6-8.

Trump, B. F., Berezesky, I. K., Chang, S. H., and Phelps, P. C. (1997). The pathways of cell death: oncosis, apoptosis, and necrosis. *Toxicol Pathol* **25**, 82-88.

Valencia, A., and Moran, J. (2004). Reactive oxygen species induce different cell death mechanisms in cultured neurons. *Free Radic Biol Med* **36**, 1112-1125.

Wagner, S. R., and Greene, F. E. (1978). Dieldrin-induced alterations in biogenic amine content of rat brain. *Toxicol Appl Pharmacol* **43**, 45-55.

Waldmeier, P. C., and Tatton, W. G. (2004). Interrupting apoptosis in neurodegenerative disease: potential for effective therapy? *Drug Discov Today* **9**, 210-218.

Walker, M. J., Jenner, P., and Marsden, C. D. (1991). A redox reaction between MPP+ and MPDP+ to produce superoxide radicals does not impair mitochondrial function. *Biochem Pharmacol* **42**, 913-919.

Weinreb, P. H., Zhen, W., Poon, A. W., Conway, K. A., and Lansbury, P. T., Jr. (1996). NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. *Biochemistry* **35**, 13709-13715.

Westerink, B. H., de Boer, P., and Damsma, G. (1990). Dopamine-acetylcholine interaction in the striatum studied by microdialysis in the awake rat: some methodological aspects. *J Neurosci Methods* **34**, 117-124.

Wilcock, G. K., Esiri, M. M., Bowen, D. M., and Smith, C. C. (1982). Alzheimer's disease. Correlation of cortical choline acetyltransferase activity with the severity of dementia and histological abnormalities. *J Neurol Sci* **57**, 407-417.

Wyllie, A. H. (1987). Apoptosis: cell death under homeostatic control. *Arch Toxicol Suppl* **11**, 3-10.

Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980). Cell death: the significance of apoptosis. *Int Rev Cytol* **68**, 251-306.

Yakovlev, A. G., Ota, K., Wang, G., Movsesyan, V., Bao, W. L., Yoshihara, K., and Faden, A. I. (2001). Differential expression of apoptotic protease-activating factor-1 and caspase-3 genes and susceptibility to apoptosis during brain development and after traumatic brain injury. *J Neurosci* **21**, 7439-7446.

Yang, S. C., Johannessen, J. N., and Markey, S. P. (1988). Metabolism of [14C]MPTP in mouse and monkey implicates MPP+, and not bound metabolites, as the operative neurotoxin. *Chem Res Toxicol* **1**, 228-233.

Zafrilla, P., Mulero, J., Xandri, J. M., Santo, E., Caravaca, G., and Morillas, J. M. (2006). Oxidative stress in Alzheimer patients in different stages of the disease. *Curr Med Chem* **13**, 1075-1083.

Zaidi, N. F., Agrawal, A. K., Anand, M., and Seth, P. K. (1985). Neonatal endosulfan neurotoxicity: behavioral and biochemical changes in rat pups. *Neurobehav Toxicol Teratol* **7**, 439-442.

Zang, L. Y., and Misra, H. P. (1992a). EPR kinetic studies of superoxide radicals generated during the autoxidation of 1-methyl-4-phenyl-2,3-dihydropyridinium, a bioactivated intermediate of parkinsonian-inducing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Biol Chem* **267**, 23601-23608.

Zang, L. Y., and Misra, H. P. (1992b). Superoxide radical production during the autoxidation of 1-methyl-4-phenyl-2,3-dihydropyridinium perchlorate. *J Biol Chem* **267**, 17547-17552.

Zhang, J., Fitsanakis, V. A., Gu, G., Jing, D., Ao, M., Amarnath, V., and Montine, T. J. (2003). Manganese ethylene-bis-dithiocarbamate and selective dopaminergic neurodegeneration in rat: a link through mitochondrial dysfunction. *J Neurochem* **84**, 336-346.

Zhang, Y., Dawson, V. L., and Dawson, T. M. (2000). Oxidative stress and genetics in the pathogenesis of Parkinson's disease. *Neurobiol Dis* **7**, 240-250.

Zhivotovsky, B. (2004). Apoptosis, necrosis and between. *Cell Cycle* **3**, 64-66.

Zhou, L. Z., Johnson, A. P., and Rando, T. A. (2001). NF kappa B and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. *Free Radic Biol Med* **31**, 1405-1416.

RESULTS

CHAPTER 3 Effects of Endosulfan and Zineb Exposure on Striatal Monoamines of Male C57Bl/6 mice

Authors: Zhenquan Jia[1] and Hara P. Misra* [1, 2].

Affiliations: [1] Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.

[2] Edward Via Virginia College of Osteopathic Medicine, Virginia Tech Corporate Research Center, 2265 Kraft Drive, Blacksburg, VA 24060, U.S.A.

Corresponding Author: * Hara P. Misra

Edward Via Virginia College of Osteopathic Medicine

Virginia Tech Corporate Research Center, 2265 Kraft Drive,

Blacksburg, VA 24060, U.S.A.

Phone: (540) 231-3693

Fax: (540) 231-5252

misra@vt.edu

Keywords: Pesticides, endosulfan, zineb, monoamines, striatum, cerebral cortex.

3.1. Abstract:

Pesticides have been suggested to play a role in the development of many neurodegenerative diseases including Parkinson's disease and Alzheimer's disease. Endosulfan and zineb are used as pesticides on a variety of crops worldwide and pose potential health risks to humans and animals. These pesticides are known to affect the nervous system. The purpose of this study was to determine if repeated exposure of mice to zineb and endosulfan, individually or in mixtures, changes the levels of monoamines and their metabolites in the brains of mice. Male C57Bl/6 mice 7-9 months old were exposed to zineb (50 and 100 mg/kg), endosulfan (1.55, 3.1 and 6.2 mg/kg) and their mixtures every other day over a 2-week period. Mice were sacrificed 7 days after the last injection. The acetylcholinesterase (AChE) activity in cerebral cortex and the levels of monoamine neurotransmitters and their metabolites in the striatum and cortex were measured. Exposure to zineb (100 mg/kg) and endosulfan (3.1 or 6.2 mg/kg) caused significantly higher levels of dopmaine accumulation in the striatum as compared to controls. Both pesticide treatment groups exhibited significantly lower norepinephrine levels in the striatum ($p < 0.005$) than the vehicle control group. Zineb at 100 mg/kg and mixtures of pesticides (zineb 100 mg + endosulfan 1.55 mg/kg) also significantly increased serotonin levels in the cerebral cortex without affecting its metabolite levels. AChE activities in the cerebral cortex were not significantly different between the pesticide treated groups and control. These data suggest that endosulfan and zineb could contribute to potential changes in the nervous system by altering the monoamine system.

3.2. Introduction:

Parkinson's disease (PD) is an age-related neurodegenerative disease, characterized by selective nigrostriatal dopaminergic degeneration. The etiology of Parkinson's disease still remains undeciphered. Recent data suggest that environmental factors play a more prominent role than genetic factors in the etiology of the Parkinson's disease phenotype (Poirier *et al.*, 1991; Holthoff *et al.*, 1994; De Michele *et al.*, 1996; Goodwin and Kite, 1998; Marder *et al.*, 1998; Tanner *et al.*, 1999). Although MPTP is not found in the environment, studies demonstrating the capability of MPTP to cause PD have stimulated a search for environmental chemicals resembling MPTP that might contribute to PD. Primates treated with MPTP exhibit behavioral, biochemical and neuropathological deficits which are very similar to those observed in human parkinsonian patients (Burns *et al.*, 1983; Chiueh *et al.*, 1984). Therefore MPTP has been used to develop animal models for PD (Tipton and Singer, 1993). Pesticides are some of the environmental chemicals investigated for correlation with PD. In a proportional mortality study, increased incidence of PD mortality was observed in rural California counties with high use of agricultural pesticides (Ritz *et al.*, 2000).

Endosulfan (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzadioxathiepin 3-oxide) is a commonly used insecticide that is effective against a variety of crop pests that infect fruit, nuts, cotton, vegetables, tea and coffee. Approximately 1.4 - 2.2 million pounds of active ingredient are used annually in the U.S (EPA, 1972). Although endosulfan is no longer produced in the United States, it is registered for use on 60 crops and is a major environmental concern in southern Florida (Baughman, 1986). The central nervous system appears to be the major target of endosulfan action in humans and animals (Dikshith *et al.*, 1984; Paul *et al.*, 1994; Balasubramaniam *et al.*, 1995; Boereboom *et al.*, 1998). Ethylene bisdithiocarbamate (EBDC) fungicides include zineb (zinc ethylene bisdithiocarbamate), maneb (manganese ethylene bisdithiocarbamate) and mancozeb (manganese zinc ethylene bisdithiocarbamate), are used worldwide in agriculture. EBDC fungicides such as maneb

possess potent dopaminergic activity and are well known to enhance the toxic effects of MPTP in mice (Takahashi *et al.*, 1989). Exposures of mice to maneb and paraquat produced selective nigrostriatal dopamine (DA) system neurotoxicity, including loss of striatal DA cell bodies in the substantia nigra pars compacta (Thiruchelvam *et al.*, 2000a; Thiruchelvam *et al.*, 2000b). Zineb alone dose-dependently reduced high affinity DA and tyrosine hydroxylase positive neurons and increased cytotoxicity in mesencephalic striatal primary co-culture (Soleo *et al.*, 1996). Since both zineb and endosulfan are widely used to protect a variety of crops against insects, human exposure to these chemicals, either concurrently or sequentially, is common due to contamination of air, water, ground and food. Recently, more emphasis has been placed on chemical mixture studies because people are actually exposed to countless mixtures of chemicals daily. The purpose of this study was to examine the capability of repeated exposure of mice to zineb and endosulfan, individually or in combination to produce adverse effects on dopaminergic or cholinergic pathways in brain of C57BL/6 mice.

3.3. Materials & Methods:

3.3.1. Animals:

C57Bl/6 male mice, 7-9 months old, weighing 30-45 g, were obtained from Charles River Laboratories (Wilmington, MA). The mice were acclimatized for 3 days and maintained under controlled conditions of temperature (22°C ± 1°C), humidity (40% to 60%) and light (12/12-hour light/dark cycle), in accordance with Virginia Polytechnic Institute and State University guidelines for animal care. Food and water were provided *ad libitum.*

3.3.2. Preparation of Pesticides:

Pesticides: Endosulfan and zineb were purchased from Chem Service (West Chester, PA). Endosulfan was dissolved in corn oil and zineb was suspended into corn oil. The pesticides were prepared and mixed just before use.

3.3.3. Treatments:

Mice were housed one per cage. C57BL/6 Mice (six/group) aged 7-9 months in each of the following groups were administered intraperitoneally (i.p) 7 times over a 2-week periodes with test chemicals or vehicle. Approximately 1/7, 1/14 or 1/28 of LD_{50} dosages of endosulfan and 1/80 and 1/40 of LD_{50} dosages of zineb were prepared by adjusting to mouse body weight, and each mouse was given the pesticides by intraperitoneal (I.P.) injection. These values were based on an LD_{50} of 43mg/kg for endosulfan and more than 4 gram mg/kg for zineb (Budavari, 1996).

1. Control (vehicle)
2. Endosulfan (EN) 1.55 mg/kg (LD_{50} =4.3 mg/kg for rat)
3. EN 3.1 mg/kg
4. EN 6.2 mg/kg
5. Zineb (ZB) 50 mg/kg (LD50 >4g/kg for rat)
6. ZB 100 mg/kg
7. ZB (50 mg/kg) + EN 1.55 mg/kg
8. ZB (50 mg/kg) + EN 3.1 mg/kg
9. ZB (50 mg/kg) + EN 6.2 mg/kg
10. ZB (100 mg/kg) + EN 1.55 mg/kg
11. ZB (100 mg/kg) + EN 3.1 mg/kg
12. ZB (100 mg/kg) + EN 6.2 mg/kg

Seven days after the last treatment, mice were sacrificed by cervical dislocation and brains were removed. The corpora striatum and cortex were dissected free and frozen in dry ice before storage at -80°C. On the day of processing, samples were allowed to thaw at room temperature. The concentration of dopamine (DA), serotonin and their metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined in the striatum and cerebral cortex. Acetylcholinesterase (AChE) activity in the cerebral cortex was also monitored.

3.3.4. Analysis of Monoamines:

Monoamines neurotransmitter and metabolite levels were determined by high performance liquid chromatography with electrochemical detection. Samples were homogenized with 250 µL of catecholamine buffer pH 4.7 (0.1 M sodium acetate, 25 mM citric acid, 134 µM EDTA, 230 mM of octanesulfonic acid and 6% methanol) containing isoproterenol 10^{-6} M. The supernatant was filtered and 100 µL of the effluent was

injected into a Beckman 344 chromatography system equipped with a Coulochem II electrochemical detector (Hewlett Packard, model 1049A) and a Phenomenex 150 × 4.6 C-18 column (Nucleosil 100 3 u, 250 × 4 mm, Macherey-Nagel, Easton, PA). The pH 3.3 mobile phase, run at 1.5ml/min, consisted of 0.01% EDTA, 0.01% NaCl and 88:12 ratio of 0.02% sodium heptane sulfonate in water: acetonitrile. System GoldTM (Beckman) ESA Coulochem II Detector software was used for data acquisition. Each sample had 10^{-6} M isoproterenol as internal standard. The concentrations of monoamines and metabolites were determined using a catecholamine standard curve and expressed as nmole per gram wet tissue.

3.3.5. Acetylcholinesterase (AChE) Analysis:

Sample were allowed to thaw on ice before being analyzed using a spectrophotometric microassay (Ellman *et al.*, 1961; Correll and Ehrich, 1991). In brief, thawed tissues were homogenized with 0.1 M sodium phosphate buffer pH 8.0 to make the stock homogenate. Then a dilution of 1:10 (100 µL stock homogenate: 900 µL 0.1 M sodium phosphate buffer pH 8.0) was made. Finally, 290 µL of the 1:10 dilution was added to 3710 µL of 0.1M sodium phosphate buffer pH 8.0 to make the working dilution. At this point, 50 µL of the working dilution was added to microplate wells along with 150 µL 0.1M sodium phosphate buffer pH 8.0, 50 µL 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and 50 µL acetylthiocholine (AcTCh), in triplicate. AChE activity was then determined spectrophotometrically at 410 nm using a molar extinction coefficient of 1.36×10^{-4} liter $M^{-1}cm^{-1}$ (Ellman *et al.*, 1961), and expressed as nmole/min/mg protein.

3.4. Protein analysis:

Protein concentrations were determined using the Bradford method with bovine serum albumin as a standard (Bio-Rad, Hercules, CA)

3.5. Statistical Analysis:

Statistical analysis was performed with analysis of variance (ANOVA) using the general linear model (GLM) procedure of SAS version 8.2 (SAS Institute Inc., Cary, NC). Data were presented as means ± standard error. After ANOVA, the Tukey-Kramer test

was used to establish differences between treatment and control groups. In addition to being compared to controls, pesticide mixture treatment results were also compared to individual pesticide treatments.

3.6. Results:

3.6.1. Body weight data:

No treatment-related changes in body weight were observed in any of the groups at the time of sacrifice of animals. The brain-to-body weight ratio was not significantly different from control in any group of pesticide-treated mice (data not shown). Body weight ranged between 40 and 46 grams; striatum to body weight ranged between 2.9×10^{-4} and 3.8×10^{-4}.

3.6.2. Monoamines and Metabolites:

The level of the monoamine neurotransmitters norepinephrine (NE), DA, and serotonin in the striatum and cerebral cortex were measured. Also, levels of monoamine metabolites dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindolacetic acid (5-HIAA) were determined. As shown in Fig. 3.1 (a-c), exposure to ZB100 mg/kg, EN 3.1, 6.2 mg/kg, and their mixtures (EN1.55 + ZB50 mg/kg, EN1.55 + 100 mg/kg, EN3.1 + ZB100 mg/kg) caused significantly higher levels of DA accumulation in the striatum as compared to controls receiving vehicle only ($\rho \leq 0.05$). However, the levels of striatal DOPAC and HVA, DA metabolites and serotonin and its metabolite, 5-HIAA were not significantly altered in any of the treatment groups ($\rho > 0.05$). Mice exposed to endosulfan and zineb individually and in mixtures exhibited significantly lower norepinephrine levels in the striatum ($p < 0.005$) than the vehicle control group (Fig. 3.1d). Exposure to ZB 100 mg/kg and mixtures of pesticides (ZB100 mg/kg + EN1.55 mg/kg) also significantly increased serotonin levels in cerebral cortex as compared to controls (Fig. 3.1e, $\rho \leq 0.05$). However, the levels of cerebral cortex DA and its major metabolites, DOPAC and HVA and 5-HIAA, a serotonin metabolite, were not significantly altered in any of the treatment groups ($\rho > 0.05$, data not shown).

3.6.3. Cerebral Cortex Acetylcholinesterase:

We monitored the activity of AChE, the enzyme responsible for acetylcholine degradation, in cerebral cortex following pesticide exposure (Fig. 3.2). AChE activities in the cerebral cortex were not significantly altered by any of the pesticide treatments as compared to controls receiving vehicle only ($\rho > 0.05$).

3.7. Discussion:

We examined the hypothesis that exposure to the organochlorine endosulfan and dithiocarbamate fungicide zineb either alone or in combination, could contribute to the disruption of the dopaminergic or cholinergic pathways in adult C57BL/6 mice. Exposure of mice to zineb and endosulfan increased levels of DA in the striatum, measured 7 days after the last injection.

Mice exposed to endosulfan showed higher levels of DA in the striatum. This contrasts with previously reported results that demonstrated decreased DA in rat brain following a single intraperitoneal injection of 40 mg/kg of endosulfan, a dose about half of the lethal dose (Ansari *et al.*, 1987). In another study, Wistar rat pups dosed with 6 mg/kg of endosulfan from postnatal days 2-25 also exhibited low levels of DA (Madepalli *et al* 1994). Involvement of the central nervous system in the neurotoxicity of endosulfan has been shown by several experimental studies conducted on small rodents (Anand *et al.*, 1980; Anand *et al.*, 1985; Anand *et al.*, 1986). Ansari (1987) suggested that changes in neurotransmitter levels (specifically serotonin, GABA, and DA) in the brain may be partly responsible for the neurotoxicity of endosulfan after observing hyperactivity, tremors, and convulsions (Ansari *et al.*, 1987). There is evidence that organochlorine pesticides up-regulate DA transport in striatal synaptosomes and affect the DA transporter (DAT) and DA release in the striatum (Kirby et al 1999, 2001; Karen et al 2001). This very likely contributes to our results. DAT is a membrane-bound carrier molecule that mediates the action of DA in the nervous synapse via the reuptake of DA into the dopaminergic neurons and the DAT is also capable of neuronal DA release (Horn,

1990). As DAT is responsible for DA re-uptake into presynaptic neurons, this would be an expected response to increased DA levels. Examination of changes in the capacity of striatal DAT might be helpful in understanding this DA increase in the striatum following endosulfan exposure.

Many dithiocarbamate fungicides such as maneb, mancozeb, metram, ziram and thiram have been reported to cause neurodegeneration and demylination of rat neurons (Ferraz *et al.*, 1988; Seaton *et al.*, 1997; McGrew *et al.*, 2000; Zhang *et al.*, 2003). Thiruchelvam et al (2000) examined the effect of maneb and paraquat in six week C57BL/6 mice on dopaminergic system toxicity. Mice were injected twice a week for 6 weeks for a total of 12 injections. Levels of striatal DA and DOPAC were significantly elevated 1-3 days after maneb (30 mg/kg) injection and decreased after 7 days. Although zineb (a zinc-substituted dithiocarbamate) is structurally similar to maneb (a manganese-substituted dithiocarbamate), studies on the action of zineb on the DA system have not appeared in the literature. In our studies, we observed a significant increase in the levels of DA in the corpora striatum following exposure to zineb after 7 days injection. This is contrast to the above study that showed decrease in striatal DA after 7 days of administration of the dithiocarbamate fungicide maneb (Thiruchelvam *et al.*, 2000a). Our mice were treated 7 times instead of 12 times during a 2-week period and 7-9 month old mice were used instead of 6 week old mice. In our study, mice were treated just for a period of two weeks, which may be too short a time to observe the dramatic decreased levels of DA and its metabolites. This difference could contribute to the increase of DA levels.

Because there was no significant change observed in DOPAC and HVA in mice following pesticide exposures, augmentation of DA degradation by MAO (monoamine oxidase) and COMT (catechol-O-methyltransferase) were unlikely to contribute to the difference.

Levels of NE were also decreased in all pesticide treatment groups. Although we were able to measure NE in this study, NE levels in corpora striatum are low and, therefore,

not easily detected in this tissue. NE is mainly synthesized and located in the locus coeruleus (Levitt et al 1979).

In our studies in adult mice, we observed a significant increase in the levels of serotonin without a correspondingly increase in its metabolite following exposure to ZB 100 mg/kg and mixtures of pesticides ZB 100 mg/kg + EN 1.55 mg/kg. Our data are consistent with a previous report that demonstrated increased of serotonin level in various brain area (olfactory bulb, hippocampus, visual cortex and brain stem) of endosulfan-treated rats (Lakshmana and Raju, 1994). Anand et al (1985) also demonstrated an increase in the levels of serotonin when adult rats were treated with 3 mg/kg of endosulfan intraperitoneally during 10 subsequent days. The serotonergic system has been implicated in endosulfan neurotoxicity in neo-natal rats by binding studies using methysergide, a 5-HT blocker (Agrawal *et al.*, 1983; Zaidi *et al.*, 1985). Paul et al. (1994) also found a correlation between the increase in serotonin and inhibition of a learning paradigm (Paul *et al.*, 1994).

In the present studies, AChE activity in cerebral cortex was not affected in both endosulfan and zineb treated mice. Acetylcholine is the major neurotransmitter affected in Alzheimer's disease. Acetylcholine is rapidly broken down by the enzyme acetylcholinesterase. If the pesticides destroy this enzyme system, it would result in the accumulation of acetylcholine. Ethylenebisdithiocarbamate fungicides such as zineb, maneb, and mancozeb have produced significant change of AChE activity indicating possible damage to the cholinergic pathway in rats (Savolainen and Hervonen, 1985; Kackar *et al.*, 1999). Exposure to a single intraperitoneal injection of endosulfan in rats also decreased brain acetylcholinesterase activity (Gupta, 1976). Kiran and Varma (1988) orally dosed rats of different age groups with 12.5 mg/kg of endosulfan. They observed age dependent decreases in brain acetylcholinesterase. However, neither Paul et al. (1994) nor Lakshmana and Raju (1994) found changes in the activity of acetylcholinesterase in the brain of rats treated with 2 mg endosulfan/kg/day for 90 days or with 6 mg/kg/day for 23 days, respectively (Lakshmana and Raju, 1994; Paul *et al.*, 1994) . Our findings are consistent with those of others (Lakshmana and Raju, 1994; Paul *et al.*, 1994).

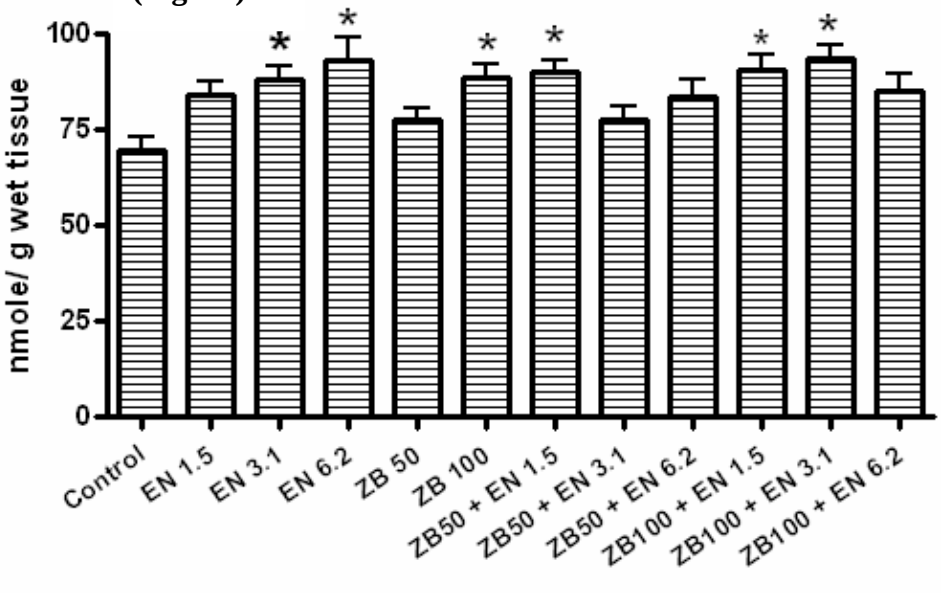
Up until recently, about 95% of all studies on the effects of chemicals were performed using individual chemicals (Groten et al. 1999). Recently research on chemical mixtures has increased due to an initiative set forth by the USEPA and NIEHS to promote a broader understanding of chemicals in mixtures and the mechanisms associated with multiple exposures (NCERQA 1998).

In summary, it appears that exposure to both pesticides (endosulfan and zineb) produced significant changes in monoamines neurotransmitters both in the striatum and cerebral cortex. Neither pesticide is associated with inhibition of AChE activities. Both pesticides might induce monoamine alterations by acting on the enzymes related to their synthesis and degradation. The study of possible effect of both pesticides on specific enzymes related to monoamine synthesis and metabolism will answer these questions. Changes in monoamines in the central nervous system may be linked to these pesticide induced neurotoxicity.

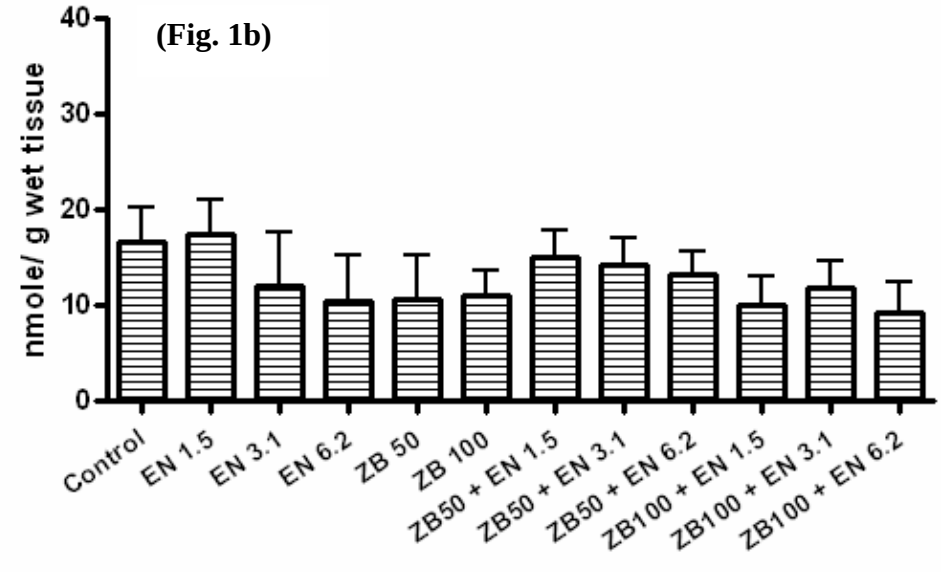
3.8. Acknowledgements:

The authors thank Dr. Marion Ehrich for reviewing the manuscript. We also gratefully acknowledge the technical assistance of Geraldine Magnin and Barbara Carson Wise for HPLC use and Dan Ward for statistical analysis. This work was supported, in part, by the Edward Via Virginia College of Osteopathic Medicine.

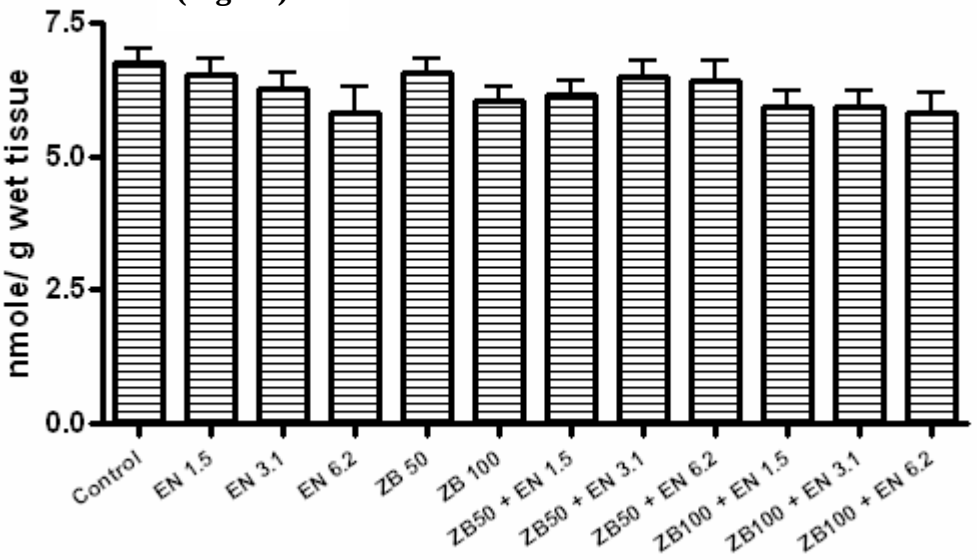
(Fig. 1a)


nmole/ g wet tissue
100
75
50
25
0
*
*
*
*
*
*
Control
EN 1.5
EN 3.1
EN 6.2
ZB 50
ZB 100
ZB50 + EN 1.5
ZB50 + EN 3.1
ZB50 + EN 6.2
ZB100 + EN 1.5
ZB100 + EN 3.1
ZB100 + EN 6.2

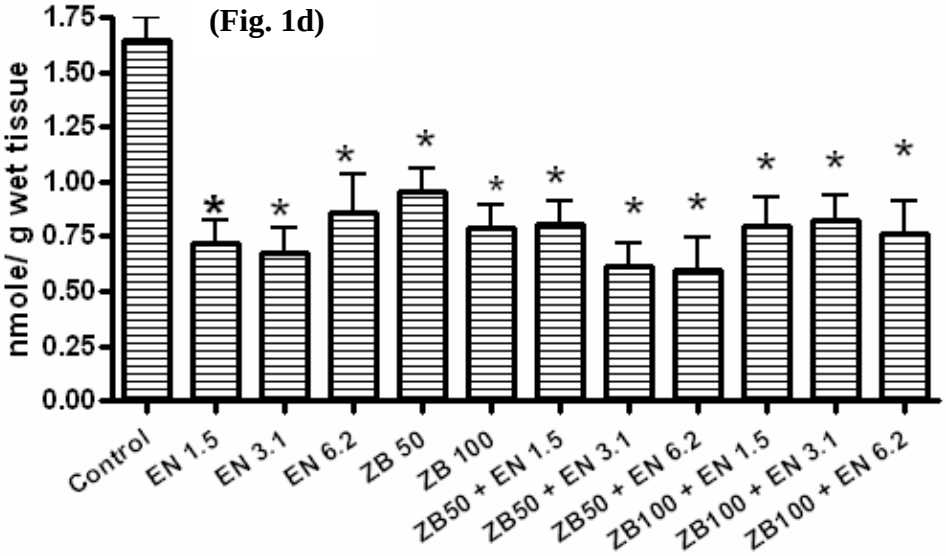
(Fig. 1b)


nmole/ g wet tissue
40
30
20
10
0
Control
EN 1.5
EN 3.1
EN 6.2
ZB 50
ZB 100
ZB50 + EN 1.5
ZB50 + EN 3.1
ZB50 + EN 6.2
ZB100 + EN 1.5
ZB100 + EN 3.1
ZB100 + EN 6.2

(Fig. 1c)


7.5
5.0
2.5
0.0
nmole/ g wet tissue
Control
EN 1.5
EN 3.1
EN 6.2
ZB 50
ZB 100
ZB50 + EN 1.5
ZB50 + EN 3.1
ZB50 + EN 6.2
ZB100 + EN 1.5
ZB100 + EN 3.1
ZB100 + EN 6.2

(Fig. 1d)


1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
nmole/ g wet tissue
Control
EN 1.5
EN 3.1
EN 6.2
ZB 50
ZB 100
ZB50 + EN 1.5
ZB50 + EN 3.1
ZB50 + EN 6.2
ZB100 + EN 1.5
ZB100 + EN 3.1
ZB100 + EN 6.2

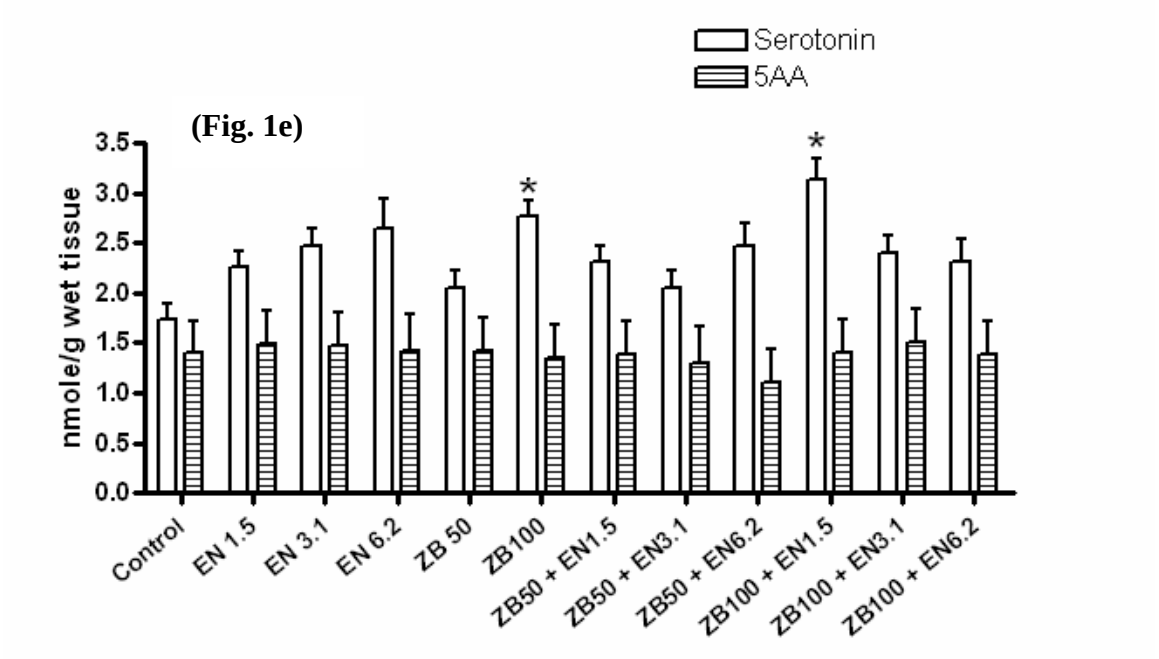


Figure 3.1: Brain neurotransmitters and metabolite levels after exposure to the pesticides, zineb (ZB), endosulfan (EN) and their combinations (EN + ZB).

Results are expressed as nmoles/mg means ± SEM of HPLC results from C57BL/6 mice 7-9 months old dosed with the following pesticides: endosulfan (EN) 1.55, 3.1, 6.2 mg/kg; zineb (ZB) 50, 100 mg/kg; and their mixtures, ZB 50 + EN 1.55, ZB 50 + EN 3.1, ZB 50 + EN 6.2, ZB 100 + EN 1.55, ZB 100 + EN 3.1 and ZB 100 + EN 6.2 mg/kg. Each of the above treatment group mice were dosed intraperitoneally 7 times every other day during a period of two weeks. Turkey's HSD post hoc test for each treatment showed differences ($p \leq 0.05$) * compared to controls (corn oil). Mixtures did not cause significant decreases compared to individual pesticide exposures. The results are reported as mean nmole/g wet tissue ± S.E., n = 6.

(Fig. 3.1a) = striatal dopamine levels

(Fig. 3.1b) = striatal DOPAC levels (no significant differences were noted)

(Fig. 3.1c) = striatal HVA levels (no significant differences were noted)

(Fig. 3.1d) = striatal norepinephrine (NE) levels

(Fig. 3.1e) = cerebral cortex indolamine levels

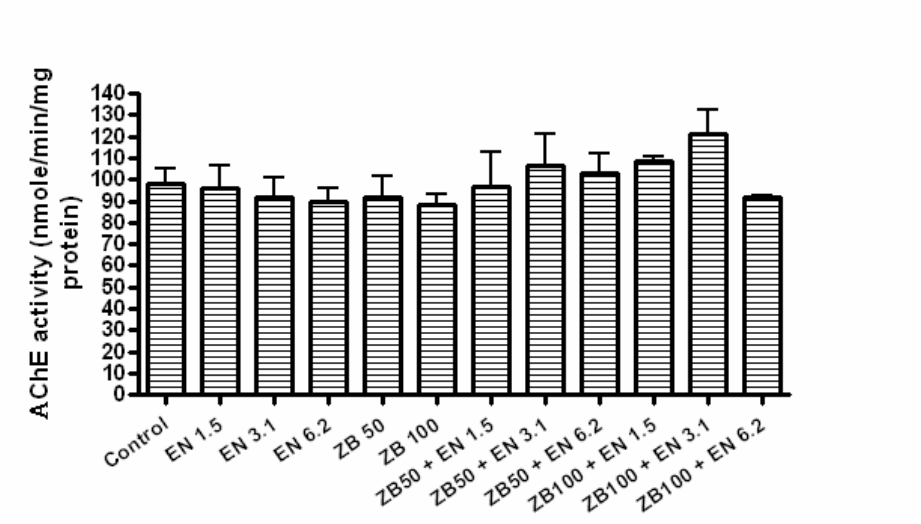


Figure 3.2: Cortex AChE activities in mouse cerebral cortex after exposure to the pesticides, zineb (ZB), endosulfan (EN) and their combinations (EN + ZB).

Results expressed as nmole/min/mg protein ± S.E from C57BL/6 mice 7-9 months old dosed with the following pesticides: endosulfan (EN) 1.55, 3.1, 6.2 mg/kg; zineb (ZB) 50, 100 mg/kg and their mixtures, ZB 50 + EN 1.55, ZB 50 + EN 3.1, ZB 50 + EN 6.2, ZB 100 + EN 1.55, ZB 100 + EN 3.1 and ZB 100 + EN 6.2 mg/kg. Each of the above treatment group mice were dosed intraperitoneally 7 times every other day during a period of two weeks. No significant differences were noted. The results are reported as mean nmole/g wet tissue ± S.E., n = 6.

3.9. Literature Cited:

Agrawal, A. K., Anand, M., Zaidi, N. F., and Seth, P. K. (1983). Involvement of serotonergic receptors in endosulfan neurotoxicity. *Biochem Pharmacol* **32**, 3591-3593.

Anand, M., Agrawal, A. K., Gopal, K., Sur, R. N., and Seth, P. K. (1986). Endosulfan and cholinergic (muscarinic) transmission: effect on electroencephalograms and [3H]quinuclidinyl benzilate in pigeon brain. *Environ Res* **40**, 421-426.

Anand, M., Khanna, R. N., Gopal, K., and Gupta, G. S. (1980). Effect of endosulfan of bioelectrical activity of brain in rats. *Vet Hum Toxicol* **22**, 385-387.

Anand, M., Mehrotra, S., Gopal, K., Sur, R. N., and Chandra, S. V. (1985). Role of neurotransmitters in endosulfan-induced aggressive behaviour in normal and lesioned rats. *Toxicol Lett* **24**, 79-84.

Ansari, R. A., Husain, K., and Gupta, P. K. (1987). Endosulfan toxicity influence on biogenic amines of rat brain. *J Environ Biol* **8**, 229-236.

Balasubramaniam, E., Paul, V., and Kazi, M. (1995). Cyclodiene insecticides-induced changes in the central depressive effect of chlorpromazine in rats. *Indian J Physiol Pharmacol* **39**, 231-236.

Baughman, D. S. (1986). The acute toxicity of inseticides associated with agricultural runoff to the grass shimp Palaemonetes Pugio. , pp. 1-70. University of Carolina, Columbia.

Boereboom, F. T., van Dijk, A., van Zoonen, P., and Meulenbelt, J. (1998). Nonaccidental endosulfan intoxication: a case report with toxicokinetic calculations and tissue concentrations. *J Toxicol Clin Toxicol* **36**, 345-352.

Budavari, S. (1996). *The Merck index : an encyclopedia of chemicals, drugs, and biologicals*. Merck & Co., Whitehouse Station, NJ.

Burns, R. S., Chiueh, C. C., Markey, S. P., Ebert, M. H., Jacobowitz, D. M., and Kopin, I. J. (1983). A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc Natl Acad Sci U S A* **80**, 4546-4550.

Chiueh, C. C., Markey, S. P., Burns, R. S., Johannessen, J. N., Jacobowitz, D. M., and Kopin, I. J. (1984). Neurochemical and behavioral effects of 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) in rat, guinea pig, and monkey. *Psychopharmacol Bull* **20**, 548-553.

Correll, L., and Ehrich, M. (1991). A microassay method for neurotoxic esterase determinations. *Fundam Appl Toxicol* **16**, 110-116.

De Michele, G., Filla, A., Volpe, G., De Marco, V., Gogliettino, A., Ambrosio, G., Marconi, R., Castellano, A. E., and Campanella, G. (1996). Environmental and genetic risk factors in Parkinson's disease: a case-control study in southern Italy. *Mov Disord* **11**, 17-23.

Dikshith, T. S., Raizada, R. B., Srivastava, M. K., and Kaphalia, B. S. (1984). Response of rats to repeated oral administration of endosulfan. *Ind Health* **22**, 295-304.

Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Feather-Stone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**, 88-95.

EPA (1972). The pollution potential in pesticide manufacturing. Pesticide Study Series 5. U.S. Environmental Protection Agency, Office of Water Products.

Ferraz, H. B., Bertolucci, P. H., Pereira, J. S., Lima, J. G., and Andrade, L. A. (1988). Chronic exposure to the fungicide maneb may produce symptoms and signs of CNS manganese intoxication. *Neurology* **38**, 550-553.

Goodwin, B. L., and Kite, G. C. (1998). Environmental MPTP as a factor in the aetiology of Parkinson's disease? *J Neural Transm* **105**, 1265-1269.

Gupta, P. K. (1976). Endosulfan-induced neurotoxicity in rats and mice. *Bull Environ Contam Toxicol* **15**, 708-713.

Holthoff, V. A., Vieregge, P., Kessler, J., Pietrzyk, U., Herholz, K., Bonner, J., Wagner, R., Wienhard, K., Pawlik, G., and Heiss, W. D. (1994). Discordant twins with Parkinson's disease: positron emission tomography and early signs of impaired cognitive circuits. *Ann Neurol* **36**, 176-182.

Horn, A. S. (1990). Dopamine uptake: a review of progress in the last decade. *Prog Neurobiol* **34**, 387-400.

Kackar, R., Srivastava, M. K., and Raizada, R. B. (1999). Assessment of toxicological effects of mancozeb in male rats after chronic exposure. *Indian J Exp Biol* **37**, 553-559.

Lakshmana, M. K., and Raju, T. R. (1994). Endosulfan induces small but significant changes in the levels of noradrenaline, dopamine and serotonin in the developing rat brain and deficits in the operant learning performance. *Toxicology* **91**, 139-150.

Marder, K., Logroscino, G., Alfaro, B., Mejia, H., Halim, A., Louis, E., Cote, L., and Mayeux, R. (1998). Environmental risk factors for Parkinson's disease in an urban multiethnic community. *Neurology* **50**, 279-281.

McGrew, D. M., Irwin, I., and Langston, J. W. (2000). Ethylenebisdithiocarbamate enhances MPTP-induced striatal dopamine depletion in mice. *Neurotoxicology* **21**, 309-312.

Paul, V., Balasubramaniam, E., and Kazi, M. (1994). The neurobehavioural toxicity of endosulfan in rats: a serotonergic involvement in learning impairment. *Eur J Pharmacol* **270**, 1-7.

Poirier, J., Kogan, S., and Gauthier, S. (1991). Environment, genetics and idiopathic Parkinson's disease. *Can J Neurol Sci* **18**, 70-76.

Ritz, B., Yu, F., Chapa, G., and Fruin, S. (2000). Effect of air pollution on preterm birth among children born in Southern California between 1989 and 1993. *Epidemiology* **11**, 502-511.

Savolainen, K., and Hervonen, H. (1985). Dithiocarbamate fungicides decrease histochemical reactivity of cholinesterases in the gut wall of the rat. *Arch Toxicol Suppl* **8**, 272-276.

Seaton, T. A., Cooper, J. M., and Schapira, A. H. (1997). Free radical scavengers protect dopaminergic cell lines from apoptosis induced by complex I inhibitors. *Brain Res* **777**, 110-118.

Soleo, L., Defazio, G., Scarselli, R., Zefferino, R., Livrea, P., and Foa, V. (1996). Toxicity of fungicides containing ethylene-bis-dithiocarbamate in serumless dissociated mesencephalic-striatal primary coculture. *Arch Toxicol* **70**, 678-682.

Takahashi, R. N., Rogerio, R., and Zanin, M. (1989). Maneb enhances MPTP neurotoxicity in mice. *Res Commun Chem Pathol Pharmacol* **66**, 167-170.

Tanner, C. M., Ottman, R., Goldman, S. M., Ellenberg, J., Chan, P., Mayeux, R., and Langston, J. W. (1999). Parkinson disease in twins: an etiologic study. *Jama* **281**, 341-346.

Thiruchelvam, M., Brockel, B. J., Richfield, E. K., Baggs, R. B., and Cory-Slechta, D. A. (2000a). Potentiated and preferential effects of combined paraquat and maneb on nigrostriatal dopamine systems: environmental risk factors for Parkinson's disease? *Brain Res* **873**, 225-234.

Thiruchelvam, M., Richfield, E. K., Baggs, R. B., Tank, A. W., and Cory-Slechta, D. A. (2000b). The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. *J Neurosci* **20**, 9207-9214.

Zaidi, N. F., Agrawal, A. K., Anand, M., and Seth, P. K. (1985). Neonatal endosulfan neurotoxicity: behavioral and biochemical changes in rat pups. *Neurobehav Toxicol Teratol* **7**, 439-442.

Zhang, J., Fitsanakis, V. A., Gu, G., Jing, D., Ao, M., Amarnath, V., and Montine, T. J. (2003). Manganese ethylene-bis-dithiocarbamate and selective dopaminergic neurodegeneration in rat: a link through mitochondrial dysfunction. *J Neurochem* **84**, 336-346.

CHAPTER 4 Developmental exposure to pesticides zineb and/or endosulfan renders the nigrostriatal dopamine system more susceptible to these environmental chemicals later in life.

Authors: Zhenquan Jia[1] and Hara P. Misra* [1, 2].

Affiliations: [1] Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.

[2] Edward Via Virginia College of Osteopathic Medicine, Virginia Tech Corporate Research Center, 2265 Kraft Drive, Blacksburg, VA 24060, U.S.A.

Corresponding Author: * Hara P. Misra
Edward Via Virginia College of Osteopathic Medicine
Virginia Tech Corporate Research Center, 2265 Kraft Drive,
Blacskburg, VA 24060, U.S.A.
Phone: (540) 231-3693
Fax: (540) 231-5252
E-mail: misra@vt.edu

Keywords: endosulfan, zineb, pesticides, chemical mixtures, developmental, neurodegeneration, dopamine, Parkinson's disease

4.1. Abstract:

Several epidemiological studies have suggested a role for environmental pesticide exposures in idiopathic Parkinson's disease. The purpose of this study was to test the hypothesis that exposure to pesticides such as endosulfan and/or zineb during critical periods of postnatal development could result in neuronal dysfunction and enhance the impact of these pesticides during exposure as adults. C57BL/6 mice, exposed daily with each of the pesticides or their mixtures from postnatal days 5 to 19, had little change in striatal dopamine, acetylcholinesterase and alpha-synuclein levels. However, mice exposed to these pesticides as juveniles and re-exposed at 8 months of age had neurotransmitters levels that were significantly altered in the striatum and brain cortex. Thus, mice re-exposed during adulthood with zineb, endosulfan and their mixtures showed a significant depletion of striatal dopamine, to 22%, 16%, and 35% of control, respectively. Acetylcholinesterase activity in the cerebral cortex was significantly increased in all pesticide treated groups ($\rho \leq 0.05$) upon repeated exposure. Mice given mixtures of pesticides also showed significantly increased levels of normal and aggregated alpha-synuclein. Collectively, these studies suggest that a silent neurotoxicity produced by developmental exposure to certain pesticides can be unmasked by challenges later in life.

4.2. Introduction:

Parkinson's disease (PD) is typically considered an aging–related neurodegenerative disorder charactered by degeneration of the nigrostriatal system. Several epidemiological studies have implicated a role for environmental factors in the etiology of this disease. Thus, there are reports correlating increased incidence of PD with pesticide exposure, such as drinking well water contaminated with pesticides, farming and rural living, and greater use of environmental chemicals in industrialized counties (Semchuk *et al.*, 1992; Gorell *et al.*, 1998). In a proportional mortality study, increased incidence of PD mortality was observed in rural California counties with high use of agricultural pesticides (Ritz *et al.*, 2000). However, a recent review (IOM, 2003) concluded evidence was insufficient to directly link pesticide exposure with PD.

Although the etiology of idiopathic PD remains an enigma, several risk factors, including pesticides, street drugs and aging have been identified. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a contaminant found in synthetic heroin, for example, causes a PD-like condition man (Langston *et al.*, 1983), and can be used in animals (Tolwani *et al.*, 1999) to cause depletion of striatal dopamine (DA), providing an accepted experimental model for PD. Discovery that MPTP could cause a PD-like disorder led to studies on increased risk of PD following exposure to other chemical substances (Betarbet *et al.*, 2000). Experiments in which animals were exposed to certain pesticides such as paraquat and rotenone supported the possibility that they could contribute to neurodegenerative diseases such as Parkinson's and Alzheimer's Diseases (Manning-Bog *et al.*, 2002; Thiruchelvam *et al.*, 2002). Paraquat, a commonly used herbicide (recently removed from the US market), has been extensively studied for its neurodegenerative effects (Rajput and Uitti, 1987; Sanchez-Ramos *et al.*, 1987; Thiruchelvam *et al.*, 2000a; Andersen, 2003). It has some structural similarity to MPP^+, the toxic metabolite of MPTP, which is formed in a two-step biotransformation process as monoamine oxidase acts on MPTP (Chiba *et al.*, 1984; Zang and Misra, 1993). Paraquat has been demonstrated to interact with other pesticides. For example, even

though maneb and paraquat are structurally dissimilar, exposure to the fungicide maneb during gestation was shown to enhance response to paraquat administered late in life. This combination provides a model of environmental PD in adult mice (Cory-Slechta *et al.*, 2005).

Many dithiocarbamate fungicides such as maneb, zineb, mancozeb, metram, ziram and thiram have been reported to cause neurodegeneration and demylination of rat neurons (Ferraz *et al.*, 1988; Seaton *et al.*, 1997; McGrew *et al.*, 2000; Zhang *et al.*, 2003). In addition, maneb appears to possess potent dopaminergic activity and is well known to enhance the toxic effects of MPTP in mice (Takahashi *et al.*, 1989). Exposures of mice to maneb were also shown to produce selective nigrostriatal DA system neurotoxicity, including loss of striatal DA and degeneration of cell bodies of DA neurons in the substantia nigra pars compacta (Thiruchelvam *et al.*, 2000a; Thiruchelvam *et al.*, 2002; Thiruchelvam *et al.*, 2003). Although zineb (a zinc- substituted dithiocarbamate) is structurally similar to maneb (a manganese-substituted dithiocarbamate), studies on the action of zineb on the DA system have not appeared in the literature.

Other pesticides may also contribute to neurodegenerative disorders. Endosulfan, a commonly used organochlorine cyclodiene pesticide, is known to cause central nervous system disorders, such as convulsions, dizziness and memory impairment in humans exposed to high concentrations (Aleksandrowicz, 1979). Cyclodiene compounds are known to antagonize the action of the neurotransmitter gamma-aminobutyric acid (GABA), which induces the uptake of chloride ions by neurons. The inhibition of this activity by cyclodiene insecticides results in only partial repolarization of the neuron and a state of uncontrolled excitation. Chronic exposure to these pesticides has been suggested to contribute to neurotoxicity leading to neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Gupta, 1976; Agrawal *et al.*, 1983; Zaidi *et al.*, 1985; Anand *et al.*, 1986; Seth *et al.*, 1986; Konno, 2003; Shin *et al.*, 2004a).

The developing nervous system is proposed to be a potentially sensitive target for pesticide exposure (Tilson, 1998; Tilson, 2000; Nakai and Satoh, 2002; Shafer *et al.*, 2005). As the dopaminergic system is mainly developed postnatally (Giorgi *et al.*, 1987;

Voorn *et al.*, 1988), the disruption of dopaminergic system by chemical exposure early in life has potential to contribute to permanent neurodegeneration and, further, to increase vulnerability to subsequent neurotoxic challenges occurring later in life (Thiruchelvam *et al.*, 2002).

Although several types of pesticides have potential to contribute to neurodegenerative diseases, the literature indicates that more than 95% of all pesticide toxicity studies were conducted on individual environmental pollutants. Recently, more emphasis has been placed on studies with multiple chemicals because exposure to mixtures of pesticides is a common occurrence (Simmons, 1995). Moreover, exposure to two or more pesticides may result in additive, synergic or antagonistic health effects mainly because the metabolism of one may affect that of the other (Iyaniwura, 1990; Hodgson and Levi, 1996).

The present study was designed to examine the possibility that postnatal exposure to endosulfan and zineb alone and in combination would result in increased susceptibility of the nigrostriatal DA system when re-challenged later in life. The rationale for studies on the combined exposure to zineb and endosulfan was based on the hypothesis that concurrent insults to the dopaminergic system by two structurally different pesticides with different modes of action would increase the vulnerability of the DA system. We report here that C57BL/6 mice exposed to endosulfan and zineb alone and in combination during postnatal days 5-19 at doses that were $1/10^{th}$ of those used for adults resulted in enhanced susceptibility to loss of nigrostriatal DA system upon re-exposure in adulthood. Furthermore, alpha-synuclein, a 140 amino acid protein which is a major component Lewy bodies, a hallmark of neurodegenerative diseases such as PD and Alzheimer's disease (Hedera *et al.*, 1995; Hardy, 2003), was quantified in the brain cortex of the mice. Exposure to mixtures of pesticides during postnatal days and subsequent re-challenge in adulthood resulted in increase of intensities of both the aggregated and the non-aggregated forms of alpha-synuclein.

4.3. Materials & Methods:

4.3.1. Animals:

C57Bl/6 male mice pups with their mothers were obtained from Charles River Laboratories (Wilmington, MA). The mice were acclimatized for 3 days and maintained under controlled conditions of temperature (22°C ± 1°C), humidity (40% to 60%) and light (12/12-hour light/dark cycle), in accordance with Virginia Polytechnic Institute and State University guidelines for animal care. Food and water were provided *ad libitum.*

4.3.2. Preparation of Pesticides:

Pesticides: Endosulfan and zineb were purchased from Chem Service (West Chester, PA). Endosulfan was dissolved in corn oil and zineb was suspended in corn oil. The pesticides were prepared and mixed just before use.

4.3.3. Treatments:

Pups were kept with their mother in groups of 6-8. Mice pups were injected by the intraperitioneal (i.p.) route daily from days 5 to 19 with either corn oil (control), endosulfan 0.155 mg/kg, zineb 5 mg/kg or a combination of endosulfan and zineb using a 0.3 ml insulin syringe. For mixture studies, two separate injections were administered. On day 19 the pups were weaned from their mother and separated into different treatment groups. They were provided feed and water *ad libitum.* At 8 months of age, groups of these mice were re-challenged with corn oil only (control), 1.55 mg/kg endosulfan (EN group), 50 mg/kg zineb (ZB group) or a combination of endosulfan + zineb, every other day for 7 injections. This yielded groups of mice exposed as juveniles (JV) and groups of mice exposed as JV + Adult. Thus, the JV group received 15 doses as juveniles and the JV + adults group received 15 doses at juveniles and were subsequently re-exposed 7 times every other day at 8 months of age. In addition, each group of mice was divided into four subsets that included vehicle control, endosulfan, zineb, and endosulfan + zineb-treated mice. The doses of pesticides in the present study were chosen based on our earlier dose-response data that showed endosulfan + zineb caused alterations in DA levels in adult C57Bl/6 mouse brain striatum (Jia and Misra, unpublished data).

Seven days after the last treatment, mice were sacrificed by cervical dislocation and after rapid decapitation brains were removed. The striatum and cortex were dissected free and frozen in dry ice before storage at -80°C. On the day of processing, samples were

allowed to thaw at room temperature. The concentration of dopamine (DA), and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined in the striatum. Acetylcholinesterase (AChE) activity and alpha-synuclein levels in the cerebral cortex were monitored using procedures described below.

4.3.4. Analysis of Dopamine and Metabolites:

Samples were homogenized with 250 µL of catecholamine buffer pH 4.7 (0.1 M sodium acetate, 25 mM citric acid, 134 µM EDTA, 230 mM of octanesulfonic acid and 6% methanol) containing isoproterenol (10^{-6} M). The supernatant was filtered and 100 µL of the effluent was injected into a Beckman 344 chromatography system equipped with a Coulochem II electrochemical detector (Hewlett Packard, model 1049A) and a Phenomenex 150 × 4.6 C-18 column (Nucleosil 100 3 u, 250 × 4 mm, Macherey-Nagel, Easton, PA). The pH 3.3 mobile phase, run at 1.5ml/min, consisted of 0.01% EDTA, 0.01% NaCl and a 88:12 ratio of 0.02% sodium heptane sulfonate in water:acetonitrile. System GoldTM (Beckman) ESA Coulochem II Detector software was used for data acquisition. Each sample had 10^{-6} M isoproterenol as internal standard. The concentrations of dopamine and its metabolites were determined using a standard curve and expressed as nmole/g wet tissue.

4.3.5. Acetylcholinesterase (AChE) Analysis:

Samples were allowed to thaw on ice before being analyzed using a spectrophotometric microassay (Ellman *et al.*, 1961; Correll and Ehrich, 1991). In brief, thawed tissues were homogenized with 0.1 M sodium phosphate buffer pH 8.0 to make the stock homogenate. Then a dilution of 1:10 (100 µL stock homogenate: 900 µL 0.1 M sodium phosphate buffer pH 8.0) was made. Finally, 290 µL of the 1:10 dilution was added to 3710 µL of 0.1M sodium phosphate buffer pH 8.0 to make the working dilution. At this point, 50 µL of the working dilution was added to microplate wells along with 150 µL 0.1M sodium phosphate buffer pH 8.0, 50 µL 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and 50 µL acetylthiocholine (AcTCh), in triplicate. AChE activity was then determined spectrophotometrically at 410 nm, and expressed as nmole/min/mg protein.

4.3.6. Alpha-synuclein Western blot studies:

Mouse cerebral cortex tissues were thawed and sonicated in PBS (phosphate buffered saline) with 0.5% Triton-100 and protease inhibitor cocktail (P8340; Sigma, Saint Louis, MO). Each homogenate was incubated on ice for 30 min to allow action of the proteases. Samples were then centrifuged at 12000 × g for 5 min at 15°C. The supernatants were thawed, mixed with sample buffer (0.5 M Tris-HCl, pH 6.5, 10% SDS, 1% bromophenol blue, glycerol, 2-mercaptoethanol) and heated for 5 min at 95°C for alpha-synuclein analysis. Thirty micrograms of protein were loaded on 15% SDS gels and electrophoresis was run using a Bio-Rad electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA). After electrophoresis was completed, proteins were electrophoretically transferred to polyvinyldiene difluoride (PVDF) membranes run at 20V for 20 min. The membrane proteins were then stained with Ponceau S dye to verify transfer. Membranes were blocked with 5% nonfat milk in 0.1% TBS Tween for 2 hours at room temperature followed by overnight incubation with primary antibodies. The blots were washed two times with TBS Tween 0.1 % (TBS + Tween- 20 0.1%), and one time with TBS buffer pH 7.5 (Tris-HCL 20 mM, NaCl 500 mM, distilled water) followed by incubation with either a goat anti-mouse or rabbit IgG horseradish peroxidase (HRP)-conjugated antibody for 2 hours. After a second washing with TBS Tween buffer, the blots were developed using a horseradish peroxidase substrate kit (Pierce Biotechnology, Rockford, IL). Protein bands on the film were quantitated using Kodak image station 440 CF (Eastman Kodak Co., Rochester, NY).

4.3.7. Protein analysis:

Protein concentrations were determined using the Bradford method with bovine serum albumin as a standard (Bio-Rad, Hercules, CA)

4.4. Statistical Analysis:

Statistical analysis was performed with analysis of variance (ANOVA) using the general linear model (GLM) procedure of SAS version 8.2 (SAS Institute Inc., Cary, NC). Data were presented as means ± standard error. After ANOVA, the Tukey-Kramer test

was used to establish differences between treatment and control groups. In addition to being compared to controls, pesticide mixture treatment results were also compared to individual pesticide treatments.

4.5. Results:

4.5.1. Body weight and pathology:

No treatment-related changes in body weight were observed in any of the groups at the time of sacrifice of animals. No gross pathological lesions were visible in heart, lungs, kidneys or liver. Body weights ranged between 39 and 45 grams. The striatum-to-body weight ratio was not significantly different from control in any group of pesticide-treated mice (data not shown).

4.5.2. Striatal Dopamine and Metabolites:

Levels of striatal DA and its metabolites DOPAC and HVA are presented in Fig. 4.1. As shown in Fig. 4.1a, the striatal DA levels in the JV groups treated with pesticides on days 5-19 days were not significantly altered as compared to the corresponding controls receiving vehicle only. When animals were exposed to pesticides both as juveniles and re-exposed at adulthood (JV + adult), endosulfan, zineb alone and the combination of pesticides significantly decreased levels of DA in the striatum ($\rho \leq 0.05$) when compared to the corn oil control of the JV + adult group. Specifically, zineb alone decreased DA levels by 22%, endosulfan alone by 16%, and endosulfan + zineb by 35% when compared to the corresponding corn oil control of JV + adult mice (Fig. 4.1a). Furthermore, zineb and zineb + endosulfan-treated mice in the JV + adult exposed group showed significantly lower DA levels compared to those exhibited by the corresponding JV only groups ($\rho \leq 0.05$).

The levels of DOPAC in the striatum appeared to follow a pattern similar to DA levels, with a significant effect of treatments ($\rho \leq 0.05$). Thus, as shown in Fig. 4.1b, the striatal

DOPAC levels in the JV groups treated with pesticides on days 5-19 days were not significantly altered as compared to the corresponding controls receiving vehicle only. When animals were exposed to pesticides both as juvenile and adults (JV + adult), endosulfan, zineb alone and the combination of endosulfan + zineb significantly decreased the levels of DOPAC in the striatum ($\rho \leq 0.05$) when compared to corn oil control of JV + adult group. The levels of HVA, another DA metabolite, were not significantly altered in any of the treatment groups ($\rho \geq 0.05$) (Fig. 4.1c).

4.5.3. Cerebral Cortex Acetylcholinesterase:

We monitored the activity of AChE, the enzyme responsible for acetylcholine degradation, in cerebral cortex following pesticide exposure (Fig. 4.2). Similar to striatal DA and DOPAC in mice treated with pesticides on days 5-19 days (JV groups), the activities of AChE in cerebral cortex in the JV groups treated with pesticides on days 5-19 days were not significantly altered as compared to the corresponding controls receiving vehicle only. When animals were exposed to pesticides both at juvenile and re-exposed at adulthood (JV + adult), endosulfan alone or the combination with zineb significantly increased cerebral cortex AChE activities ($\rho \leq 0.05$) when compared to the corn oil control of JV + adult group (Fig. 4.2). Exposure of mice to mixtures of pesticides in the JV + adult group resulted in a significant increase on AChE level (30%) as compared to the corresponding control ($\rho \leq 0.05$).

4.5.4. Alpha-synuclein:

Alpha-synuclein, a hallmark of neurodegenerative diseases, was detected and quantified by Western blotting. Representative Western blots of alpha-synuclein are presented in Fig. 4.3. For all treatment protocols, exposure of mice to endosulfan and zineb alone or in the combination resulted in two protein bands, ~ 17 kDa and ~25 kDa. Normal alpha-synuclein detected as a ~17 kDa band is clearly visible in all groups including the control (Fig. 4.3). Aggregates of alpha-synuclein in these studies migrated at an apparent molecular mass around 25 kDa. As shown in Fig. 4.3b and Fig. 4.3c, neither endosulfan nor zineb alone significantly alter the intensity of either the 17 kDa or 25 kDa alpha-synuclein bands in the JV and JV + adult groups. However, exposure of mice to mixtures

of pesticides both as juvenile and again at adulthood (JV + adult) resulted in a significantly increased intensity of both the normal band (Fig. 4.3b) and the aggregated band (Fig. 4.3c) of the protein when compared to corresponding corn oil controls and compared to corresponding mixture treatment at JV only.

4.6. Discussion:

Although the etiology of idiopathic Parkinson's disease is not known, several studies suggest that environmental factors, particularly pesticide exposure, may play an important role in the pathogenesis of this disorder (Semchuk *et al.*, 1992; Butterfield *et al.*, 1993; Gorell *et al.*, 1998). This may be especially true if exposures are to the developing nervous system. It is known that some neuronal connections are plastic only transiently during development, indicating that this may be a critical period for normal brain development (Cook, 1991). Disturbances during this period may lead to long-lasting consequences for functional brain development (Kalil, 1989; Lipton and Kater, 1989; Shatz, 1990). For example, the developing mammalian brain has been reported to be more susceptible than the adult to acute high doses of organophosphate pesticides (Benke and Murphy, 1975; Harbison, 1975; Mendoza, 1976) and there are reports that exposure of neonatal animals can be detrimental later in life (Carr *et al.*, 2001).

The present study indicated that exposure to the pesticides zineb and endosulfan as neonates and re-exposure as adults could result in neurochemical changes that did not occur when the exposure was as neonates only. In this study, as reported in the earlier work with maneb (Thiruchelvam *et al.*, 2002), we demonstrated that juvenile mice exposed only to endosulfan and re-exposed to the same chemical at adulthood (JV + adult group) had little change in striatal DA levels compared to animals treated as juveniles only. However, a marked change in striatal DA levels occurred when mice were exposed to zineb or mixtures with endosulfan as both juvenile and adults. This is interesting in light of the recent work by Thiruchelvam (2002) which showed that similar changes in DA level occurred when mice were exposed to maneb and paraquat. In addition, present studies showed that levels of one of the main DA metabolites, DOPAC, were not

significantly altered when animals were exposed as juveniles only as compared to controls. However, there was a significant decrease in DOPAC levels, parallel to those seen in DA levels, in JV + adult groups given endosulfan and zineb when compared to vehicle treated controls. The biochemical mechanisms underlying or preceding these changes are unknown. One can hypothesize that the cells were undergoing degeneration at these concentration of pesticides. However, because we did not observe any gross behavioral or motor changes, this may not be the most likely reason for the above observation. Because there was no significant change observed in HVA levels with any treatment, and there was decrease in both the DA and DOPAC levels by the treatments, augmentation of DA degradation by MAO (monoamine oxidase) and COMT (catechol-O-methyltransferase) were unlikely to contribute to the difference. Other investigators have also shown decreased DA levels in the striatum following pesticide exposures. Thus, Thiruchelvam et al (2002) found decreased levels of striatal DA after developmental exposure to maneb (MB) and paraquat (PQ) in C57BL/6 mice. They also noted significant reduction of DA following adult re-challenge (Thiruchelvam *et al.*, 2002). In contrast, our studies showed no significant change in striatal DA levels in mice as adults after they were exposed to endosulfan, zineb or their combination only as juveniles.

It has been suggested that neonatal exposure to certain other pesticides such as the chlorinated hydrocarbons DDT and the pyrethroid deltamethrin during brain growth affects neuro-electrical activity and alters transmitter concentration at the synapse (Eriksson *et al.*, 1990; Eriksson and Nordberg, 1990; Eriksson *et al.*, 1992). Postnatal exposure to other chlorinated hydrocarbons such as lindane and heptachlor also has been reported to produce changes in motor activity and other behavioral endpoints (Rivera *et al.*, 1990; Rivera *et al.*, 1998; Moser *et al.*, 2001). DA levels were shown to decrease at 10 and 25 days, after dosing rat pups orally with 6 mg/kg of endosulfan, to 42 and 45% of control, respectively (Lakshmana and Raju, 1994). Although behavioral endpoints were not assessed in the present study, our data show that JV groups treated with pesticides on days 5-19 days had little change in the levels of DA and its metabolites, DOPAC and HVA, in the corpora striatum at adulthood. One likely factor in this age-related sensitivity is the difference in the dose of pesticide used. Our studies were

intentionally designed to use low concentrations for exposure as it is more realistic for most organisms to be exposed to pesticides at this level.

Acetylcholine, which is hydrolyzed by AChE, is the major neurotransmitter affected in Alzheimer's disease. Current treatment of Alzheimer's disease involves use of AChE inhibitors (Savolainen and Hervonen, 1985). Previous studies demonstrated that ethylenebisdithiocarbamate fungicides (such as zineb) produced significant decreases in AChE activity in rats (Savolainen and Hervonen, 1985; Kackar *et al.*, 1999). In contrast to the present study, exposure of rats to a single intraperitoneal injection of endosulfan has been shown to decrease brain AChE activity (Gupta, 1976). Other pesticide-induced effects on the cholinergic system of exposed neonates have also been reported. For example, DDT and the pyrethroids bioallethrin and deltamethrin affect muscarinic cholinergic receptors in the neonatal mouse brain, leading to permanent changes in the cholinergic system and behavior in adult animals (Marchi *et al.*, 1983; Mattson *et al.*, 1988; Ahlbom *et al.*, 1994). In our study, mice exposed to a mixture of pesticides both as juveniles and again as adults had an increase in AChE and a decrease in DA levels, suggesting that a decrease in DA levels by pesticide exposure may, in part, be partially compensated by increased AChE activity. It has been proposed that DA controls the neurotransmission of acetylcholine in a reciprocally symmetric manner through stimulatory D1 and inhibitory D2 receptors (Di Chiara et al 1994). The stimulation of D1 receptor increases acetylcholine release while the stimulation of D2 decreases acetylcholine. Examination of DA receptors (D1 and D2) and muscarinic cholinergic receptors might help in understanding possible effect on the control of acetylcholine release in the striatum and cortex following pesticides exposure.

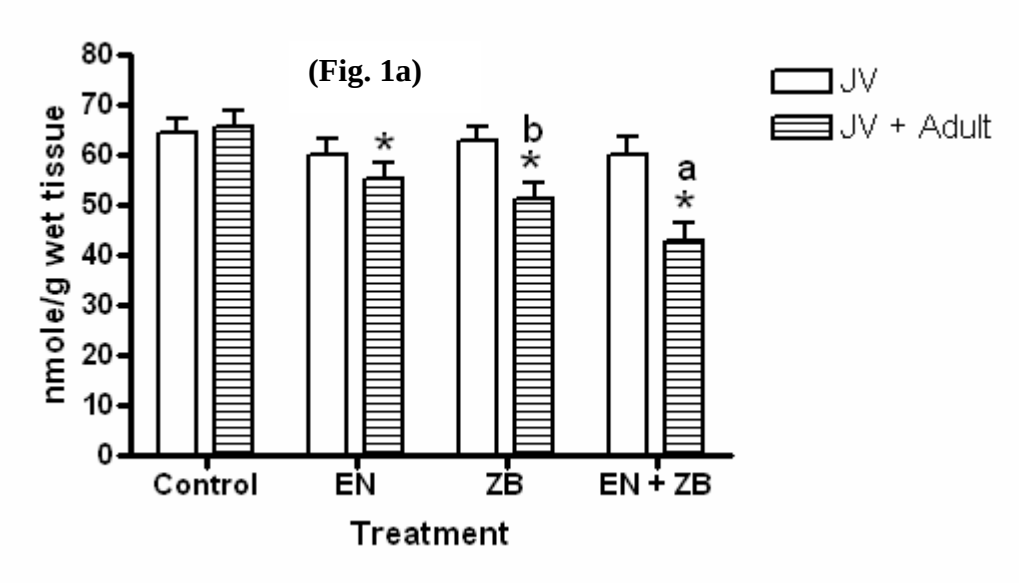
The present study also demonstrated an increase in expression of alpha-synulcein in pesticide-exposed animals. Accumulation of alpha-synuclein in Lewy bodies and neurites is a pathological hallmark of Parkinson's disease. Abnormal aggregation and accumulation of alpha-synuclein are associated with plaque formation in Alzheimer's disease and Lewy body formation in Lewy body dementias (LBD) (Hashimoto and Masliah, 1999; Campbell *et al.*, 2001; el-Agnaf and Irvine, 2002; Fasano *et al.*, 2003;

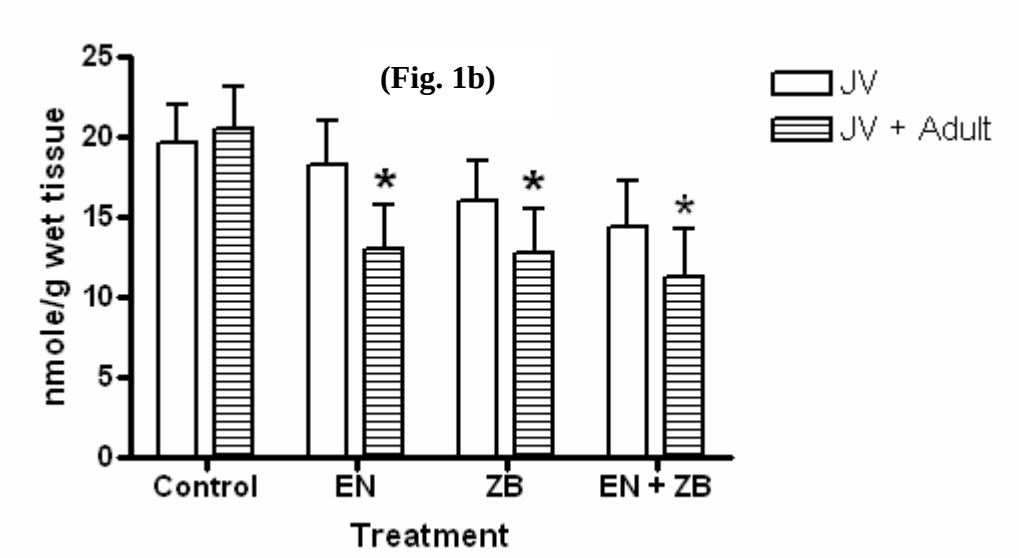
Surgucheva *et al.*, 2005). In the present study, mice treated with pesticides both as JV and as JV + adults showed a distinct protein band on Western blots at ~25 kDa in addition to the normal alpha-synuclein (~17 kDa). This indicates that aggregation of this protein occurs in mice exposed to the pesticides. Furthermore, the net intensities of both the aggregated (~25 kDa) and the non-aggregated (~17 kDa) proportion of the alpha-synuclein in Western blots were significantly increased in the mice treated with a pesticide mixture as JV + adult compared to JV. However, mice dosed as juveniles only did not appear to be affected when compared with controls indicating that a change in neurotransmitters can only be unmasked by later challenge. The role of alpha-synuclein in mitigating neuropathy leading to Dopaminergic neuronal death remains undetermined. It has been suggested that oxidative stress and free radical generation play a major role in causing the destruction of Dopaminergic neurons via mutation or overexpression of alpha-synuclein (Lotharius and Brundin, 2002). Alpha-synuclein could modulate intracellular DA handling through interactions with proteins that regulate DA synthesis and uptake (Lee *et al.*, 2001; Perez *et al.*, 2002). The impaired DA neurotransmitter storage arising from mutations in alpha-synuclein could lead to reduce vesicular storage of DA and increased DA release into synapse. The breakdown of this labile DA neurotransmitter in the cytoplasm could, in turn, promote oxidative stress and metabolic dysfunction, which in turn could lead to the death of DA containing neurons. Because oxidative stress has been implicated in both chemical metabolism and apoptosis, measuring these endpoints may offer insight to the oxidative status of cells undergoing apoptosis (Buttke and Sandstrom, 1994; Slater *et al.*, 1995). Studies are in progress in our laboratory to better understand the relationship among the above factors. Recent studies indicated that one of the normal functions of alpha-synulcein is to modulate DA transporter function, regulating the synaptic tone of DA (Sidhu *et al.*, 2004). The results of the present studies indicate that increased net intensity and aggregation of alpha-synuclein is associated with the decreased levels of DA and DOPAC. The changes of alpha-synuclein content and its structure could correlate with changes in intracellular and extracellular DA content, which could ultimately contribute to neurodegeneation as seen in PD patients.

In summary, mice exposed to zineb and endosulfan as juveniles and re-exposed at 8 months had a significant depletion of striatal DA, its metabolite DOPAC and increased AChE activity in the cerebral cortex. Treatment with both zineb + endosulfan in the JV + adult group also resulted in significantly decreased DA and DOPAC and increased intensity of both the normal band and the aggregated band of alpha-synuclein. However, mice exposed only as juveniles had little change in striatal DA, AChE and alpha-synuclein levels. Taken together, these findings support our hypothesis that exposure to pesticides such as endosulfan and zineb during critical periods of postnatal development could contribute to neurotransmitters changes when re-challenged later in life. This study could make a potential contribution toward the identification of environmental toxicants as risk factors in the etiology of neurodegenerative diseases. The lack of data on chemical mixtures and the neurotoxicity make this study unique.

4.7. Acknowledgements:

The authors thank Dr. Marion Ehrich for reviewing the manuscript. We also gratefully acknowledge the technical assistance of Geraldine Magnin and Barbara Carson Wise for HPLC use and Dan Ward for statistical analysis. This work was supported, in part, by the Edward Via Virginia College of Osteopathic Medicine.


(Fig. 1a)
nmole/g wet tissue
80
70
60
50
40
30
20
10
0
Control
EN
ZB
EN + ZB
Treatment
JV
JV + Adult
*
b
*
a
*


(Fig. 1b)
nmole/g wet tissue
25
20
15
10
5
0
Control
EN
ZB
EN + ZB
Treatment
JV
JV + Adult
*
*
*

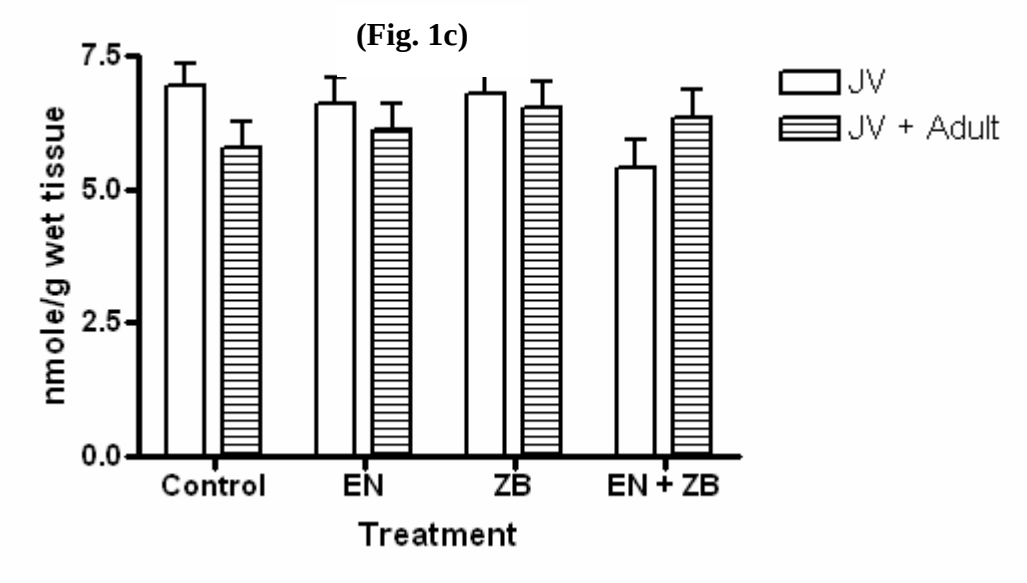


Figure 4.1: Levels of dopamine, DOPAC and HVA in the striatum of C57BL/6 mice after exposure to the pesticides, zineb (ZB), endosulfan (EN) and their combination (EN + ZB). Exposure was carried out from postnatal days 5-19, with adult re-challenge occurring at 8 months of age as described in Methods. EN = animal administered with 0.155 mg/kg endosulfan as juveniles (JV) and 1.55 mg/kg as adults (JV + Adult); ZB = animals administered 0.5 mg/kg zineb as JV and 5 mg/kg at adulthood. Tukey's HSD post hoc test for each treatment indicated differences ($\rho \leq 0.05$): * compared to control JV+ adults; a: compared to JV in ZB + EN mg/kg group. b: compared to JV in ZB group. Mixtures did not cause significant decreases compared to individual pesticide exposures. The results are reported as mean nmole/g wet tissue ± S.E., n = 8.

(Fig. 4.1a) = dopamine levels
(Fig. 4.1b) = DOPAC levels
(Fig. 4.1c) = HVA levels

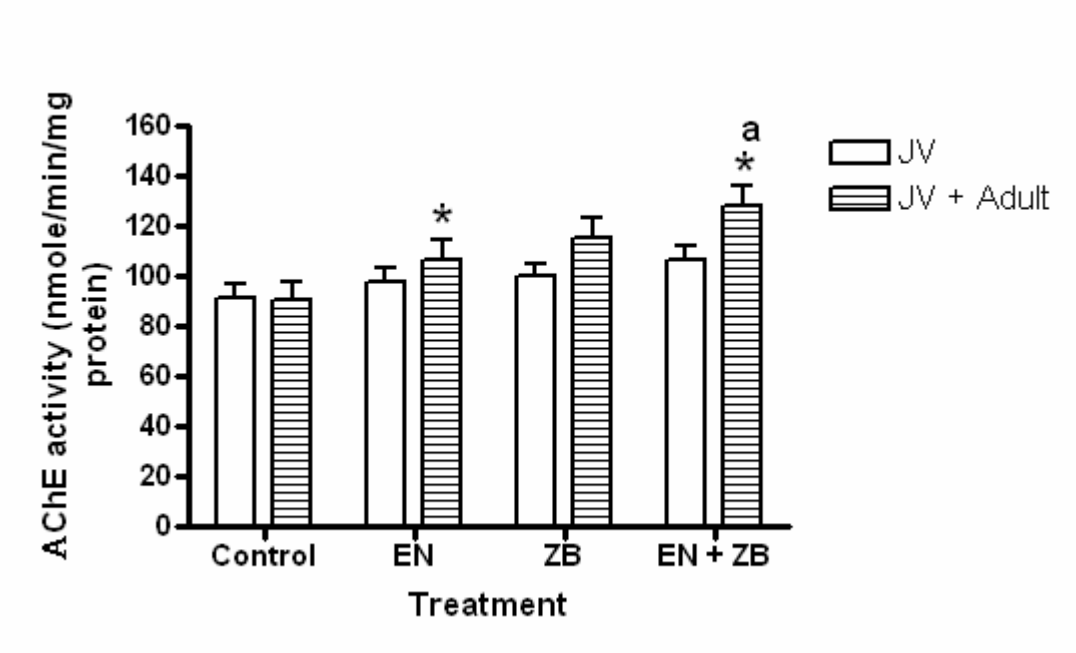
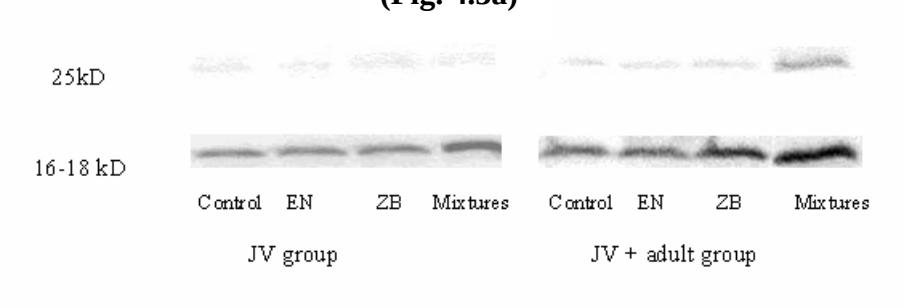


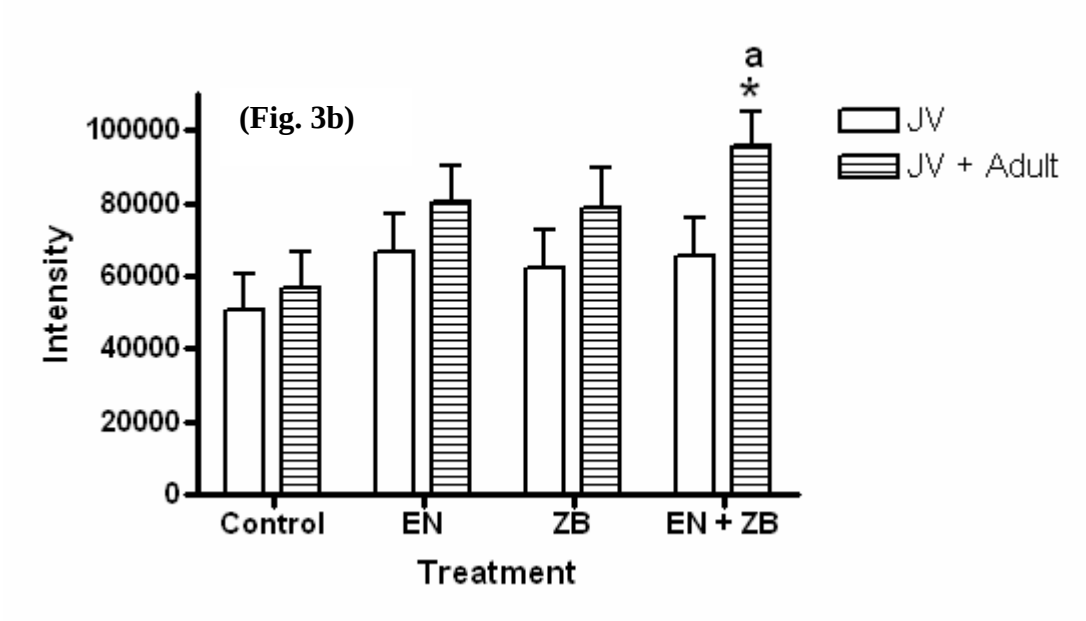
Figure 4.2: AChE activities in mouse cerebral cortex after exposure to the pesticides ZB and EN and in their combination.

* indicates significant differences compared to JV + adult controls; a: significant differences compared to the JV ZB group. The mixtures did not cause significant increase compared to individual pesticide exposures. The results are reported as mean nmole/min/mg protein ± S.E., n = 8.

(Fig. 4.3a)



(Fig. 3b)



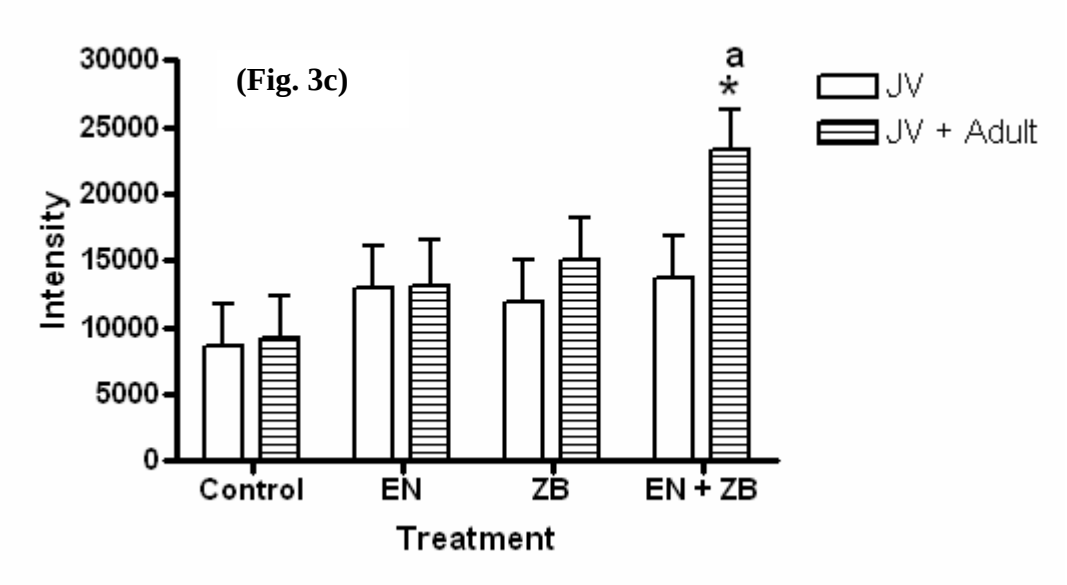


Figure 4.3. Effects of EN, ZB and their combination on densitometric analysis of antibody-labeled alpha-synuclein in western blots of cerebral cortex tissue taken from C57BL/6 mouse brain.

Experimental conditions are described in legend to Figure 4.1. The conditions for the alpha-synuclein assay are described in Materials and Methods. Turkey's HSD post hoc test for each treatment showed differences ($\rho \leq 0.05$): * significant differences compared to (JV + adult) controls; a: significant differences compared to JV in ZB + EN groups. The mixture did not cause significant increase compared to individual pesticide exposure, n = 8.

(Fig. 4.3a) = a representative western blot of cortical alpha-synuclein

(Fig. 4.3b) = the intensity of alpha-synuclein in (~17 kDa) band

(Fig. 4.3c) = the intensity of alpha-synuclein aggregation band (~25 kDa)

4.8. Literature Cited

Agrawal, A. K., Anand, M., Zaidi, N. F., and Seth, P. K. (1983). Involvement of serotonergic receptors in endosulfan neurotoxicity. *Biochem Pharmacol* **32**, 3591-3593.

Ahlbom, J., Fredriksson, A., and Eriksson, P. (1994). Neonatal exposure to a type-I pyrethroid (bioallethrin) induces dose-response changes in brain muscarinic receptors and behaviour in neonatal and adult mice. *Brain Res* **645**, 318-324.

Aleksandrowicz, D. R. (1979). Endosulfan poisoning and chronic brain syndrome. *Arch Toxicol* **43**, 65-68.

Anand, M., Agrawal, A. K., Gopal, K., Sur, R. N., and Seth, P. K. (1986). Endosulfan and cholinergic (muscarinic) transmission: effect on electroencephalograms and [3H]quinuclidinyl benzilate in pigeon brain. *Environ Res* **40**, 421-426.

Andersen, J. K. (2003). Paraquat and iron exposure as possible synergistic environmental risk factors in Parkinson's disease. *Neurotox Res* **5**, 307-313.

Benke, G. M., and Murphy, S. D. (1975). The influence of age on the toxicity and metabolism of methyl parathion and parathion in male and female rats. *Toxicol Appl Pharmacol* **31**, 254-269.

Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., and Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* **3**, 1301-1306.

Butterfield, P. G., Valanis, B. G., Spencer, P. S., Lindeman, C. A., and Nutt, J. G. (1993). Environmental antecedents of young-onset Parkinson's disease. *Neurology* **43**, 1150-1158.

Buttke, T. M., and Sandstrom, P. A. (1994). Oxidative stress as a mediator of apoptosis. *Immunol Today* **15**, 7-10.

Campbell, B. C., McLean, C. A., Culvenor, J. G., Gai, W. P., Blumbergs, P. C., Jakala, P., Beyreuther, K., Masters, C. L., and Li, Q. X. (2001). The solubility of alpha-synuclein in multiple system atrophy differs from that of dementia with Lewy bodies and Parkinson's disease. *J Neurochem* **76**, 87-96.

Carr, R. L., Chambers, H. W., Guarisco, J. A., Richardson, J. R., Tang, J., and Chambers, J. E. (2001). Effects of repeated oral postnatal exposure to chlorpyrifos on open-field behavior in juvenile rats. *Toxicol Sci* **59**, 260-267.

Chiba, K., Trevor, A., and Castagnoli, N., Jr. (1984). Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. *Biochem Biophys Res Commun* **120**, 574-578.

Cook, J. E. (1991). Correlated activity in the CNS: a role on every timescale? *Trends Neurosci* **14**, 397-401.

Correll, L., and Ehrich, M. (1991). A microassay method for neurotoxic esterase determinations. *Fundam Appl Toxicol* **16**, 110-116.

Cory-Slechta, D. A., Thiruchelvam, M., Richfield, E. K., Barlow, B. K., and Brooks, A. I. (2005). Developmental pesticide exposures and the Parkinson's disease phenotype. *Birth Defects Res A Clin Mol Teratol* **73**, 136-139.

el-Agnaf, O. M., and Irvine, G. B. (2002). Aggregation and neurotoxicity of alpha-synuclein and related peptides. *Biochem Soc Trans* **30**, 559-565.

Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Feather-Stone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**, 88-95.

Eriksson, P., Ahlbom, J., and Fredriksson, A. (1992). Exposure to DDT during a defined period in neonatal life induces permanent changes in brain muscarinic receptors and behaviour in adult mice. *Brain Res* **582**, 277-281.

Eriksson, P., Nilsson-Hakansson, L., Nordberg, A., Aspberg, A., and Fredriksson, A. (1990). Neonatal exposure to DDT and its fatty acid conjugate: effects on cholinergic and behavioural variables in the adult mouse. *Neurotoxicology* **11**, 345-354.

Eriksson, P., and Nordberg, A. (1990). Effects of two pyrethroids, bioallethrin and deltamethrin, on subpopulations of muscarinic and nicotinic receptors in the neonatal mouse brain. *Toxicol Appl Pharmacol* **102**, 456-463.

Fasano, M., Giraudo, S., Coha, S., Bergamasco, B., and Lopiano, L. (2003). Residual substantia nigra neuromelanin in Parkinson's disease is cross-linked to alpha-synuclein. *Neurochem Int* **42**, 603-606.

Ferraz, H. B., Bertolucci, P. H., Pereira, J. S., Lima, J. G., and Andrade, L. A. (1988). Chronic exposure to the fungicide maneb may produce symptoms and signs of CNS manganese intoxication. *Neurology* **38**, 550-553.

Giorgi, O., De Montis, G., Porceddu, M. L., Mele, S., Calderini, G., Toffano, G., and Biggio, G. (1987). Developmental and age-related changes in D1-dopamine receptors and dopamine content in the rat striatum. *Brain Res* **432**, 283-290.

Gorell, J. M., Johnson, C. C., Rybicki, B. A., Peterson, E. L., and Richardson, R. J. (1998). The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. *Neurology* **50**, 1346-1350.

Gupta, P. K. (1976). Endosulfan-induced neurotoxicity in rats and mice. *Bull Environ Contam Toxicol* **15**, 708-713.

Harbison, R. D. (1975). Comparative toxicity of some selected pesticides in neonatal and adult rats. *Toxicol Appl Pharmacol* **32**, 443-446.

Hardy, J. (2003). The relationship between Lewy body disease, Parkinson's disease, and Alzheimer's disease. *Ann N Y Acad Sci* **991**, 167-170.

Hashimoto, M., and Masliah, E. (1999). Alpha-synuclein in Lewy body disease and Alzheimer's disease. *Brain Pathol* **9**, 707-720.

Hedera, P., Lerner, A. J., Castellani, R., and Friedland, R. P. (1995). Concurrence of Alzheimer's disease, Parkinson's disease, diffuse Lewy body disease, and amyotrophic lateral sclerosis. *J Neurol Sci* **128**, 219-224.

Hodgson, E., and Levi, P. E. (1996). Pesticides: an important but underused model for the environmental health sciences. *Environ Health Perspect* **104 Suppl 1**, 97-106.

IOM (2003). In *Institute of Medicine (IOM), Gulf War and Health: Volume 2, Insecticides and Solvents* pp. 412-418. The National Academics Press, Washington, DC.

Iyaniwura, T. T. (1990). Mammalian toxicity and combined exposure to pesticides. *Vet Hum Toxicol* **32**, 58-62.

Kackar, R., Srivastava, M. K., and Raizada, R. B. (1999). Assessment of toxicological effects of mancozeb in male rats after chronic exposure. *Indian J Exp Biol* **37**, 553-559.

Kalil, R. E. (1989). Synapse formation in the developing brain. *Sci Am* **261**, 76-79, 82-75.

Konno, N. (2003). [Neurotoxicity of organophosphorus and dithiocarbamate compounds]. *Nippon Eiseigaku Zasshi* **57**, 645-654.

Lakshmana, M. K., and Raju, T. R. (1994). Endosulfan induces small but significant changes in the levels of noradrenaline, dopamine and serotonin in the developing rat brain and deficits in the operant learning performance. *Toxicology* **91**, 139-150.

Langston, J. W., Ballard, P., Tetrud, J. W., and Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**, 979-980.

Lee, F. J., Liu, F., Pristupa, Z. B., and Niznik, H. B. (2001). Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. *Faseb J* **15**, 916-926.

Lipton, S. A., and Kater, S. B. (1989). Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *Trends Neurosci* **12**, 265-270.

Lotharius, J., and Brundin, P. (2002). Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. *Nat Rev Neurosci* **3**, 932-942.

Manning-Bog, A. B., McCormack, A. L., Li, J., Uversky, V. N., Fink, A. L., and Di Monte, D. A. (2002). The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. *J Biol Chem* **277**, 1641-1644.

Marchi, M., Caviglia, A., Paudice, P., and Raiteri, M. (1983). Calcium-dependent [3H]acetylcholine release and muscarinic autoreceptors in rat cortical synaptosomes during development. *Neurochem Res* **8**, 621-628.

Mattson, M. P., Dou, P., and Kater, S. B. (1988). Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *J Neurosci* **8**, 2087-2100.

McGrew, D. M., Irwin, I., and Langston, J. W. (2000). Ethylenebisdithiocarbamate enhances MPTP-induced striatal dopamine depletion in mice. *Neurotoxicology* **21**, 309-312.

Mendoza, C. E. (1976). Toxicity and effects of malathion on esterases of suckling albino rats. *Toxicol Appl Pharmacol* **35**, 229-238.

Moser, V. C., Shafer, T. J., Ward, T. R., Meacham, C. A., Harris, M. W., and Chapin, R. E. (2001). Neurotoxicological outcomes of perinatal heptachlor exposure in the rat. *Toxicol Sci* **60**, 315-326.

Nakai, K., and Satoh, H. (2002). Developmental neurotoxicity following prenatal exposures to methylmercury and PCBs in humans from epidemiological studies. *Tohoku J Exp Med* **196**, 89-98.

Perez, R. G., Waymire, J. C., Lin, E., Liu, J. J., Guo, F., and Zigmond, M. J. (2002). A role for alpha-synuclein in the regulation of dopamine biosynthesis. *J Neurosci* **22**, 3090-3099.

Rajput, A. H., and Uitti, R. J. (1987). Paraquat and Parkinson's disease. *Neurology* **37**, 1820-1821.

Ritz, B., Yu, F., Chapa, G., and Fruin, S. (2000). Effect of air pollution on preterm birth among children born in Southern California between 1989 and 1993. *Epidemiology* **11**, 502-511.

Rivera, S., Rosa, R., Martinez, E., Sunol, C., Serrano, M. T., Vendrell, M., Rodriguez-Farre, E., and Sanfeliu, C. (1998). Behavioral and monoaminergic changes after lindane exposure in developing rats. *Neurotoxicol Teratol* **20**, 155-160.

Rivera, S., Sanfeliu, C., and Rodriguez-Farre, E. (1990). Behavioral changes induced in developing rats by an early postnatal exposure to lindane. *Neurotoxicol Teratol* **12**, 591-595.

Sanchez-Ramos, J. R., Hefti, F., and Weiner, W. J. (1987). Paraquat and Parkinson's disease. *Neurology* **37**, 728.

Savolainen, K., and Hervonen, H. (1985). Dithiocarbamate fungicides decrease histochemical reactivity of cholinesterases in the gut wall of the rat. *Arch Toxicol Suppl* **8**, 272-276.

Seaton, T. A., Cooper, J. M., and Schapira, A. H. (1997). Free radical scavengers protect dopaminergic cell lines from apoptosis induced by complex I inhibitors. *Brain Res* **777**, 110-118.

Semchuk, K. M., Love, E. J., and Lee, R. G. (1992). Parkinson's disease and exposure to agricultural work and pesticide chemicals. *Neurology* **42**, 1328-1335.

Seth, P. K., Saidi, N. F., Agrawal, A. K., and Anand, M. (1986). Neurotoxicity of endosulfan in young and adult rats. *Neurotoxicology* **7**, 623-635.

Shafer, T. J., Meyer, D. A., and Crofton, K. M. (2005). Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. *Environ Health Perspect* **113**, 123-136.

Shatz, C. J. (1990). Impulse activity and the patterning of connections during CNS development. *Neuron* **5**, 745-756.

Shin, E. J., Jhoo, J. H., Kim, W. K., Jhoo, W. K., Lee, C., Jung, B. D., and Kim, H. C. (2004). Protection against kainate neurotoxicity by pyrrolidine dithiocarbamate. *Clin Exp Pharmacol Physiol* **31**, 320-326.

Sidhu, A., Wersinger, C., and Vernier, P. (2004). alpha-Synuclein regulation of the dopaminergic transporter: a possible role in the pathogenesis of Parkinson's disease. *FEBS Lett* **565**, 1-5.

Simmons, J. E. (1995). Chemical mixtures: challenge for toxicology and risk assessment. *Toxicology* **105**, 111-119.

Slater, A. F., Nobel, C. S., and Orrenius, S. (1995). The role of intracellular oxidants in apoptosis. *Biochim Biophys Acta* **1271**, 59-62.

Surgucheva, I., Park, B. C., Yue, B. Y., Tomarev, S., and Surguchov, A. (2005). Interaction of myocilin with gamma-synuclein affects its secretion and aggregation. *Cell Mol Neurobiol* **25**, 1009-1033.

Takahashi, R. N., Rogerio, R., and Zanin, M. (1989). Maneb enhances MPTP neurotoxicity in mice. *Res Commun Chem Pathol Pharmacol* **66**, 167-170.

Thiruchelvam, M., Brockel, B. J., Richfield, E. K., Baggs, R. B., and Cory-Slechta, D. A. (2000). Potentiated and preferential effects of combined paraquat and maneb on nigrostriatal dopamine systems: environmental risk factors for Parkinson's disease? *Brain Res* **873**, 225-234.

Thiruchelvam, M., McCormack, A., Richfield, E. K., Baggs, R. B., Tank, A. W., Di Monte, D. A., and Cory-Slechta, D. A. (2003). Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. *Eur J Neurosci* **18**, 589-600.

Thiruchelvam, M., Richfield, E. K., Goodman, B. M., Baggs, R. B., and Cory-Slechta, D. A. (2002). Developmental exposure to the pesticides paraquat and maneb and the Parkinson's disease phenotype. *Neurotoxicology* **23**, 621-633.

Tilson, H. A. (1998). Developmental neurotoxicology of endocrine disruptors and pesticides: identification of information gaps and research needs. *Environ Health Perspect* **106 Suppl 3**, 807-811.

Tilson, H. A. (2000). Neurotoxicology risk assessment guidelines: developmental neurotoxicology. *Neurotoxicology* **21**, 189-194.

Tolwani, R. J., Jakowec, M. W., Petzinger, G. M., Green, S., and Waggie, K. (1999). Experimental models of Parkinson's disease: insights from many models. *Lab Anim Sci* **49**, 363-371.

Voorn, P., Kalsbeek, A., Jorritsma-Byham, B., and Groenewegen, H. J. (1988). The pre- and postnatal development of the dopaminergic cell groups in the ventral mesencephalon and the dopaminergic innervation of the striatum of the rat. *Neuroscience* **25**, 857-887.

Zaidi, N. F., Agrawal, A. K., Anand, M., and Seth, P. K. (1985). Neonatal endosulfan neurotoxicity: behavioral and biochemical changes in rat pups. *Neurobehav Toxicol Teratol* **7**, 439-442.

Zang, L. Y., and Misra, H. P. (1993). Generation of reactive oxygen species during the monoamine oxidase-catalyzed oxidation of the neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Biol Chem* **268**, 16504-16512.

Zhang, J., Fitsanakis, V. A., Gu, G., Jing, D., Ao, M., Amarnath, V., and Montine, T. J. (2003). Manganese ethylene-bis-dithiocarbamate and selective dopaminergic neurodegeneration in rat: a link through mitochondrial dysfunction. *J Neurochem* **84**, 336-346.

CHAPTER 5 Exposure to Mixtures of endosulfan and Zineb Induces Apoptotic and necrotic Cell Death in SH-SY5Y Neuronal Cells, *In Vitro*:

Authors: Zhen-Quan Jia[1], and Hara P. Misra* [1, 2].

Affiliations: [1] Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.

[2] Edward Via Virginia College of Osteopathic Medicine, Virginia Tech Corporate Research Center, 2265 Kraft Drive, Blacksburg, VA 24060, U.S.A.

Corresponding Author: * Hara P. Misra
Edward Via Virginia College of Osteopathic Medicine
Virginia Tech Corporate Research Center, 2265 Kraft Drive,
Blacksburg, VA 24060, U.S.A.
Phone: (540) 231-3693
Fax: (540) 231-5252
misra@vt.edu

Keywords: Pesticides, Neuronal cell, endosulfan, zineb, cytotoxicity, apoptosis, necrosis, neurodegeneration.

5.1. Abstract:

A number of epidemiological studies have demonstrated a strong association between the incidence of Parkinson's disease and pesticide exposure. We and others have demonstrated that exposure to the pesticides endosulfan and zineb, alone and in combination, cause neurodegeneration *in vivo*. We hypothesized that these pesticides cause neurotoxicity, in part, by enhancing apoptotic cell death. SH-SY5Y human neuroblastoma cells, which retain a catecholaminergic phenotype, were exposed to endosulfan, zineb or a combination of these chemicals, *in vitro*. Cytotoxicity was evaluated using lactate dehydrogenase release. A dose-dependent response was obtained by exposing to individual pesticides. For mixture studies, concentrations of pesticides (100 μM each) were chosen based on LC_{25} (lethal concentration) that would result in minimum cell death. Exposure to mixture of pesticides (endosulfan 100 μM + zineb 100 μM) exhibited significantly ($p \leq 0.05$) higher toxicity, more than additive effect, in this assay. Both pesticides were found to cause apoptotic cell death that was concentration (50-400 μM) dependent. A flow cytometric (7-aminoactinomycin D) assay was used to distinguish live, early apoptotic and late apoptotic/necrotic populations. Exposure to mixtures of the pesticides enhanced both the early apoptosis and late apoptosis/necrosis compared to either chemical alone. Visual evaluation using DNA ladder assay and fluorescence Annexin V/PI assay confirmed the contribution of both apoptotic and necrotic processes. These findings suggest that the cytotoxicity of endosulfan and zineb, both individually and in mixtures, is associated with the occurrence of early and late apoptotic/necrotic processes in SH-SY5Y human neuroblastoma cells and support our contention that the pesticide-induced neuronal cell death leading to neurodegenerative disease may, at least in part, be associated with early and late apoptosis of dopaminergic neurons. Understanding the mechanism of action of pesticides and their combination could make a potential contribution for risk assessment in the etiology of neurodegenerative disease.

5.2. Introduction:

Numerous studies have suggested exposure to pesticide has a negative impact on public health (Rhile *et al.*, 1996; Hampton and Orrenius, 1998; Tebourbi *et al.*, 1998; Thiruchelvam *et al.*, 2000b; Camacho *et al.*, 2001; Thiruchelvam *et al.*, 2002; Thiruchelvam *et al.*, 2004). Each year about 5 billion pounds of pesticides are used worldwide (Smith, 2001). An estimated 85-90% of pesticides used in agriculture do not reach their target organisms but contaminate the air, water and soil, potentially predisposing humans and animals to various toxic agents. (Repetto and Baliga, 1996). Chronic exposure to certain pesticides has been linked to neurodegenerative disease such as Parkinson's and Alzheimer's Diseases (Manning-Bog *et al.*, 2002; Thiruchelvam *et al.*, 2002). In a proportional mortality study, an increased Parkinson's disease and mortality was observed in rural California with the use of high levels of agricultural pesticides (Ritz et al., 2000). Nigrostriatal neurodegeneration via depletion of striatal dopamine production using the chemical 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) is an accepted experimental model for Parkinson's-like syndrome in animals and has been linked to Parkinson's disease in man. (Langston *et al.*, 1983; Tolwani *et al.*, 1999).

Endosulfan is a chlorinated hydrocarbon cyclodiene insecticide used primarily on a wide variety of food crops including tea, coffee, fruits, and vegetables, as well as on rice, cereals, maize, sorghum, or other grains. In humans with endosulfan poisoning, overstimulation of the central nervous system is the major characteristic (Aleksandrowicz, 1979; Chugh *et al.*, 1998). Cyclodiene pesticides antagonize the action of the neurotransmitter gamma-aminobutyric acid (GABA), which induces the uptake of chloride ions by neurons. The blockage of this activity by cyclodiene insecticides results in only partial repolarization of the neuron and a state of uncontrolled excitation. The dithiocarbamate fungicides, such as zineb, maneb, mancozeb, metram, ziram and thiram, are known to possess potent dopaminergic activity and enhance the toxic effects of MPTP in mice (Takahashi *et al.*, 1989). The exposure of maneb to mice produced selective nigrostriatal dopamine (DA) system neurotoxicity, including loss of striatal DA

and loss of cell bodies of DA neurons in the substantia nigra pars compacta (Thiruchelvam *et al.*, 2002). Although adverse effects of exposure to both endosulfan and zineb have been reported (Gupta, 1976; Zaidi *et al.*, 1985; Seth *et al.*, 1986; Guven *et al.*, 1999; Vaccari *et al.*, 1999; Schmuck *et al.*, 2002), the biochemical mechanisms by which endosulfan and zineb cause neurotoxicity are still largely unknown. Our current studies were designed to assess the cytotoxicity of endosulfan and zineb, alone and in combination, as well as to investigate the characteristics of cell death (apoptosis and necrosis) in the SH-SY5Y cell line.

5.3. Materials and Method:

5.3.1. Cell culture and treatment:

Culture Medium: RPMI-1640 media with phenol red was used for culturing the cells. Supplemented media was prepared by adding 10% fetal bovine serum, 1% MEM non-essential amino acids, 1% penicillin/streptomycin, 1% L-glutamine and 1% HEPES. All chemicals and media were purchased from Sigma-Aldrich (St. Louis, MO). Human neuroblastoma SH-SY5Y cells (ATCC, Manassas, VA) were seeded in flasks (75 cm^2) and grown for 4-5 days until 90% confluence in a 95% air, 5% CO_2 humidified incubator at 37 ºC. Prior to confluence, cells were harvested using 0.25% trypsin Sigma-Aldrich (St. Louis, MO) and seeded into 96-well microtiter plates or 25 cm^2 flasks. The cells were then allowed to grow at 37°C and 5% CO_2 for 1-2 days prior to treatment.

Preparation of pesticides: Endosulfan and zineb were obtained from ChemService (West Chester, PA). Stock solutions of endosulfan and zineb (100 mM) were prepared using 100 % dimethylsulfoxide. These stock solutions were serially diluted with incomplete media, RPMI-1640, to prepare 4X-working solutions.

5.3.2. LDH Assay:

The release of lactate dehydrogenase (LDH) from cells, during the culturing in presence or absence of pesticides, was assessed by a non-radioactive protocol using the LDH cytotox kit (Cat. #1644 793: Boehringer-Mannheim GmbG, Biochemica). SH-SY5Y cells

were treated with 50, 100, 150, 200, 250, 300 and 400 μM of zineb or endosulfan separately in order to obtain a dose-dependent response for each pesticide exposure. After treatment, cells were centrifuged at 300 × g, 4°C, for 7 min. fifty μL/well of each cell-free supernatant was removed from samples and placed in flat-bottom of 96 well tissue culture plates (Costar, Corning, NY). Fifty μL of reaction mixture containing dye (idotetrazolium chloride and sodium lactate) was added to each well. The reaction was allowed to proceed for 30 minutes and then stopped by adding 50 μl of 1 N HCl. The development of pink color was read at 490 nm using a microplate reader (VERSAmax tunable, Molecular Devices Corp., and Sunnyvale, CA). The values of media-only background were subtracted from each well reading. The release of LDH from cells into the medium during 16 hours exposure to pesticides was calculated as the percent of total LDH activity that was present in an equivalent number of cells lysed with Triton X-100 with the following formula:

LDH release (%) = (pesticide exposed samples) / (Maximum release) × 100

5.3.3. 7-AAD staining:

7-AAD staining was used to differentiate alive, early apoptosis, late apoptosis and necrotic cells by measuring staining intensity via flow cytometry: 7-AAD^{dull}, live cells; 7-AAD^{intermediate}, apoptotic cells; and 7-AAD^{bright}, late apoptotic / necrotic cells as previously described (Olgun *et al.*, 2004). After treatments, cells were rinsed with PBS and centrifuged at 200 x g, for 10 minutes at 4°C. The media was removed and 100 μL of 10 μg/mL 7-AAD DNA binding dye (Molecular Probes, Eugene OR) in a supplemented buffer (0.15% sodium azide and 2% bovine serum albumin in PBS) was added to all wells. The plate was incubated for no more than 30 minutes on ice, in the dark. Cells were measured on the Beckman-Coulter Epics XL/MCL flow cytometer (Hialeah, FL). Apoptotic cells were identified and quantified based on the published method (Schmid *et al.*, 1994a; Schmid *et al.*, 1994b)

5.3.4. FITC-Annexin-V/propidium iodide Staining Assay:

Apoptosis and necrosis were assessed with the "ApoAlert" Annexin-V Apoptosis by flow cytometry and fluorescence microscopy according to the manufacturer's protocol (Annexin-V–FLUOS Staining Kit, Roche Diagnostics Co., Indianapolis, IN). Flow cytometric analysis was performed using a Coulter Epics XL/MXL flow cytometer (Hialeah, FL). Fluorescence was detected at excitation wavelength of 488 nm and emission wavelengths of 525 and 625 nm for the green fluorescence (FITC) and red fluorescence (PI), respectively.

For the fluorescence microscopy, cells were plated at a density of 2.5×10^4 cells/well onto glass cover-slips and exposed to pesticides as above. After treatment, cells were washed with PBS and then incubated with staining buffer containing 2 μL /ml PI and 2 μL /ml Annexin- V-FITC, for 10-15 min at room temperature.

5.3.5. DNA ladder Assay:

To assess apoptosis we examined DNA laddering using agarose and ethidium bromide gel electrophoresis. Briefly, after 10 and 16 hours of incubation with or without pesticides, the cells were detached by 0.25 g/L trypsin-0.1 g/L EDTA and washed once with PBS. The DNA was immediately extracted using Boehringer Mannheim's Apoptotic DNA Ladder Kit (Boehringer Mannheim, GmbH, Mannheim). The cells were then centrifuged at 200 × g, 4 °C, for 5 min and the pellet was resuspended in 200 μl cold PBS. Cells were lysed with binding buffer and DNA was separated from intact chromatin by centrifugation. Residual impurities were removed by a wash step and subsequently DNA was eluted in elution buffer. The samples were electrophoresed on 1% agarose gel. The DNA was detected with ethidium bromide and viewed under UV light.

5.4. Statistical Analysis:

Analysis of variance (ANOVA) was used with GLM procedure of the SAS system (Version 8.2, SAS Institute Inc., Cary, NC) for statistical analysis of the data. Further statistical analysis for post hoc comparisons was performed using the Tukey-Kramer test. All differences of $p \leq 0.05$ were considered as significant.

5.5. Results:

5.5.1. Effects of pesticides on LDH release:

Cellular toxicity of the pesticides endosulfan and zineb on SH-SY5Y cells were assessed by monitoring LDH release. SH-SY5Y cells were treated with 50, 100, 150, 200, 250, 300 and 400 μM zineb or endosulfan in order to obtain a dose-dependent response for each pesticide exposure. As shown in Figs 5.1 and 5.2, the release of LDH from SH-SY5Y cells treated with zineb or endosulfan at various concentrations for 16 hrs show a dose-dependent response to chemical treatment. Thus, as shown in these figures, there was an increased LDH release from cells as the concentration of zineb or endosulfan was increased. No significant differences were observed in LDH release when cells were treated with 50 μM of either endosulfan or zineb (Figs. 5.1 and 5.2).

Having established a concentration response curve for both zineb and endosulfan to trigger cell death, we next sought to determine if the two pesticides would have an additive, subtractive or synergistic effect on cytotoxicity. The doses with the minimum toxic effects (less than LC_{25}) were chosen for mixture studies. The minimal concentration of zineb or endosulfan to cause a significant release of LDH beyond control levels was 100 μM. Mixture study was performed with 100 μM endosulfan or 100 μM zineb alone and in combination to examine if there was an additive or synergistic effect. As shown in figure 5.3, the mixtures of pesticides caused significantly higher toxicity ($P \leq 0.05$) compared to individual pesticides. These data indicate that exposure to multiple pesticides simultaneously can cause more than additive effects damaging plasma membrane of neuronal cells.

5.5.2. Effects of pesticides on cell morphology:

The morphological characteristics of the cells were observed by phase-contrast microscopy (Fig. 5.4). The unstimulated controls showed a typical ellipsoid-shape homogenous morphology with neutrite extension, characteristics of neuronal cells (Fig. 5.4a). Cells treated with 100 μM of either endosulfan (Fig. 5.4b) or zineb (Fig. 5.4c) for

16 hours displayed significant changes in cell morphology including the loss of uniformity and detritus surrounding the cell clusters. The surviving cells were more circular in shape and neutrite extension was virtually lost. However, most cells remain attached to the culture flask. When the SH-SY5Y cells were treated with endosulfan and zineb (100 μM endosulfan and 100 μM zineb), the cells showed significant apoptotic features such as cell blebbing, cell shrinkage, and apoptotic body formation and exhibited decreased adhesion to the tissue flask. Some were found to be detached from the culture flask and floated into the growth medium after 16 hours of incubation with pesticide mixtures.

5.5.3. Effect of pesticides on 7-AAD staining assay:

To examine the mechanism of cell death caused from endosulfan and zineb exposure, the apoptotic and necrotic pathways of cell death were investigated by flow cytometric analysis in combination with 7-AAD staining (Figs. 5.5, 5.6 and 5.7). Figure 5.5 illustrates representative flow cytometric charts when cells were exposed to pesticides for 16 hours. As shown in this figure, exposure to endosulfan (100 μM) or zineb (100 μM) caused an increase in shift of cells towards both early apoptotic and later apoptotic/necrotic gates (Fig. 5.5b and c) compared to solvent control (Fig. 5.5a). There was an intense shift towards necrosis gate observed when cells were exposed to mixtures of pesticides (Fig. 5.5d). Figure 5.6 illustrates cells exposed to 100 μM endosulfan, zineb or mixtures for 16 hours had a significant increase in early apoptosis compared to solvent control ($p \leq 0.05$). Exposure to mixture of 100 μM each of endosulfan and zineb significantly ($p \leq 0.05$) increased the number of apoptotic cells compared to treatment of zineb at 100 μM. As shown in this figure, zineb or endosulfan at 100 μM each had no elicited remarkable changes in late apoptosis/necrosis compared to vehicle controls (Fig. 5.7). However, as shown in Figure 5.7, there was a dramatic increase in late apoptosis/necrosis when mixtures of pesticides were applied to the cells. The increase in numbers of late apoptotic/necrotic cells observed in mixture of pesticide treatment was significantly ($p \leq 0.01$) higher than the individual pesticide treatment and the effect was found to be more than additive.

5.5.4. Effect of Pesticides on FITC-Annexin-V/ Propidium Idodide Staining Assay:

Effects of pesticides on SH-SY5Y cells were further examined for the presence of apoptotic and necrotic cells using FITC-Annexin-V/Propidium Iodide staining assay. In apoptotic cells, phosphatidyl serine (PS) normally, located on the inner leaflet of the plasma membrane, translocate to the extra cellular side. The Ca^{2+}-dependent phospholipid-binding protein Annexin V has high affinity for PS and has been used for the detection of apoptosis. In addition, Annexin V can enter necrotic cells that have lost membrane integrity and bind to the PS attached to the inner leaflet of membrane. Staining simultaneously with propidium iodide (PI), a DNA stain that is not cell membrane permeable, distinguishes apoptotic from necrotic cells. Thus, the apoptotic cells that stain Annexin V positive but PI negative and necrotic cells will stain positive for both Annexin V and PI because cell membrane is not intact. Based on this principle, the apoptotic and necrotic actions on SH-SY5Y cells were visualized by Annexin-V/PI double staining. As shown in Figure 5.8 a, only 4% of cells appear to be apoptotic in DMSO (solvent) treated cells. Cells incubated with pesticides were found to have increased apoptotic and necrotic cells populations (Figs. 5.8 b-d). Thus, as illustrated in Figure 5.8 b-d, the apoptotic cell populations were 15.9, 10.3, and 22.4% when treated with 100 μM endosulfan, 100 μM zineb and 100 μM each of endosulfan + zineb, respectively. From these results, it is evident that mixture of pesticides had an additive effect on induction of apoptosis on SH-SY5Y cells. This conclusion was derived by subtracting the values for DMSO (solvent) from the treated groups. A similar result on cell necrosis (dead cells) was observed when cells were treated with individual pesticides. More intense cell death was noted when mixtures of pesticides were applied to the cells. Thus, after subtracting the number of dead cells in DMSO (solvent) from pesticide- treated cells, 100 μM each of endosulfan, zineb and their mixture were found to have approximately 11, 10 and 47% dead cells, respectively. These data indicate that mixtures of pesticides caused intensive cell death (4 times higher) than individual pesticides.

Apoptotic and necrotic cells were further identified by visualization under fluorescence microscope in combination with Annexin-V-FITC-propidium iodide staining. The Annexin-V (green color) detects apoptosis at an early stage, revealing the translocation of phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. As illustrated in Figure 5.9 a, most of solvent-treated cells were unstained. However, increased number of cells was stained with Annexin-V when treated with endosulfan or zineb (Fig. 5.9 b, c). Cells treated with mixture of endosulfan 100 μM + zineb 100 μM were found to be more intensively stained with (green fluorescence) Annexin-V, confirming the cellular death via apoptosis. Similarly, only few cells in Figure 5.9 (b & c) were stained with PI (red color) compared to solvent control (Figure 5.9 a). However, there were more cells stained with propidium iodide (red color) in pesticide mixture-treated cells confirming that SH-SY5Y cells exposed to pesticides die via necrosis as well.

5.5.5. Effects of pesticides on DNA fragmentation:

To further confirm the effects of pesticides on SH-SY5Y cells on the induction of apoptosis, fragmentation of DNA (a hallmark of apoptosis) was examined by agarose/ethidium bromide gel electrophoresis. The DNA from cells treated with pesticides or solvent (control) was purified and DNA cleavage into oligo-nucleosomal fragments induced by pesticide exposure was visualized on the gels. Figure 5.10 (a & b) illustrates the effects of pesticides on cellular DNA when cells were treated with 100 μM each of endosulfan or zineb alone or in combination. As shown in Figure 5.10a, lanes 3 and 4, fragmentation of DNA in the endosulfan- and zineb- treated cells was more pronounced than the controls (lane 2) when cells were incubated for 10 hours. These data indicate that cell populations exposed to 100 μM each of endosulfan or zineb undergo some degree of apoptosis. SH-SY5Y cells treated with mixtures of 100 μM each of endosulfan and zineb (lane 5) show significantly less genomic (high molecular weight) DNA after 10 hours incubation. The smearing of the gel indicates the presence of more necrotic cells. However, DNA laddering is not clearly visible after 16 hours of exposure to 100 μM zineb or endosulfan alone or in combination (Figure 5.10 b). The presence of less genomic DNA at the top of the gels, in 100 μM endosulfan (lane 3) and mixture (lane 5) studies indicates DNA degradation into smaller fragments. The smearing

observed in Figure. 5.10 b, lane 3, 4 supports the earlier flow cytometric observation (Fig. 5.5, 5.7, 5.8) of necrotic cell death.

5.6. Discussion:

The toxicity of complex chemical mixtures may differ from the toxicity observed when individual components are tested as pure chemicals. Our knowledge in assessing potential interactive effects among chemicals is not well investigated. Much concern has been expressed regarding the toxicological interactions of environmental chemicals exposure. The Presidential Commission on Risk Assessment and Risk Management (CRARM, 1997) has recommended the need to focus on chemical mixtures to which public might be exposed concurrently or sequentially. Certain chemicals such as methylcyclopentadienyl manganese tricarbonyl, paraquat, dieldrin and chlorpyrifos are known to induce neurotoxicity by triggering neuronal cell dealth via necrosis or apoptosis (Cappelletti *et al.*, 1998; Fabisiak *et al.*, 1998; Auman *et al.*, 2000; Anantharam *et al.*, 2002; Hallegue *et al.*, 2002). Endosulfan, an organo-chlorine insecticide belonging to the cyclodiene group (Gupta and Gupta, 1979), is one of the most commonly used pesticides in agriculture worldwide. In a population-based study, significantly higher frequency of sister chromatid exchanges and cell cycle delay in subjects who handled the pesticide has been reported (Rupa *et al.*, 1991). Exposure to endosulfan has been monitored to induce apoptotic cell death via mitochrondrial dysfunction and oxidative stress in human-T cell and leukemic cells *in vitro* (Kannan *et al.*, 2000; Kang *et al.*, 2001). Dithiocarbamates such as thiram, disulfiram, and ethylenebisdithiocarbamates (such as nabam, maneb, and zineb) are widely used for eradication of fungal infection on fruit plants and vegetables. These compounds potently augment the striatal DA depletion induced by Parkinson's Disease causing chemicals such as rotenone and MPTP that inhibit mitochondrial electron transport at the level of complex I (Corsini *et al.*, 1985; Irwin *et al.*, 1987; Bachurin *et al.*, 1996). In the present study, we investigated the mechanism of cell death upon exposure to endosulfan and zineb individually and in mixture on SH-SY5Y cell lines. These cells are generally classified as dopaminergic neurons (Pahlman *et al.*, 1984), which posses many qualities of substaintial nigra neuron with high levels of dopamine

hydroxylase and tyrosine hydroxylase, the key enzymes in catecholaminergic neurotransmitter synthesis. (Oyarce and Fleming, 1991; Takahashi *et al.*, 1994).

In this study, we hypothesized that both endosulfan and zineb are neurotoxic and the pesticide-induced neurotoxicity may be associated with both apoptotic and necrotic cell death. Our goal in the present study was to investigate the potential cytotoxicty of endosulfan in the presence or absence of a second pesticide zineb on human neuronal cells (SH-SY5Y) in culture.

Apoptosis plays an important role in maintaining body's homeostasis as well as in the development of the nervous system (Meier *et al.*, 2000). Several lines of evidence have suggested the apoptotic cell death may contribute to various pathological conditions, such as cerebral ischemia (Chen *et al.*, 1998; Namura *et al.*, 1998) and neurogenerative disorders, such as Alzheimer disease and Parkinson's disease, Huntington disease and amyotrophic lateral sclerosis (Honig and Rosenberg, 2000; Mattson, 2000; Martin, 2001). Therefore, it was critical to identify some environmental compounds that would cause apoptosis leading to the neurodegenerative diseases. We believe this is the first report describing that endosulfan, zineb and their mixtures can induce apoptosis and necrosis in a human neuroblastoma (SH-SY5Y) cell line that may have direct relevance to neurodegenerative diseases caused by environmental chemicals.

Using multiple approaches, we have demonstrated that exposure of endosulfan and zineb alone causes both apoptosis and necrosis and in mixtures, predominantly necrosis. Visual evaluation using DNA ladder assay and fluorescence Annexin-V/PI assay confirmed the contribution of both apoptotic and necrotic processes. These results suggest that both apoptosis and necrosis are the major events involved in pesticide-induced cytotoxicity in SH-SY5Y cells.

Furthermore, the release of LDH from cells has been used as a sensitive marker of the chemical-induced cell injury (Bonfoco *et al.*, 1995). Our data clearly indicate that release of LDH from SH-SY5Y cells exposed to endosulfan and zineb show a dose-dependent

response to chemical treatment (Figures 5.1, 5.2). A significantly greater release of LDH ($P \leq 0.05$) was observed when cells were exposed to mixtures of these pesticides. Sinha, *et a.l*, 1997 observed increased LDH release in reproductive (testis) cells of male growing rats exposed to endosulfan (Sinha *et al.*, 1997). We concur this finding in SH-SY5Y cells.

The type of cell death as apoptotic or necrotic was further identified by using flow cytometry in combination with either 7-AAD or Annexin-V fluorescein staining. Identification and quantitation of apoptotic or necrotic cell death by flow cytometry is generally based on a single parameter reflecting the change in molecular or biochemical events, representative of that particular process (Gorczyca, 1999). The 7-AAD staining method has been shown as a reliable assay in the quantification of apoptosis (Schmid *et al.*, 1994a; Schmid *et al.*, 1994b; Lecoeur *et al.*, 1997; Donner *et al.*, 1999). Using this method, we have observed that exposure to 100μM endosulfan or zineb caused an increase in shift of cells towards early apoptotic gates, and treatment with pesticide mixtures caused a greater portion of the cells to be necrosis compared to the individual pesticide treatments. Kannan *et al.,* (2000) demonstrated similar changes in endosulfan induces apoptosis in Jurkat cells, a human T-cell leukemic line (Kannan *et al.*, 2000). Hence, our data are consistent with this above report.

DNA fragmentation has been suggested to be one of the initial irreversible events to trigger cell apoptosis (Duke *et al.*, 1983; Golstein *et al.*, 1991) and DNA ladder assay, which monitors fragmented DNA, is hallmark for apoptosis. In our studies, fragmentation of DNA was clearly visible when SH-SY5Y cells are exposed to 100μM endosulfan and zineb alone as early as 10 hours indicating the cells undergoing early apoptosis. Exposing the cells for 16 hours with above pesticides caused smearing effects in the gels indicating late apoptosis or necrosis. Hence, we propose that most early events of cell death could be due to apoptosis and at later time the predominant form of cell death is due to necrotic evident.

In addition, morphological evaluation and flow cytometric analysis using FITC-Annexin-V/PI iodide staining assays were conducted to differentiate the apoptotic and

necrotic events. Our results from both these assays (Figures 5.8 and 5.9) indicate that both apoptotic and necrotic cell death processes occur when cells are exposed to peticides for 16 hours. The results are consistent with the results observed using 7-AAD staining and DNA ladder assays.

In conclusion, our results help to better characterize pesticide-induced neuronal cell death. Both zineb and endosulfan were found to induce cytotoxicity in SH-SY5Y cells via both apoptotic and necrotic pathways. Further studies on the mechanism of apoptotic neuronal cell death induced by these environmental chemicals will clarify the pathogenesis involved in neurodegenerative process. It is interesting to note that when the results of solvent controls were subtracted from pesticide exposed cells, the mixtures of endosulfan and zineb were found to cause more than two-times higher necrotic cell death (Fig. 5.7) than the total cell death caused by individual pesticides. Whereas, the results of early apoptotic cell death (Fig. 5.6) was found to be additive in nature. The additive effects of zineb and endosulfan on early apoptotic cell death suggest that the two pesticides may operate in two different/independent mechanisms. However, the late apoptotic cell death as monitored by 7-AAD staining technique showed more than additive effect indicating some related pathway, rather than by totally independent mechanisms of cell death by these pesticides. Together with evidence provided in this study, it appears likely that endosulfan and zineb alone or in combination play a role in the cell death in an *in vitro* model system that may, at least in part, be involved in the degeneration of dopaminergic neurons and may be relevant for the pathogenesis of some of these neurodegenerative diseases.

5.7. Acknowledgements:

The authors gratefully acknowledge the technical assistance of Joan Kalnitsky for flow cytometry use and Dan Ward for statistical analysis. This work was supported, in part, by the Edward Via Virginia College of Osteopathic Medicine.

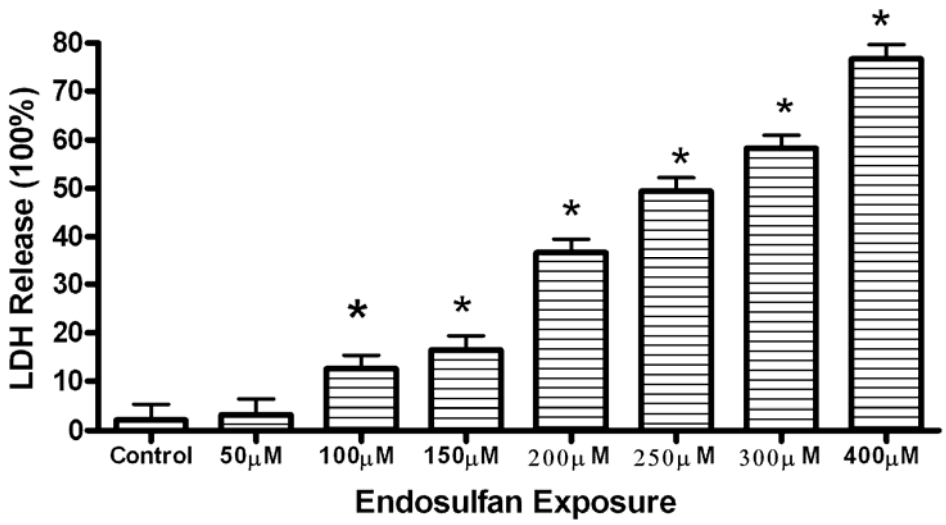


Figure 5.1: LDH release from SH-SY5Y cells incubations (3×10^4) with various concentrations of endosulfan for 16 hours.

* Statistically significant difference ($P \leq 0.05$) when compared to control. Significant ($P \leq 0.05$) of LDH release was observed in cells treated with 100 μM or higher endosulfan than the untreated cells. Data are presented as mean ± S.E.

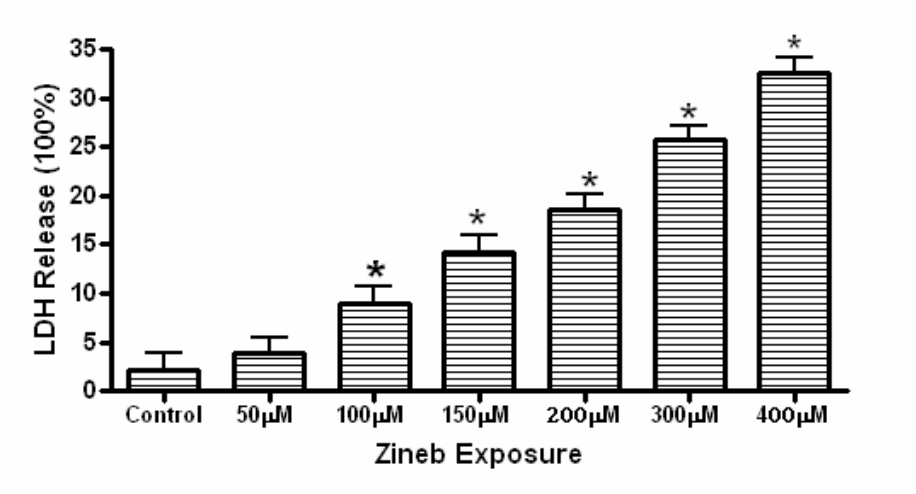


Figure 5.2: LDH release from SH-SY5Y cells incubations (3×10^4) with various concentrations of zineb for 16 hours.

Statistically significant difference ($P \leq 0.05$) when compared to control. Significant ($P \leq 0.05$) of LDH release was observed in cells treated with 100 µM or higher zineb than the untreated cells. Data are presented as mean ± S.E.

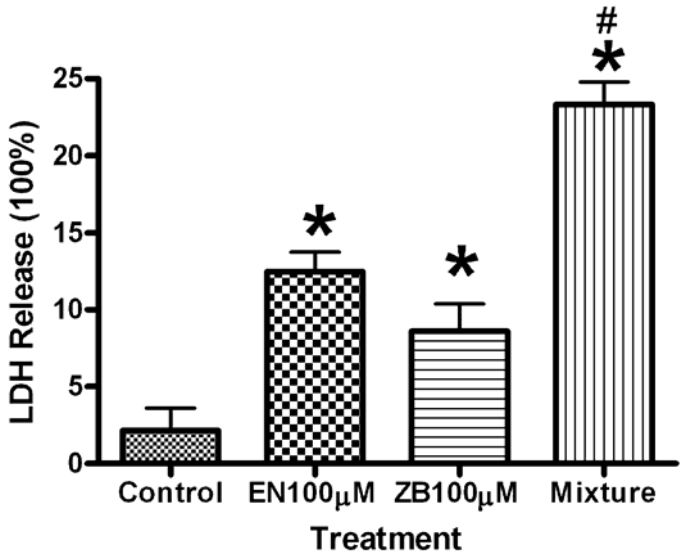


Figure 5.3: LDH release from SH-SY5Y cells (3×10^4) incubations with endosulfan 100 μM, zineb 100 μM and mixture for 16 hours.

Greater release of LDH ($P \leq 0.05$) was seen in cells treated with mixtures. Data are presented as mean ± S.E. * statistically significant difference ($P \leq 0.05$) when compared to control. # Statistically significant difference ($P \leq 0.05$) when compared to single pesticide treatments.

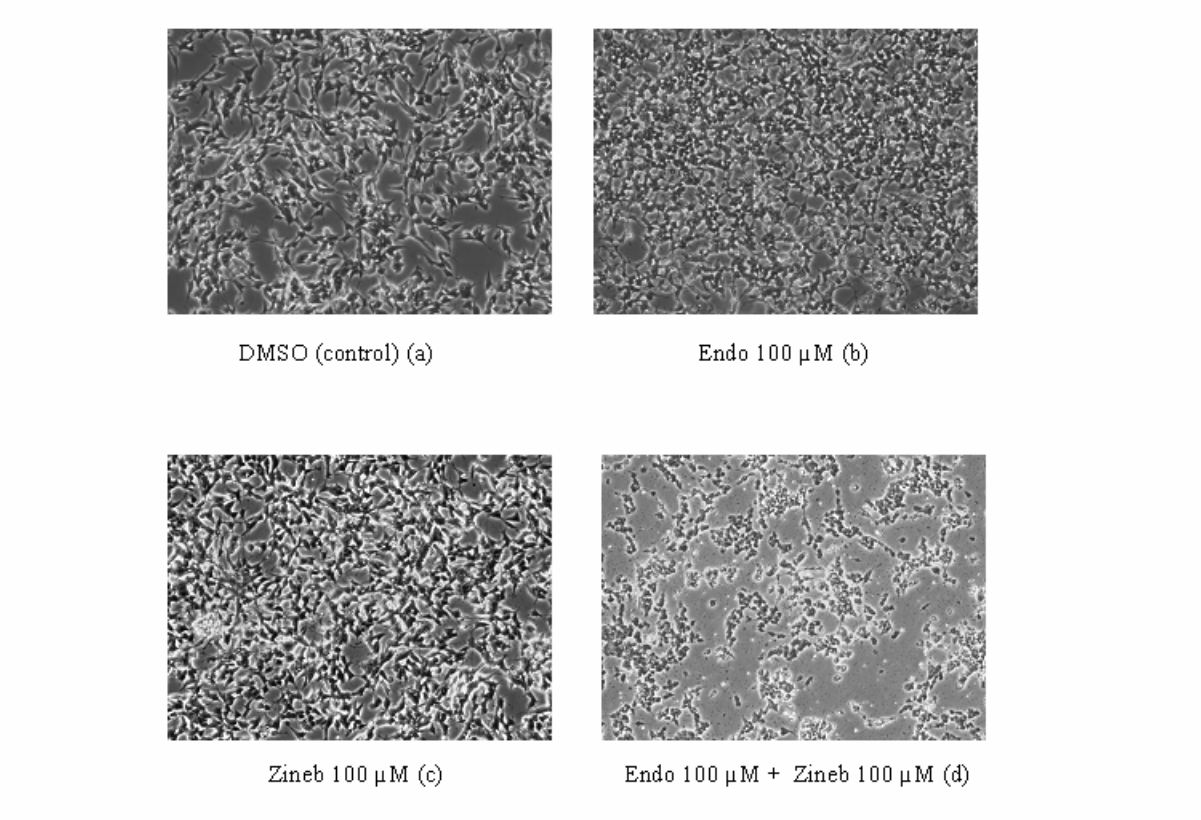


Figure 5.4: Phase contrast images of SH-SY5Y cells with and without treatment of pesticides or pesticides mixture for 16 h, magnification 10X.

Under control conditions, the cells had a homogenous morphology: they had an ellipsoid-shaped surface with neurite extension. After exposure to pesticides, cell detritus surrounded clusters of cell; the neurite extensions completely disappeared and the surviving cell were circular shape. Cells treated with mixtures also had decreased adhesion to the tissue flask and emerge into the growth medium.

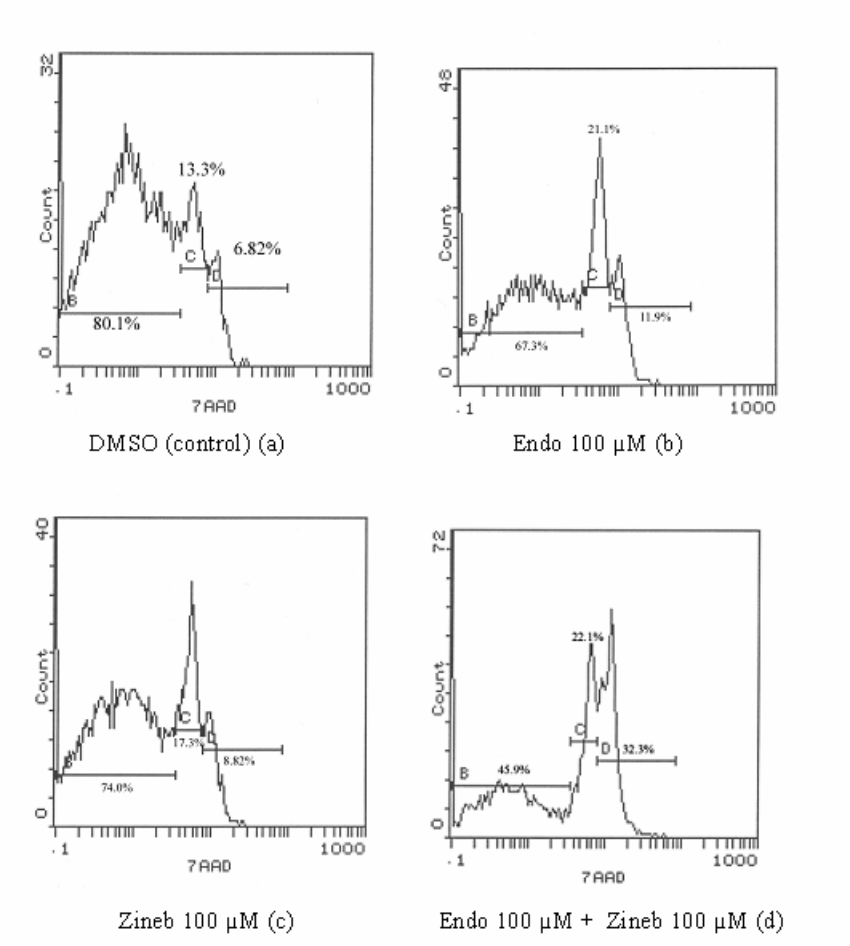


Figure 5.5: Representative flow cytometric analysis of control SH-SY5Y cells and cells exposed to pesticides or pesticide mixtures.

Cells were treated with zineb, endosulfan or their mixtures for 16 h then stained with 7-AAD and analyzed with flow cytometry. B: live cells (7-AAD^{dull}); C: apoptotic cells (7-AAD^{intermediate}); D: late apoptotic/necrotic cells (7-AAD^{bright}). Exposure to endosulfan (100 µM) or zineb (100 µM) caused an increased in shift of cells towards both early apoptotic and later apoptotic/necrotic gates.

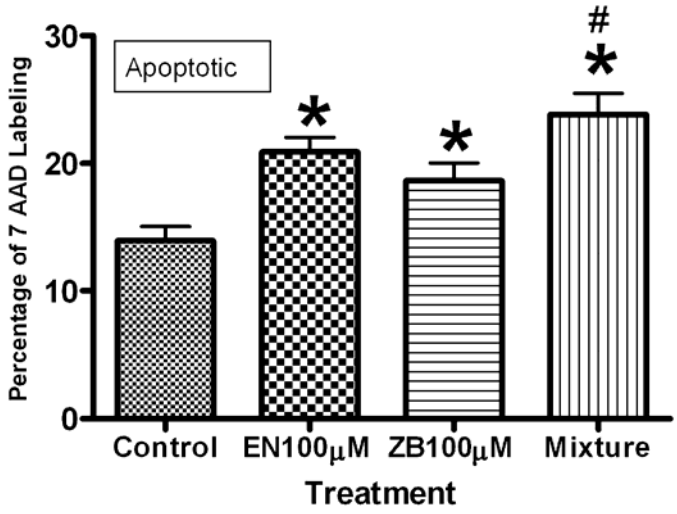


Figure 5.6: The percent of early apoptotic cells following treatment with pesticides or pesticide mixtures was measured on the flow cytometry using the 7-AAD Staining assay.

SH-SY5Y cells were treated with endosulfan 100 μM, zineb 100 μM alone or mixture for 16 hours. Data are presented as mean ± S.E. * Statistically significant difference ($P \leq 0.05$) when compared to control. # Statistically significant difference ($P \leq 0.05$) when compared to zineb100 μ treatments.

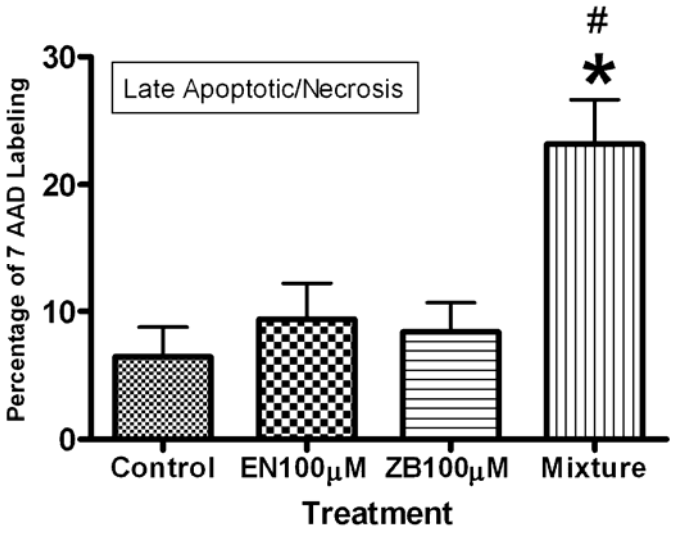


Figure 5.7: The percent of late apoptotic / necrotic cells following treatment with pesticides or pesticide mixtures was measured on the flow cytometry using the 7-AAD Staining assay.

SH-SY5Y cells were treated with endosulfan 100 μM, zineb 100 μM and mixture for 16 hours. Data are presented as mean ± S.E. * Statistically significant difference ($P \leq 0.05$) when compared to control. # Statistically significant difference ($P \leq 0.05$) when compared to single pesticide treatments.

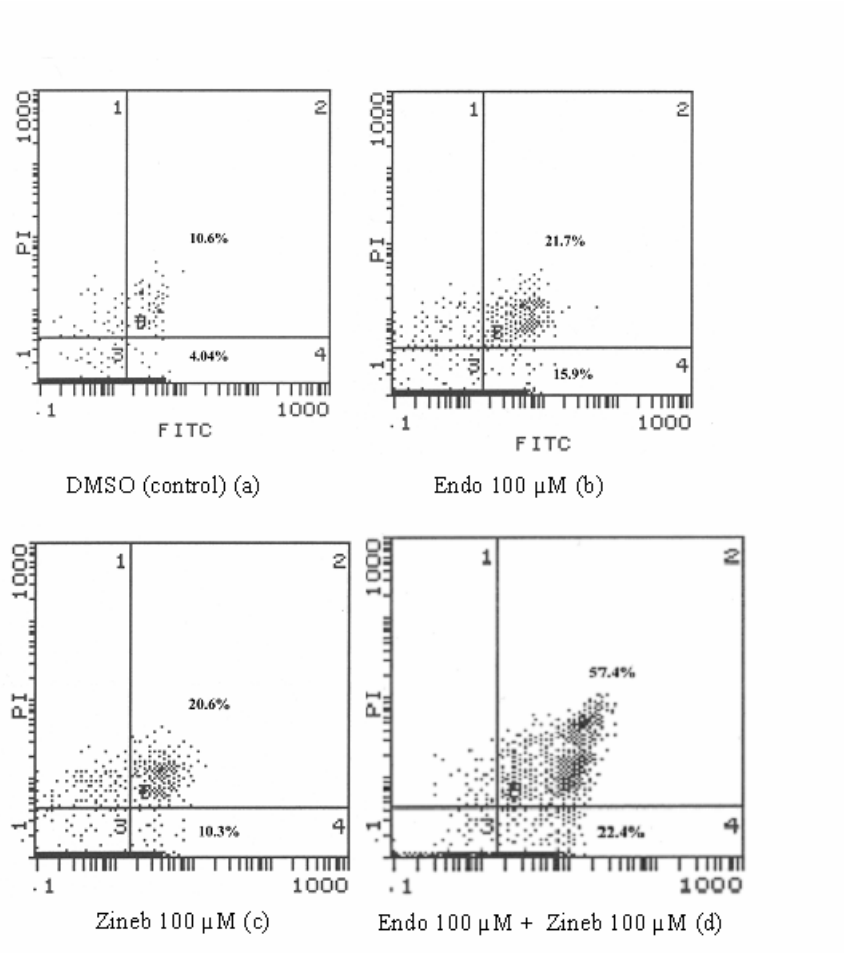


Figure 5.8: Evaluation of apoptotic and necrotic cell population of endosulfan and zineb and their mixtures as assessed by the Annexin-V Staining assay.

SH-SY5Y cells were treated with endosulfan 100 µM, zineb 100 µM alone or mixture for 16 hours. The percent of cells stained as Annexin-V^-/PI^- (live), Annexin-V^+/PI^- (early apoptotic) and Annexin-V^+/PI^+ (dead) is presented. Viable cells are in the bottom left quadrant, apoptotic cells in the bottom right quadrant, and necrotic and late apoptotic cells in the upper right quadrant. Numbers in each quadrant are percentage of cells they contain.

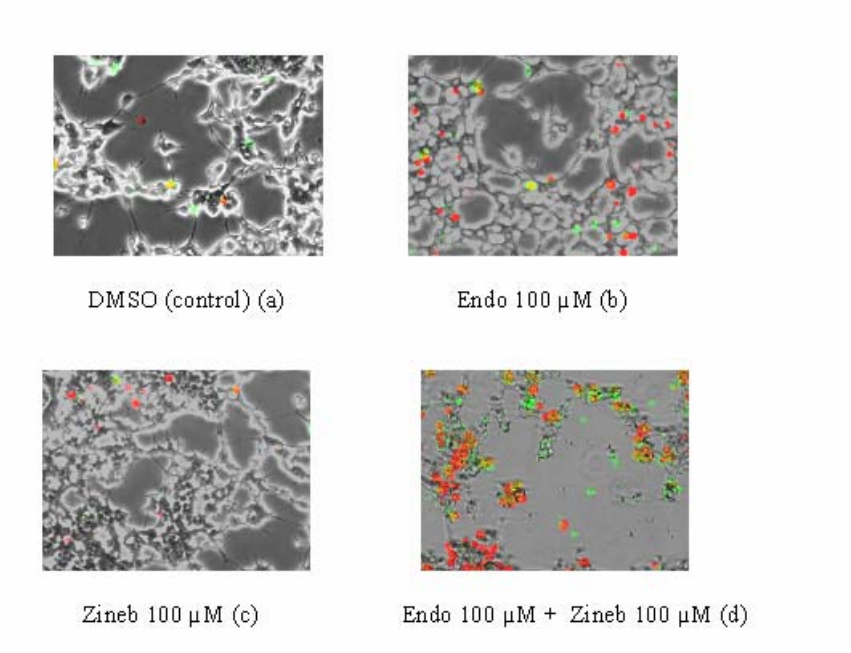


Figure 5.9: Fluorescence photomicrographs of SH-SY5Y cells after Aannexin V-FITC staining and propidium iodide staining (10X).

SH-SY5Y cells were treated with endosulfan 100 µM, zineb 100 µM alone or mixture for 16 hours and then stained with annexin V-FITC, Propidium Iodide. Cells were photographed with an inverted fluorescence microscope. In (a, solvent control), most of the cells are unstained; In (b, c), a few cells are stained with annexin V-FITC (green colour), PI (red colour) compared to solvent control (a). In (d), most of the cells are stained with annexin V-FITC (green colour) and more cells are stained with propidium iodide (red colour).

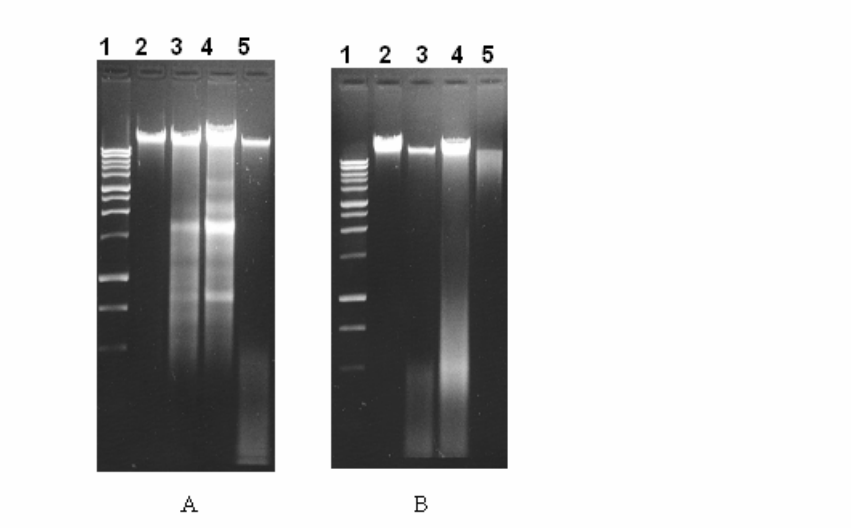


Figure 5.10: DNA laddering of SH-SY5Y cells after incubation with endosulfan, zineb or their mixtures:

Agarose gel electrophoresis to detect DNA ladder pattern in endosulfan and zineb induced apoptosis. Cells (2×10^6) were untreated or treated with either endosulfan or zineb or mixtures for 10 hours (A) or 16hours (B). A 10 μl of isolated DNA per treatment was electrophoresed on a 1% agarose gel. The lanes represent: A 1 = 1kb DNA ladder; 2 = vehicle control (DMSO); 3 = Endosulfan 100 μM; 4 = zineb 100 μM; 5 = mixture; B 1 = 1kb DNA ladder; 2 = vehicle control (DMSO); 3 = endosulfan 100 μM; 4 = Zineb 100 μM; 5 = mixture; These experiments were repeated 3 more times with the similar results; DNA ladder assay shows that treatment with 100 μM either zineb or endosulfan shows greater DNA Ladder after 10 hours exposure. The genomic DNA in the top of the gel is indicative of viable cells. Viable cell population of combination of endosulfan 100 μM with 100 μM zineb is greatly reduced after 10 hours and 16 hours.

5.8. Literature Cited:

Aleksandrowicz, D. R. (1979). Endosulfan poisoning and chronic brain syndrome. *Arch Toxicol* **43**, 65-68.

Anantharam, V., Kitazawa, M., Wagner, J., Kaul, S., and Kanthasamy, A. G. (2002). Caspase-3-dependent proteolytic cleavage of protein kinase Cdelta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese tricarbonyl. *J Neurosci* **22**, 1738-1751.

Auman, J. T., Seidler, F. J., and Slotkin, T. A. (2000). Neonatal chlorpyrifos exposure targets multiple proteins governing the hepatic adenylyl cyclase signaling cascade: implications for neurotoxicity. *Brain Res Dev Brain Res* **121**, 19-27.

Bachurin, S. O., Shevtzova, E. P., Lermontova, N. N., Serkova, T. P., and Ramsay, R. R. (1996). The effect of dithiocarbamates on neurotoxic action of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) and on mitochondrial respiration chain. *Neurotoxicology* **17**, 897-903.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., and Lipton, S. A. (1995). Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci U S A* **92**, 7162-7166.

Camacho, I. A., Hassuneh, M. R., Nagarkatti, M., and Nagarkatti, P. S. (2001). Enhanced activation-induced cell death as a mechanism of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity in peripheral T cells. *Toxicology* **165**, 51-63.

Cappelletti, G., Maggioni, M. G., and Maci, R. (1998). Apoptosis in human lung epithelial cells: triggering by paraquat and modulation by antioxidants. *Cell Biol Int* **22**, 671-678.

Chen, J., Nagayama, T., Jin, K., Stetler, R. A., Zhu, R. L., Graham, S. H., and Simon, R. P. (1998). Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. *J Neurosci* **18**, 4914-4928.

Chugh, S. N., Dhawan, R., Agrawal, N., and Mahajan, S. K. (1998). Endosulfan poisoning in Northern India: a report of 18 cases. *Int J Clin Pharmacol Ther* **36**, 474-477.

Corsini, G. U., Pintus, S., Chiueh, C. C., Weiss, J. F., and Kopin, I. J. (1985). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in mice is enhanced by pretreatment with diethyldithiocarbamate. *Eur J Pharmacol* **119**, 127-128.

CRARM (1997). *Risk Assessment and Risk Management in Regulatory Decision-Making, Final Report*. Washington, D.C.

Donner, K. J., Becker, K. M., Hissong, B. D., and Ahmed, S. A. (1999). Comparison of multiple assays for kinetic detection of apoptosis in thymocytes exposed to dexamethasone or diethylstilbesterol. *Cytometry* **35**, 80-90.

Duke, R. C., Chervenak, R., and Cohen, J. J. (1983). Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytolysis. *Proc Natl Acad Sci U S A* **80**, 6361-6365.

Fabisiak, J. P., Kagan, V. E., Tyurina, Y. Y., Tyurin, V. A., and Lazo, J. S. (1998). Paraquat-induced phosphatidylserine oxidation and apoptosis are independent of activation of PLA2. *Am J Physiol* **274**, L793-802.

Golstein, P., Ojcius, D. M., and Young, J. D. (1991). Cell death mechanisms and the immune system. *Immunol Rev* **121**, 29-65.

Gorczyca, W. (1999). Cytometric analyses to distinguish death processes. *Endocr Relat Cancer* **6**, 17-19.

Gupta, P. K. (1976). Endosulfan-induced neurotoxicity in rats and mice. *Bull Environ Contam Toxicol* **15**, 708-713.

Gupta, P. K., and Gupta, R. C. (1979). Pharmacology, toxicology and degradation of endosulfan A review. *Toxicology* **13**, 115-130.

Guven, K., Power, R. S., Avramides, S., Allender, R., and de Pomerai, D. I. (1999). The toxicity of dithiocarbamate fungicides to soil nematodes, assessed using a stress-inducible transgenic strain of Caenorhabditis elegans. *J Biochem Mol Toxicol* **13**, 324-333.

Hallegue, D., Ben Rhouma, K., Krichah, R., and Sakly, M. (2002). Dieldrin initiates apoptosis in rat thymocytes. *Indian J Exp Biol* **40**, 1147-1150.

Hampton, M. B., and Orrenius, S. (1998). Redox regulation of apoptotic cell death. *Biofactors* **8**, 1-5.

Honig, L. S., and Rosenberg, R. N. (2000). Apoptosis and neurologic disease. *Am J Med* **108**, 317-330.

Irwin, I., Wu, E. Y., DeLanney, L. E., Trevor, A., and Langston, J. W. (1987). The effect of diethyldithiocarbamate on the biodisposition of MPTP: an explanation for enhanced neurotoxicity. *Eur J Pharmacol* **141**, 209-217.

Kang, K. S., Park, J. E., Ryu, D. Y., and Lee, Y. S. (2001). Effects and neuro-toxic mechanisms of 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl and endosulfan in neuronal stem cells. *J Vet Med Sci* **63**, 1183-1190.

Kannan, K., Holcombe, R. F., Jain, S. K., Alvarez-Hernandez, X., Chervenak, R., Wolf, R. E., and Glass, J. (2000). Evidence for the induction of apoptosis by endosulfan in a human T-cell leukemic line. *Mol Cell Biochem* **205**, 53-66.

Langston, J. W., Ballard, P., Tetrud, J. W., and Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**, 979-980.

Lecoeur, H., Ledru, E., Prevost, M. C., and Gougeon, M. L. (1997). Strategies for phenotyping apoptotic peripheral human lymphocytes comparing ISNT, annexin-V and 7-AAD cytofluorometric staining methods. *J Immunol Methods* **209**, 111-123.

Manning-Bog, A. B., McCormack, A. L., Li, J., Uversky, V. N., Fink, A. L., and Di Monte, D. A. (2002). The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. *J Biol Chem* **277**, 1641-1644.

Martin, L. J. (2001). Neuronal cell death in nervous system development, disease, and injury (Review). *Int J Mol Med* **7**, 455-478.

Mattson, M. P. (2000). Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol* **1**, 120-129.

Meier, P., Finch, A., and Evan, G. (2000). Apoptosis in development. *Nature* **407**, 796-801.

Namura, S., Zhu, J., Fink, K., Endres, M., Srinivasan, A., Tomaselli, K. J., Yuan, J., and Moskowitz, M. A. (1998). Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *J Neurosci* **18**, 3659-3668.

Olgun, S., Gogal, R. M., Jr., Adeshina, F., Choudhury, H., and Misra, H. P. (2004). Pesticide mixtures potentiate the toxicity in murine thymocytes. *Toxicology* **196**, 181-195.

Oyarce, A. M., and Fleming, P. J. (1991). Multiple forms of human dopamine beta-hydroxylase in SH-SY5Y neuroblastoma cells. *Arch Biochem Biophys* **290**, 503-510.

Pahlman, S., Ruusala, A. I., Abrahamsson, L., Mattsson, M. E., and Esscher, T. (1984). Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. *Cell Differ* **14**, 135-144.

Repetto, R., and Baliga, S. S. (1996). Pesticides and the immune system: the public health risks. Executive summary. *Cent Eur J Public Health* **4**, 263-265.

Rhile, M. J., Nagarkatti, M., and Nagarkatti, P. S. (1996). Role of Fas apoptosis and MHC genes in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity of T cells. *Toxicology* **110**, 153-167.

Ritz, B., Yu, F., Chapa, G., and Fruin, S. (2000). Effect of air pollution on preterm birth among children born in Southern California between 1989 and 1993. *Epidemiology* **11**, 502-511.

Rupa, D. S., Reddy, P. P., Sreemannarayana, K., and Reddi, O. S. (1991). Frequency of sister chromatid exchange in peripheral lymphocytes of male pesticide applicators. *Environ Mol Mutagen* **18**, 136-138.

Schmid, I., Uittenbogaart, C. H., and Giorgi, J. V. (1994a). Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. *Cytometry* **15**, 12-20.

Schmid, I., Uittenbogaart, C. H., Keld, B., and Giorgi, J. V. (1994b). A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. *J Immunol Methods* **170**, 145-157.

Schmuck, G., Ahr, H. J., Mihail, F., Stahl, B., and Kayser, M. (2002). Effects of the dithiocarbamate fungicide propineb in primary neuronal cell cultures and skeletal muscle cells of the rat. *Arch Toxicol* **76**, 414-422.

Seth, P. K., Saidi, N. F., Agrawal, A. K., and Anand, M. (1986). Neurotoxicity of endosulfan in young and adult rats. *Neurotoxicology* **7**, 623-635.

Sinha, N., Narayan, R., and Saxena, D. K. (1997). Effect of endosulfan on the testis of growing rats. *Bull Environ Contam Toxicol* **58**, 79-86.

Smith, C. (2001). Pesticide exports from U.S. ports, 1997-2000. *Int J Occup Environ Health* **7**, 266-274.

Takahashi, R. N., Rogerio, R., and Zanin, M. (1989). Maneb enhances MPTP neurotoxicity in mice. *Res Commun Chem Pathol Pharmacol* **66**, 167-170.

Takahashi, T., Deng, Y., Maruyama, W., Dostert, P., Kawai, M., and Naoi, M. (1994). Uptake of a neurotoxin-candidate, (R)-1,2-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline into human dopaminergic neuroblastoma SH-SY5Y cells by dopamine transport system. *J Neural Transm Gen Sect* **98**, 107-118.

Tebourbi, O., Rhouma, K. B., and Sakly, M. (1998). DDT induces apoptosis in rat thymocytes. *Bull Environ Contam Toxicol* **61**, 216-223.

Thiruchelvam, M., Richfield, E. K., Baggs, R. B., Tank, A. W., and Cory-Slechta, D. A. (2000). The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. *J Neurosci* **20**, 9207-9214.

Thiruchelvam, M., Richfield, E. K., Goodman, B. M., Baggs, R. B., and Cory-Slechta, D. A. (2002). Developmental exposure to the pesticides paraquat and maneb and the Parkinson's disease phenotype. *Neurotoxicology* **23**, 621-633.

Thiruchelvam, M. J., Powers, J. M., Cory-Slechta, D. A., and Richfield, E. K. (2004). Risk factors for dopaminergic neuron loss in human alpha-synuclein transgenic mice. *Eur J Neurosci* **19**, 845-854.

Tolwani, R. J., Jakowec, M. W., Petzinger, G. M., Green, S., and Waggie, K. (1999). Experimental models of Parkinson's disease: insights from many models. *Lab Anim Sci* **49**, 363-371.

Vaccari, A., Saba, P., Mocci, I., and Ruiu, S. (1999). Dithiocarbamate pesticides affect glutamate transport in brain synaptic vesicles. *J Pharmacol Exp Ther* **288**, 1-5.

Zaidi, N. F., Agrawal, A. K., Anand, M., and Seth, P. K. (1985). Neonatal endosulfan neurotoxicity: behavioral and biochemical changes in rat pups. *Neurobehav Toxicol Teratol* **7**, 439-442.

CHAPTER 6 Reactive Oxygen Species in In Vitro Pesticide-induced Neuronal Cell (SH-SY5Y) Cytotoxicity,: Role of NFkappaB and Caspase-3

Authors: Zhenquan Jia[1], and Hara P. Misra* [1, 2].

Affiliations: [1] Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.

[2] Edward Via Virginia College of Osteopathic Medicine, Virginia Tech Corporate Research Center, 2265 Kraft Drive, Blacksburg, VA 24060, U.S.A.

Corresponding Author: * Hara P. Misra

Edward Via Virginia College of Osteopathic Medicine

Virginia Tech Corporate Research Center, 2265 Kraft Drive,

Blacksburg, VA 24060, U.S.A.

Phone: (540) 231-3693

Fax: (540) 231-5252

misra@vt.edu

Keywords: Pesticides, Neuronal cell, endosulfan, zineb, reactive oxygen species, oxidative stress, caspase-3, NFkappaB, superoxide dismutase, catalase, glutathione peroxidase

6.1. Abstract:

Oxidative stress has been implicated in pesticide-induced neurotoxicity, based on its role in the cascade of biochemical changes that lead to dopaminergic neuronal cell death. We have, therefore, examined the role of oxidative stress caused by the pesticides endosulfan and zineb in human neuroblastoma cells (SH-SY5Y) in culture. Upon treatment with 50-200μM concentrations of either of these pesticides, SH-SY5Y cells generated both superoxide anion and hydrogen peroxide in a dose- and time-dependent manner. Mixtures of the pesticides significantly enhanced the production of these reactive oxygen species compared to individual pesticide exposures. The effects of these pesticides on levels of antioxidant enzymes were also investigated. Pesticide treatment decreased superoxide dismutase, glutathione peroxidase, and catalase levels in SH-SY5Y cells. Additionally, these pesticides induced lipid peroxide (thiobarbituric acid reactive products) formation in these cells. While both pesticides individually (at 100 μM) increased caspase-3 activity, cells exposed to a mixture of the pesticides exhibited significantly low levels of this enzyme, probably due to excessive necrotic cell death. Furthermore, exposure to these pesticides increased nuclear NFkappaB activity. Taken together, these findings support the above hypothesis and suggest that the cytotoxicity of endosulfan and zineb, both individually and in mixtures may, at least in part, be associated with the generation of reactive oxygen species with concomitant increased expression of NFkappaB.

6.2. Introduction:

Parkinson's disease (PD) is a chronic, progressive neurodegenerative movement disorder (Sandyk *et al.*, 1988; Leenders and Oertel, 2001) resulting, in part, from the progressive loss of dopamine neurons in substantia nigra parts compacts. Although the etiology of idiopathic PD is not known, most studies strongly suggest that environmental factors, particularly pesticides exposure, may play an important role in the pathogenesis of this disorder (Semchuk *et al.*, 1992; Butterfield *et al.*, 1993; Gorell *et al.*, 1998). Our laboratory has demonstrated that mice exposure to endosulfan (an organochlorine cyclodiene pesticide) and zineb (an ethylenebisdithiocarbamate (EBDC) fungicide) as juveniles and re-exposed at 8 months of age showed loss of dopamine in the striatum. Mixtures of these pesticides also showed significantly increased levels of alpha-synuclein, a major component of Lewy bodies and a hallmark of neurodegenerative diseases such as PD and Alzheimer's disease (AD). In addition, we have demonstrated that mice exposed to these pesticides during critical period of brain development enhance the susceptibility to the same chemicals later in life (Jia and Misra, 2006a). These pesticides are widely used in agriculture and in some countries in public health (Pokharkar and Dethe, 1981). In human with endosulfan poisoning, overstimulation of the central nervous system is the major characteristic (Boyd and Dobos, 1969; Boyd *et al.*, 1970; Aleksandrowicz, 1979; Blanco-Coronado *et al.*, 1992; Gilbert and Mack, 1995; Boereboom *et al.*, 1998; Chugh *et al.*, 1998). Studies in animals have shown changes in neurotransmitter levels and alterations in neurobehavioral processes after exposure to endosulfan (Lakshmana and Raju, 1994; Paul *et al.*, 1995). EBDC have been implicated as potential risk factors for the PD phenotype (Ferraz *et al.*, 1988; McGrew *et al.*, 2000; Zhang *et al.*, 2003). Zineb was reported to cause reduction of high affinity dopamine and number of tyrosine hydroxylase positive neurons in mesencephalic striatal primary co-culture (Soleo *et al.*, 1996). Recently, our laboratory has demonstrated that exposure to endosulfan and zineb caused both apoptosis and necrotic cell death in SH-SY5Y neuroblama cells (Jia and Misra, 2006b). However, the mechanism(s) of neuronal cell death caused by these pesticides is not known.

Several hypothesis have linked enviromental toxicant exposure, particularly pesticides, with caspases (cysteinyl-aspartate-specific proteinases) in the induction of cytotoxicity. Thus, caspase-3 was suggested to play a critical role in both upstream and downstream pathways in dieldrin-induced apoptosis in dopaminergic PC12 cells (Kitazawa *et al.*, 2001; Kitazawa *et al.*, 2003). Caspase-3 activation was also observed with high concentrations of heptachlor, an organochlorine insecticide (Rought *et al.*, 2000).

An alternative hypothesis postulates that oxidative stress plays an important role in pesticide-induced neurotoxicity and has been suggested to play an important role in the pathogenesis of neurodegenerative diseases, such as PD and AD (Jenner, 1991; Jenner *et al.*, 1992; Gotz *et al.*, 1994; Owen *et al.*, 1996; Christen, 2000; Rottkamp *et al.*, 2000; Zhang *et al.*, 2000; Pratico *et al.*, 2002; Zafrilla *et al.*, 2006). It has been suggested that many pesticides are capable of inducing oxidative stress by overwhelming or modulating cellular drug metabolizing system (Dikshith, 1991; Bachowski et al., 1998; (Gauthier *et al.*, 2001); (Bagchi *et al.*, 2000). Oxidative stress has been implicated as a key aspect in Mn-EBDC-induced neurotoxicity (Fitsanakis *et al.*, 2002; Zhou *et al.*, 2004). Oxidative stress-inducing effects of endosulfan have been reported in rats (Hincal *et al.*, 1995), human cell lines (Kannan *et al.*, 2000; Sohn *et al.*, 2004), and fish (Pandey *et al.*, 2001; Dorval and Hontela, 2003; Dorval *et al.*, 2003).

The role of oxidative stress in neuronal cells with exposure to the combination of these pesticides is not known. Because NFkappaB, an eukaryotic transcription factor, is activated upon oxidative stress (Schreck *et al.*, 1991), and plays a key role in the regulation of numerous genes involved in pathogen responses and cellular defense mechanisms during the programmed cell death (Guerrini *et al.*, 1995), we explore further the mechanism(s) of pesticide-induced oxidative stress as a possible mechanism of neuronal cell cytotoxicity in SH-SY5Y cells following pesticides exposure in culture.

In the present study, we examined the effects of endosulfan and zineb individually and in combination for their potential to stimulate oxidative stress in human neuroblastoma cells (SH-SY5Y) *in vitro.* We also monitored the fate of expression of NFkappaB and Caspase-3

in SH-SY5Y cells upon exposure to these pesticides. We report here that exposure of SH-SY5Y cells to endosulfan and zineb augmented production of superoxide and hydrogen peroxide and decreased the level of certain antioxidant enzymes. We also show that exposure to these pesticides altered caspase-3 activity in the cells and increased the NFkappaB activity in the cell nucleus. These new findings add to the growing body of knowledge that reactive oxygen species generated during the metabolism of environmental chemicals may be involved in the degeneration of dopaminergic neurons in idiopathic PD.

6.3. Material and Methods:

6.3.1. Cell culture and treatment:

RPMI-1640 media with phenol red was used for culturing the cells. The complete media was prepared by adding 10% fetal bovine serum, 1% MEM non-essential amino acids, 1% penicillin/streptomycin, 1% L-glutamine and 1% HEPES to the RPMI-1640 media. All these chemicals and media were purchased from Sigma-Aldrich (St. Louis, MO). Human neuroblastoma SH-SY5Y cells were purchased from ATCC (Manassas, VA) at 23 passages and seeded in flasks (75 cm^2) and grown for 4-5 days until 90% confluence in a 95% air, 5% CO_2 humidified incubator at 37 ºC. Prior to confluence, cells were harvested using 0.25% trypsin (Sigma-Aldrich, St. Louis, MO) and seeded into 96-well microtiter plates or 75 cm^2 flasks. The cells were then allowed to grow at 37°C and 5% CO_2 for 1-2 days prior to treatment.

6.3.2. Preparation of pesticides:

Endosulfan and zineb were obtained from Chem Service (West Chester, PA). Stock solutions of endosulfan and zineb (100 mM) were prepared using 100% dimethylsulfoxide (DMSO). These stock solutions were serially diluted with incomplete media, RPMI-1640, to prepare 4X-working solutions.

6.3.3. DCF-DA assay

Chloromethyl-DCF-DA (2',7'-dichlorofluorescin diacetate (Molecular Probes, Inc., Eugene, Oregon.) was used to monitored intracellular H_2O_2 production. The generation of H_2O_2 is known to convert the non-fluorescent compound DCF-DA to a fluorescent

compound DCF that can be measured by Gemini XPS Microplate Spectrofluorometer (Molecular Devices Corporation, Sunnyvale, California) using an excitation wavelength of 492 nm and emission wavelength of 527 nm. Briefly, Chloromethyl-DCF-DA was dissolved in DMSO at the concentration of 5 mM. SH-SY5Y cells were seeded onto 96-well plates (Corning Costar) and allowed to adhere for at least 24 hrs. The cells were then incubated in Hanks' Balanced Salt Solution (HBSS) containing 5 μM chloromethyl-DCF-DA for 30 minutes at 37^0C in the dark to allow dye loading into the cells. After incubation with dye, excess dye was removed by separation and SH-SY5Y cells were treated with phenol free RPMI containing 0, 50, 100 and 200 μM of zineb or endosulfan separately in order to obtain a dose response for each pesticide exposure. The reaction was initiated by the addition of pesticides into cells and incubated at 37°C up to 20 hrs. A cell sample without DCF-DA was included as a negative control. The generation of peroxides was measured and reported as relative fluorescence intensity.

6.3.4. Hydroethidium assay:

Hydroethidine (HE), a sodium borohydride-reduced derivative of ethidium bromide, was used to detect $O_2^{-\bullet}$. Once $O_2^{-\bullet}$ is generated, it converts hydroethidine to ethidium bromide and increases red fluorescence. The increase in fluorescence can be measured through the Gemini XPS Microplate Spectrofluorometer (Molecular Devices Corporation, Sunnyvale, California) using an excitation wavelength of 510 nm and emission wavelength of 590 nm. SH-SY5Y cells were seeded onto 96-well plates (Corning) and allowed to adhere for at least 24 hrs. Cells were then stained with 5 μM HE for 30 minutes prior to addition of pesticides into cells to initiate the reaction. Following the addition of pesticides, $O_2^{-\bullet}$ generation was measured as relative fluorescence intensity.

6.3.5. Antioxidant enzyme assays:

6.3.5.1. Preparation of cell extracts for enzyme assays:

cells (8×10^6) were incubated for 16 hrs with various concentrations of the test pesticides, pooled into a tube, centrifuged at 300 x g, 4^0C, for 5 min, and the cell pellet was washed once with phosphate buffered saline (PBS) and resuspended in 120 μl of PBS. This

suspension of cells was freeze-thawed twice, and centrifuged at 20,000 x g, 4^0C, for 10 min. The supernatant was collected for various enzyme assays.

6.3.5.2. Superoxide dismutase (SOD):

Total SOD levels in treated and untreated cells were measured on an automated oxidative stress analyzer (OxyScan^{TM}; Oxis Health Products Inc., Portland, OR, USA) using quantitative, colorimetric assay test kits: Bioxytech® SOD-525TM. A 50 µl sample was used for measuring the SOD activity (units/ ml of sample) using the method of Nebot (Nebot *et al.*, 1993). Specific activity of SOD was measured as units/mg protein.

6.3.5.3. Glutathione peroxidase (GPX):

Total GPX levels in treated and untreated cells were measured on an automated oxidative stress analyzer (OxyScanTM; Oxis Health Products Inc., Portland, OR, USA) using quantitative, colorimetric assay test kits: Bioxytech® Oxyscan^{TM} GPX-340TM Kit. GPX catalyzes the reduction of various organic hydroperoxides and H_2O_2 using reduced glutathione (GSH) as donor.

$$2GSH + H_2O_2 \text{ ------> } GSSG + 2H_2O$$

By coupling this reaction with glutathione reductase, the specific activity of this enzyme can be calculated by measuring the consumption of NADH in the reaction spectrophotometrically at 340nm (Paglia and Valentine, 1967). A 50 µl sample was used to measure the GPX activity (units/ ml of sample). Specific activity of GPX was measured as units/mg protein.

6.3.5.4. Catalase (CAT):

Catalase activity was determined by the kinetic assay adopted from Beers and Sizer (Beers and Sizer, 1952). The measurement of catalase activity is based in the quantification of the hydrogen peroxide breakdown; thus, we define one unit of catalase as the amount of enzyme required to decompose 1 µmole of H_2O_2 per minute at 25 °C. The rate of decrease in absorbance at 240 nm was measured on a Shimazu UV-visible spectrophotometer at 25°C. The concentration of H_2O_2 were determined on the basis of a molar extinction coefficient : 43.6 $M^{-1}cm^{-1}$ at 240 nm (Beers and Sizer, 1952)

Units/ml =

$$\frac{\Delta A/\text{min at 240 nm} \times \text{cuvette volume (ml)} \times \text{dilution factor} \times 1\text{ cm lightpath}}{43.6\ M^{-1}\ cm^{-1} \times \text{sample volume used (ml)}}$$

$$\text{Specific activity} = \frac{\text{units/ml}}{\text{mg protein /ml}}.$$

6.3.6. Lipid peroxidation:

Lipid peroxides were detected as malondialdehyde (MDA) reacting with thiobarbituric acid (TBA) (Oxi-Tek, Zeptometric, Buffalo, NY) to form a 1:2 adduct. The MDA-TBA colored complex (TBARS) was measured by spectrofluorometric analysis. Briefly, following 16 hrs incubation with pesticides, cells (2×10^7) were collected by centrifuging at 300 x g, 4^0C, for 5 min, and cell pellet was resuspended with 200 µl of PBS. This suspension of cells was sonicated on ice for three-5 second intervals at setting 7.0 (Fisher Scientific Sonic Dismembrator F550, USA). Whole homogenates was used to quantify lipid peroxides using the TBARS assay. Briefly, cell homogenates or standard (MDA) were mixed with 100 µl of Sodium Dodecyl Sulfate (SDS) and each mixture was added to tube and swirled to mix. Then, 2.5 ml (TBA/Buffer reagent) was added by pouring down the side of each tube. The mixtures were incubated in a boiling water bath for 60 min. Marbles were placed on the tops of tubes during the incubation period to avoid excessive evaporation of the reaction mixture. After cooling the tubes on ice, the reaction mixture was centrifuged at 3000 × g for 15 min. The fluorescence of supernatant was measured with a Gemini XPS Microplate Spectrofluorometer (Molecular Devices Corporation, Sunnyvale, California) with an excitation wavelength of 530 nm and emission wavelength of 550 nm. The concentrations of TBARS were calculated using MDA as a reference standard. The quantities of TBARS were expressed in terms of amount (ρmol) per mg protein.

6.3.7. Caspase-3 activity:

Caspase-3 activity was measured by a colorimetric assay (Thornberry, 1994). The release of p-nitroaniline (pNA) from the substrate ((Ac-DEVD-pNA) upon cleavage by caspase-3 was detected by following the absorbance at 405nm. Briefly, after pesticides treatment for 16 hrs, cells were lysed by freeze-thraw and the resulting supernatants were incubated with the reaction buffer (CaspACE ^{TM} Assay system colorimetric, Promega Corporation, Madison) for 4 hrs at 37°C. The absorbance of pNA was measured at 405 nm against the reagent blank devoid of recombinant caspase-3. The caspase-3 activity was expressed in terms of absorbance at 405 nm (Thornberry, 1994). The assay was run in triplicate and repeated 5 times.

6.3.8. Quantitative analysis of NFkappaB by ELISA:

The DNA-binding capacity of NFkappaB was assayed using the Trans-AM NFkappaB p50 transcription factor assay kit (Active Motif, Carlshad, CA) following the manufacturer's instructions. Quantitative analysis of NFkappaB/p50 was performed using ELISA. For this assay, the nuclear extract of SH-SY5Y cells was prepared using the Nuclear Extraction Kit (Active Motif) according to the manufacturer's protocol. According to the manufacture, this Trans-AM detection ELISA kit is 10-fold more sensitive and 40-fold faster than the electromobility shift assay (EMSA). The assay was done in triplicate and absorbance read at 450 nm with reference taken at 650 nm. The assay was repeated 5 times.

6.3.9. Protein analysis:

Protein concentrations were determined using the Bradford method with bovine serum albumin as a standard (BioRad, Hercules, CA)

6.4. Statistical Analysis:

Analysis of variance (ANOVA) was used with general linear models (GLM) procedure of the SAS system (Version 8.2, SAS Institute Inc., Cary, NC) for statistical analysis of the data. Further statistical analysis for post hoc comparisons was performed using the Tukey-Kramer test. Standard residual plots were used to assess model adequacy. All differences of $p \leq 0.05$ were considered as significant.

6.5. Results:

6.5.1. Effects of pesticide on hydrogen peroxide generation:

Intracellular production of H_2O_2 in SH-SY5Y cells following pesticide exposure was analyzed by spectrofluorometric analysis using DCF-DA dye as previously described (Shen *et al.*, 1996). SH-SY5Y cells were treated with 50, 100, 200 μM of zineb or endosulfan for 1, 6, 8, 20 hrs and analyzed for the accumulation of DCF. As shown in Figs. 6.1a and 6.1b, H_2O_2 generation from SH-SY5Y cells treated with zineb or endosulfan at various concentrations for different time periods show both a dose- and time-dependent responses to chemical exposure. Thus, as shown in these figures, there was an increased H_2O_2 generation in cells as the concentrations endosulfan (Fig. 6.1a) or zineb (Fig. 6.1b) were increased. There was a significant increase in H_2O_2 production observed in cells exposure to as little as 50 μM endosulfan for 20 hrs. Exposure to 100 μM or 200 μM of endosulfan caused a significant increase in this ROS production as early as 1 hrs of exposure to this chemical compared to control ($p \leq 0.05$) (Fig. 6.1a). Cells treated with as little as 50 μM of zineb exhibited a significant increase in endogenous H_2O_2 production in 1 hrs of exposure time ($p \leq 0.05$). Based on these results, all subsequent studies were designed to investigate endosulfan and zineb-mediated oxidative stress in cells exposed to 100 μM each of pesticides individually and in mixtures for 16 hrs. As shown in figure 6.1c, the mixtures of pesticides caused significantly higher levels of production of H_2O_2 ($p \leq 0.05$) compared to individual pesticides and control ($p \leq 0.05$).

6.5.2. Effects of pesticides on superoxide generation:

Superoxide anion ($O_2^{-\bullet}$) production in pesticide-treated SH-SY5Y cells was monitored by spectrofluorometric analysis in combination with hydroethidine (HE) dye. Figures 6.2a and 6.2b show the amount of $O_2^{-\bullet}$ generated as a function of time and concentration of endosulfan and zineb, respectively. As shown in these figures, SH-SY5Y cells treated with as little as 50 μM zineb or endosulfan for15 hrs showed a significant increase in $O_2^{-\bullet}$ production when compared to control ($p \leq 0.05$). Cells exposure to 100 μM or 200 μM endosulfan also caused a significant increase in this radical production as early as 3 hrs ($p \leq 0.05$) (Fig. 6.2a). However, this concentration zineb had little effect when cells were exposed for 3 hrs. Cells treated with 200 μM zineb exhibited a significant increase in superoxide anion production at all time periods (1-20 hrs) tested when compared to control ($p \leq 0.05$). Mixture study was performed with 100 μM endosulfan and 100 μM zineb to examine if there was an additive or synergistic effect. As shown in Fig. 6.2c, the mixtures of pesticides caused significant augmentation of superoxide anion production ($p \leq 0.05$) compared to individual pesticides and control. When the results of solvent controls were subtracted from pesticide exposed cells, the mixtures of endosulfan and zineb were found to cause more than additive effects in superoxide anion generation when compared to that of individual pesticides. Thus, there was an increase in superoxide anion production of ~9% by each of the pesticides and over 57% increase by exposing to mixture of these chemicals compared to control. These data indicate that exposure to multiple pesticides simultaneously can cause more than additive effects in superoxide anion production in neuronal cells.

6.5.3. Effects of pesticides on antioxidant enzyme levels:

Intracellular levels of antioxidant enzymes such as SOD, CAT and GPX in SH-SY5Y cells following exposure to endosulfan or zineb individually and in mixtures for 16 hrs were measured by spectrophotometric analysis. The results are presented in Table 1. As shown in Table 1, SH-SY5Y cells exposed to 100 μM zineb showed a significant decrease in the specific activities of SOD (27%), CAT (35%) and GPX (31%) when

compared to solvent control ($p \leq 0.05$). Cells exposure to 100 μM endosulfan also caused a significant decrease in CAT (37%) activity ($p \leq 0.05$) (Table 1). This dose of endosulfan had no significant effects on SOD and GPX levels. Exposure to mixture of 100 μM each of endosulfan and zineb significantly ($p \leq 0.05$) decreased the activities of SOD (39%), CAT (33%) and GPX (42%) as compared to controls.

6.5.4. Effects of pesticides on lipid peroxide levels:

The levels of MDA, an indicator of lipid peroxidation, in SH-SY5Y cells following exposure to endosulfan or zineb individually and in mixtures for 16 hrs were measured using TBARS assay. Figure 6.3 illustrates cells exposed to 100 μM each of endosulfan and zineb or mixtures of pesticides for 16 hrs exhibited a significant increase on MDA levels. As shown in Fig. 6.3, exposure to individual pesticides or their mixtures caused significantly higher levels of MDA production compared to solvent control. MDA levels in the mixture of pesticides treated cells were found to be significantly higher than individual pesticide treatment ($p \leq 0.05$).

6.5.5. Effects of endosulfan and zineb on caspase-3 activity:

Because ROS has been implicated for activation of caspase-3 (Matsura *et al.*, 1999), we measured for caspase-3 activity in SH-SY5Y cells after 16 hours incubation with 100 μM each of endosulfan and zineb individually or in mixtures. As shown in Fig. 6.4, cells exposed to endosulfan or zineb were found to have significantly higher levels of caspase-3 activity compared to controls ($p \leq 0.05$). Thus, when cells were exposed to endosulfan and zineb individually, the activity of caspase-3 was increased by 60% and 56%, respectively. This was based on results of subtracting the values of controls from pesticides exposed cells. However, mixture of pesticides (100 μM endosulfan + 100 μM zineb) significantly decreased caspase-3 activity to 50% of controls. This decrease of caspase-3 activity will be discussed later.

6.5.6. Effects of endosulfan and zineb on NFkappaB p50 subunit activity:

Several transcription factors are known to be modulated by ROS. To further confirm the cells exposed to pesticides undergoing oxidative stress, we monitored the activity of NFkappaB, a ubiquitous transcript factor, as an indicator of oxidative stress. Expression of NFkappaB was investigated in SH-SY5Y cells following exposure to endosulfan or zineb individually and in mixtures for 16 hrs by ELISA analysis. As shown in Figure 6.5, cells exposed to 100 μM each of endosulfan and zineb individually or in combination caused significant increase in the expression of NFkappaB compared to solvent control ($p \leq 0.05$). However, when cells were exposed to mixture of 100 μM each of endosulfan and zineb, no significant differences were observed in the nuclear translocation of the NFkappaB p50 transcription factor activity as compared to treatment of individual pesticides ($p > 0.05$).

6.6. Discussion:

Accumulated evidence supports that oxidative stress as one of the important pathways leading to neuronal cell death in Parkinson's disease (Jenner, 1991; Jenner *et al.*, 1992; Gotz *et al.*, 1994; Owen *et al.*, 1996; Christen, 2000; Rottkamp *et al.*, 2000; Zhang *et al.*, 2000; Pratico *et al.*, 2002; Zafrilla *et al.*, 2006). Known neurotoxic agents, such as MPTP, paraquat, rotenone have been shown to induce apoptosis through a cellular signaling mechanisms (Huang *et al.*, 1997; Li *et al.*, 2003; Shimoke *et al.*, 2003; McCarthy *et al.*, 2004; Peng *et al.*, 2004; Przedborski *et al.*, 2004). In the present study, we have shown that neuronal cells (human neuroblastoma SH-SY5Y) exposed to individual pesticides (endosulfan and zineb) increased the production of hydrogen peroxide as well as superoxide anion in a dose-dependent manner. The mixtures of pesticides caused more than additive effects in producing of superoxide anion. Furthermore, cells treated with pesticides showed decreased levels of SOD GPX, and CAT. Pesticides also induced lipid peroxides (thiobarbituric acid reactive products) formation in SH-SY5Y cells. These results indicate that cytotoxic action of these pesticides to SH-SY5Y cells is mediated, at least in part, through an oxyradical mechanism involving over production of ROS and inactivation of certain key antioxidant enzymes such as SOD, GPX, and CAT. Both events would lead to oxidative stress in the pesticide-exposed cells. The increased activity of NFkappaB in the nucleus seems to be a

consequence of oxidative stress. Because SH-SY5Y cells are human catecholaminergic neuroblastoma cell line which has been proposed as suitable *in vitro* model of human dopaminergic neurons (Pahlman *et al.*, 1990), the results of this study provide an *in vitro* evidence that oxidative stress is involved in pesticide-induced neuron cell death.

Hydrogen peroxide can induce both apoptotic and necrotic forms of cell death (Lennon *et al.*, 1991; Escargueil-Blanc *et al.*, 1994). High concentration of hydrogen peroxide is known to cause rapid cell death with no evidence of apoptosis. Moderate concentrations of hydrogen peroxide induce DNA cleavage and is associate with morphologic evidence of apoptosis (Gardner *et al.*, 1997). Hydrogen peroxide also was shown to induce neuronal cell death with more or less necrotic and/or apoptotic characteristics depending on its concentration (Cole and Perez-Polo, 2002; Valencia and Moran, 2004). Our present study showed that hydrogen peroxide generation from SH-SY5Y cells treated with zineb or endosulfan at various concentrations for different time periods (1-20 hrs) show a dose- and time dependent response to chemical treatment. Mixtures of pesticides augmented hydrogen peroxide generation ($P \leq 0.05$) compared to individual pesticide treatment (Fig. 6.3). In the earlier studies, we have demonstrated that exposure of endosulfan and zineb at similar doses causes both apoptosis and necrosis and in higher dose (>100μM) or mixtures predominantly necrosis in SH-SY5Y cells. The accumulation of hydrogen peroxide production as demonstrated here seems to correlate with increased number of apoptotic and necrotic cells reported earlier (Jia and Misra, 2006b). It is interesting to note that cells concurrently exposed to endosulfan and zineb generated more superoxide anion than the total amount of these radicals cumulatively generated by individual pesticides. Although superoxide anion lacks the ability to penetrate lipid membranes, it can react with hydrogen peroxide by Fe^{3+} to generate the more reactive hydroxyl radicals to damage lipid, DNA and protein ((Feierman *et al.*, 1985; Jackson *et al.*, 1989). The present study did not delineate the exact nature of ROS causing apoptosis/necrosis in neuronal cells. However, it was of interest to note that there was indeed augmentation of ROS production by exposing cells to these pesticides.

To minimize oxidative damage to cellular components, cells have adaptive mechanisms to increase antioxidant defenses (Zhou, 2001). Antioxidant enzymes including isoforms of SOD, GPX, and CAT, have been found to be inducible in transgenic mice in response to certain pesticide exposure oxidative stress (Thiruchelvam *et al.*, 2005). The antioxidant enzyme systems that were monitored in the present study are SOD, CAT, and GPX. These enzymes are known to protect against the ROS produced during the metabolism of various drugs and toxicants. Results of this study shows that SH-SY5Y cells exposed to 100 μM zineb or mixture with 100 μM endosulfan showed a significant decrease in the specific activities of SOD, GPX and CAT when compared to solvent control ($p \leq 0.05$). These results are consistent with a recent report of decrease in both SOD and GPX in lung in endosulfan-exposed rats (Bebe and Panemangalore, 2003). However, in the same report the authors noted increased liver GPX in the endosulfan-exposed rats. These results suggest that different cells may show differential GPX enzyme activity following endosulfan exposure. Reduced levels of GPX and CAT activity has also been reported in fish model following endosulfan exposure (Pandey *et al.*, 2001; Dorval and Hontela, 2003; Dorval *et al.*, 2003). Inhibition of Cu,Zn SOD activity was observed by N-N'-diethyl-dithiocarbamate (DDC) in human umbilical vein endothelial cells (Maitre *et al.*, 1993). Hence, our data on lowered levels of antioxidant enzymes are inconsistent with the above reports.

Oxidative stress occurs when the rate at which the ROS are generated exceeds the capacity of the cell to be removed by antioxidants. Because the above antioxidant enzymes are part of vital defense mechanism against ROS-induced tissue damage, the production of ROS and reduction of antioxidant enzymes in pesticide-exposed cells could cause oxidative stress including enzyme inactivation, protein degradation, DNA damage and lipid peroxidation leading to spontaneous apoptosis (Perandones *et al.*, 1993; Reiter *et al.*, 2001; Djordjevic, 2004). Lipid peroxidation is one of the major outcomes of free radical-mediated injury that directly damages membranes and contribute to DNA damage (Marnett, 1999a; Marnett, 1999b; Mutlu-Turkoglu *et al.*, 2003). Accumulation of

MDA is regarded as good biomarkers of age and of the degree of oxidative stress (Requena *et al.*, 1996; Kedziora-Kornatowska *et al.*, 2000). Results of this studies showed that cells exposed to 100 μM endosulfan, zineb or mixtures for 16 hours had a significant increase in MDA level compared to solvent control ($p \leq 0.05$). There are number of reports that show both endosulfan and metal-EBDC pesticides induce lipid peroxidation in various cell systems both *in vivo and in vitro* (Agarwal *et al.*, 1978; Narayan *et al.*, 1985; Singh and Pandey, 1989; Hincal *et al.*, 1995; Ayub *et al.*, 2003; Thiruchelvam *et al.*, 2005; Patel *et al.*, 2006). Hence, our data are in accord with this above report that both endosulfan and zineb are potent neurotoxicants and exposure to pesticides would lead to oxidative stress in neuronal cells, such as SH-SY5Y cells.

To determine if the caspase pathways are involved in endosulfan and zineb-induced apoptosis in SH-SY5Y cells, we examined the caspase-3 activity in these cells. Caspase-3 appears to play an important role in developmentally regulated cell death in the brain (de Bilbao *et al.*, 1999; Shimohama *et al.*, 2001; Yakovlev *et al.*, 2001). Activation of caspase-3 is a necessary step for neuronal apoptosis after exposure to neurotoxins such as MPP^+ (Fall and Bennett, 1999). Activation of caspase-3 has been implicated in PD and AD (Stadelmann *et al.*, 1999; Hartmann *et al.*, 2000; Jellinger, 2000; Jordan *et al.*, 2000; Rideout and Stefanis, 2001) This suggests that caspase-3 may act as a common downstream effectors of cell death in many neurodegenerative disorders. Results of this study showed exposure to 100μM endosulfan or zineb for 16 hours had a significant increase in caspase-3 activities. However, mixture of endosulfan and zineb at 100 μM each decreased caspase-3 activity indicating that necrosis replaces apoptosis as the dominant form of cell death in SH-SY5Y cells under these conditions. Most, if not all, chemicals tested seem to be capable of causing apoptosis at low doses and necrosis at higher doses (Wyllie, 1987; Lennon *et al.*, 1991; Raffray and Cohen, 1991). Our earlier study demonstrated high necrotic cell death in exposing cells to mixtures of chemicals, the lower level of caspase-3 activity observed here by exposing cells to mixtures of chemicals can be contribute to predominately necrotic process.

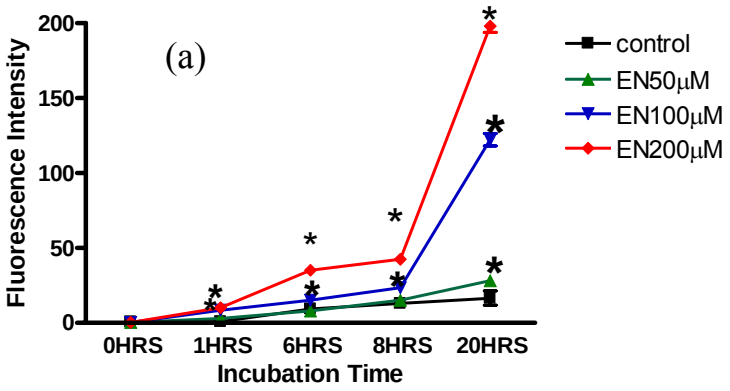
ROS was also thought to serve as common messengers in activation of NFkappaB (Bowie and O'Neill, 2000; Foncea *et al.*, 2000) and NFkappaB is suggested to be primarily an oxidative stress-responsive transcription factor (Schreck *et al.*, 1992a). Activation of NFkappaB has been implicated as an important pathway in the regulation of many genes that code for mediators of the immune and acute phase of inflammatory response (Wooten, 1999; Giuliani *et al.*, 2001; Feinman *et al.*, 2004; Dolcet *et al.*, 2005). The inactive form of NFkappaB is present in the cytoplasm and it is a heterodimer of 50 kDa DNA-binding (p50) and 65 kDa-binding (p65) subunits bound with an inhibitory (IkB) monomers. The activation of NFkappaB in response to oxidative stress involves release of the inhibitory subunit (IkB) from a cytoplasmic complex with heterodimer and translocate to the nucleus to bind to their DNA sequences (Schulze-Osthoff *et al.*, 1995; Muller *et al.*, 1997; Zhou *et al.*, 2001). The p50 homodimers are the most common dimmers found in NFkappaB signaling pathway. In this study, exposure to 100 μM endosulfan, zineb or mixtures for 16 hours significantly increase in expression of NFkappaB in the nucleus when compared to solvent control ($p \leq 0.05$; Fig. 6.5). Hence, our results confirmed that the oxidative stress induced by pesticide exposure to cells contribute, at least in part, to the activation of NFkappaB. The mechanism(s) by which NFkappaB activation induces cell death is not well understood. JNK/SAPK signaling pathway has been suggested as one of the apoptosis inducing pathways (Cahill *et al.*, 1996; Johnson *et al.*, 1996). Blocking activation of JNK/SAPK protected against PC12 dopaminergic neuronal cell apoptosis (Xia *et al.*, 1995). NFkappaB may also function through the activation of various kinases including MAPK and Bcl-2 induced pathway (Jang and Surh, 2004; Lee *et al.*, 2004; Shin *et al.*, 2004b). Bcl-2 families such as Bax, Bcl-xl and Bad was affected following exposure to paraquat and maneb in transgenic mice (Thiruchelvam *et al.*, 2005). MAPK activity was decreased in neuronal stem cells following endosulfan exposure (Kang *et al.*, 2001). So, examination of Bcl-2 and MAPK activity might shed some light to fully understand pesticides induced neuronal cell death.

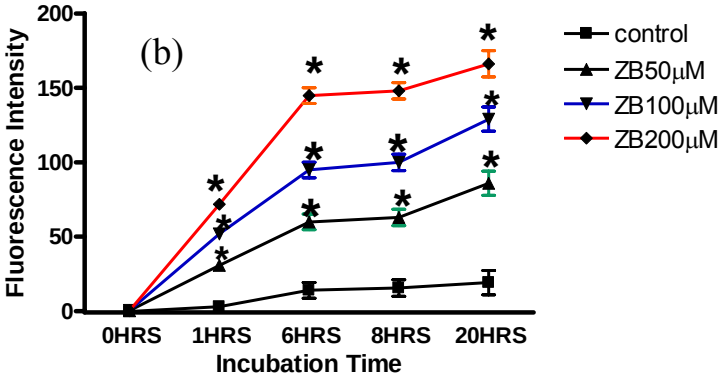
In summary, the results of present study demonstrated that neuronal cells exposed to endosulfan and zineb individually or in mixture increase the production of hydrogen peroxide as well as superoxide anion, decrease SOD GPX, and CAT enzyme levels and

increase lipid peroxide levels. It was also demonstrated exposure to these pesticides also altered caspase-3 activity in the cells and increased the NFkappaB activity in the nucleus. We believe this is the first report demonstrating pesticide-induced oxidative stress that may play a key mediator in neuronal activation of redox-sensitive transcription factor NFkappaB and caspase-3 in causing cell death and holds tremendous implication for the derivation of risk assessment guidelines for human exposure.

6.7. Acknowledgements:

The authors gratefully acknowledge Dr. Korinn Saker for allowing me to use OxyScan equipment in her lab and Mr. Jimmy Martin for his technical assistance in this equipment use and Dan Ward for statistical analysis. This work was supported, in part, by the Edward Via Virginia College of Osteopathic Medicine.


(a)
Fluorescence Intensity
200
150
100
50
0
0HRS
1HRS
6HRS
8HRS
20HRS
Incubation Time
control
EN50μM
EN100μM
EN200μM


(b)
Fluorescence Intensity
200
150
100
50
0
0HRS
1HRS
6HRS
8HRS
20HRS
Incubation Time
control
ZB50μM
ZB100μM
ZB200μM

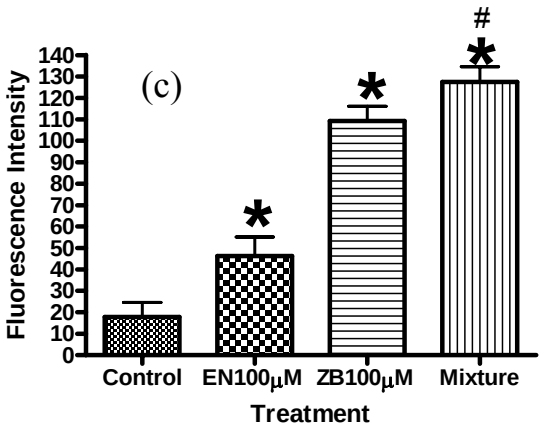


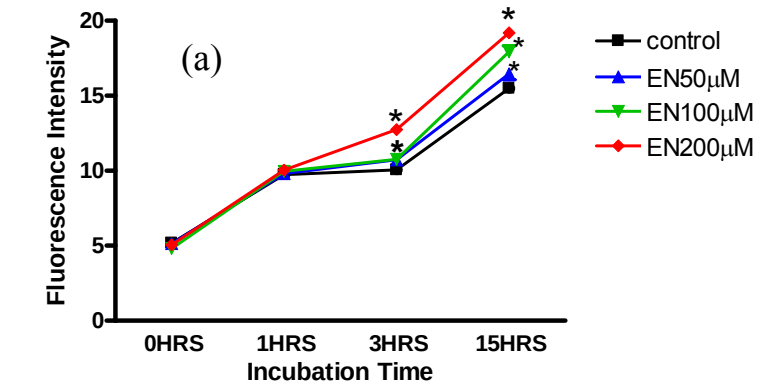
Figure 6.1: Intracellular hydrogen peroxide production in SH-SY5Y cells following exposure to endosulfan (EN) and/or zineb (ZB).

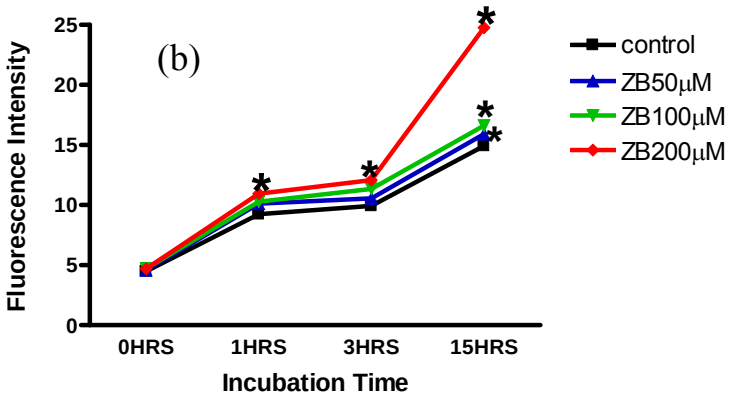
SH-SY5Y (3×10^4) cells were seeded in 96-well plates and incubated with 5 μM of DCF-DA for 30 min before indicated concentration of pesticides was added and further incubated for various time periods. Generation of DCF was measured by a spectrofluometric analysis at excitation wave length 492 nm and emission wave length 527 nm. Turkey's HSD post hoc test for each treatment indicated difference ($p \leq 0.05$): * compared to control; # = compared to EN. The data are presented as the mean ± SEM. N = 8.

(a) = cells exposed to indicated concentrations of EN

(b) = cells exposed to indicated concentrations of ZB

(c) cells exposed to EN (100 μM) or ZB (100 μM) individually or in mixture for 16 hrs


(a)
Fluorescence Intensity
20
15
10
5
0
0HRS
1HRS
3HRS
15HRS
Incubation Time
control
EN50μM
EN100μM
EN200μM
*


(b)
Fluorescence Intensity
25
20
15
10
5
0
0HRS
1HRS
3HRS
15HRS
Incubation Time
control
ZB50μM
ZB100μM
ZB200μM
*

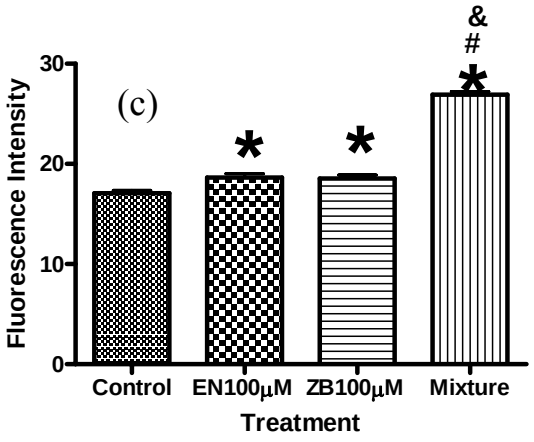


Figure 6.2: Intracellular superoxide anion production in SH-SY5Y cells following exposure to EN and/or ZB.

SH-SY5Y (3×10^4) cells were seeded on 96-well plates and incubated with 5 μM of hydroethidium (HE) for 30 min before indicated concentration of pesticides was added and further incubated for various time periods. Generation of ethidium bromide was measured by a spectrofluometric analysis at excitation wave length 510 nm and emission wave length 590 nm. Turkey's HSD post hoc test for each treatment indicated difference ($p \leq 0.05$): * compared to control; # = compared to EN; & = compared to ZB. The data are presented as the mean ± SEM. N = 8.

a) = cells exposed to indicated concentrations of EN
b) = cells exposed to indicated concentrations of ZB
c) cells exposed to EN (100 μM) or ZB (100 μM) individually or in mixture for 16 hrs

Table 6.1: Change in superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) in SH-SY5Y cells following exposure to EN, ZB and their mixtures.

Treatment	SOD *(U/mg protein)*	Catalase *(mU/mg protein)*	GPX *(mU/mg protein)*
Control	5.2± 0.4	2.7±0.2	63.1± 2.9
EN 100 µM	4.6± 0.4	1.7± 0.2[a]	57.7± 3.2
ZB 100 µM	3.8± 0.4[a]	1.7± 0.2[a]	43.6± 3.2[a]
EN + ZB	3.2± 0.4[ab]	1.3± 0.2[a]	36.4± 3.2[ab]

Specific Activity of SOD, catalase and GPX in SH-SY5Y following exposure to endosulfan, zineb and their mixture for 16 hrs. The specific activities of enzyme in cells homogenate treated with pesticides were assayed as described in the Materials and Methods. Turkey's HSD post hoc test for each treatment indicated difference ($p \leq 0.05$): a = data compared to control; b = compared to EN. N = 5. Results are expressed as the mean ± SEM. Treatments with same letters are not different from each other ($p \leq 0.05$).

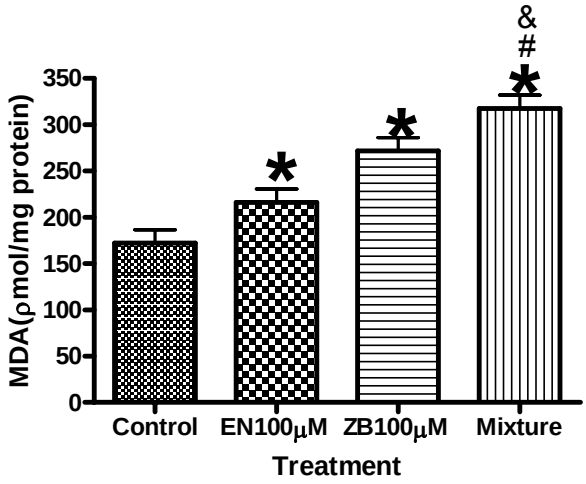


Figure 6.3: Production of lipid peroxides in SH-SY5Y following exposure to endosulfan, zineb and their mixture for 16 hrs.

Lipid peroxidation was evaluated using a TBARS assay kit as described in the Materials and Methods. TBARS are expressed as ρmol malondialdehyde (MDA)/mg protein. Turkey's HSD post hoc test for each treatment indicated difference ($p \leq 0.05$): * compared to control; # = compared to EN; & = compared to ZB. N = 5. Results are expressed as the mean ± SEM.

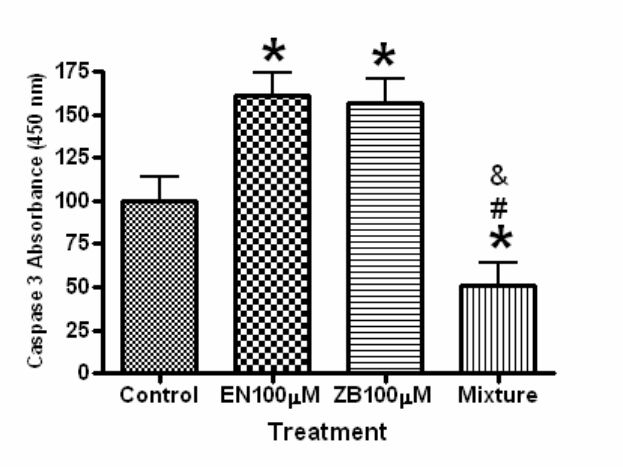


Figure 6.4: Caspase-3 activity in SH-SY5Y cells in SH-SY5Y following exposure to endosulfan, zineb and their mixture for 16 hrs.

Caspase-3 activity was measured by a colorimetric assay as described in the Materials and Methods. The caspase-3 activity was expressed in terms of absorbance at 405 nm. Turkey's HSD post hoc test for each treatment indicated difference ($p \leq 0.05$): * compared to control; # = compared to EN; & = compared to ZB. N = 5. Results are expressed as the mean ± SEM.

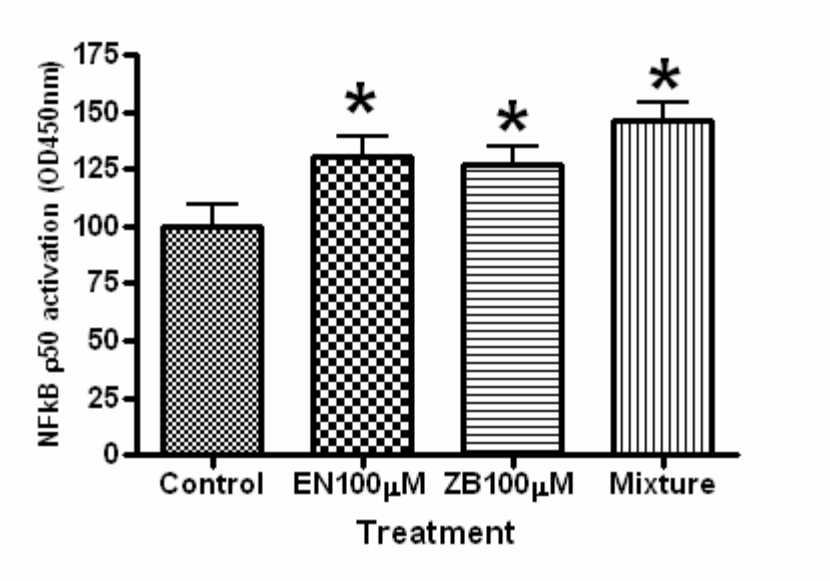


Figure 6.5: NFkappaB p50 subunit expression in pesticide exposed SH-SY5Y following exposure to endosulfan, zineb and their mixture for 16 hrs.

NFkappaB activation was measured using TransAM NFkappaB p50 kit as described in the Materials and Methods. Turkey's HSD post hoc test for each treatment indicated difference ($p \leq 0.05$): * compared to control N = 5. Results are expressed as the mean ± SEM.

6.8. Cited literature:

Agrawal, A. K., Anand, M., Zaidi, N. F., and Seth, P. K. (1983). Involvement of serotonergic receptors in endosulfan neurotoxicity. *Biochem Pharmacol* **32**, 3591-3593.

Ahlbom, J., Fredriksson, A., and Eriksson, P. (1994). Neonatal exposure to a type-I pyrethroid (bioallethrin) induces dose-response changes in brain muscarinic receptors and behaviour in neonatal and adult mice. *Brain Res* **645**, 318-324.

Aleksandrowicz, D. R. (1979). Endosulfan poisoning and chronic brain syndrome. *Arch Toxicol* **43**, 65-68.

Anand, M., Agrawal, A. K., Gopal, K., Sur, R. N., and Seth, P. K. (1986). Endosulfan and cholinergic (muscarinic) transmission: effect on electroencephalograms and [3H]quinuclidinyl benzilate in pigeon brain. *Environ Res* **40**, 421-426.

Andersen, J. K. (2003). Paraquat and iron exposure as possible synergistic environmental risk factors in Parkinson's disease. *Neurotox Res* **5**, 307-313.

Benke, G. M., and Murphy, S. D. (1975). The influence of age on the toxicity and metabolism of methyl parathion and parathion in male and female rats. *Toxicol Appl Pharmacol* **31**, 254-269.

Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., and Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* **3**, 1301-1306.

Butterfield, P. G., Valanis, B. G., Spencer, P. S., Lindeman, C. A., and Nutt, J. G. (1993). Environmental antecedents of young-onset Parkinson's disease. *Neurology* **43**, 1150-1158.

Buttke, T. M., and Sandstrom, P. A. (1994). Oxidative stress as a mediator of apoptosis. *Immunol Today* **15**, 7-10.

Campbell, B. C., McLean, C. A., Culvenor, J. G., Gai, W. P., Blumbergs, P. C., Jakala, P., Beyreuther, K., Masters, C. L., and Li, Q. X. (2001). The solubility of alpha-synuclein in multiple system atrophy differs from that of dementia with Lewy bodies and Parkinson's disease. *J Neurochem* **76**, 87-96.

Carr, R. L., Chambers, H. W., Guarisco, J. A., Richardson, J. R., Tang, J., and Chambers, J. E. (2001). Effects of repeated oral postnatal exposure to chlorpyrifos on open-field behavior in juvenile rats. *Toxicol Sci* **59**, 260-267.

Chiba, K., Trevor, A., and Castagnoli, N., Jr. (1984). Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. *Biochem Biophys Res Commun* **120**, 574-578.

Cook, J. E. (1991). Correlated activity in the CNS: a role on every timescale? *Trends Neurosci* **14**, 397-401.

Correll, L., and Ehrich, M. (1991). A microassay method for neurotoxic esterase determinations. *Fundam Appl Toxicol* **16**, 110-116.

Cory-Slechta, D. A., Thiruchelvam, M., Richfield, E. K., Barlow, B. K., and Brooks, A. I. (2005). Developmental pesticide exposures and the Parkinson's disease phenotype. *Birth Defects Res A Clin Mol Teratol* **73**, 136-139.

el-Agnaf, O. M., and Irvine, G. B. (2002). Aggregation and neurotoxicity of alpha-synuclein and related peptides. *Biochem Soc Trans* **30**, 559-565.

Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Feather-Stone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**, 88-95.

Eriksson, P., Ahlbom, J., and Fredriksson, A. (1992). Exposure to DDT during a defined period in neonatal life induces permanent changes in brain muscarinic receptors and behaviour in adult mice. *Brain Res* **582**, 277-281.

Eriksson, P., Nilsson-Hakansson, L., Nordberg, A., Aspberg, A., and Fredriksson, A. (1990). Neonatal exposure to DDT and its fatty acid conjugate: effects on cholinergic and behavioural variables in the adult mouse. *Neurotoxicology* **11**, 345-354.

Eriksson, P., and Nordberg, A. (1990). Effects of two pyrethroids, bioallethrin and deltamethrin, on subpopulations of muscarinic and nicotinic receptors in the neonatal mouse brain. *Toxicol Appl Pharmacol* **102**, 456-463.

Fasano, M., Giraudo, S., Coha, S., Bergamasco, B., and Lopiano, L. (2003). Residual substantia nigra neuromelanin in Parkinson's disease is cross-linked to alpha-synuclein. *Neurochem Int* **42**, 603-606.

Ferraz, H. B., Bertolucci, P. H., Pereira, J. S., Lima, J. G., and Andrade, L. A. (1988). Chronic exposure to the fungicide maneb may produce symptoms and signs of CNS manganese intoxication. *Neurology* **38**, 550-553.

Giorgi, O., De Montis, G., Porceddu, M. L., Mele, S., Calderini, G., Toffano, G., and Biggio, G. (1987). Developmental and age-related changes in D1-dopamine receptors and dopamine content in the rat striatum. *Brain Res* **432**, 283-290.

Gorell, J. M., Johnson, C. C., Rybicki, B. A., Peterson, E. L., and Richardson, R. J. (1998). The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. *Neurology* **50**, 1346-1350.

Gupta, P. K. (1976). Endosulfan-induced neurotoxicity in rats and mice. *Bull Environ Contam Toxicol* **15**, 708-713.

Harbison, R. D. (1975). Comparative toxicity of some selected pesticides in neonatal and adult rats. *Toxicol Appl Pharmacol* **32**, 443-446.

Hardy, J. (2003). The relationship between Lewy body disease, Parkinson's disease, and Alzheimer's disease. *Ann N Y Acad Sci* **991**, 167-170.

Hashimoto, M., and Masliah, E. (1999). Alpha-synuclein in Lewy body disease and Alzheimer's disease. *Brain Pathol* **9**, 707-720.

Hedera, P., Lerner, A. J., Castellani, R., and Friedland, R. P. (1995). Concurrence of Alzheimer's disease, Parkinson's disease, diffuse Lewy body disease, and amyotrophic lateral sclerosis. *J Neurol Sci* **128**, 219-224.

Hodgson, E., and Levi, P. E. (1996). Pesticides: an important but underused model for the environmental health sciences. *Environ Health Perspect* **104 Suppl 1**, 97-106.

IOM (2003). In *Institute of Medicine (IOM), Gulf War and Health: Volume 2, Insecticides and Solvents* pp. 412-418. The National Academics Press, Washington, DC.

Iyaniwura, T. T. (1990). Mammalian toxicity and combined exposure to pesticides. *Vet Hum Toxicol* **32**, 58-62.

Kackar, R., Srivastava, M. K., and Raizada, R. B. (1999). Assessment of toxicological effects of mancozeb in male rats after chronic exposure. *Indian J Exp Biol* **37**, 553-559.

Kalil, R. E. (1989). Synapse formation in the developing brain. *Sci Am* **261**, 76-79, 82-75.
Konno, N. (2003). [Neurotoxicity of organophosphorus and dithiocarbamate compounds]. *Nippon Eiseigaku Zasshi* **57**, 645-654.
Lakshmana, M. K., and Raju, T. R. (1994). Endosulfan induces small but significant changes in the levels of noradrenaline, dopamine and serotonin in the developing rat brain and deficits in the operant learning performance. *Toxicology* **91**, 139-150.
Langston, J. W., Ballard, P., Tetrud, J. W., and Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**, 979-980.
Lee, F. J., Liu, F., Pristupa, Z. B., and Niznik, H. B. (2001). Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. *Faseb J* **15**, 916-926.
Lipton, S. A., and Kater, S. B. (1989). Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *Trends Neurosci* **12**, 265-270.
Lotharius, J., and Brundin, P. (2002). Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. *Nat Rev Neurosci* **3**, 932-942.
Manning-Bog, A. B., McCormack, A. L., Li, J., Uversky, V. N., Fink, A. L., and Di Monte, D. A. (2002). The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. *J Biol Chem* **277**, 1641-1644.
Marchi, M., Caviglia, A., Paudice, P., and Raiteri, M. (1983). Calcium-dependent [3H]acetylcholine release and muscarinic autoreceptors in rat cortical synaptosomes during development. *Neurochem Res* **8**, 621-628.
Mattson, M. P., Dou, P., and Kater, S. B. (1988). Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *J Neurosci* **8**, 2087-2100.
McGrew, D. M., Irwin, I., and Langston, J. W. (2000). Ethylenebisdithiocarbamate enhances MPTP-induced striatal dopamine depletion in mice. *Neurotoxicology* **21**, 309-312.
Mendoza, C. E. (1976). Toxicity and effects of malathion on esterases of suckling albino rats. *Toxicol Appl Pharmacol* **35**, 229-238.
Moser, V. C., Shafer, T. J., Ward, T. R., Meacham, C. A., Harris, M. W., and Chapin, R. E. (2001). Neurotoxicological outcomes of perinatal heptachlor exposure in the rat. *Toxicol Sci* **60**, 315-326.
Nakai, K., and Satoh, H. (2002). Developmental neurotoxicity following prenatal exposures to methylmercury and PCBs in humans from epidemiological studies. *Tohoku J Exp Med* **196**, 89-98.
Perez, R. G., Waymire, J. C., Lin, E., Liu, J. J., Guo, F., and Zigmond, M. J. (2002). A role for alpha-synuclein in the regulation of dopamine biosynthesis. *J Neurosci* **22**, 3090-3099.
Rajput, A. H., and Uitti, R. J. (1987). Paraquat and Parkinson's disease. *Neurology* **37**, 1820-1821.
Ritz, B., Yu, F., Chapa, G., and Fruin, S. (2000). Effect of air pollution on preterm birth among children born in Southern California between 1989 and 1993. *Epidemiology* **11**, 502-511.
Rivera, S., Rosa, R., Martinez, E., Sunol, C., Serrano, M. T., Vendrell, M., Rodriguez-Farre, E., and Sanfeliu, C. (1998). Behavioral and monoaminergic changes after lindane exposure in developing rats. *Neurotoxicol Teratol* **20**, 155-160.

Rivera, S., Sanfeliu, C., and Rodriguez-Farre, E. (1990). Behavioral changes induced in developing rats by an early postnatal exposure to lindane. *Neurotoxicol Teratol* **12**, 591-595.

Sanchez-Ramos, J. R., Hefti, F., and Weiner, W. J. (1987). Paraquat and Parkinson's disease. *Neurology* **37**, 728.

Savolainen, K., and Hervonen, H. (1985). Dithiocarbamate fungicides decrease histochemical reactivity of cholinesterases in the gut wall of the rat. *Arch Toxicol Suppl* **8**, 272-276.

Seaton, T. A., Cooper, J. M., and Schapira, A. H. (1997). Free radical scavengers protect dopaminergic cell lines from apoptosis induced by complex I inhibitors. *Brain Res* **777**, 110-118.

Semchuk, K. M., Love, E. J., and Lee, R. G. (1992). Parkinson's disease and exposure to agricultural work and pesticide chemicals. *Neurology* **42**, 1328-1335.

Seth, P. K., Saidi, N. F., Agrawal, A. K., and Anand, M. (1986). Neurotoxicity of endosulfan in young and adult rats. *Neurotoxicology* **7**, 623-635.

Shafer, T. J., Meyer, D. A., and Crofton, K. M. (2005). Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. *Environ Health Perspect* **113**, 123-136.

Shatz, C. J. (1990). Impulse activity and the patterning of connections during CNS development. *Neuron* **5**, 745-756.

Shin, E. J., Jhoo, J. H., Kim, W. K., Jhoo, W. K., Lee, C., Jung, B. D., and Kim, H. C. (2004). Protection against kainate neurotoxicity by pyrrolidine dithiocarbamate. *Clin Exp Pharmacol Physiol* **31**, 320-326.

Sidhu, A., Wersinger, C., and Vernier, P. (2004). alpha-Synuclein regulation of the dopaminergic transporter: a possible role in the pathogenesis of Parkinson's disease. *FEBS Lett* **565**, 1-5.

Simmons, J. E. (1995). Chemical mixtures: challenge for toxicology and risk assessment. *Toxicology* **105**, 111-119.

Slater, A. F., Nobel, C. S., and Orrenius, S. (1995). The role of intracellular oxidants in apoptosis. *Biochim Biophys Acta* **1271**, 59-62.

Surgucheva, I., Park, B. C., Yue, B. Y., Tomarev, S., and Surguchov, A. (2005). Interaction of myocilin with gamma-synuclein affects its secretion and aggregation. *Cell Mol Neurobiol* **25**, 1009-1033.

Takahashi, R. N., Rogerio, R., and Zanin, M. (1989). Maneb enhances MPTP neurotoxicity in mice. *Res Commun Chem Pathol Pharmacol* **66**, 167-170.

Thiruchelvam, M., Brockel, B. J., Richfield, E. K., Baggs, R. B., and Cory-Slechta, D. A. (2000). Potentiated and preferential effects of combined paraquat and maneb on nigrostriatal dopamine systems: environmental risk factors for Parkinson's disease? *Brain Res* **873**, 225-234.

Thiruchelvam, M., McCormack, A., Richfield, E. K., Baggs, R. B., Tank, A. W., Di Monte, D. A., and Cory-Slechta, D. A. (2003). Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. *Eur J Neurosci* **18**, 589-600.

Thiruchelvam, M., Richfield, E. K., Goodman, B. M., Baggs, R. B., and Cory-Slechta, D. A. (2002). Developmental exposure to the pesticides paraquat and maneb and the Parkinson's disease phenotype. *Neurotoxicology* **23**, 621-633.

Tilson, H. A. (1998). Developmental neurotoxicology of endocrine disruptors and pesticides: identification of information gaps and research needs. *Environ Health Perspect* **106 Suppl 3**, 807-811.

Tilson, H. A. (2000). Neurotoxicology risk assessment guidelines: developmental neurotoxicology. *Neurotoxicology* **21**, 189-194.

Tolwani, R. J., Jakowec, M. W., Petzinger, G. M., Green, S., and Waggie, K. (1999). Experimental models of Parkinson's disease: insights from many models. *Lab Anim Sci* **49**, 363-371.

Voorn, P., Kalsbeek, A., Jorritsma-Byham, B., and Groenewegen, H. J. (1988). The pre- and postnatal development of the dopaminergic cell groups in the ventral mesencephalon and the dopaminergic innervation of the striatum of the rat. *Neuroscience* **25**, 857-887.

Zaidi, N. F., Agrawal, A. K., Anand, M., and Seth, P. K. (1985). Neonatal endosulfan neurotoxicity: behavioral and biochemical changes in rat pups. *Neurobehav Toxicol Teratol* **7**, 439-442.

Zang, L. Y., and Misra, H. P. (1993). Generation of reactive oxygen species during the monoamine oxidase-catalyzed oxidation of the neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Biol Chem* **268**, 16504-16512.

Zhang, J., Fitsanakis, V. A., Gu, G., Jing, D., Ao, M., Amarnath, V., and Montine, T. J. (2003). Manganese ethylene-bis-dithiocarbamate and selective dopaminergic neurodegeneration in rat: a link through mitochondrial dysfunction. *J Neurochem* **84**, 336-346.

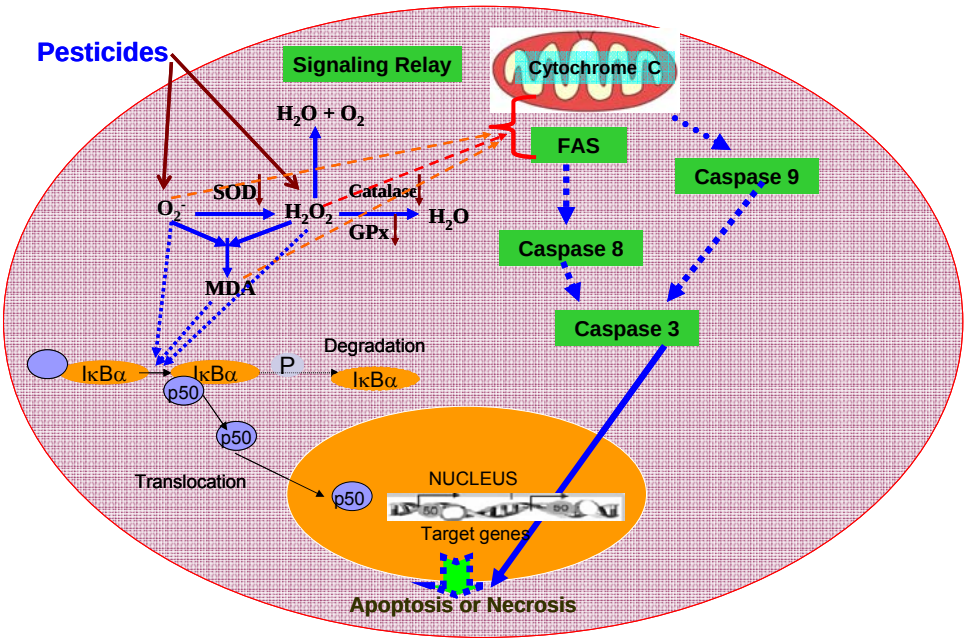
Chapter 7: General Discussion:

The present study is the first to demonstrate the effects of endosulfan, zineb and their mixtures on the dopaminergic and cholinergic systems in C57BL/6 mice. The *in vitro* studies using SH-SY5Y human neuroblastoma cells improved our understanding on the role of ROS and oxidative stress in the mechanisms by which concurrent exposure to theses pesticides could cause neuronal cell death (apoptosis and necrosis).

Studies have shown that exposure to a variety of toxic chemicals in the environment can affect initial growth and development. The potential for adverse effects of pesticide exposures to infants and children is of significant concern (Kimmel and Makris, 2001; Dietert *et al.*, 2002). The parameters of fetal, infant, and childhood growth may be predictors of disease in later life (Osmond and Barker, 2000). Exposure to certain pesticides during childhood could later affect the brain, whose immature development could make these children more susceptible to neurotoxicity later in life. Our *in vivo* studies using C57BL/6 mice demonstrated that exposure to endosulfan, zineb and their mixtures at juvenile age (postnatal days 5 to 19) and re-exposed at 8 months of age enhances the vulnerability to subsequent neurotoxic challenges occurring later in life suggesting that a silent neurotoxicity produced by developmental exposure to these pesticides can be unmasked by challenges later in life.

The SH-SY5Y cell lines have been used extensively as *in vitro* cellular convenient model for studying neurotoxicity of chemicals. The human neuroblastoma clone SH-SY5Y was derived from a human sympathetic ganglion (Ross and Biedler, 1985). It is generally classified as dopaminergic neurons and is known to retain catecholaminergic phenotype (Pahlman *et al.*, 1984). Using this model, we identified the mechanism of cells death following exposure to these pesticides. As presented in a schematic diagram (Scheme.1), the SH-SY5Y cells treated with both pesticides could enhance the generation of superoxide anion and hydrogen peroxide. This was observed when cells were exposed to pesticides individually or in combination and ROS generation was found to be dependent on the dose of pesticides used as well as time of exposure. Lipid peroxide formation, an indicator of

oxidative stress, was also evident in the cells exposed to these pesticides. Moreover, cells exposed to these pesticides were found to increase the expression of NFkappaB activity in nucleus. As NFkappaB is suggested to be primarily an oxidative stress-responsive transcription factor (Schreck *et al.*, 1992a) and activation of NFkappaB has been implicated as an important pathway in the regulation of many genes that code for mediators of the immune and acute phase of inflammatory response (Wooten, 1999; Giuliani *et al.*, 2001; Feinman *et al.*, 2004; Dolcet *et al.*, 2005), the role of oxidative stress in pesticide-induced cells death was further investigated. NFkappaB has also been reported to be activated by triggers of necrotic cell death (Li *et al.*, 1997) and plays a key role the programmed cell death (Guerrini *et al.*, 1995). The characteristic of cell death in SH-SY5Y human neuroblastoma cells was examined using multiple approaches. We have demonstrated that exposure of endosulfan and zineb alone causes both apoptosis and necrosis and their combination cause predominantly necrosis. Visual evaluation using DNA ladder assay and fluorescence Annexin-V/PI assay confirmed the contribution of both apoptotic and necrotic processes. Because caspases, intracellular cysteine proteases, are involved in the process of programmed cell death, we examined if caspase-3, a down stream caspase, is involved in the apoptotic pathways of SH-SY5Y cells exposed to individual pesticides or pesticide



Scheme 1. Possible pathways of cell death induced by environmental pesticides exposure.

mixtures. Endosulfan and zineb alone and in combination altered the caspase-3 activity indicating that exposure to both pesticides exert their apoptotic effect via the caspase-3 pathway (Scheme 1). As proposed in the Scheme 1, exposure to pesticides could cause oxidative stress in neuroblastoma cells. Excess free radical generation would act as signaling molecules that most probably act via mitochondrial pathways or FAS death receptor pathway to activate caspase-3 leading to apoptosis. Alternatively, these radicals could activate NFkappaB. The inactive form of NFkappaB in the cytoplasm, heterodimer of 50 kDa DNA-binding (p50) subunits after dissociated from the inhibitory (IκB) unit, could translocate to the nucleus and to bind to their DNA sequences leading to apoptosis or necrosis. The results of our finding are consistent with the above proposed mechanisms. Further studies on caspases-8 and 9, mitogen activated protein kinase (MAPK), Bcl-2 or change in mitochondrial potential might better clarify pesticide-induced neuronal cell death.

Predicting the effects of pesticides and pesticide mixtures in humans on the basis of our *in vitro* experiments is tempting but is a long shot. Therefore, until further studies are performed, it is difficult to extrapolate the *in vivo* relevance of the concentrations employed in the current *in vitro* studies. Although no epidemiological study has been reported yet, there is, however, likelihood that occupational hazards may occur in certain individuals to be exposed to high levels of these pesticides. Furthermore, simultaneous introduction of certain chemicals to a body system is known to cause decrease in clearance and increase in bio-availability (von Moltke *et al.*, 1998). These chemicals may also interact while crossing cell membranes (hepatic, renal), when binding to plasma proteins, or at the receptor level (Kremers, 2002). All of these possibilities as well as duration of exposure and age of the individuals may, at least in part, contribute to the level of tissue damage that occurs. Our findings suggest that the cytotoxicity of endosulfan and zineb, both individually and in mixtures, is associated with the occurrence of early and late apoptotic/necrotic processes in SH-SY5Y human neuroblastoma cells. The results of these studies are important because they demonstrate the possible pathway of neuronal cells death to these xenobiotics. These studies also strongly suggest that

alterations in oxidative status of cells were crucial for the enhanced toxicity observed with combined pesticide exposure.

CHAPTER 8 Conclusion

We have investigated the effects of endosulfan and zineb, alone or in combination, on the dopaminergic and cholinergic systems in C57BL/6 mice. The mechanistic studies were conducted *in vitro* using SH-SY5Y human neuroblastoma cells.

Thus, in these studies, we found the following:

1. C57Bl/6 mice (7-9 months) exposed to endosulfan and/or zineb showed increased levels of monoamine neurotransmitters both in the striatum and cerebral cortex.
2. C57BL/6 mice exposed to endosulfan or zineb alone, and in combination during postnatal days (5-19 days old) resulted in enhanced susceptibility to loss of nigrostriatal DA system upon re-exposure as adults. Exposure to mixtures of pesticides during postnatal days and subsequent re-challenge in adulthood resulted increased intensities of both the aggregated and the non-aggregated forms of alpha-synuclein. These studies show that a silent neurotoxicity produced by developmental exposure to certain pesticides can be unmasked by challenges later in life.
3. Exposure of endosulfan or zineb alone causes both apoptosis and necrosis in SH-SY5Y human neuroblastoma cells as confirmed by visual evaluation using DNA ladder assay and fluorescence Annexin-V/PI assay. These pesticides induce more than additive effects in inducing necrosis when used in combinations compared to the corresponding individual pesticide treatments as assessed by 7-AAD and Annexin-V/PI assay.
4. The production of lipid peroxides and intracellular reactive oxygen intermediates (superoxide and hydrogen peroxide) were found to be augmented and the levels of certain antioxidant enzymes (SOD, CAT and GPX) were decreased following pesticide and pesticide mixture exposures. These effects certainly induce oxidative stress in pesticide-exposed cells. Exposure to these pesticides also altered caspase-3 activity in the cells and increased the NFkappaB activity in the nucleus.

Taken together, our findings suggest that (1) endosulfan and zineb could alter nigrostrial function by altering the monoamine system, (2) a silent neurotoxicity produced by developmental insults to these pesticides can be unmasked by challenges later in life, (3) both apoptosis and necrosis are the major events involved in pesticide-induced cytotoxicity in SH-SY5Y cells, and (4) endosulfan and zineb induce neuronal cell death, at least in part, through the induction oxidative stress that can activate the redox-sensitive transcription factor NFkappaB and caspase-3.

CHAPTER 9 Future studies

The specific enzymes related to monoamine synthesis and metabolism such as, tyrosine hydroxylase, monoamine oxidase and catechol-O-methyltransferase need be conducted to further understand the pesticide-induced changes on monoamine neurotransmitters. A study of dopamine synthesis and uptake and their relationship with alpha-synuclein will help understand the role of alpha-synuclein in mitigating neuropathy leading to dopaminergic neuronal cell death caused by these pesticides. Future research should also focus on the study of the dopamine receptor, (D1, and D2) and muscarinic cholinergic receptor to study their possible effect on the control of acetylcholine release in the striatum and cortex. Studying of locomotor activity may shed some light on the pathological consequences of pesticide exposure.

In order to delineate the role of ROS on the pathological consequences of pesticide exposure, the glutathione pathway needs to be more closely examined. The results of the intracellular redox status (GSH/GSSG ratio) of the cells might provide some insights regarding the cells' oxidative status. Future studies should include investigation of alternative mechanisms involving apoptotic cell death caused by ROS, including involvement of caspases-8 and 9, mitogen activated protein kinase (MAPK), Bcl-2 or change in mitochondrial potential. Examination of MAPK and Bcl-2 activity might better clarify pesticide-induced neuronal cell death. Studies on the effects of pesticides in transgenic mice with overexpressed SOD and GPX may further explore the mechanism(s) and the role of antioxidant enzymes in the neurotoxicity associated with these pesticides. The use of antioxidants in ameliorating neuronal cell dysfunction will also confirm the role of oxidative stress imposed by these pesticides.

CHAPTER 10 Complete list of references

Abalis, I. M., Eldefrawi, M. E., and Eldefrawi, A. T. (1986). Effects of insecticides on GABA-induced chloride influx into rat brain microsacs. *J Toxicol Environ Health* **18**, 13-23.

Abdollahi, M., Ranjbar, A., Shadnia, S., Nikfar, S., and Rezaiee, A. (2004). Pesticides and oxidative stress: a review. *Med Sci Monit* **10**, RA141-147.

Abercrombie, E. D., and DeBoer, P. (1997). Substantia nigra D1 receptors and stimulation of striatal cholinergic interneurons by dopamine: a proposed circuit mechanism. *J Neurosci* **17**, 8498-8505.

Adams, J. D., Jr., Klaidman, L. K., and Leung, A. C. (1993). MPP+ and MPDP+ induced oxygen radical formation with mitochondrial enzymes. *Free Radic Biol Med* **15**, 181-186.

Agarwal, D. K., Seth, P. K., and Gupta, P. K. (1978). Effect of endosulfan on drug metabolizing enzymes and lipid peroxidation in rat. *J Environ Sci Health C* **13**, 49-62.

Agil, A., Duran, R., Barrero, F., Morales, B., Arauzo, M., Alba, F., Miranda, M. T., Prieto, I., Ramirez, M., and Vives, F. (2006). Plasma lipid peroxidation in sporadic Parkinson's disease. Role of the L-dopa. *J Neurol Sci* **240**, 31-36.

Agrawal, A. K., Anand, M., Zaidi, N. F., and Seth, P. K. (1983). Involvement of serotonergic receptors in endosulfan neurotoxicity. *Biochem Pharmacol* **32**, 3591-3593.

Ahlbom, J., Fredriksson, A., and Eriksson, P. (1994). Neonatal exposure to a type-I pyrethroid (bioallethrin) induces dose-response changes in brain muscarinic receptors and behaviour in neonatal and adult mice. *Brain Res* **645**, 318-324.

Ahmadi, F. A., Linseman, D. A., Grammatopoulos, T. N., Jones, S. M., Bouchard, R. J., Freed, C. R., Heidenreich, K. A., and Zawada, W. M. (2003). The pesticide rotenone induces caspase-3-mediated apoptosis in ventral mesencephalic dopaminergic neurons. *J Neurochem* **87**, 914-921.

Alam, M., and Schmidt, W. J. (2004). Mitochondrial complex I inhibition depletes plasma testosterone in the rotenone model of Parkinson's disease. *Physiol Behav* **83**, 395-400.

Albin, R. L., Young, A. B., and Penney, J. B. (1989). The functional anatomy of basal ganglia disorders. *Trends Neurosci* **12**, 366-375.

Albin, R. L., Young, A. B., and Penney, J. B. (1995). The functional anatomy of disorders of the basal ganglia. *Trends Neurosci* **18**, 63-64.

Aleksandrowicz, D. R. (1979). Endosulfan poisoning and chronic brain syndrome. *Arch Toxicol* **43**, 65-68.

Alexander, G. E., DeLong, M. R., and Strick, P. L. (1986). Parallel organization of functionally segregated circuits linking basal ganglia and cortex. *Annu Rev Neurosci* **9**, 357-381.

Anand, M., Agrawal, A. K., Gopal, K., Sur, R. N., and Seth, P. K. (1986). Endosulfan and cholinergic (muscarinic) transmission: effect on electroencephalograms and [3H]quinuclidinyl benzilate in pigeon brain. *Environ Res* **40**, 421-426.

Anand, M., Khanna, R. N., Gopal, K., and Gupta, G. S. (1980). Effect of endosulfan of bioelectrical activity of brain in rats. *Vet Hum Toxicol* **22**, 385-387.

Anand, M., Mehrotra, S., Gopal, K., Sur, R. N., and Chandra, S. V. (1985). Role of neurotransmitters in endosulfan-induced aggressive behaviour in normal and lesioned rats. *Toxicol Lett* **24**, 79-84.

Anantharam, V., Kitazawa, M., Wagner, J., Kaul, S., and Kanthasamy, A. G. (2002). Caspase-3-dependent proteolytic cleavage of protein kinase Cdelta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese tricarbonyl. *J Neurosci* **22**, 1738-1751.

Andersen, J. K. (2003). Paraquat and iron exposure as possible synergistic environmental risk factors in Parkinson's disease. *Neurotox Res* **5**, 307-313.

Andrews, A. M., Ladenheim, B., Epstein, C. J., Cadet, J. L., and Murphy, D. L. (1996). Transgenic mice with high levels of superoxide dismutase activity are protected from the neurotoxic effects of 2'-NH2-MPTP on serotonergic and noradrenergic nerve terminals. *Mol Pharmacol* **50**, 1511-1519.

Ansari, R. A., Husain, K., and Gupta, P. K. (1987). Endosulfan toxicity influence on biogenic amines of rat brain. *J Environ Biol* **8**, 229-236.

Arora, P. K., Riachi, N. J., Fiedler, G. C., Singh, M. P., Abdallah, F., Harik, S. I., and Sayre, L. M. (1990). Structure-neurotoxicity trends of analogues of 1-methyl-4-phenylpyridinium (MPP+), the cytotoxic metabolite of the dopaminergic neurotoxin MPTP. *Life Sci* **46**, 379-390.

Asanuma, M., Ogawa, N., Haba, K., Hirata, H., and Mori, A. (1992). Effects of chronic catecholamine depletions on muscarinic M1-receptor and its mRNA in rat brain. *J Neurol Sci* **110**, 205-214.

Aschner, M. (2000). Manganese: brain transport and emerging research needs. *Environ Health Perspect* **108 Suppl 3**, 429-432.

Auman, J. T., Seidler, F. J., and Slotkin, T. A. (2000). Neonatal chlorpyrifos exposure targets multiple proteins governing the hepatic adenylyl cyclase signaling cascade: implications for neurotoxicity. *Brain Res Dev Brain Res* **121**, 19-27.

Ayub, S., Verma, J., and Das, N. (2003). Effect of endosulfan and malathion on lipid peroxidation, nitrite and TNF-alpha release by rat peritoneal macrophages. *Int Immunopharmacol* **3**, 1819-1828.

Bachowski, S., Xu, Y., Stevenson, D. E., Walborg, E. F., Jr., and Klaunig, J. E. (1998). Role of oxidative stress in the selective toxicity of dieldrin in the mouse liver. *Toxicol Appl Pharmacol* **150**, 301-309.

Bachurin, S. O., Shevtzova, E. P., Lermontova, N. N., Serkova, T. P., and Ramsay, R. R. (1996). The effect of dithiocarbamates on neurotoxic action of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) and on mitochondrial respiration chain. *Neurotoxicology* **17**, 897-903.

Bagchi, D., Balmoori, J., Bagchi, M., Ye, X., Williams, C. B., and Stohs, S. J. (2000). Role of p53 tumor suppressor gene in the toxicity of TCDD, endrin, naphthalene, and chromium (VI) in liver and brain tissues of mice. *Free Radic Biol Med* **28**, 895-903.

Bagetta, G., Corasaniti, M. T., Iannone, M., Nistico, G., and Stephenson, J. D. (1992). Production of limbic motor seizures and brain damage by systemic and intracerebral injections of paraquat in rats. *Pharmacol Toxicol* **71**, 443-448.

Balasubramaniam, E., Paul, V., and Kazi, M. (1995). Cyclodiene insecticides-induced changes in the central depressive effect of chlorpromazine in rats. *Indian J Physiol Pharmacol* **39**, 231-236.

Baptista, M. J., O'Farrell, C., Daya, S., Ahmad, R., Miller, D. W., Hardy, J., Farrer, M. J., and Cookson, M. R. (2003). Co-ordinate transcriptional regulation of dopamine synthesis genes by alpha-synuclein in human neuroblastoma cell lines. *J Neurochem* **85**, 957-968.

Barbeau, A. (1967). The "pink spot", 3,4-dimethoxyphenylethylamine and dopamine. Relationship to Parkinson's disease and to schizophrenia. *Rev Can Biol* **26**, 55-79.

Barbeau, A. (1968). Dopamine and dopamine metabolites in Parkinson's disease-a review. *Proc Aust Assoc Neurol* **5**, 95-100.

Barlow, B. K., Lee, D. W., Cory-Slechta, D. A., and Opanashuk, L. A. (2005). Modulation of antioxidant defense systems by the environmental pesticide maneb in dopaminergic cells. *Neurotoxicology* **26**, 63-75.

Baughman, D. S. (1986). The acute toxicity of inseticides associated with agricultural runoff to the grass shimp Palaemonetes Pugio. , pp. 1-70. University of Carolina, Columbia.

Bebe, F. N., and Panemangalore, M. (2003). Exposure to low doses of endosulfan and chlorpyrifos modifies endogenous antioxidants in tissues of rats. *J Environ Sci Health B* **38**, 349-363.

Beers, R. F., Jr., and Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem* **195**, 133-140.

Benke, G. M., and Murphy, S. D. (1975). The influence of age on the toxicity and metabolism of methyl parathion and parathion in male and female rats. *Toxicol Appl Pharmacol* **31**, 254-269.

Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., and Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* **3**, 1301-1306.

Bezard, E., Gross, C. E., Fournier, M. C., Dovero, S., Bloch, B., and Jaber, M. (1999). Absence of MPTP-induced neuronal death in mice lacking the dopamine transporter. *Exp Neurol* **155**, 268-273.

Bird, T. D., Stranahan, S., Sumi, S. M., and Raskind, M. (1983). Alzheimer's disease: choline acetyltransferase activity in brain tissue from clinical and pathological subgroups. *Ann Neurol* **14**, 284-293.

Blanco-Coronado, J. L., Repetto, M., Ginestal, R. J., Vicente, J. R., Yelamos, F., and Lardelli, A. (1992). Acute intoxication by endosulfan. *J Toxicol Clin Toxicol* **30**, 575-583.

Blandini, F., Nappi, G., Tassorelli, C., and Martignoni, E. (2000). Functional changes of the basal ganglia circuitry in Parkinson's disease. *Prog Neurobiol* **62**, 63-88.

Bloom, S. E., Lemley, A. T., and Muscarella, D. E. (2006). Potentiation of apoptosis by heat stress plus pesticide exposure in stress resistant human B-lymphoma cells and its attenuation through interaction with follicular dendritic cells: role for c-Jun N-terminal kinase signaling. *Toxicol Sci* **89**, 214-223.

Boereboom, F. T., van Dijk, A., van Zoonen, P., and Meulenbelt, J. (1998). Nonaccidental endosulfan intoxication: a case report with toxicokinetic calculations and tissue concentrations. *J Toxicol Clin Toxicol* **36**, 345-352.

Boissiere, F., Faucheux, B., Agid, Y., and Hirsch, E. C. (1997). Choline acetyltransferase mRNA expression in the striatal neurons of patients with Alzheimer's disease. *Neurosci Lett* **225**, 169-172.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., and Lipton, S. A. (1995). Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci U S A* **92**, 7162-7166.

Bowie, A., and O'Neill, L. A. (2000). Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* **59**, 13-23.

Boyd, E. M., and Dobos, I. (1969). Protein deficiency and tolerated oral doses of endosulfan. *Arch Int Pharmacodyn Ther* **178**, 152-165.

Boyd, E. M., Dobos, I., and Krijnen, C. J. (1970). Endosulfan toxicity and dietary protein. *Arch Environ Health* **21**, 15-19.

Brion, J. P. (1998). Neurofibrillary tangles and Alzheimer's disease. *Eur Neurol* **40**, 130-140.

Brion, J. P. (1999). [Neurofibrillary tangles and early modification of the neuronal cytoskeleton in Alzheimer's disease and in experimental models]. *Bull Mem Acad R Med Belg* **154**, 287-294.

Brooks, A. I., Chadwick, C. A., Gelbard, H. A., Cory-Slechta, D. A., and Federoff, H. J. (1999). Paraquat elicited neurobehavioral syndrome caused by dopaminergic neuron loss. *Brain Res* **823**, 1-10.

Budavari, S. (1996). *The Merck index : an encyclopedia of chemicals, drugs, and biologicals*. Merck & Co., Whitehouse Station, NJ.

Buja, L. M., Eigenbrodt, M. L., and Eigenbrodt, E. H. (1993). Apoptosis and necrosis. Basic types and mechanisms of cell death. *Arch Pathol Lab Med* **117**, 1208-1214.

Burkitt, M. J., Bishop, H. S., Milne, L., Tsang, S. Y., Provan, G. J., Nobel, C. S., Orrenius, S., and Slater, A. F. (1998). Dithiocarbamate toxicity toward thymocytes involves their copper-catalyzed conversion to thiuram disulfides, which oxidize glutathione in a redox cycle without the release of reactive oxygen species. *Arch Biochem Biophys* **353**, 73-84.

Burns, R. S., Chiueh, C. C., Markey, S. P., Ebert, M. H., Jacobowitz, D. M., and Kopin, I. J. (1983). A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc Natl Acad Sci U S A* **80**, 4546-4550.

Bush, A. I., Multhaup, G., Moir, R. D., Williamson, T. G., Small, D. H., Rumble, B., Pollwein, P., Beyreuther, K., and Masters, C. L. (1993). A novel zinc(II) binding site modulates the function of the beta A4 amyloid protein precursor of Alzheimer's disease. *J Biol Chem* **268**, 16109-16112.

Butterfield, P. G., Valanis, B. G., Spencer, P. S., Lindeman, C. A., and Nutt, J. G. (1993). Environmental antecedents of young-onset Parkinson's disease. *Neurology* **43**, 1150-1158.

Buttke, T. M., and Sandstrom, P. A. (1994). Oxidative stress as a mediator of apoptosis. *Immunol Today* **15**, 7-10.

Cahill, M. A., Peter, M. E., Kischkel, F. C., Chinnaiyan, A. M., Dixit, V. M., Krammer, P. H., and Nordheim, A. (1996). CD95 (APO-1/Fas) induces activation of SAP kinases downstream of ICE-like proteases. *Oncogene* **13**, 2087-2096.

Calabresi, P., Stefani, A., Mercuri, N. B., and Bernardi, G. (1989). Acetylcholine-dopamine balance in striatum: is it still a target for antiparkinsonian therapy? *Exs* **57**, 315-321.

Calo, M., Iannone, M., Passafaro, M., and Nistico, G. (1990). Selective vulnerability of hippocampal CA3 neurones after microinfusion of paraquat into the rat substantia nigra or into the ventral tegmental area. *J Comp Pathol* **103**, 73-78.

Calviello, G., Piccioni, E., Boninsegna, A., Tedesco, B., Maggiano, N., Serini, S., Wolf, F. I., and Palozza, P. (2006). DNA damage and apoptosis induction by the pesticide Mancozeb in rat cells: involvement of the oxidative mechanism. *Toxicol Appl Pharmacol* **211**, 87-96.

Camacho, I. A., Hassuneh, M. R., Nagarkatti, M., and Nagarkatti, P. S. (2001). Enhanced activation-induced cell death as a mechanism of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity in peripheral T cells. *Toxicology* **165**, 51-63.

Campbell, B. C., McLean, C. A., Culvenor, J. G., Gai, W. P., Blumbergs, P. C., Jakala, P., Beyreuther, K., Masters, C. L., and Li, Q. X. (2001). The solubility of alpha-synuclein in multiple system atrophy differs from that of dementia with Lewy bodies and Parkinson's disease. *J Neurochem* **76**, 87-96.

Cappelletti, G., Maggioni, M. G., and Maci, R. (1998). Apoptosis in human lung epithelial cells: triggering by paraquat and modulation by antioxidants. *Cell Biol Int* **22**, 671-678.

Carlson, K., Jortner, B. S., and Ehrich, M. (2000). Organophosphorus compound-induced apoptosis in SH-SY5Y human neuroblastoma cells. *Toxicol Appl Pharmacol* **168**, 102-113.

Carr, R. L., Chambers, H. W., Guarisco, J. A., Richardson, J. R., Tang, J., and Chambers, J. E. (2001). Effects of repeated oral postnatal exposure to chlorpyrifos on open-field behavior in juvenile rats. *Toxicol Sci* **59**, 260-267.

Chang, G. D., and Ramirez, V. D. (1987). Effects on dopamine metabolism of MPTP and MPP+ infused through a push-pull cannula into the caudate nucleus of awake adult male rats. *Brain Res* **424**, 49-57.

Chen, J., Nagayama, T., Jin, K., Stetler, R. A., Zhu, R. L., Graham, S. H., and Simon, R. P. (1998). Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. *J Neurosci* **18**, 4914-4928.

Chiba, K., Trevor, A., and Castagnoli, N., Jr. (1984). Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. *Biochem Biophys Res Commun* **120**, 574-578.

Chiueh, C. C., Huang, S. J., and Murphy, D. L. (1992). Enhanced hydroxyl radical generation by 2'-methyl analog of MPTP: suppression by clorgyline and deprenyl. *Synapse* **11**, 346-348.

Chiueh, C. C., Markey, S. P., Burns, R. S., Johannessen, J. N., Jacobowitz, D. M., and Kopin, I. J. (1984). Neurochemical and behavioral effects of 1-methyl-4-phenyl-

1,2,3,6- tetrahydropyridine (MPTP) in rat, guinea pig, and monkey. *Psychopharmacol Bull* **20**, 548-553.
Chiueh, C. C., Miyake, H., and Peng, M. T. (1993). Role of dopamine autoxidation, hydroxyl radical generation, and calcium overload in underlying mechanisms involved in MPTP-induced parkinsonism. *Adv Neurol* **60**, 251-258.
Chiueh, C. C., and Rauhala, P. (1998). Free radicals and MPTP-induced selective destruction of substantia nigra compacta neurons. *Adv Pharmacol* **42**, 796-800.
Christen, Y. (2000). Oxidative stress and Alzheimer disease. *Am J Clin Nutr* **71**, 621S-629S.
Chugh, S. N., Dhawan, R., Agrawal, N., and Mahajan, S. K. (1998). Endosulfan poisoning in Northern India: a report of 18 cases. *Int J Clin Pharmacol Ther* **36**, 474-477.
Cicchetti, R., and Argentin, G. (2003). The role of oxidative stress in the in vitro induction of micronuclei by pesticides in mouse lung fibroblasts. *Mutagenesis* **18**, 127-132.
Clayton, D. F., and George, J. M. (1998). The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends Neurosci* **21**, 249-254.
Clayton, D. F., and George, J. M. (1999). Synucleins in synaptic plasticity and neurodegenerative disorders. *J Neurosci Res* **58**, 120-129.
Clayton, R., Clark, J. B., and Sharpe, M. (2005). Cytochrome c release from rat brain mitochondria is proportional to the mitochondrial functional deficit: implications for apoptosis and neurodegenerative disease. *J Neurochem* **92**, 840-849.
Cohen, G. M. (2005). Caspase inactivation of the proteasome. *Cell Death Differ* **12**, 1218.
Cohen, Z., Wilson, J., Ritter, L., and McDonagh, P. (2004). Caspase inhibition decreases both platelet phosphatidylserine exposure and aggregation: caspase inhibition of platelets. *Thromb Res* **113**, 387-393.
Cole, K. K., and Perez-Polo, J. R. (2002). Poly(ADP-ribose) polymerase inhibition prevents both apoptotic-like delayed neuronal death and necrosis after H(2)O(2) injury. *J Neurochem* **82**, 19-29.
Cole, L. M., and Casida, J. E. (1986). Polychlorocycloalkane insecticide-induced convulsions in mice in relation to disruption of the GABA-regulated chloride ionophore. *Life Sci* **39**, 1855-1862.
Columbano, A. (1995). Cell death: current difficulties in discriminating apoptosis from necrosis in the context of pathological processes in vivo. *J Cell Biochem* **58**, 181-190.
Constantinidis, J. (1990). [Alzheimer's disease and the zinc theory]. *Encephale* **16**, 231-239.
Cook, J. E. (1991). Correlated activity in the CNS: a role on every timescale? *Trends Neurosci* **14**, 397-401.
Cooper, B. (1991). The epidemiology of primary degenerative dementia and related neurological disorders. *Eur Arch Psychiatry Clin Neurosci* **240**, 223-233.
Correll, L., and Ehrich, M. (1991). A microassay method for neurotoxic esterase determinations. *Fundam Appl Toxicol* **16**, 110-116.

Corsini, G. U., Pintus, S., Chiueh, C. C., Weiss, J. F., and Kopin, I. J. (1985). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in mice is enhanced by pretreatment with diethyldithiocarbamate. *Eur J Pharmacol* **119**, 127-128.

Cory-Slechta, D. A., Thiruchelvam, M., Richfield, E. K., Barlow, B. K., and Brooks, A. I. (2005). Developmental pesticide exposures and the Parkinson's disease phenotype. *Birth Defects Res A Clin Mol Teratol* **73**, 136-139.

CRARM (1997). *Risk Assessment and Risk Management in Regulatory Decision-Making, Final Report*. Washington, D.C.

Crossman, A. R. (1987). Primate models of dyskinesia: the experimental approach to the study of basal ganglia-related involuntary movement disorders. *Neuroscience* **21**, 1-40.

Cuajungco, M. P., and Faget, K. Y. (2003). Zinc takes the center stage: its paradoxical role in Alzheimer's disease. *Brain Res Brain Res Rev* **41**, 44-56.

Cuajungco, M. P., and Lees, G. J. (1997). Zinc and Alzheimer's disease: is there a direct link? *Brain Res Brain Res Rev* **23**, 219-236.

Curtain, C. C., Ali, F., Volitakis, I., Cherny, R. A., Norton, R. S., Beyreuther, K., Barrow, C. J., Masters, C. L., Bush, A. I., and Barnham, K. J. (2001). Alzheimer's disease amyloid-beta binds copper and zinc to generate an allosterically ordered membrane-penetrating structure containing superoxide dismutase-like subunits. *J Biol Chem* **276**, 20466-20473.

D'Amato, R. J., Lipman, Z. P., and Snyder, S. H. (1986). Selectivity of the parkinsonian neurotoxin MPTP: toxic metabolite MPP+ binds to neuromelanin. *Science* **231**, 987-989.

Da Prada, M., and Kettler, R. (1986). Uptake, metabolism and subcellular localization of MPTP and MPP+ in blood platelets. *Clin Neuropharmacol* **9 Suppl 4**, 347-349.

Dawson, V. L., Dawson, T. M., and Wamsley, J. K. (1990). Muscarinic and dopaminergic receptor subtypes on striatal cholinergic interneurons. *Brain Res Bull* **25**, 903-912.

de Bilbao, F., Guarin, E., Nef, P., Vallet, P., Giannakopoulos, P., and Dubois-Dauphin, M. (1999). Postnatal distribution of cpp32/caspase 3 mRNA in the mouse central nervous system: an in situ hybridization study. *J Comp Neurol* **409**, 339-357.

de la Fuente-Fernandez, R., and Calne, D. B. (2002). Evidence for environmental causation of Parkinson's disease. *Parkinsonism Relat Disord* **8**, 235-241.

De Michele, G., Filla, A., Volpe, G., De Marco, V., Gogliettino, A., Ambrosio, G., Marconi, R., Castellano, A. E., and Campanella, G. (1996). Environmental and genetic risk factors in Parkinson's disease: a case-control study in southern Italy. *Mov Disord* **11**, 17-23.

DeLong, M. R. (1990). Primate models of movement disorders of basal ganglia origin. *Trends Neurosci* **13**, 281-285.

Denaro, A., Tamminga, C. A., Ruggieri, S., Chase, T. N., and Agnoli, A. (1984). State of DA/ACh balance after chronic treatment with haloperidol and with THIP in rats. *Adv Neurol* **40**, 141-147.

Dexter, D., Carter, C., Agid, F., Agid, Y., Lees, A. J., Jenner, P., and Marsden, C. D. (1986). Lipid peroxidation as cause of nigral cell death in Parkinson's disease. *Lancet* **2**, 639-640.

Dexter, D. T., Carter, C. J., Wells, F. R., Javoy-Agid, F., Agid, Y., Lees, A., Jenner, P., and Marsden, C. D. (1989). Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. *J Neurochem* **52**, 381-389.

Di Rosa, G., Puzzo, D., Sant'Angelo, A., Trinchese, F., and Arancio, O. (2003). Alpha-synuclein: between synaptic function and dysfunction. *Histol Histopathol* **18**, 1257-1266.

Dietert, R. R., Lee, J. E., and Bunn, T. L. (2002). Developmental immunotoxicology: emerging issues. *Hum Exp Toxicol* **21**, 479-485.

Dikshith, T. S., Raizada, R. B., Srivastava, M. K., and Kaphalia, B. S. (1984). Response of rats to repeated oral administration of endosulfan. *Ind Health* **22**, 295-304.

Dikshith, T. S. S. (1991). Pesticides. In *Pesticides of Toxicology in Animals* (T. S. S. Dikshith, Ed.), pp. 1-41. CRC Press, Boca Raton, FL.

Dineley, K. E., Votyakova, T. V., and Reynolds, I. J. (2003). Zinc inhibition of cellular energy production: implications for mitochondria and neurodegeneration. *J Neurochem* **85**, 563-570.

Djordjevic, V. B. (2004). Free radicals in cell biology. *Int Rev Cytol* **237**, 57-89.

Dolcet, X., Llobet, D., Pallares, J., and Matias-Guiu, X. (2005). NF-kB in development and progression of human cancer. *Virchows Arch* **446**, 475-482.

Donner, K. J., Becker, K. M., Hissong, B. D., and Ahmed, S. A. (1999). Comparison of multiple assays for kinetic detection of apoptosis in thymocytes exposed to dexamethasone or diethylstilbesterol. *Cytometry* **35**, 80-90.

Dorval, J., and Hontela, A. (2003). Role of glutathione redox cycle and catalase in defense against oxidative stress induced by endosulfan in adrenocortical cells of rainbow trout (Oncorhynchus mykiss). *Toxicol Appl Pharmacol* **192**, 191-200.

Dorval, J., Leblond, V. S., and Hontela, A. (2003). Oxidative stress and loss of cortisol secretion in adrenocortical cells of rainbow trout (Oncorhynchus mykiss) exposed in vitro to endosulfan, an organochlorine pesticide. *Aquat Toxicol* **63**, 229-241.

Dostert, P., and Strolin Benedetti, M. (1988). [The bases of MPTP neurotoxicity]. *Encephale* **14**, 399-412.

Double, K. L., Maywald, M., Schmittel, M., Riederer, P., and Gerlach, M. (1998). In vitro studies of ferritin iron release and neurotoxicity. *J Neurochem* **70**, 2492-2499.

Duke, R. C., Chervenak, R., and Cohen, J. J. (1983). Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytolysis. *Proc Natl Acad Sci U S A* **80**, 6361-6365.

Eberling, J. L., Bankiewicz, K. S., Pivirotto, P., Bringas, J., Chen, K., Nowotnik, D. P., Steiner, J. P., Budinger, T. F., and Jagust, W. J. (1999). Dopamine transporter loss and clinical changes in MPTP-lesioned primates. *Brain Res* **832**, 184-187.

Ebmeier, K. P., Besson, J. A., Cherryman, G., and Smith, F. (1988). [Parkinson's disease and Alzheimer's disease--similarities in magnetic resonance imaging parameters and their possible explanation]. *Z Gerontol* **21**, 142-145.

el-Agnaf, O. M., and Irvine, G. B. (2002). Aggregation and neurotoxicity of alpha-synuclein and related peptides. *Biochem Soc Trans* **30**, 559-565.

Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Feather-Stone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**, 88-95.

Enz, A., Amstutz, R., Boddeke, H., Gmelin, G., and Malanowski, J. (1993). Brain selective inhibition of acetylcholinesterase: a novel approach to therapy for Alzheimer's disease. *Prog Brain Res* **98**, 431-438.

EPA (1972). The pollution potential in pesticide manufacturing. Pesticide Study Series 5. U.S. Environmental Protection Agency, Office of Water Products.

Eriksson, P., Ahlbom, J., and Fredriksson, A. (1992). Exposure to DDT during a defined period in neonatal life induces permanent changes in brain muscarinic receptors and behaviour in adult mice. *Brain Res* **582**, 277-281.

Eriksson, P., Nilsson-Hakansson, L., Nordberg, A., Aspberg, A., and Fredriksson, A. (1990). Neonatal exposure to DDT and its fatty acid conjugate: effects on cholinergic and behavioural variables in the adult mouse. *Neurotoxicology* **11**, 345-354.

Eriksson, P., and Nordberg, A. (1990). Effects of two pyrethroids, bioallethrin and deltamethrin, on subpopulations of muscarinic and nicotinic receptors in the neonatal mouse brain. *Toxicol Appl Pharmacol* **102**, 456-463.

Escargueil-Blanc, I., Salvayre, R., and Negre-Salvayre, A. (1994). Necrosis and apoptosis induced by oxidized low density lipoproteins occur through two calcium-dependent pathways in lymphoblastoid cells. *Faseb J* **8**, 1075-1080.

Exner, C., Koschack, J., and Irle, E. (2002). The differential role of premotor frontal cortex and basal ganglia in motor sequence learning: evidence from focal basal ganglia lesions. *Learn Mem* **9**, 376-386.

Fabisiak, J. P., Kagan, V. E., Tyurina, Y. Y., Tyurin, V. A., and Lazo, J. S. (1998). Paraquat-induced phosphatidylserine oxidation and apoptosis are independent of activation of PLA2. *Am J Physiol* **274**, L793-802.

Fall, C. P., and Bennett, J. P., Jr. (1999). Characterization and time course of MPP+ -induced apoptosis in human SH-SY5Y neuroblastoma cells. *J Neurosci Res* **55**, 620-628.

Farber, E. (1994). Programmed cell death: necrosis versus apoptosis. *Mod Pathol* **7**, 605-609.

Fasano, M., Giraudo, S., Coha, S., Bergamasco, B., and Lopiano, L. (2003). Residual substantia nigra neuromelanin in Parkinson's disease is cross-linked to alpha-synuclein. *Neurochem Int* **42**, 603-606.

Feany, M. B., and Bender, W. W. (2000). A Drosophila model of Parkinson's disease. *Nature* **404**, 394-398.

Fearnley, J. M., and Lees, A. J. (1991). Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain* **114 (Pt 5)**, 2283-2301.

Feierman, D. E., Winston, G. W., and Cederbaum, A. I. (1985). Ethanol oxidation by hydroxyl radicals: role of iron chelates, superoxide, and hydrogen peroxide. *Alcohol Clin Exp Res* **9**, 95-102.

Feinman, R., Siegel, D. S., and Berenson, J. (2004). Regulation of NF-kB in multiple myeloma: therapeutic implications. *Clin Adv Hematol Oncol* **2**, 162-166.

Ferraz, H. B., Bertolucci, P. H., Pereira, J. S., Lima, J. G., and Andrade, L. A. (1988). Chronic exposure to the fungicide maneb may produce symptoms and signs of CNS manganese intoxication. *Neurology* **38**, 550-553.

Fitsanakis, V. A., Amarnath, V., Moore, J. T., Montine, K. S., Zhang, J., and Montine, T. J. (2002). Catalysis of catechol oxidation by metal-dithiocarbamate complexes in pesticides. *Free Radic Biol Med* **33**, 1714-1723.

Fitzgerald, D. J. (1995). Zinc and Alzheimer's disease. *Science* **268**, 1920; author reply 1921-1923.

Foncea, R., Carvajal, C., Almarza, C., and Leighton, F. (2000). Endothelial cell oxidative stress and signal transduction. *Biol Res* **33**, 89-96.

Forno, L. S. (1992). Neuropathologic features of Parkinson's, Huntington's, and Alzheimer's diseases. *Ann N Y Acad Sci* **648**, 6-16.

Foster, N. L., Tamminga, C. A., O'Donohue, T. L., Tanimoto, K., Bird, E. D., and Chase, T. N. (1986). Brain choline acetyltransferase activity and neuropeptide Y concentrations in Alzheimer's disease. *Neurosci Lett* **63**, 71-75.

Fridovich, I. (1995). Superoxide radical and superoxide dismutases. *Annu Rev Biochem* **64**, 97-112.

Gainetdinov, R. R., Fumagalli, F., Jones, S. R., and Caron, M. G. (1997). Dopamine transporter is required for in vivo MPTP neurotoxicity: evidence from mice lacking the transporter. *J Neurochem* **69**, 1322-1325.

Gao, H. M., Hong, J. S., Zhang, W., and Liu, B. (2003). Synergistic dopaminergic neurotoxicity of the pesticide rotenone and inflammogen lipopolysaccharide: relevance to the etiology of Parkinson's disease. *J Neurosci* **23**, 1228-1236.

Gardner, A. M., Xu, F. H., Fady, C., Jacoby, F. J., Duffey, D. C., Tu, Y., and Lichtenstein, A. (1997). Apoptotic vs. nonapoptotic cytotoxicity induced by hydrogen peroxide. *Free Radic Biol Med* **22**, 73-83.

Gauthier, M., Roberge, C. J., Pelletier, M., Tessier, P. A., and Girard, D. (2001). Activation of human neutrophils by technical toxaphene. *Clin Immunol* **98**, 46-53.

George, J. M. (2002). The synucleins. *Genome Biol* **3**, REVIEWS3002.

Gerfen, C. R., Engber, T. M., Mahan, L. C., Susel, Z., Chase, T. N., Monsma, F. J., Jr., and Sibley, D. R. (1990). D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* **250**, 1429-1432.

Gerfen, C. R., Keefe, K. A., and Gauda, E. B. (1995). D1 and D2 dopamine receptor function in the striatum: coactivation of D1- and D2-dopamine receptors on separate populations of neurons results in potentiated immediate early gene response in D1-containing neurons. *J Neurosci* **15**, 8167-8176.

Gilbert, M. E., and Mack, C. M. (1995). Seizure thresholds in kindled animals are reduced by the pesticides lindane and endosulfan. *Neurotoxicol Teratol* **17**, 143-150.

Gillette, J. S., and Bloomquist, J. R. (2003). Differential up-regulation of striatal dopamine transporter and alpha-synuclein by the pyrethroid insecticide permethrin. *Toxicol Appl Pharmacol* **192**, 287-293.

Giorgi, O., De Montis, G., Porceddu, M. L., Mele, S., Calderini, G., Toffano, G., and Biggio, G. (1987). Developmental and age-related changes in D1-dopamine receptors and dopamine content in the rat striatum. *Brain Res* **432**, 283-290.

Giuliani, C., Napolitano, G., Bucci, I., Montani, V., and Monaco, F. (2001). [Nf-kB transcription factor: role in the pathogenesis of inflammatory, autoimmune, and neoplastic diseases and therapy implications]. *Clin Ter* **152**, 249-253.

Goedert, M., Sisodia, S. S., and Price, D. L. (1991). Neurofibrillary tangles and beta-amyloid deposits in Alzheimer's disease. *Curr Opin Neurobiol* **1**, 441-447.
Golstein, P., Ojcius, D. M., and Young, J. D. (1991). Cell death mechanisms and the immune system. *Immunol Rev* **121**, 29-65.
Goodwin, B. L., and Kite, G. C. (1998). Environmental MPTP as a factor in the aetiology of Parkinson's disease? *J Neural Transm* **105**, 1265-1269.
Gorczyca, W. (1999). Cytometric analyses to distinguish death processes. *Endocr Relat Cancer* **6**, 17-19.
Gorell, J. M., Johnson, C. C., Rybicki, B. A., Peterson, E. L., and Richardson, R. J. (1998). The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. *Neurology* **50**, 1346-1350.
Gotz, M. E., Kunig, G., Riederer, P., and Youdim, M. B. (1994). Oxidative stress: free radical production in neural degeneration. *Pharmacol Ther* **63**, 37-122.
Graybiel, A. M. (1996). Basal ganglia: new therapeutic approaches to Parkinson's disease. *Curr Biol* **6**, 368-371.
Greenamyre, J. T., Betarbet, R., and Sherer, T. B. (2003). The rotenone model of Parkinson's disease: genes, environment and mitochondria. *Parkinsonism Relat Disord* **9 Suppl 2**, S59-64.
Guerrini, L., Blasi, F., and Denis-Donini, S. (1995). Synaptic activation of NF-kappa B by glutamate in cerebellar granule neurons in vitro. *Proc Natl Acad Sci U S A* **92**, 9077-9081.
Gupta, P. K. (1976). Endosulfan-induced neurotoxicity in rats and mice. *Bull Environ Contam Toxicol* **15**, 708-713.
Gupta, P. K., and Gupta, R. C. (1979). Pharmacology, toxicology and degradation of endosulfan A review. *Toxicology* **13**, 115-130.
Guven, K., Power, R. S., Avramides, S., Allender, R., and de Pomerai, D. I. (1999). The toxicity of dithiocarbamate fungicides to soil nematodes, assessed using a stress-inducible transgenic strain of Caenorhabditis elegans. *J Biochem Mol Toxicol* **13**, 324-333.
Hallegue, D., Ben Rhouma, K., Krichah, R., and Sakly, M. (2002). Dieldrin initiates apoptosis in rat thymocytes. *Indian J Exp Biol* **40**, 1147-1150.
Hampton, M. B., and Orrenius, S. (1998). Redox regulation of apoptotic cell death. *Biofactors* **8**, 1-5.
Harbison, R. D. (1975). Comparative toxicity of some selected pesticides in neonatal and adult rats. *Toxicol Appl Pharmacol* **32**, 443-446.
Hardy, J. (2003). The relationship between Lewy body disease, Parkinson's disease, and Alzheimer's disease. *Ann N Y Acad Sci* **991**, 167-170.
Harris, E. D. (1992). Regulation of antioxidant enzymes. *Faseb J* **6**, 2675-2683.
Hartmann, A., Hunot, S., Michel, P. P., Muriel, M. P., Vyas, S., Faucheux, B. A., Mouatt-Prigent, A., Turmel, H., Srinivasan, A., Ruberg, M., Evan, G. I., Agid, Y., and Hirsch, E. C. (2000). Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. *Proc Natl Acad Sci U S A* **97**, 2875-2880.
Hasegawa, E., Takeshige, K., Oishi, T., Murai, Y., and Minakami, S. (1990). 1-Methyl-4-phenylpyridinium (MPP+) induces NADH-dependent superoxide formation and

enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. *Biochem Biophys Res Commun* **170**, 1049-1055.
Hashimoto, M., and Masliah, E. (1999). Alpha-synuclein in Lewy body disease and Alzheimer's disease. *Brain Pathol* **9**, 707-720.
Haslett, C. (1992). Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. *Clin Sci (Lond)* **83**, 639-648.
Hedera, P., Lerner, A. J., Castellani, R., and Friedland, R. P. (1995). Concurrence of Alzheimer's disease, Parkinson's disease, diffuse Lewy body disease, and amyotrophic lateral sclerosis. *J Neurol Sci* **128**, 219-224.
Heikkila, R. E., Manzino, L., Cabbat, F. S., and Duvoisin, R. C. (1984). Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine by monoamine oxidase inhibitors. *Nature* **311**, 467-469.
Heinz, G. H., Hill, E. F., and Contrera, J. F. (1980). Dopamine and norepinephrine depletion in ring doves fed DDE, dieldrin, and Aroclor 1254. *Toxicol Appl Pharmacol* **53**, 75-82.
Hincal, F., Gurbay, A., and Giray, B. (1995). Induction of lipid peroxidation and alteration of glutathione redox status by endosulfan. *Biol Trace Elem Res* **47**, 321-326.
Hipkiss, A. R. (2005). Could carnosine suppress zinc-mediated proteasome inhibition and neurodegeneration? Therapeutic potential of a non-toxic but non-patentable dipeptide. *Biogerontology* **6**, 147-149.
Hodgson, E., and Levi, P. E. (1996). Pesticides: an important but underused model for the environmental health sciences. *Environ Health Perspect* **104 Suppl 1**, 97-106.
Hogan, K. A., Staal, R. G., and Sonsalla, P. K. (2000). Analysis of VMAT2 binding after methamphetamine or MPTP treatment: disparity between homogenates and vesicle preparations. *J Neurochem* **74**, 2217-2220.
Holthoff, V. A., Vieregge, P., Kessler, J., Pietrzyk, U., Herholz, K., Bonner, J., Wagner, R., Wienhard, K., Pawlik, G., and Heiss, W. D. (1994). Discordant twins with Parkinson's disease: positron emission tomography and early signs of impaired cognitive circuits. *Ann Neurol* **36**, 176-182.
Honig, L. S., and Rosenberg, R. N. (2000). Apoptosis and neurologic disease. *Am J Med* **108**, 317-330.
Horn, A. S. (1990). Dopamine uptake: a review of progress in the last decade. *Prog Neurobiol* **34**, 387-400.
Hornykiewicz, O. (1989). Ageing and neurotoxins as causative factors in idiopathic Parkinson's disease--a critical analysis of the neurochemical evidence. *Prog Neuropsychopharmacol Biol Psychiatry* **13**, 319-328.
Huang, T. T., Yasunami, M., Carlson, E. J., Gillespie, A. M., Reaume, A. G., Hoffman, E. K., Chan, P. H., Scott, R. W., and Epstein, C. J. (1997). Superoxide-mediated cytotoxicity in superoxide dismutase-deficient fetal fibroblasts. *Arch Biochem Biophys* **344**, 424-432.
Ince, E., Ciliax, B. J., and Levey, A. I. (1997). Differential expression of D1 and D2 dopamine and m4 muscarinic acetylcholine receptor proteins in identified striatonigral neurons. *Synapse* **27**, 357-366.

IOM (2003). In *Institute of Medicine (IOM), Gulf War and Health: Volume 2, Insecticides and Solvents* pp. 412-418. The National Academics Press, Washington, DC.

Irwin, I., Wu, E. Y., DeLanney, L. E., Trevor, A., and Langston, J. W. (1987). The effect of diethyldithiocarbamate on the biodisposition of MPTP: an explanation for enhanced neurotoxicity. *Eur J Pharmacol* **141**, 209-217.

Iyaniwura, T. T. (1990). Mammalian toxicity and combined exposure to pesticides. *Vet Hum Toxicol* **32**, 58-62.

Jackson, J. H., Gajewski, E., Schraufstatter, I. U., Hyslop, P. A., Fuciarelli, A. F., Cochrane, C. G., and Dizdaroglu, M. (1989). Damage to the bases in DNA induced by stimulated human neutrophils. *J Clin Invest* **84**, 1644-1649.

Jackson, J. H., Schraufstatter, I. U., Hyslop, P. A., Vosbeck, K., Sauerheber, R., Weitzman, S. A., and Cochrane, C. G. (1987). Role of hydroxyl radical in DNA damage. *Trans Assoc Am Physicians* **100**, 147-157.

Jang, J. H., and Surh, Y. J. (2004). Bcl-2 attenuation of oxidative cell death is associated with up-regulation of gamma-glutamylcysteine ligase via constitutive NF-kappaB activation. *J Biol Chem* **279**, 38779-38786.

Jarvis, M. F., and Wagner, G. C. (1985). Neurochemical and functional consequences following 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) and methamphetamine. *Life Sci* **36**, 249-254.

Jellinger, K. A. (2000). Cell death mechanisms in Parkinson's disease. *J Neural Transm* **107**, 1-29.

Jenner, P. (1991). Oxidative stress as a cause of Parkinson's disease. *Acta Neurol Scand Suppl* **136**, 6-15.

Jenner, P., Dexter, D. T., Sian, J., Schapira, A. H., and Marsden, C. D. (1992). Oxidative stress as a cause of nigral cell death in Parkinson's disease and incidental Lewy body disease. The Royal Kings and Queens Parkinson's Disease Research Group. *Ann Neurol* **32 Suppl**, S82-87.

Jia, Z. Q., and Misra, H. P. (2006a). Developmental exposure to pesticides zineb and/or endosulfan renders the nigrostriatal dopamine system more susceptible to these environmental chemicals later in life (in preparation).

Jia, Z. Q., and Misra, H. P. (2006b). Exposure to Mixtures of Endosulfan and Zineb Induces Apoptotic and necrotic Cell Death in SH-SY5Y Neuronal Cells, *In Vitro* (in preparation).

Johnson, N. L., Gardner, A. M., Diener, K. M., Lange-Carter, C. A., Gleavy, J., Jarpe, M. B., Minden, A., Karin, M., Zon, L. I., and Johnson, G. L. (1996). Signal transduction pathways regulated by mitogen-activated/extracellular response kinase kinase kinase induce cell death. *J Biol Chem* **271**, 3229-3237.

Jordan, J., Galindo, M. F., Cena, V., and Gonzalez-Garcia, C. (2000). [Cysteine proteinase and neurodegeneration]. *Rev Neurol* **31**, 333-340.

Jueptner, M., and Krukenberg, M. (2001). Motor system: cortex, basal ganglia, and cerebellum. *Neuroimaging Clin N Am* **11**, 203-219, viii.

Kackar, R., Srivastava, M. K., and Raizada, R. B. (1999). Assessment of toxicological effects of mancozeb in male rats after chronic exposure. *Indian J Exp Biol* **37**, 553-559.

Kalil, R. E. (1989). Synapse formation in the developing brain. *Sci Am* **261**, 76-79, 82-75.

Kang, K. S., Park, J. E., Ryu, D. Y., and Lee, Y. S. (2001). Effects and neuro-toxic mechanisms of 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl and endosulfan in neuronal stem cells. *J Vet Med Sci* **63**, 1183-1190.

Kannan, K., Holcombe, R. F., Jain, S. K., Alvarez-Hernandez, X., Chervenak, R., Wolf, R. E., and Glass, J. (2000). Evidence for the induction of apoptosis by endosulfan in a human T-cell leukemic line. *Mol Cell Biochem* **205**, 53-66.

Kannan, K., and Jain, S. K. (2003). Oxygen radical generation and endosulfan toxicity in Jurkat T-cells. *Mol Cell Biochem* **247**, 1-7.

Kaplan, B., Ratner, V., and Haas, E. (2003). Alpha-synuclein: its biological function and role in neurodegenerative diseases. *J Mol Neurosci* **20**, 83-92.

Karen, D. J., Li, W., Harp, P. R., Gillette, J. S., and Bloomquis, J. R. (2001). Striatal dopaminergic pathways as a target for the insecticides permethrin and chlorpyrifos. *Neurotoxicology* **22**, 811-817.

Kedziora-Kornatowska, K. Z., Luciak, M., and Paszkowski, J. (2000). Lipid peroxidation and activities of antioxidant enzymes in the diabetic kidney: effect of treatment with angiotensin convertase inhibitors. *IUBMB Life* **49**, 303-307.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-257.

Kilbourn, M. R., Kuszpit, K., and Sherman, P. (2000). Rapid and differential losses of in vivo dopamine transporter (DAT) and vesicular monoamine transporter (VMAT2) radioligand binding in MPTP-treated mice. *Synapse* **35**, 250-255.

Kilinc, A., Yalcin, A. S., Yalcin, D., Taga, Y., and Emerk, K. (1988). Increased erythrocyte susceptibility to lipid peroxidation in human Parkinson's disease. *Neurosci Lett* **87**, 307-310.

Kim, K. W., Suh, Y. J., Park, W. Y., Jhoo, J. H., Lee, D. Y., Youn, J. C., Lee, K. H., Seo, J. S., and Woo, J. I. (2004). Choline acetyltransferase G +4 A polymorphism confers a risk for Alzheimer's disease in concert with Apolipoprotein E epsilon4. *Neurosci Lett* **366**, 182-186.

Kimmel, C. A., and Makris, S. L. (2001). Recent developments in regulatory requirements for developmental toxicology. *Toxicol Lett* **120**, 73-82.

Kitazawa, M., Anantharam, V., Kanthasamy, A., and Kanthasamy, A. G. (2004). Dieldrin promotes proteolytic cleavage of poly(ADP-ribose) polymerase and apoptosis in dopaminergic cells: protective effect of mitochondrial anti-apoptotic protein Bcl-2. *Neurotoxicology* **25**, 589-598.

Kitazawa, M., Anantharam, V., and Kanthasamy, A. G. (2001). Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptopic cell death in dopaminergic cells. *Free Radic Biol Med* **31**, 1473-1485.

Kitazawa, M., Anantharam, V., and Kanthasamy, A. G. (2003). Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase Cdelta in dopaminergic cells: relevance to oxidative stress and dopaminergic degeneration. *Neuroscience* **119**, 945-964.

Klucken, J., McLean, P. J., Gomez-Tortosa, E., Ingelsson, M., and Hyman, B. T. (2003). Neuritic alterations and neural system dysfunction in Alzheimer's disease and dementia with Lewy bodies. *Neurochem Res* **28**, 1683-1691.

Konno, N. (2003). [Neurotoxicity of organophosphorus and dithiocarbamate compounds]. *Nippon Eiseigaku Zasshi* **57**, 645-654.
Kotake, Y., and Ohta, S. (2003). MPP+ analogs acting on mitochondria and inducing neuro-degeneration. *Curr Med Chem* **10**, 2507-2516.
Kovacic, P. (2003). Mechanism of organophosphates (nerve gases and pesticides) and antidotes: electron transfer and oxidative stress. *Curr Med Chem* **10**, 2705-2709.
Kremers, P. (2002). In vitro tests for predicting drug-drug interactions: the need for validated procedures. *Pharmacol Toxicol* **91**, 209-217.
Kurosaki, R., Muramatsu, Y., Watanabe, H., Michimata, M., Matsubara, M., Imai, Y., and Araki, T. (2003). Role of dopamine transporter against MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) neurotoxicity in mice. *Metab Brain Dis* **18**, 139-146.
Lakshmana, M. K., and Raju, T. R. (1994). Endosulfan induces small but significant changes in the levels of noradrenaline, dopamine and serotonin in the developing rat brain and deficits in the operant learning performance. *Toxicology* **91**, 139-150.
Langston, J. W., Ballard, P., Tetrud, J. W., and Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**, 979-980.
Latchoumycandane, C., and Mathur, P. P. (2002). Induction of oxidative stress in the rat testis after short-term exposure to the organochlorine pesticide methoxychlor. *Arch Toxicol* **76**, 692-698.
Lecoeur, H., Ledru, E., Prevost, M. C., and Gougeon, M. L. (1997). Strategies for phenotyping apoptotic peripheral human lymphocytes comparing ISNT, annexin-V and 7-AAD cytofluorometric staining methods. *J Immunol Methods* **209**, 111-123.
Lee, F. J., Liu, F., Pristupa, Z. B., and Niznik, H. B. (2001). Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. *Faseb J* **15**, 916-926.
Lee, R. G. (1987). Physiology of the basal ganglia and pathophysiology of Parkinson's disease. *Can J Neurol Sci* **14**, 373-380.
Lee, Y. J., Kang, I. J., Bunger, R., and Kang, Y. H. (2004). Enhanced survival effect of pyruvate correlates MAPK and NF-kappaB activation in hydrogen peroxide-treated human endothelial cells. *J Appl Physiol* **96**, 793-801; discussion 792.
Leenders, K. L., and Oertel, W. H. (2001). Parkinson's disease: clinical signs and symptoms, neural mechanisms, positron emission tomography, and therapeutic interventions. *Neural Plast* **8**, 99-110.
Lennon, S. V., Martin, S. J., and Cotter, T. G. (1991). Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Prolif* **24**, 203-214.
Lester, J., Fink, S., Aronin, N., and DiFiglia, M. (1993). Colocalization of D1 and D2 dopamine receptor mRNAs in striatal neurons. *Brain Res* **621**, 106-110.
Li, N., Ragheb, K., Lawler, G., Sturgis, J., Rajwa, B., Melendez, J. A., and Robinson, J. P. (2003). Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J Biol Chem* **278**, 8516-8525.

Li, Y., Zhang, W., Mantell, L. L., Kazzaz, J. A., Fein, A. M., and Horowitz, S. (1997). Nuclear factor-kappaB is activated by hyperoxia but does not protect from cell death. *J Biol Chem* **272**, 20646-20649.

Liou, H. H., Chen, R. C., Tsai, Y. F., Chen, W. P., Chang, Y. C., and Tsai, M. C. (1996). Effects of paraquat on the substantia nigra of the wistar rats: neurochemical, histological, and behavioral studies. *Toxicol Appl Pharmacol* **137**, 34-41.

Lipton, S. A., and Kater, S. B. (1989). Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *Trends Neurosci* **12**, 265-270.

Lotharius, J., Barg, S., Wiekop, P., Lundberg, C., Raymon, H. K., and Brundin, P. (2002). Effect of mutant alpha-synuclein on dopamine homeostasis in a new human mesencephalic cell line. *J Biol Chem* **277**, 38884-38894.

Lotharius, J., and Brundin, P. (2002). Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. *Nat Rev Neurosci* **3**, 932-942.

Maitre, B., Jornot, L., and Junod, A. F. (1993). Effects of inhibition of catalase and superoxide dismutase activity on antioxidant enzyme mRNA levels. *Am J Physiol* **265**, L636-643.

Majno, G., and Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* **146**, 3-15.

Mann, D. M., and Yates, P. O. (1982). Is the loss of cerebral cortical choline acetyl transferase activity in Alzheimer's disease due to degeneration of ascending cholinergic nerve cells. *J Neurol Neurosurg Psychiatry* **45**, 936.

Manning-Bog, A. B., McCormack, A. L., Li, J., Uversky, V. N., Fink, A. L., and Di Monte, D. A. (2002). The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. *J Biol Chem* **277**, 1641-1644.

Marchi, M., Caviglia, A., Paudice, P., and Raiteri, M. (1983). Calcium-dependent [3H]acetylcholine release and muscarinic autoreceptors in rat cortical synaptosomes during development. *Neurochem Res* **8**, 621-628.

Marder, K., Logroscino, G., Alfaro, B., Mejia, H., Halim, A., Louis, E., Cote, L., and Mayeux, R. (1998). Environmental risk factors for Parkinson's disease in an urban multiethnic community. *Neurology* **50**, 279-281.

Marnett, L. J. (1999a). Chemistry and biology of DNA damage by malondialdehyde. *IARC Sci Publ*, 17-27.

Marnett, L. J. (1999b). Lipid peroxidation-DNA damage by malondialdehyde. *Mutat Res* **424**, 83-95.

Maroni, M., Colosio, C., Ferioli, A., and Fait, A. (2000). Biological Monitoring of Pesticide Exposure: a review. Introduction. *Toxicology* **143**, 1-118.

Marsden, C. D. (1982). The mysterious motor function of the basal ganglia: the Robert Wartenberg Lecture. *Neurology* **32**, 514-539.

Marsden, C. D., and Obeso, J. A. (1994). The functions of the basal ganglia and the paradox of stereotaxic surgery in Parkinson's disease. *Brain* **117 (Pt 4)**, 877-897.

Martin, L. J. (2001). Neuronal cell death in nervous system development, disease, and injury (Review). *Int J Mol Med* **7**, 455-478.

Maruyama, S. (1998). [Necrosis and apoptosis]. *Masui* **47 Suppl**, S77-80.

Masoud, L., Vijayasarathy, C., Fernandez-Cabezudo, M., Petroianu, G., and Saleh, A. M. (2003). Effect of malathion on apoptosis of murine L929 fibroblasts: a possible mechanism for toxicity in low dose exposure. *Toxicology* **185**, 89-102.

Masters, C. L., and Beyreuther, K. (1990). Protein abnormalities in neurofibrillary tangles: their relation to the extracellular amyloid deposits of the A4 protein in Alzheimer's disease. *Adv Neurol* **51**, 151-161.

Matsura, T., Kai, M., Fujii, Y., Ito, H., and Yamada, K. (1999). Hydrogen peroxide-induced apoptosis in HL-60 cells requires caspase-3 activation. *Free Radic Res* **30**, 73-83.

Mattson, M. P. (2000). Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol* **1**, 120-129.

Mattson, M. P., Dou, P., and Kater, S. B. (1988). Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *J Neurosci* **8**, 2087-2100.

McCarthy, S., Somayajulu, M., Sikorska, M., Borowy-Borowski, H., and Pandey, S. (2004). Paraquat induces oxidative stress and neuronal cell death; neuroprotection by water-soluble Coenzyme Q10. *Toxicol Appl Pharmacol* **201**, 21-31.

McGinty, J. F. (1999). Regulation of neurotransmitter interactions in the ventral striatum. *Ann N Y Acad Sci* **877**, 129-139.

McGrew, D. M., Irwin, I., and Langston, J. W. (2000). Ethylenebisdithiocarbamate enhances MPTP-induced striatal dopamine depletion in mice. *Neurotoxicology* **21**, 309-312.

Meco, G., Bonifati, V., Vanacore, N., and Fabrizio, E. (1994). Parkinsonism after chronic exposure to the fungicide maneb (manganese ethylene-bis-dithiocarbamate). *Scand J Work Environ Health* **20**, 301-305.

Meier, P., Finch, A., and Evan, G. (2000). Apoptosis in development. *Nature* **407**, 796-801.

Mendoza, C. E. (1976). Toxicity and effects of malathion on esterases of suckling albino rats. *Toxicol Appl Pharmacol* **35**, 229-238.

Merello, M., and Cammarota, A. (2000). [Functional anatomy of the basal ganglia]. *Rev Neurol* **30**, 1055-1060.

Meyer, M., Schreck, R., and Baeuerle, P. A. (1993). H2O2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *Embo J* **12**, 2005-2015.

Miller, G. W., Kirby, M. L., Levey, A. I., and Bloomquist, J. R. (1999). Heptachlor alters expression and function of dopamine transporters. *Neurotoxicology* **20**, 631-637.

Mizuno, Y., Sone, N., Suzuki, K., and Saitoh, T. (1988). Studies on the toxicity of 1-methyl-4-phenylpyridinium ion (MPP+) against mitochondria of mouse brain. *J Neurol Sci* **86**, 97-110.

Mizuno, Y., Suzuki, K., Sone, N., and Saitoh, T. (1987). Inhibition of ATP synthesis by 1-methyl-4-phenylpyridinium ion (MPP+) in isolated mitochondria from mouse brains. *Neurosci Lett* **81**, 204-208.

Mocchegiani, E., Bertoni-Freddari, C., Marcellini, F., and Malavolta, M. (2005). Brain, aging and neurodegeneration: role of zinc ion availability. *Prog Neurobiol* **75**, 367-390.

Mohanakumar, K. P., de Bartolomeis, A., Wu, R. M., Yeh, K. J., Sternberger, L. M., Peng, S. Y., Murphy, D. L., and Chiueh, C. C. (1994). Ferrous-citrate complex

and nigral degeneration: evidence for free-radical formation and lipid peroxidation. *Ann N Y Acad Sci* **738**, 392-399.

Moser, V. C., Shafer, T. J., Ward, T. R., Meacham, C. A., Harris, M. W., and Chapin, R. E. (2001). Neurotoxicological outcomes of perinatal heptachlor exposure in the rat. *Toxicol Sci* **60**, 315-326.

Muindi, J. R., Sinha, B. K., Gianni, L., and Myers, C. E. (1984). Hydroxyl radical production and DNA damage induced by anthracycline-iron complex. *FEBS Lett* **172**, 226-230.

Muller-Vahl, K. R., Kolbe, H., and Dengler, R. (1999). Transient severe parkinsonism after acute organophosphate poisoning. *J Neurol Neurosurg Psychiatry* **66**, 253-254.

Muller, J. M., Rupec, R. A., and Baeuerle, P. A. (1997). Study of gene regulation by NF-kappa B and AP-1 in response to reactive oxygen intermediates. *Methods* **11**, 301-312.

Muller, K., and Gurster, D. (1993). Hydroxyl radical damage to DNA sugar and model membranes induced by anthralin (dithranol). *Biochem Pharmacol* **46**, 1695-1704.

Mutlu-Turkoglu, U., Ilhan, E., Oztezcan, S., Kuru, A., Aykac-Toker, G., and Uysal, M. (2003). Age-related increases in plasma malondialdehyde and protein carbonyl levels and lymphocyte DNA damage in elderly subjects. *Clin Biochem* **36**, 397-400.

Mytilineou, C., Cohen, G., and Heikkila, R. E. (1985). 1-Methyl-4-phenylpyridine (MPP+) is toxic to mesencephalic dopamine neurons in culture. *Neurosci Lett* **57**, 19-24.

Nagai, T., McGeer, P. L., Peng, J. H., McGeer, E. G., and Dolman, C. E. (1983). Choline acetyltransferase immunohistochemistry in brains of Alzheimer's disease patients and controls. *Neurosci Lett* **36**, 195-199.

Nakai, K., and Satoh, H. (2002). Developmental neurotoxicity following prenatal exposures to methylmercury and PCBs in humans from epidemiological studies. *Tohoku J Exp Med* **196**, 89-98.

Namura, S., Zhu, J., Fink, K., Endres, M., Srinivasan, A., Tomaselli, K. J., Yuan, J., and Moskowitz, M. A. (1998). Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *J Neurosci* **18**, 3659-3668.

Narayan, S., Bajpai, A., Chauhan, S. S., and Misra, U. K. (1985). Lipid peroxidation in lung and liver of rats given DDT and endosulfan intratracheally. *Bull Environ Contam Toxicol* **34**, 63-67.

Nebot, C., Moutet, M., Huet, P., Xu, J. Z., Yadan, J. C., and Chaudiere, J. (1993). Spectrophotometric assay of superoxide dismutase activity based on the activated autoxidation of a tetracyclic catechol. *Anal Biochem* **214**, 442-451.

Novikova, L., Garris, B. L., Garris, D. R., and Lau, Y. S. (2006). Early signs of neuronal apoptosis in the substantia nigra pars compacta of the progressive neurodegenerative mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/probenecid model of Parkinson's disease. *Neuroscience*.

O.T.A. (1990). *United States. Congress. Office of Technology Assessment. Neurotoxicity : identifying and controlling poisons of the nervous system : summary : new developments in neuroscience*. Congress of the U.S. For sale by the Supt. of Docs., U.S. G.P.O., Washington DC.

Obata, T., and Yamanaka, Y. (2001). [Parkinsonism induced by MPTP and free radical generation]. *Nippon Yakurigaku Zasshi* **117**, 105-110.

Olgun, S., Gogal, R. M., Jr., Adeshina, F., Choudhury, H., and Misra, H. P. (2004). Pesticide mixtures potentiate the toxicity in murine thymocytes. *Toxicology* **196**, 181-195.

Osmond, C., and Barker, D. J. (2000). Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women. *Environ Health Perspect* **108 Suppl 3**, 545-553.

Owen, A. D., Schapira, A. H., Jenner, P., and Marsden, C. D. (1996). Oxidative stress and Parkinson's disease. *Ann N Y Acad Sci* **786**, 217-223.

Oyarce, A. M., and Fleming, P. J. (1991). Multiple forms of human dopamine beta-hydroxylase in SH-SY5Y neuroblastoma cells. *Arch Biochem Biophys* **290**, 503-510.

Ozoe, Y., and Matsumura, F. (1986). Structural requirements for bridged bicyclic compounds acting on
picrotoxinin receptor. *J Agric Food Chem* **34**, 126-134.

Paglia, D. E., and Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* **70**, 158-169.

Pahlman, S., Mamaeva, S., Meyerson, G., Mattsson, M. E., Bjelfman, C., Ortoft, E., and Hammerling, U. (1990). Human neuroblastoma cells in culture: a model for neuronal cell differentiation and function. *Acta Physiol Scand Suppl* **592**, 25-37.

Pahlman, S., Ruusala, A. I., Abrahamsson, L., Mattsson, M. E., and Esscher, T. (1984). Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. *Cell Differ* **14**, 135-144.

Pall, H. S., Williams, A. C., Blake, D. R., Winyard, P., and Lunec, J. (1986). Lipid peroxidation and Parkinson's disease. *Lancet* **2**, 870-871.

Pandey, S., Ahmad, I., Parvez, S., Bin-Hafeez, B., Haque, R., and Raisuddin, S. (2001). Effect of endosulfan on antioxidants of freshwater fish Channa punctatus Bloch: 1. Protection against lipid peroxidation in liver by copper preexposure. *Arch Environ Contam Toxicol* **41**, 345-352.

Patel, S., Singh, V., Kumar, A., Gupta, Y. K., and Singh, M. P. (2006). Status of antioxidant defense system and expression of toxicant responsive genes in striatum of maneb- and paraquat-induced Parkinson's disease phenotype in mouse: Mechanism of neurodegeneration. *Brain Res* **1081**, 9-18.

Paul, V., Balasubramaniam, E., Jayakumar, A. R., and Kazi, M. (1995). A sex-related difference in the neurobehavioral and hepatic effects following chronic endosulfan treatment in rats. *Eur J Pharmacol* **293**, 355-360.

Paul, V., Balasubramaniam, E., and Kazi, M. (1994). The neurobehavioural toxicity of endosulfan in rats: a serotonergic involvement in learning impairment. *Eur J Pharmacol* **270**, 1-7.

Peng, J., Mao, X. O., Stevenson, F. F., Hsu, M., and Andersen, J. K. (2004). The herbicide paraquat induces dopaminergic nigral apoptosis through sustained activation of the JNK pathway. *J Biol Chem* **279**, 32626-32632.

Penney, J. B., Jr., and Young, A. B. (1983). Speculations on the functional anatomy of basal ganglia disorders. *Annu Rev Neurosci* **6**, 73-94.

Perandones, C. E., Illera, V. A., Peckham, D., Stunz, L. L., and Ashman, R. F. (1993). Regulation of apoptosis in vitro in mature murine spleen T cells. *J Immunol* **151**, 3521-3529.

Perez, R. G., Waymire, J. C., Lin, E., Liu, J. J., Guo, F., and Zigmond, M. J. (2002). A role for alpha-synuclein in the regulation of dopamine biosynthesis. *J Neurosci* **22**, 3090-3099.

Perier, C., Bove, J., Vila, M., and Przedborski, S. (2003). The rotenone model of Parkinson's disease. *Trends Neurosci* **26**, 345-346.

Perry, J. C., Da Cunha, C., Anselmo-Franci, J., Andreatini, R., Miyoshi, E., Tufik, S., and Vital, M. A. (2004). Behavioural and neurochemical effects of phosphatidylserine in MPTP lesion of the substantia nigra of rats. *Eur J Pharmacol* **484**, 225-233.

Perry, T. L., Godin, D. V., and Hansen, S. (1982). Parkinson's disease: a disorder due to nigral glutathione deficiency? *Neurosci Lett* **33**, 305-310.

Piccirilli, M., Piccinin, G. L., and Agostini, L. (1984). Dopamine deficiency in Parkinson's disease. *Neurology* **34**, 265-266.

Poirier, J., Kogan, S., and Gauthier, S. (1991). Environment, genetics and idiopathic Parkinson's disease. *Can J Neurol Sci* **18**, 70-76.

Pokharkar, D. S., and Dethe, M. D. (1981). Gas-liquid chromatographic studies on residues of endosulfan on chilli fruits. *J Environ Sci Health B* **16**, 439-451.

Pomes, A., Rodriguez-Farre, E., and Sunol, C. (1994). Disruption of GABA-dependent chloride flux by cyclodienes and hexachlorocyclohexanes in primary cultures of cortical neurons. *J Pharmacol Exp Ther* **271**, 1616-1623.

Pradhan, S. N. (1974). Proceedings: Balance between acetylcholine, serotonin, norepinephrine, and dopamine in self-stimulation. *Psychopharmacol Bull* **10**, 47-48.

Pratico, D., Clark, C. M., Liun, F., Rokach, J., Lee, V. Y., and Trojanowski, J. Q. (2002). Increase of brain oxidative stress in mild cognitive impairment: a possible predictor of Alzheimer disease. *Arch Neurol* **59**, 972-976.

Przedborski, S., Tieu, K., Perier, C., and Vila, M. (2004). MPTP as a mitochondrial neurotoxic model of Parkinson's disease. *J Bioenerg Biomembr* **36**, 375-379.

Raffray, M., and Cohen, G. M. (1991). Bis(tri-n-butyltin)oxide induces programmed cell death (apoptosis) in immature rat thymocytes. *Arch Toxicol* **65**, 135-139.

Rajput, A. H., and Uitti, R. J. (1987). Paraquat and Parkinson's disease. *Neurology* **37**, 1820-1821.

Ramsay, R. R., and Singer, T. P. (1992). Relation of superoxide generation and lipid peroxidation to the inhibition of NADH-Q oxidoreductase by rotenone, piericidin A, and MPP+. *Biochem Biophys Res Commun* **189**, 47-52.

Reiter, R. J., Acuna-Castroviejo, D., Tan, D. X., and Burkhardt, S. (2001). Free radical-mediated molecular damage. Mechanisms for the protective actions of melatonin in the central nervous system. *Ann N Y Acad Sci* **939**, 200-215.

Repetto, R., and Baliga, S. S. (1996). Pesticides and the immune system: the public health risks. Executive summary. *Cent Eur J Public Health* **4**, 263-265.

Requena, J. R., Fu, M. X., Ahmed, M. U., Jenkins, A. J., Lyons, T. J., and Thorpe, S. R. (1996). Lipoxidation products as biomarkers of oxidative damage to proteins during lipid peroxidation reactions. *Nephrol Dial Transplant* **11 Suppl 5**, 48-53.

Rhile, M. J., Nagarkatti, M., and Nagarkatti, P. S. (1996). Role of Fas apoptosis and MHC genes in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity of T cells. *Toxicology* **110**, 153-167.

Rideout, H. J., and Stefanis, L. (2001). Caspase inhibition: a potential therapeutic strategy in neurological diseases. *Histol Histopathol* **16**, 895-908.

Rinne, U. K. (1982). Parkinson's disease as a model for changes in dopamine receptor dynamics with aging. *Gerontology* **28 Suppl 1**, 35-52.

Ritz, B., Yu, F., Chapa, G., and Fruin, S. (2000). Effect of air pollution on preterm birth among children born in Southern California between 1989 and 1993. *Epidemiology* **11**, 502-511.

Rivera, S., Rosa, R., Martinez, E., Sunol, C., Serrano, M. T., Vendrell, M., Rodriguez-Farre, E., and Sanfeliu, C. (1998). Behavioral and monoaminergic changes after lindane exposure in developing rats. *Neurotoxicol Teratol* **20**, 155-160.

Rivera, S., Sanfeliu, C., and Rodriguez-Farre, E. (1990). Behavioral changes induced in developing rats by an early postnatal exposure to lindane. *Neurotoxicol Teratol* **12**, 591-595.

Romo, R., and Schultz, W. (1992). Role of primate basal ganglia and frontal cortex in the internal generation of movements. III. Neuronal activity in the supplementary motor area. *Exp Brain Res* **91**, 396-407.

Rondot, P., Bianco, C., and de Recondo, J. (1986). Ageing and Parkinson's disease. *Gerontology* **32 Suppl 1**, 102-105.

Rosa, R., Rodriguez-Farre, E., and Sanfeliu, C. (1996). Cytotoxicity of hexachlorocyclohexane isomers and cyclodienes in primary cultures of cerebellar granule cells. *J Pharmacol Exp Ther* **278**, 163-169.

Rosenstock, T. R., Carvalho, A. C., Jurkiewicz, A., Frussa-Filho, R., and Smaili, S. S. (2004). Mitochondrial calcium, oxidative stress and apoptosis in a neurodegenerative disease model induced by 3-nitropropionic acid. *J Neurochem* **88**, 1220-1228.

Ross, R. A., and Biedler, J. L. (1985). Expression of a melanocyte phenotype in human neuroblastoma cells in vitro. *Prog Clin Biol Res* **175**, 249-259.

Rottkamp, C. A., Nunomura, A., Raina, A. K., Sayre, L. M., Perry, G., and Smith, M. A. (2000). Oxidative stress, antioxidants, and Alzheimer disease. *Alzheimer Dis Assoc Disord* **14 Suppl 1**, S62-66.

Rought, S. E., Yau, P. M., Guo, X. W., Chuang, L. F., Doi, R. H., and Chuang, R. Y. (2000). Modulation of CPP32 activity and induction of apoptosis in Human CEM X 174 lymphocytes by heptachlor, a chlorinated hydrocarbon insecticide. *J Biochem Mol Toxicol* **14**, 42-50.

Rupa, D. S., Reddy, P. P., Sreemannarayana, K., and Reddi, O. S. (1991). Frequency of sister chromatid exchange in peripheral lymphocytes of male pesticide applicators. *Environ Mol Mutagen* **18**, 136-138.

Russ, H., Mihatsch, W., Gerlach, M., Riederer, P., and Przuntek, H. (1991). Neurochemical and behavioural features induced by chronic low dose treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the common marmoset: implications for Parkinson's disease? *Neurosci Lett* **123**, 115-118.

Russ, H., Staust, K., Martel, F., Gliese, M., and Schomig, E. (1996). The extraneuronal transporter for monoamine transmitters exists in cells derived from human central nervous system glia. *Eur J Neurosci* **8**, 1256-1264.

Saggu, H., Cooksey, J., Dexter, D., Wells, F. R., Lees, A., Jenner, P., and Marsden, C. D. (1989). A selective increase in particulate superoxide dismutase activity in parkinsonian substantia nigra. *J Neurochem* **53**, 692-697.

Sanchez-Ramos, J. R., Hefti, F., and Weiner, W. J. (1987). Paraquat and Parkinson's disease. *Neurology* **37**, 728.

Sanchez-Ramos, J. R., Song, S., Facca, A., Basit, A., and Epstein, C. J. (1997). Transgenic murine dopaminergic neurons expressing human Cu/Zn superoxide dismutase exhibit increased density in culture, but no resistance to methylphenylpyridinium-induced degeneration. *J Neurochem* **68**, 58-67.

Sandyk, R., Bamford, C. R., and Iacono, R. P. (1988). Pain and sensory symptoms in Parkinson's disease. *Int J Neurosci* **39**, 15-25.

Savolainen, K., and Hervonen, H. (1985). Dithiocarbamate fungicides decrease histochemical reactivity of cholinesterases in the gut wall of the rat. *Arch Toxicol Suppl* **8**, 272-276.

Schenk, H., Klein, M., Erdbrugger, W., Droge, W., and Schulze-Osthoff, K. (1994). Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-kappa B and AP-1. *Proc Natl Acad Sci U S A* **91**, 1672-1676.

Scherman, D., Desnos, C., Darchen, F., Pollak, P., Javoy-Agid, F., and Agid, Y. (1989). Striatal dopamine deficiency in Parkinson's disease: role of aging. *Ann Neurol* **26**, 551-557.

Schmid, I., Uittenbogaart, C. H., and Giorgi, J. V. (1994a). Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. *Cytometry* **15**, 12-20.

Schmid, I., Uittenbogaart, C. H., Keld, B., and Giorgi, J. V. (1994b). A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. *J Immunol Methods* **170**, 145-157.

Schmidt, N., and Ferger, B. (2001). Neurochemical findings in the MPTP model of Parkinson's disease. *J Neural Transm* **108**, 1263-1282.

Schmidt, W. J. (1995). Balance of transmitter activities in the basal ganglia loops. *J Neural Transm Suppl* **46**, 67-76.

Schmuck, G., Ahr, H. J., Mihail, F., Stahl, B., and Kayser, M. (2002). Effects of the dithiocarbamate fungicide propineb in primary neuronal cell cultures and skeletal muscle cells of the rat. *Arch Toxicol* **76**, 414-422.

Schreck, R., Albermann, K., and Baeuerle, P. A. (1992a). Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic Res Commun* **17**, 221-237.

Schreck, R., Meier, B., Mannel, D. N., Droge, W., and Baeuerle, P. A. (1992b). Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. *J Exp Med* **175**, 1181-1194.

Schreck, R., Rieber, P., and Baeuerle, P. A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *Embo J* **10**, 2247-2258.

Schulze-Osthoff, K., Los, M., and Baeuerle, P. A. (1995). Redox signalling by transcription factors NF-kappa B and AP-1 in lymphocytes. *Biochem Pharmacol* **50**, 735-741.

Seaton, T. A., Cooper, J. M., and Schapira, A. H. (1997). Free radical scavengers protect dopaminergic cell lines from apoptosis induced by complex I inhibitors. *Brain Res* **777**, 110-118.

Sechi, G., Deledda, M. G., Bua, G., Satta, W. M., Deiana, G. A., Pes, G. M., and Rosati, G. (1996). Reduced intravenous glutathione in the treatment of early Parkinson's disease. *Prog Neuropsychopharmacol Biol Psychiatry* **20**, 1159-1170.

Segawa, M. (2000). Development of the nigrostriatal dopamine neuron and the pathways in the basal ganglia. *Brain Dev* **22 Suppl 1**, S1-4.

Semchuk, K. M., Love, E. J., and Lee, R. G. (1992). Parkinson's disease and exposure to agricultural work and pesticide chemicals. *Neurology* **42**, 1328-1335.

Seth, P. K., Saidi, N. F., Agrawal, A. K., and Anand, M. (1986). Neurotoxicity of endosulfan in young and adult rats. *Neurotoxicology* **7**, 623-635.

Shafer, T. J., Meyer, D. A., and Crofton, K. M. (2005). Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. *Environ Health Perspect* **113**, 123-136.

Sharma, R. P., Winn, D. S., and Low, J. B. (1976). Toxic, neurochemical and behavioral effects of dieldrin exposure in mallard ducks. *Arch Environ Contam Toxicol* **5**, 43-53.

Shastry, B. S. (2001). Parkinson disease: etiology, pathogenesis and future of gene therapy. *Neurosci Res* **41**, 5-12.

Shatz, C. J. (1990). Impulse activity and the patterning of connections during CNS development. *Neuron* **5**, 745-756.

Shavali, S., Combs, C. K., and Ebadi, M. (2006). Reactive macrophages increase oxidative stress and alpha-synuclein nitration during death of dopaminergic neuronal cells in co-culture: relevance to Parkinson's disease. *Neurochem Res* **31**, 85-94.

Shen, H. M., Shi, C. Y., Shen, Y., and Ong, C. N. (1996). Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin B1. *Free Radic Biol Med* **21**, 139-146.

Shen, Z. X. (1994). Acetylcholinesterase provides deeper insights into Alzheimer's disease. *Med Hypotheses* **43**, 21-30.

Sherer, T. B., Betarbet, R., Kim, J. H., and Greenamyre, J. T. (2003). Selective microglial activation in the rat rotenone model of Parkinson's disease. *Neurosci Lett* **341**, 87-90.

Shimohama, S., Tanino, H., and Fujimoto, S. (2001). Differential expression of rat brain caspase family proteins during development and aging. *Biochem Biophys Res Commun* **289**, 1063-1066.

Shimoke, K., Kudo, M., and Ikeuchi, T. (2003). MPTP-induced reactive oxygen species promote cell death through a gradual activation of caspase-3 without expression of GRP78/Bip as a preventive measure against ER stress in PC12 cells. *Life Sci* **73**, 581-593.

Shin, E. J., Jhoo, J. H., Kim, W. K., Jhoo, W. K., Lee, C., Jung, B. D., and Kim, H. C. (2004a). Protection against kainate neurotoxicity by pyrrolidine dithiocarbamate. *Clin Exp Pharmacol Physiol* **31**, 320-326.

Shin, M. H., Jang, J. H., and Surh, Y. J. (2004b). Potential roles of NF-kappaB and ERK1/2 in cytoprotection against oxidative cell death induced by tetrahydropapaveroline. *Free Radic Biol Med* **36**, 1185-1194.

Sidhu, A., Wersinger, C., and Vernier, P. (2004). alpha-Synuclein regulation of the dopaminergic transporter: a possible role in the pathogenesis of Parkinson's disease. *FEBS Lett* **565**, 1-5.

Simmons, J. E. (1995). Chemical mixtures: challenge for toxicology and risk assessment. *Toxicology* **105**, 111-119.

Singer, T. P., Ramsay, R. R., McKeown, K., Trevor, A., and Castagnoli, N. E., Jr. (1988). Mechanism of the neurotoxicity of 1-methyl-4-phenylpyridinium (MPP+), the toxic bioactivation product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Toxicology* **49**, 17-23.

Singh, S. K., and Pandey, R. S. (1989). Toxicity of endosulfan on kidney of male rats in relation to drug metabolizing enzymes and microsomal lipid peroxidation. *Indian J Exp Biol* **27**, 725-728.

Sinha, B. K., Singh, Y., and Krishna, G. (1986). Formation of superoxide and hydroxyl radicals from 1-methyl-4-phenylpyridinium ion (MPP+): reductive activation by NADPH cytochrome P-450 reductase. *Biochem Biophys Res Commun* **135**, 583-588.

Sinha, N., Narayan, R., and Saxena, D. K. (1997). Effect of endosulfan on the testis of growing rats. *Bull Environ Contam Toxicol* **58**, 79-86.

Sirinathsinghji, D. J., Whittington, P. E., and Audsley, A. R. (1986). Neurochemical changes in the substantiae nigrae and caudate nuclei following acute unilateral intranigral infusions of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Brain Res* **399**, 339-345.

Slater, A. F., Nobel, C. S., and Orrenius, S. (1995). The role of intracellular oxidants in apoptosis. *Biochim Biophys Acta* **1271**, 59-62.

Smith, C. (2001). Pesticide exports from U.S. ports, 1997-2000. *Int J Occup Environ Health* **7**, 266-274.

Smith, M. T., Sandy, M. S., and Di Monte, D. (1987). Free radicals, lipid peroxidation, and Parkinson's disease. *Lancet* **1**, 38.

Sofic, E., Lange, K. W., Jellinger, K., and Riederer, P. (1992). Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. *Neurosci Lett* **142**, 128-130.

Sohn, H. Y., Kwon, C. S., Kwon, G. S., Lee, J. B., and Kim, E. (2004). Induction of oxidative stress by endosulfan and protective effect of lipid-soluble antioxidants against endosulfan-induced oxidative damage. *Toxicol Lett* **151**, 357-365.

Soleo, L., Defazio, G., Scarselli, R., Zefferino, R., Livrea, P., and Foa, V. (1996). Toxicity of fungicides containing ethylene-bis-dithiocarbamate in serumless dissociated mesencephalic-striatal primary coculture. *Arch Toxicol* **70**, 678-682.

Souza, J. M., Giasson, B. I., Chen, Q., Lee, V. M., and Ischiropoulos, H. (2000). Dityrosine cross-linking promotes formation of stable alpha -synuclein polymers.

Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J Biol Chem* **275**, 18344-18349.
Spencer, J. P., Jenner, P., and Halliwell, B. (1995). Superoxide-dependent depletion of reduced glutathione by L-DOPA and dopamine. Relevance to Parkinson's disease. *Neuroreport* **6**, 1480-1484.
Spillantini, M. G., Crowther, R. A., and Goedert, M. (1996). Comparison of the neurofibrillary pathology in Alzheimer's disease and familial presenile dementia with tangles. *Acta Neuropathol (Berl)* **92**, 42-48.
Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997). Alpha-synuclein in Lewy bodies. *Nature* **388**, 839-840.
Stadelmann, C., Deckwerth, T. L., Srinivasan, A., Bancher, C., Bruck, W., Jellinger, K., and Lassmann, H. (1999). Activation of caspase-3 in single neurons and autophagic granules of granulovacuolar degeneration in Alzheimer's disease. Evidence for apoptotic cell death. *Am J Pathol* **155**, 1459-1466.
Storey, K. B. (1996). Oxidative stress: animal adaptations in nature. *Braz J Med Biol Res* **29**, 1715-1733.
Sun, X. M., Butterworth, M., MacFarlane, M., Dubiel, W., Ciechanover, A., and Cohen, G. M. (2004). Caspase activation inhibits proteasome function during apoptosis. *Mol Cell* **14**, 81-93.
Sundstrom, E., Fredriksson, A., and Archer, T. (1990). Chronic neurochemical and behavioral changes in MPTP-lesioned C57BL/6 mice: a model for Parkinson's disease. *Brain Res* **528**, 181-188.
Surgucheva, I., Park, B. C., Yue, B. Y., Tomarev, S., and Surguchov, A. (2005). Interaction of myocilin with gamma-synuclein affects its secretion and aggregation. *Cell Mol Neurobiol* **25**, 1009-1033.
Suri, R. E., Albani, C., and Glattfelder, A. H. (1997). A dynamic model of motor basal ganglia functions. *Biol Cybern* **76**, 451-458.
Tai, K. K., and Truong, D. D. (2002). Activation of adenosine triphosphate-sensitive potassium channels confers protection against rotenone-induced cell death: therapeutic implications for Parkinson's disease. *J Neurosci Res* **69**, 559-566.
Takahashi, N., Miner, L. L., Sora, I., Ujike, H., Revay, R. S., Kostic, V., Jackson-Lewis, V., Przedborski, S., and Uhl, G. R. (1997). VMAT2 knockout mice: heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. *Proc Natl Acad Sci U S A* **94**, 9938-9943.
Takahashi, R. N., Rogerio, R., and Zanin, M. (1989). Maneb enhances MPTP neurotoxicity in mice. *Res Commun Chem Pathol Pharmacol* **66**, 167-170.
Takahashi, T., Deng, Y., Maruyama, W., Dostert, P., Kawai, M., and Naoi, M. (1994). Uptake of a neurotoxin-candidate, (R)-1,2-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline into human dopaminergic neuroblastoma SH-SY5Y cells by dopamine transport system. *J Neural Transm Gen Sect* **98**, 107-118.
Talesa, V. N. (2001). Acetylcholinesterase in Alzheimer's disease. *Mech Ageing Dev* **122**, 1961-1969.
Tanner, C. M., Ottman, R., Goldman, S. M., Ellenberg, J., Chan, P., Mayeux, R., and Langston, J. W. (1999). Parkinson disease in twins: an etiologic study. *Jama* **281**, 341-346.

Tariq, M., Arshaduddin, M., Biary, N., Al Deeb, S., and Al Moutaery, K. (2000). Diethyldithiocarbamate (DEDC) impairs neuronal recovery following sciatic nerve injury in rats. *Restor Neurol Neurosci* **17**, 135-141.

Tatton, W. G., and Olanow, C. W. (1999). Apoptosis in neurodegenerative diseases: the role of mitochondria. *Biochim Biophys Acta* **1410**, 195-213.

Taylor, P. (1998). Development of acetylcholinesterase inhibitors in the therapy of Alzheimer's disease. *Neurology* **51**, S30-35; discussion S65-37.

Tebourbi, O., Rhouma, K. B., and Sakly, M. (1998). DDT induces apoptosis in rat thymocytes. *Bull Environ Contam Toxicol* **61**, 216-223.

Testa, C. M., Sherer, T. B., and Greenamyre, J. T. (2005). Rotenone induces oxidative stress and dopaminergic neuron damage in organotypic substantia nigra cultures. *Brain Res Mol Brain Res* **134**, 109-118.

Thiruchelvam, M., Brockel, B. J., Richfield, E. K., Baggs, R. B., and Cory-Slechta, D. A. (2000a). Potentiated and preferential effects of combined paraquat and maneb on nigrostriatal dopamine systems: environmental risk factors for Parkinson's disease? *Brain Res* **873**, 225-234.

Thiruchelvam, M., McCormack, A., Richfield, E. K., Baggs, R. B., Tank, A. W., Di Monte, D. A., and Cory-Slechta, D. A. (2003). Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. *Eur J Neurosci* **18**, 589-600.

Thiruchelvam, M., Prokopenko, O., Cory-Slechta, D. A., Richfield, E. K., Buckley, B., and Mirochnitchenko, O. (2005). Overexpression of superoxide dismutase or glutathione peroxidase protects against the paraquat + maneb-induced Parkinson disease phenotype. *J Biol Chem* **280**, 22530-22539.

Thiruchelvam, M., Richfield, E. K., Baggs, R. B., Tank, A. W., and Cory-Slechta, D. A. (2000b). The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. *J Neurosci* **20**, 9207-9214.

Thiruchelvam, M., Richfield, E. K., Goodman, B. M., Baggs, R. B., and Cory-Slechta, D. A. (2002). Developmental exposure to the pesticides paraquat and maneb and the Parkinson's disease phenotype. *Neurotoxicology* **23**, 621-633.

Thiruchelvam, M., Richfield, E. K., Goodman, B. M., Baggs, R. B., and Cory-Slechta, D. A. (2002b). Developmental exposure to the pesticides paraquat and maneb and the Parkinson's disease phenotype. *Neurotoxicology* **23**, 621-633.

Thiruchelvam, M. J., Powers, J. M., Cory-Slechta, D. A., and Richfield, E. K. (2004). Risk factors for dopaminergic neuron loss in human alpha-synuclein transgenic mice. *Eur J Neurosci* **19**, 845-854.

Thornberry, N. A. (1994). Interleukin-1 beta converting enzyme. *Methods Enzymol* **244**, 615-631.

Thornberry, N. A., and Lazebnik, Y. (1998). Caspases: enemies within. *Science* **281**, 1312-1316.

Tilson, H. A. (1998). Developmental neurotoxicology of endocrine disruptors and pesticides: identification of information gaps and research needs. *Environ Health Perspect* **106 Suppl 3**, 807-811.

Tilson, H. A. (2000). Neurotoxicology risk assessment guidelines: developmental neurotoxicology. *Neurotoxicology* **21**, 189-194.

Tolwani, R. J., Jakowec, M. W., Petzinger, G. M., Green, S., and Waggie, K. (1999). Experimental models of Parkinson's disease: insights from many models. *Lab Anim Sci* **49**, 363-371.

Trojanowski, J. Q. (2003). Rotenone neurotoxicity: a new window on environmental causes of Parkinson's disease and related brain amyloidoses. *Exp Neurol* **179**, 6-8.

Trump, B. F., Berezesky, I. K., Chang, S. H., and Phelps, P. C. (1997). The pathways of cell death: oncosis, apoptosis, and necrosis. *Toxicol Pathol* **25**, 82-88.

Vaccari, A., Saba, P., Mocci, I., and Ruiu, S. (1999). Dithiocarbamate pesticides affect glutamate transport in brain synaptic vesicles. *J Pharmacol Exp Ther* **288**, 1-5.

Valencia, A., and Moran, J. (2004). Reactive oxygen species induce different cell death mechanisms in cultured neurons. *Free Radic Biol Med* **36**, 1112-1125.

von Moltke, L. L., Greenblatt, D. J., Schmider, J., Wright, C. E., Harmatz, J. S., and Shader, R. I. (1998). In vitro approaches to predicting drug interactions in vivo. *Biochem Pharmacol* **55**, 113-122.

Voorn, P., Kalsbeek, A., Jorritsma-Byham, B., and Groenewegen, H. J. (1988). The pre- and postnatal development of the dopaminergic cell groups in the ventral mesencephalon and the dopaminergic innervation of the striatum of the rat. *Neuroscience* **25**, 857-887.

Wagner, S. R., and Greene, F. E. (1978). Dieldrin-induced alterations in biogenic amine content of rat brain. *Toxicol Appl Pharmacol* **43**, 45-55.

Waldmeier, P. C., and Tatton, W. G. (2004). Interrupting apoptosis in neurodegenerative disease: potential for effective therapy? *Drug Discov Today* **9**, 210-218.

Walker, M. J., Jenner, P., and Marsden, C. D. (1991). A redox reaction between MPP+ and MPDP+ to produce superoxide radicals does not impair mitochondrial function. *Biochem Pharmacol* **42**, 913-919.

Weinreb, P. H., Zhen, W., Poon, A. W., Conway, K. A., and Lansbury, P. T., Jr. (1996). NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. *Biochemistry* **35**, 13709-13715.

Westerink, B. H., de Boer, P., and Damsma, G. (1990). Dopamine-acetylcholine interaction in the striatum studied by microdialysis in the awake rat: some methodological aspects. *J Neurosci Methods* **34**, 117-124.

Wilcock, G. K., Esiri, M. M., Bowen, D. M., and Smith, C. C. (1982). Alzheimer's disease. Correlation of cortical choline acetyltransferase activity with the severity of dementia and histological abnormalities. *J Neurol Sci* **57**, 407-417.

Wooten, M. W. (1999). Function for NF-kB in neuronal survival: regulation by atypical protein kinase C. *J Neurosci Res* **58**, 607-611.

Wyllie, A. H. (1987). Apoptosis: cell death under homeostatic control. *Arch Toxicol Suppl* **11**, 3-10.

Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980). Cell death: the significance of apoptosis. *Int Rev Cytol* **68**, 251-306.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270**, 1326-1331.

Yakovlev, A. G., Ota, K., Wang, G., Movsesyan, V., Bao, W. L., Yoshihara, K., and Faden, A. I. (2001). Differential expression of apoptotic protease-activating factor-1 and caspase-3 genes and susceptibility to apoptosis during brain development and after traumatic brain injury. *J Neurosci* **21**, 7439-7446.

Yang, S. C., Johannessen, J. N., and Markey, S. P. (1988). Metabolism of [14C]MPTP in mouse and monkey implicates MPP+, and not bound metabolites, as the operative neurotoxin. *Chem Res Toxicol* **1**, 228-233.

Zafrilla, P., Mulero, J., Xandri, J. M., Santo, E., Caravaca, G., and Morillas, J. M. (2006). Oxidative stress in Alzheimer patients in different stages of the disease. *Curr Med Chem* **13**, 1075-1083.

Zaidi, N. F., Agrawal, A. K., Anand, M., and Seth, P. K. (1985). Neonatal endosulfan neurotoxicity: behavioral and biochemical changes in rat pups. *Neurobehav Toxicol Teratol* **7**, 439-442.

Zang, L. Y., and Misra, H. P. (1992a). EPR kinetic studies of superoxide radicals generated during the autoxidation of 1-methyl-4-phenyl-2,3-dihydropyridinium, a bioactivated intermediate of parkinsonian-inducing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Biol Chem* **267**, 23601-23608.

Zang, L. Y., and Misra, H. P. (1992b). Superoxide radical production during the autoxidation of 1-methyl-4-phenyl-2,3-dihydropyridinium perchlorate. *J Biol Chem* **267**, 17547-17552.

Zang, L. Y., and Misra, H. P. (1993). Generation of reactive oxygen species during the monoamine oxidase-catalyzed oxidation of the neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Biol Chem* **268**, 16504-16512.

Zhang, J., Fitsanakis, V. A., Gu, G., Jing, D., Ao, M., Amarnath, V., and Montine, T. J. (2003). Manganese ethylene-bis-dithiocarbamate and selective dopaminergic neurodegeneration in rat: a link through mitochondrial dysfunction. *J Neurochem* **84**, 336-346.

Zhang, Y., Dawson, V. L., and Dawson, T. M. (2000). Oxidative stress and genetics in the pathogenesis of Parkinson's disease. *Neurobiol Dis* **7**, 240-250.

Zhivotovsky, B. (2004). Apoptosis, necrosis and between. *Cell Cycle* **3**, 64-66.

Zhou, L. Z., Johnson, A. P., and Rando, T. A. (2001). NF kappa B and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. *Free Radic Biol Med* **31**, 1405-1416.

Zhou, Y., Shie, F. S., Piccardo, P., Montine, T. J., and Zhang, J. (2004). Proteasomal inhibition induced by manganese ethylene-bis-dithiocarbamate: relevance to Parkinson's disease. *Neuroscience* **128**, 281-291.