Immunotoxic and Oxidative Effects of Endosulfan and Permethrin on Murine Splenocytes, *in vitro*

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ABSTRACT

Indiscriminate use of pesticides appears to alter immune response in non-target organisms such as humans and other animals. Thus, immune modulation is considered as one of the potential risks and consequences following exposure to these chemicals. Because of the widespread usage, exposure to mixtures of pesticides during the lifetime of individuals is unavoidable and can result in potentiation of the toxic effects. Because immune cells are more susceptible to toxic insults at a lower dose than most other cell types, the effects of pesticides and their mixtures on murine splenocytes were evaluated. C57BL/6 male mouse splenocytes, *in vitro*, were exposed to permethrin and endosulfan, individually and in-combination (25-200 μM). The immunotoxic potential of these pesticides was monitored using a flow cytometric technique in combination with 7-Amino Actinomycin D (7-AAD) staining. Endosulfan exposures (25-150 μM) resulted in time- and dose-dependent increase in apoptotic and necrotic cell death in murine splenocytes, *in vitro*. Permethrin exposure (50-200 μM) resulted in neither a time-dependent/dose-dependent loss of splenocyte viability nor induction of apoptosis in splenocytes. With mixtures of permethrin and endosulfan, depressed viability and enhanced early apoptosis and late apoptosis/necrosis were observed. Exposure to mixtures of 50 μM endosulfan with 50 or 100 μM permethrin increased late apoptosis/necrosis compared to exposure to either chemical alone. DNA fragmentation, a

hall mark of apoptosis was observed by DNA ladder technique, confirming the occurrence of apoptosis. Morphological observation using cytospun slides was also carried out to further confirm the presence of apoptosis and necrosis. These findings suggest that the immunotoxicity of endosulfan both individually and in mixtures with permethrin is associated with the occurrence of apoptotic and necrotic processes.

Further, the ability of these pesticides to alter the oxidative status of the cells, via reactive oxygen species (ROS) generation and modulation of intracellular antioxidant enzymes levels, was investigated. We monitored the generation of ROS such as hydrogen peroxide (H_2O_2) with 2′, 7′- dichlorofluorescin diacetate (DCFH-DA) assay and superoxide anion (O_2^-) with hydroethidine (HE) assay in combination with flow cytometry. Spectrophotometric techniques were used to measure antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPX). Results of the analyses revealed that individual pesticides increased the production of H_2O_2 in a time and dose-dependent manner. Both time and dose-dependent increases in O_2^- production were caused by permethrin; whereas endosulfan exposure resulted in only a dose-dependent increase. However, exposure to mixtures of these pesticides had little or no effect on the generation of H_2O_2 and O_2^- radicals as compared to individual pesticides. The levels of SOD and GPX in pesticide-treated splenocytes were found to be not different from solvent control. An increase in GR and CAT levels in cells was noticed with permethrin (100 μM) exposure. These findings suggest that permethrin and endosulfan have the ability to affect the cellular oxidative status and can cause toxicity in immune cells, *in vitro*.

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DECLARATION OF WORK PERFORMED

I declare that I, Vimala Vemireddi performed all the work herein, except for the portions of flow cytometric analysis.

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LIST OF ABBREVIATIONS

7-AAD – 7- Aminoactinomycin-D
AT – 3 –amino -2, 3, 4-triazole
ATP – Adenosine Triphosphate
CAT – Catalase
CMI – Cell-mediated immunity
CNS – Central nervous system
Cu/Zn SOD – Copper-Zinc Superoxide Dismutase
DCF – 2',7'- Diochloroflourescein
DCFH-DA – 2',7'- Diochloroflourescin diacetate
DDT – 1,1 bis (p- chlorophenyl) 2,2,2 trichloroethane
DEET – N, N Diethyl m-Toluamide
DNA – Deoxyribonucleic acid
e^- – Electron
EDTA – Ethylenediaminetetracetic acid
Fe^{2+} – Ferrous ion
Fe^{3+} – Ferric ion
GABA – Gamma amino butyric acid
GPX – Glutathione Peroxidase
GR – Glutathione Reductase
GSH – Reduced glutathione
GSSG – Oxidized glutathione
H^+ – Proton
HE – Hydroethidine
H_2O – Water
H_2O_2 – Hydrogen peroxide
IgG – Immunoglobulin G
IgM – Immunoglobulin M
kDa – Kilo Daltons
L• – Lipid radical
LO• – Lipid alkoxyl radical
LOO• – Lipid peroxyl radical
LOOH – Lipid hydroperoxide
Mn-SOD – Manganese superoxide dismutase
NADP – Nicotenamide adenine dinucleotide phosphate
NADPH – Nicotenamide adenine dinucleotide phosphate-reduced form
NK – Natural killer cell
O_2 – Oxygen
O_2^- – Superoxide radical
$^{\cdot}OH$ – Hydroxyl radical
P – Statistical probability
PBS – Phosphate buffered saline
RBC – Red blood cell
ROH – Organic alcohol
ROOH – Organic peroxide

ROS – Reactive oxygen species
RPMI-1640 – Roosevelt Park Memorial Institute Medium
SEM – Standard Error of Mean
SOD – Superoxide Dismutase
TCDD – 2,3,7,8- tetrachloro-p-benzo dioxin
U.S.EPA – United States Environmental Protection Agency
UV – Ultraviolet
WHO – World Health Organization

Chapter 1. INTRODUCTION

1.1. Study Hypothesis:

This study was designed to examine the effect of pesticides and their mixtures on oxidative status and cytotoxicity of murine splenocytes, individually and in simple mixtures, *in vitro.* Oxidative damage to tissues caused by ROS has been proposed to play a role in the induction of variety of pesticide-induced toxic manifestations. It was hypothesized that the pesticide mixtures might potentiate the cytotoxicity in immune cells as compared to individual pesticides.

1.2. Study Rationale:

Because of ever-increasing use in every aspect of life, humans are exposed to a large number of chemicals through various routes of exposure either concurrently or sequentially (Simmons, 1995). The daily exposure to mixtures of chemicals is real and and there is paucity of data about the effects of various chemicals in mixtures. The sensitivity of the immune system to low dose chemical exposures when major systems are not affected (Sharma et al., 1987), further warrants investigation of pesticide mixtures role in immune toxicity. It has been suggested that the ability of certain pesticides to alter oxidative status of the cell might be responsible, at least in part, for the proposed cellular dysfunction

1.3. Study Objective:

The overall goal of this study was to understand the role of pesticides, singly and in mixtures in inducing cytoxicity and their ability to alter oxidative (both pro-oxidant and anti-oxidant) status of C57BL6 mice splenocytes, *in vitro*.

1.4. Specific Aims:

There were three specific aims in this study.

1) To investigate the cytotoxic effects of endosulfan, permethrin and their mixtures on C57BL/6 murine splenocytes, *in vitro*

2) To investigate the role of the pesticides and their mixtures in inducing ROS generation in murine splenocytes, *in vitro*

3) To investigate the ability of pesticides and their mixtures in modulating intracellular antioxidant enzyme systems

With these specific aims we intend to gain a better understanding of pesticide-induced oxidative stress as a possible mechanism of immune-cell cytotoxicity.

Chapter 2. LITERATURE REVIEW

2.1. Introduction

Pesticides are xenobiotics, intended to destroy or repel unwanted plants, insects and animal species. Pesticides are used both in agriculture and in public hygiene for protection against pests. Globally, close to 5.6 million pounds of pesticides, worth $33.5 billion dollars were used annually in years 1998 and 1999 (USEPA, 2002). Because of indiscriminate use and poor biodegradability, many of these chemicals have become major environmental pollutants. Pesticides are designed to be target specific, nonetheless has the potential to affect the well being of other non-target species as well. Although vital in increasing food production and eliminating diseases, exposure to pesticides can also be harmful to humans. The ill effects may follow from short- or long-term and low- or high-level exposures through various routes of entry in to the body.

Pesticides residues have been detected in diverse types of environmental samples (Meulenberg et al., 1995). Numerous studies have demonstrated that many of the commonly used pesticides/chemicals can suppress the normal response of the human immune system to invading microorganisms, parasites, and tumors. The immune system is the primary line of defense against organic insults, so suppression of this system could increase the toll of infections/diseases. Most pesticides now on the market have been inadequately tested in the laboratory for their immunotoxic effects. In view of the widespread use, there is an urgent need to investigate the immunotoxicological effects of pesticides in mammals.

The current study was designed to monitor the immunotoxic potential of endosulfan and permethrin in C57BL/6 mouse splenocytes, *in vitro*. The role of pesticides in inducing oxidative stress was also monitored.

2.2. Pesticides

2.2.1. 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 polycyclic chlorinated hydrocarbon insecticide that belongs to the cyclodiene subgroup (Fig.1). This is a highly toxic pesticide and a potential endocrine disruptor. 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 Environmental Protection Agency (EPA) has classified endosulfan as a toxicity class Ib chemical. Though it is no longer made in the US, it is registered for use on 60 crops and is a major environmental concern because of its persistent nature.

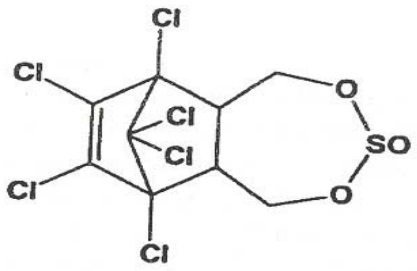


Figure1: The chemical structure of endosulfan

Endosulfan is commonly used to control insect pests that attack food and non-food crops. Approximately 1.4 - 2.2 million pounds of active ingredient are used annually in the U.S. Almost 50% of this amount is used only on three crops: pecans, honeydew melons and strawberries. It is also used on a wide range of crops including cereals, cotton, fruits,

oilseeds, vegetables and maize (Insect control guide, 1991). So food contamination with endosulfan may be one of the main sources of exposure to the public. Endosulfan is metabolized to endosulfan sulfate (oxidation) or endosulfan diol (hydrolysis), which further gets metabolized to endosulfan lactone, hydroxy ether and ether. Absorption of endosulfan in mammals is by inhalation, ingestion and percutaneous absorption. The lipophilic nature of endosulfan enables it to get deposited in fatty tissues initially and finally in liver and kidney.

Endosulfan causes lethality in target species by affecting the nervous system. It is a known gamma amino butyric acid (GABA) antagonist, similar to other organochlorine compounds (Gupta, 1976; Seth et al., 1986). GABA is a major inhibitory neurotransmitter in the central nervous system. This amino acid binds to its receptor and stimulates the flux of chloride ions, causing the reduction of neuronal membrane electrical activity followed by inhibition of neurons. Acute exposure to endosulfan stimulates CNS resulting in lack of coordination, gagging, vomiting, diarrhea, agitation, convulsions and loss of consciousness. Apart from CNS, it has been shown to affect renal, hepatic, respiratory, reproductive and immune systems in mammals, (Singh and Pandey, 1989; Lo, 1995; Rawat et al., 2002; Srikanth and Seth, 1990; Kiran and Varma, 1988; Choudhary and Joshi, 2003; Banerjee and Hussain, 1986; 1987; Pistl et al., 2001). Endosulfan depresses humoral and cell mediated immunity in albino rats at doses that do not show any other signs of toxicity (Banerjee and Hussain, 1987). Along with immunotoxic effects, endosulfan also causes genotoxic effects in peripheral sheep leukocytes, *in vitro* (Pistl et al., 2001). Long-term exposure of endosulfan significantly decreases serum concentrations of IgG, IgM and gamma globulin, as well as tetanus

toxoid titer levels in rats (Akay et al., 1999). Moreover, endosulfan has been shown to induce toxicity in the human T- cell leukemic line by apoptosis (Kannan et al., 2000). Although there have been studies about endosulfan effects on immune system, there have been very few studies that consider this insecticide effects when combined with another pesticide.

2.2.2. Permethrin: Permethrin (cyclopropane carboxylic acid, 3-(2, 2-dichloroethenyl) 2, 2-dimethyl- (3-phenoxyphenyl) methyl ester), a broad-spectrum pyrethroid insecticide, is a synthetic form of the natural insecticide pyrethrum, originally derived from chrysanthemum flowers (Fig. 2).

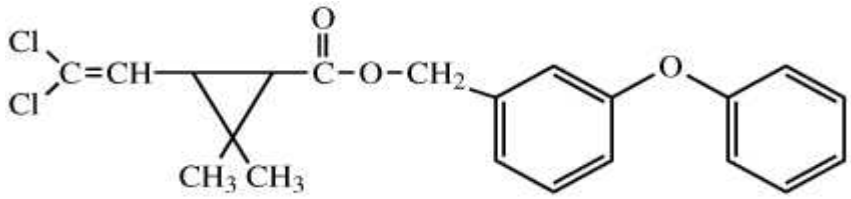


Figure 2: The chemical structure of permethrin.

This type I synthetic pyrethroid (without ∝-cyano group) is used for killing pests and insects in agriculture, homes, forests and in public health programs including lice and tick control in humans and animals. Pyrethroid insecticides gained popularity because of their effectiveness, low toxicity and rapid biodegradability (Kale et al., 1998). Permethrin is used mostly on cotton, accounting for 60% of the worldwide usage (WHO, 1990). In the

U.S, permethrin is used on corn, wheat and alfalfa, approximating 70% of the country's agricultural usage (Gianessi and Anderson, 1995). Because of permethrin's adverse effects on aquatic life, the EPA restricted its commercial usage (USEPA, 1987).

Permethrin exists in cis and trans forms. Toxicity depends on the ratio of cis-trans isomers in the formulation, the former being more toxic. Exposure of permethrin to non-target organisms is mainly through diet and environmental contamination. Oral absorption of permethrin is rapid after which it is deposited in adipose tissue followed by liver, kidney and brain. The potential toxic effects of this pesticide in humans were first recognized when military personnel who wore insecticide treated (N, N Diethyl m-Toluamide (DEET), and permethrin) uniforms in the Pesrsian Gulf War, started showing a series of symptoms and illnesses (Gulf War Syndrome).

Pyrethroids are neurotoxic by nature. Permethrin binds to sodium channels in peripheral nerve fibers resulting in repetitive firing, depolarization, and eventually block of excitation (Vijverberg and Van den Bercken, 1990). The type I pyrethroids, including permethrin, cause a syndrome characterized by tremors, uncoordinated muscle twitching and hyper excitability. Occupational exposure in humans sometimes results in parasthesia and respiratory irritation (Vijverberg and Van den Bercken, 1990). Pyrethroids are readily detoxified by esterases in mammals leading to quick recovery from the toxicity. Nonetheless, in case of immune suppressed individuals and in persons with ailments that affect drug detoxification, this may not be true. Although permethrin is considered as relatively safe to use, the effects of indiscriminate use of this insecticide on non-target organisms cannot be considered as harmless. In animal studies, topical permethrin

exposure has been shown to affect the antibody production and function of macrophages in C57BL/6N mice (Punareewattana et al., 2001). Even single topical exposure to permethrin has been shown to have adverse effects on spleen and thymus function (Prater et al., 2002). Permethrin also suppresses cellular response in BALB/c mice (Blaylock et al., 1995). Despite permethrin's role in causing immune dysfunction, there are not many studies explaining the plausible mechanisms behind its toxicity or its effects when combined with a different class of insecticide.

2.3. Immunotoxicity

Through evolution, vertebrates, especially mammals, developed an efficient defense mechanism called the immune system for protection against harmful pathogens and potential neoplastic events (Voccia et al., 1999). The mammalian immune system is a complex network of organs, tissues, cells and cell products, which function in an orderly manner to generate and sustain proper defense responses and homeostasis (Voccia et al., 1999). The immune system carries out effective immune surveillance by connecting immune cell production centers with peripheral components of the body (Huston, 1997). These peripheral components include blood, thymus, spleen, skin-mucosa and lymphatic system. The spleen, located in the upper left quadrant of the abdomen in mammals is the largest lymphoid organ. Splenocytes include B-lymphocytes, T-lymphocytes, macrophages, dendritic cells, natural killer cells and red blood cells (RBC). RBC free splenocytes from mice were used in this study to investigate the effects of pesticides and their mixtures.

Imbalance or perturbations in immune function might result upon exposure to various xenobiotics/chemicals, owing to the altered physiological or biochemical or cellular parameters. These alterations may lead to increased susceptibility to infection, prolonged recovery periods, ineffective immune surveillance and decreased response to antibiotics or vaccines (Voccia et al., 1999). Human and ecologic exposures to chemicals are never limited to a single chemical. Almost 95% of toxicology studies conducted has been with single chemical exposures (Yang et al., 1994). This makes the assessment of health risks in chemical mixture exposures, a difficult task. However, studies aimed at determining the impact of chemical mixtures on human and animal health have increased over the past few years. Each component of a chemical mixture has certain toxic potential and might influence the toxicity of others by affecting their toxico-kinetics or toxico-dynamics (Simmons, 1994).

It has been apparent from animal studies that a variety of drugs and environmental chemicals have the potential to unintentionally impair components of the immune system. These xenobiotics may affect the complex immune system by a variety of mechanisms. Of all, pesticides were among the first investigated environmental chemicals for their immunomodulatory effects (Wassermann et al., 1969; Ercegovich, 1973; Street and Sharma, 1975). Pesticide–induced oxidative stress is considered as one of those possible mechanisms (Koner et al., 1997; 1998; Ray and Banerjee, 1998). The metabolism of certain pesticides is known to result in production of pro-oxidants or reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radicals ($^{\cdot}OH$) (Auclair et al., 1978; Almeida et al., 1997; Ali et al., 2000; Kannan and Jain, 2003). Though the body maintains an effective balance between pro-

oxidants and anti-oxidants, an imbalance favoring the former may lead to oxidative stress.

Toxic insult to the cell can result in necrosis or apoptosis. Necrosis is characterized by cell swelling, rupture of plasma membrane along with leakage of cellular contents in to the surrounding extra cellular space (Gores et al., 1990). Cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation and formation of apoptotic bodies characterize apoptosis (Kerr et al., 1972; Wyllie et al., 1980; McConkey et al., 1988). Pesticide-induced oxidative stress may result in a dysfunctional immune system. Increased apoptotic cell death and loss of immune cells also has been linked to the impairment (Kerkvliet et al., 1994; Mc Conkey et al., 1994; Enan et al., 1996), suggesting that both processes might be involved. Similarly, apoptotic processes have been accompanied by ROS increase and a concomitant decrease in glutathione (GSH) levels (Tan et al., 1998). Further, the protective role of antioxidants like catalase and N-acetyl cysteine in inhibiting apoptosis caused by other diverse agents other than oxidants supports the role of oxidative stress in this mode of cell death (Butteke and Sandstrom, 1994).

2.4. The ROS and Oxidative Stress

The “oxygen paradox” is that it is essential to the human life and toxic at the same time. Oxidation of typical organic molecules by oxygen during the metabolic pathways is a major source of energy for many life forms. In this oxidation process, oxygen gets reduced to water which is a thermodynamically favorable reaction. However, the two unpaired and parallel spinned electrons in the outer orbit of the oxygen do not allow

direct reduction with a spin matched pair of electrons because of high activation energy (Velker, 1996). Instead, oxygen due to its electronic structure undergoes one-, or two-, or three-electron reduction (step wise reduction) and allows reducing power of oxygen to be coupled to ATP generation during electron transport (McCord, 2000). This step wise reduction can lead to the successive generation of O_2^- (superoxide radical) or H_2O_2 (hydrogen peroxide), or $^{\cdot}OH$ (Hydroxyl radical).

$$O_2 + e^- \rightarrow O_2^- \quad \text{.....(i)}$$

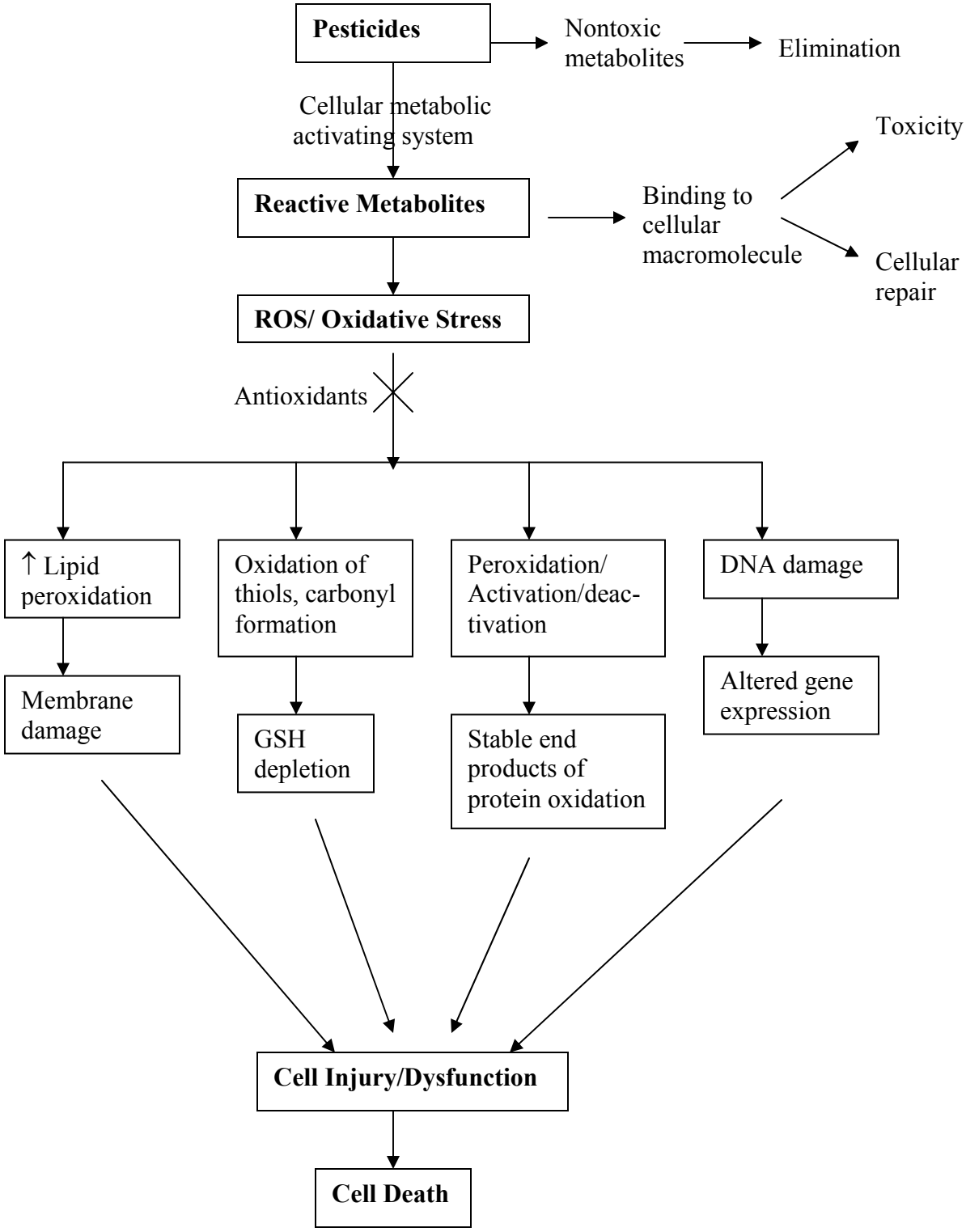
$$O_2^- + e^- + 2H^+ \rightarrow H_2O_2 \quad \text{.....(ii)}$$

$$H_2O_2 + e^- + H^+ \rightarrow {}^{\cdot}OH + H_2O \quad \text{.....(iii)}$$

$$^{\cdot}OH + e^- + H^+ \rightarrow H_2O \quad \text{.....(iv)}$$

Of the above reactions, formation of $^{\cdot}OH$ (reaction iii), believed to be catalyzed by redox metals is the most toxic event and most of the cell's protective mechanisms are aimed at preventing the formation of this radical. The generation of these radicals can occur during 1) mitochondrial consumption of oxygen, 2) hydrogen peroxide production in peroxisomes, 3) phagocyte activation during respiratory bust, and 4) cytochrome P_{450} induction (Chance al., 1979; Ames et al., 1995). However, various exogenous factors like radiation, chemicals and pesticides have been shown to enhance some of these metabolic processes thereby causing marked increase in ROS production (Videla et al., 1990). Low levels of ROS have physiological roles in cellular processes like cell signaling and phagocytosis. In contrast, high levels might result in potential cell damage. Below is a

schematic representation of pesticide-induced tissue injury initiated by free radicals (Scheme 1).



Scheme 1: Pesticide-induced tissue injury initiated by free radicals (with permission from Freund Publishing House from Banerjee et al., 2001. Pesticide-induced oxidative stress: Perspectives and trends. Rev. Environ. Health. 16(1): 1-40).

As depicted in the Scheme 1, high levels of ROS can overwhelm the cellular antioxidants and protective mechanisms, leading to several direct and indirect health effects. Direct effects include chain of peroxidation reactions involving lipids and other macromolecules and indirect effects include, modified metabolic pathways and altered pathophysiology of the organ systems due to the damage (Miller et al., 1993).

The initiation of lipid peroxidation starts when $^{\cdot}OH$ abstracts hydrogen from an unsaturated fatty acyl chain resulting in a carbon-centered lipid radical ($L^{\bullet}$). This $L^{\bullet}$ combines with O_2 to form a lipid peroxyl radical ($LOO^{\bullet}$) (Halliwell and Gutteridge, 1989). Once formed, $LOO^{\bullet}$ can initiate a chain reaction by abstraction of hydrogen atoms from nearby unsaturated fatty acids. The lipid hydroperoxide (LOOH) formed, further decomposes into lipid alkoxyl radical ($LO^{\bullet}$) and other reactive species causing more damage (Nakazawa et al., 1996). Membrane phospholipid peroxidation leads to loss of membrane integrity followed by cell destruction. $^{\cdot}OH$ can also oxidize functional groups of proteins along with lipids. Oxidative attack results in charge modifications and new disulfide and/or covalent and/or hydrophobic intermolecular bond formations in functional groups of proteins (Thomas et al., 1988). The damaged proteins eventually undergo proteolytic burst (Pacific and Davies, 1990). Along with proteins and lipids, ribo- and deoxyribo-nucleic acids are also attacked by ROS resulting in strand breaks, sister chromatid exchanges, DNA-DNA and DNA-protein cross links, and base modifications (Teebor et al., 1988). The strand breaks might be the reason for the laddering appearance of DNA that is characteristic of apoptosis.

2.5. Cytotoxicity and Apoptosis

Cell death is a natural process during development. Cell death can follow 2 pathways, apoptosis or necrosis. Apoptotic cell death differs from necrosis both morphologically (cell shrinkage and DNA fragmentation) and biochemically (requires ATP), (Tomei and Cope, 1991). Old cells in organ systems (skin & intestinal lining) are replaced by new cells in order to maintain homeostasis. Physiological death of cells that are not required (resorption of tadpole tail) or that are malfunctional (cancer) is essential in normal vertebrate development and health. In many of the systems studied this cell death is programmed and occurs via apoptosis (Wood and Youle, 1994). Nonetheless, apoptosis is also associated with several pathological disorders in addition to normal physiology, for example, neurodegenerative diseases may be a result of too much apoptosis whereas cancer may be due to lack of apoptosis (Wood and Youle, 1994).

Single dose topical exposure of permethrin resulted in decreased thymic weight and cellularity in C57BL/6N mice due to possible apoptotic mechanism (Prater et al., 2002). Permethrin with DEET and pyridostigmine bromide appear to be responsible for testicular germ cell apoptosis in stressed rats (Abou-Donia et al., 2003). Studies indicate that endosulfan also induces apoptosis in a human T-cell leukemic line (Kannan et al., 2000). Earlier studies in our laboratory further demonstrate that endosulfan and permethrin cause thymocyte dysfunction by apoptosis (Olgun et al., 2004). Thus, pesticide-induced immune cytotoxicity may be linked to modulation of apoptosis in immune cells.

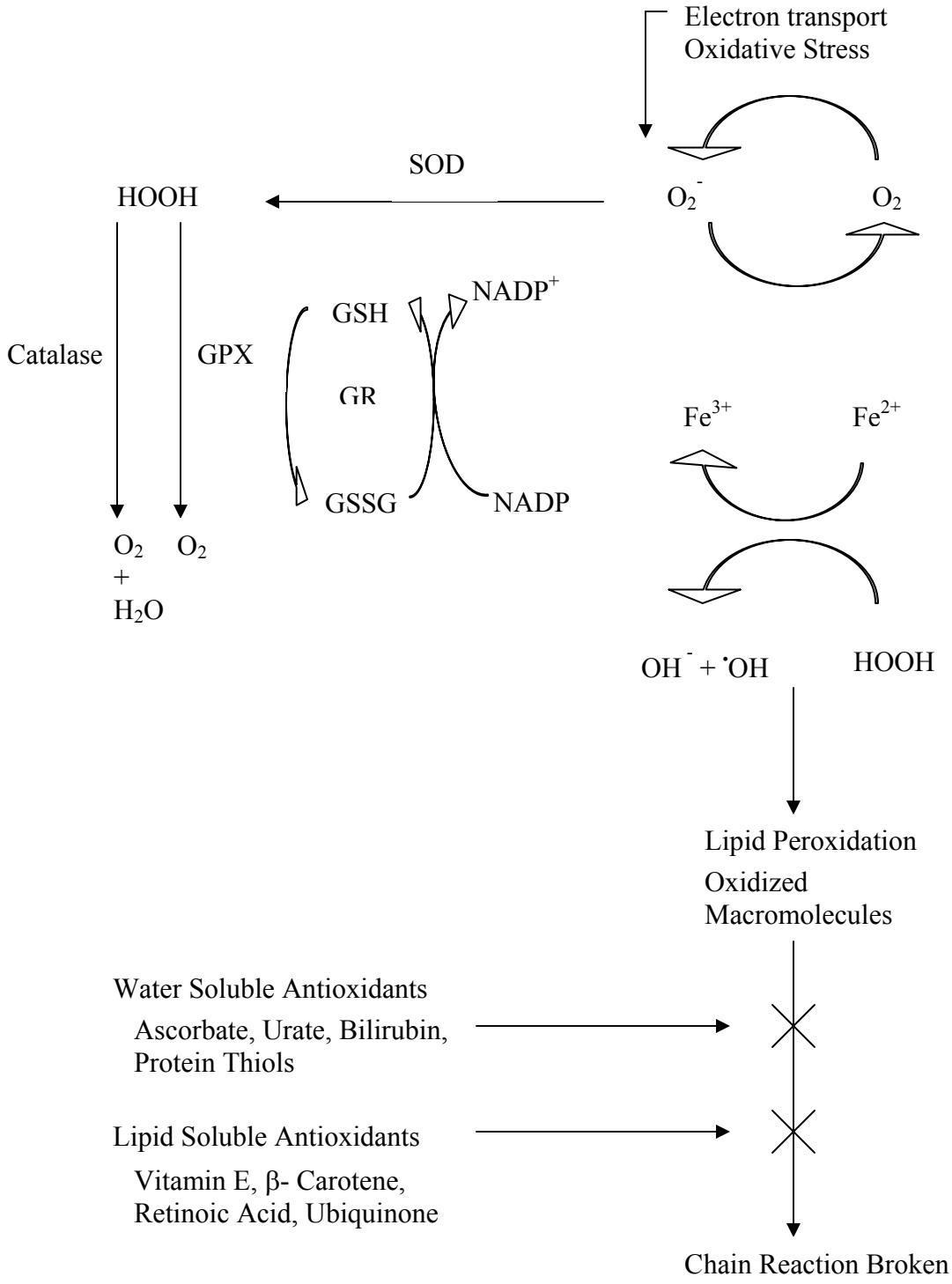
Reactive oxygen metabolites when generated in excess are considered as potential threats to the survival of a living organism. In most cases, cellular defenses can overcome oxidative insults. However, sustained or overwhelming free radical generation may ultimately result in cell death or dysfunction. Several mechanisms have been proposed regarding ROS involvement in cell death. One way they can cause cell death is via apoptosis induction. Free radical activity in several toxicities and their role in triggering apoptosis have been studied (Ozben, 1988; Rice-Evans and Diplock, 1993; Halliwell and Gutteridge, 1989). Hampton and Orrenis (1997) disproved the old notion that oxidants (H_2O_2 or redox active quinines) induce necrosis exclusively, by showing that the lower doses of these oxidants can cause apoptosis. This was also supported by the fact that endosulfan induces apoptosis at lower concentrations and necrosis at higher concentrations in a human T-cell leukemic line (Kannan et al., 2000). So, it appears that the severity of toxic insult dictates the type of cell death to follow.

Further more, mitochondrial dysfunction and oxidative stress were shown to be responsible for endosulfan-induced toxicity in Jurkat T-cells (Kannan and Jain, 2003). Endosulfan's ability to cause lipid peroxidation and alteration of glutathione redox status contributes to cerebral and hepatic toxicity of male albino Wistar rats (Hincal, 1995). Reports suggest that pyrethroid exposure also leads to peroxidative damage and oxidative stress (Maiti et al., 1995; Kale et al., 1999). Besides, permethrin alone and in combination with pyridostigmine bromide and DEET (N, N-diethyl-m-toluamide) was shown to induce the urinary excretion of 3-nitrotyrosine, a marker of oxidative stress upon dermal exposure in rats (Abu-Qare et al., 2001). In addition, ROS-induced apoptosis is cited in association with radiation and chemotherapeutic drug toxicity (Zamzami et al., 1996). We

therefore propose that in part immune dysfunction might is a consequence of oxidative stress.

2.6. Antioxidant Enzymes

Generally, aerobic organisms have utilized various defense or anti-oxidative mechanisms to counteract the damage caused by ROS and their reactive products. These defense mechanisms either act by preventing the chain reaction initiated by ROS or by breaking the chain reaction after its initiation. Metal binding macromolecules (transferrin, ceruloplasmin and albumin) and antioxidant enzymes are classified as preventive antioxidants. Lipid soluble vitamin E, ubiquitone, and β carotene and water-soluble GSH, ascorbate and urate are grouped under chain breaking antioxidants (Miller et al., 1993). Given below is a schematic representation of the body's defenses against free radicals (Scheme 2).



Scheme 2: Systems for protection against reactive oxygen species (adapted from Miller, J.K., Brzezinska-Slebodzinska, E., and Madsen, F.C., 1993. Oxidative stress, antioxidants, and animal function. J. Dairy Sci. 76(9): 2812-2823).

Among the enzymatic anti-oxidants, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione reductase (GR) are considered important.

2.6.1. Superoxide dismutase (SOD): SOD catalyzes the dismutation of superoxide in to peroxide and oxygen.

$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$

This reaction catalysed by SOD is extremely efficient and maintains the concentrations of O_2^- in tissues at very low orders of magnitude. Mammalian cells contain 3 forms of SOD, the mitochondrial, tetrameric, 96-kDa Mn-SOD, the cytosolic, dimeric, 64-kDa Cu/Zn SOD, and the tetrameric extracellular SOD. It was proposed that Mn-SOD is more essential for survival of cells than Cu/Zn SOD (Li et al., 1995). The presence of SOD in the organs helps to dismutate O_2^- immediately upon its generation and thus protects the cells from oxidant-induced stress.

2.6.2. Catalase (CAT): Catalase is a heme-containing, tetrameric, 240-kDa enzyme located mainly in peroxisomes. CAT reduces H_2O_2 to H_2O and O_2 as shown below.

$2H_2O_2 \rightarrow O_2 + 2H_2O$

Through this reaction CAT makes the H_2O_2 unavailable for further reaction and plays an important role in adaptive cells to overcome the oxidative stress. It has been shown that Injection of SOD and CAT to rats before and while getting exposed to 100% O_2 increased their survival rate (Turrens et al., 1984).

2.6.3. Glutathione peroxidase (GPX): The selenium containing, tetrameric 80-kDa enzyme catalyzes the reduction of peroxides using GSH.

$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$

Five isomers of GPX were found in mammalian tissues. Most tissues contain GPX1 and GPX4. Cytosolic and mitochondrial GPX1 reduces both H_2O_2 and fattyacid hydroperoxides. The GPX4 Found in cytosol and membrane fractions can reduce the phospholipid, cholesterol and fattyacid hydroperoxides. GPX1 is predominant in erythrocytes, kidney and liver whereas GPX4 is in renal epithelial cells and testes. Most tissues, except for gastrointestinal tract and kidney are low in cytosolic GPX2 and extracellular GPX3 (Mates et al., 1999). Because it is the only enzyme that can react with lipid and other organic peroxides, GPX serves as major antioxidant against low levels of oxidative stress.

2.6.4. Glutathione reductase (GR): GR uses reducing equalents from NADPH to regenerate reduced GSH from the oxidized GSH (GSSG) produced during the reduction of peroxides by GPX.

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

This enzyme is essential for the GSH redox cycle that maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is necessary for protection against oxidative stress.

Overall, antioxidant enzymes protect the cells from toxic insults by scavenging the generated free radicals. From a functional point of view, modulation of cellular antioxidant enzyme levels by pro-oxidant pesticides/chemicals will influence the resulting toxicity. Several *in vivo* and *in vitro* studies suggested that pesticides alter enzymatic antioxidants levels in different tissues (Junqueira et al., 1986; 1988; Chhabra et al., 1993). Organophosphate pesticides have been reported to inhibit SOD and GPX activities in brain and liver homogenates (Bagchi et al., 1993; Gupta et al., 1999). Even

pyrethroid pesticides are said to modify intracellular antioxidants in erythrocytes of rats (Maiti et al., 1995; Kale et al., 1999). Low doses of endosulfan are shown to modify endogeneous antioxidants, SOD, GPX and GSH in erythrocytes, liver and lungs of rats (Bebe and Panemangalore, 2003). Hence, pesticide-mediated immune toxicity might be the consequence of insufficient antioxidant potential.

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3.1. Abstract

The pesticides endosulfan and permethrin negatively affect the immune system in non-target organisms. We hypothesized that these pesticides cause immune suppression, at least in part, by enhanced apoptotic cell death. In order to test this hypothesis, splenocytes from adult C57BL/6 male mice were exposed to endosulfan, or permethrin or mixtures of these chemicals, *in vitro*. Cytotoxicity was evaluated using flow cytometric technique in combination with 7-amino-actinomycin D (7-AAD) staining. Endosulfan (25 - 150 μM) decreased the viability (47 - 22%) with a corresponding increase in early apoptotic (28 - 32%) and late apoptotic/necrotic (25 - 46%) cell populations in a dose-dependent manner when exposed for 12 hours. Endosulfan at 100 μM also caused a time-dependent (4 - 12 h) decrease in live cells (71 - 30%), and an increase in early apoptotic (15 - 33%) and late apoptotic/necrotic (14 - 36%) populations. Permethrin (50-200 μM) exposure resulted in neither a time-dependent/dose-dependent loss of splenocyte viability nor induction of apoptosis in these cells. With mixtures of permethrin and endosulfan, depressed viability (47 - 31 %) and enhanced early apoptosis (30 - 34%) and late apoptosis/necrosis (22 – 35 %) were also observed in these cells. In addition, exposure to mixtures of 50 μM endosulfan with 50 or 100 μM permethrin increased the late apoptosis/necrosis compared to the results of exposure to either chemical alone. Visual evaluation, using DNA ladder assay, confirmed the contribution of both apoptotic and necrotic processes. These findings suggest that the immunotoxicity of endosulfan both individually and in mixtures with permethrin is associated with the occurrence of early apoptosis and late apoptotic/necrotic processes.

In order to distinguish the late apoptotic and necrotic cells microscopic examination of cytospun slides was carried out. This study confirmed the presence of predominantly necrotic populations in endosulfan and mixture exposed splenocytes. These findings suggest that the immunotoxicity of endosulfan both individually and in mixtures with permethrin is associated with the occurrence of apoptotic and necrotic processes.

3.2. Introduction

Increasing amounts of pesticides are used in agriculture and public health. Along with target species (pests, insects) non target organisms including humans are subjected to the exposure. Due to the widespread use of different classes of pesticides, exposure to mixtures of pesticides in one's lifetime has become unavoidable. Although vital in increasing food production and eliminating diseases, over exposure to pesticides can result in severe health conditions including cardiovascular diseases, hypertension, skin diseases and cancer (Klaunig et al., 1998). The immunotoxic potential of the pesticides has gained recognition over the last decade as immune dysfunction can influence the survival of the organism in numerous ways (Banerjee et al., 1986). Organochlorines and pyrethroids are two different groups of widely used insecticides that target the nervous system. Organochlorines act on permeability of sodium and potassium channels across the nerve membrane whereas pyrethroids target the permeability of the sodium channels only. These pesticides, at sublethal concentrations seem to affect the immune system in non-target animals (Koner et al., 1998; Madsen et al., 1996).

Topical permethrin exposure has been shown to affect immune cell parameters in C57BL/6N mice (Punareewattana et al., 2001). Even single dose topical exposure of

permethrin has been shown to adversely affect the function of immune organs like spleen and thymus (Prater et al., 2002). Similarly, endosulfan depresses humoral and cell-mediated immunity (CMI) in rodents at doses that do not show any other overt signs of toxicity (Banerjee et al., 1986; 1987). Genotoxic and immunotoxic effects were observed in peripheral sheep leukocytes, *in vitro*, upon endosulfan exposure (Pistl et al., 2001). Despite a considerable amount of work done on several individual pesticides in the field, the effects of mixtures of pesticides on immune cell function are lacking in the literature. In this study we investigated the cytotoxic effects of endosulfan and permethrin in splenocytes of C57BL/6 mice, *in vitro*.

Instead of being directly cytotoxic, many compounds cause sublethal damage which may trigger a sequence of events that could lead to cell suicide or apoptosis (Hampton and Orrenis, 1998). Previous studies have shown that DDT causes thymic atrophy via apoptosis. Moreover, short-term exposure to TCDD can result in thymus and spleen atrophy, as well as cellular depletion in bone marrow due to accelerated cell suicide or apoptosis (Kerkvliet et al., 1994; McConkey et al., 1994). Similarly, deltamethrin, a synthetic pyrethroid induces brain and testicular apoptosis in rats (Wu et al., 2000; El-Gohary et al., 1999). Furthermore deltamethrin can cause thymus atrophy in male Balb/c mice (Enan et al., 1996). Based on these observations we hypothesized that, endosulfan and permethrin induced immune dysfunction may be linked to their ability to modulate apoptosis and thus being cytotoxic to immune cells.

3.3. Material and Methods

3.3.1. Animals: All animals in this experiment were treated as blocks. Eight to twelve weeks old C57BL/6 male mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were acclimatized for 1 week 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. Splenocyte isolation: Animals were euthanized by cervical dislocation and body weights were recorded. The average body weight of mice was 27 ± 0.41 g and average spleen weight was 250 ± 3.3 mg. The spleens were removed, weighed and placed individually in a 60 mm × 15 mm petri dish with phenol red-depleted RPMI-1640 media (Gibco BRL, Grand Island, NY). Splenocytes were isolated by dissociating spleen on 60 μm mesh Seives screens (Sigma, St. Louis, MO) and lysing of erythrocytes was carried out with ACK lysis buffer. Splenocytes were washed and diluted in supplemented RPMI-1640 media to obtain a final concentration of 10^7cells/ ml. A million cells treated with or without pesticides were incubated at 37°C, 5% CO_2 and 95% humidity in 96 well plates.

3.3.3. Preparation of pesticides: Permethrin and endosulfan were purchased from Chem Service (West Chester, PA) and stock solutions (200 mM) were prepared using 100 % acetone. These stock solutions were serially diluted with RPMI-1640 media to prepare 4X working solutions. Final concentrations were 25, 50, 100, 150 μM for endosulfan; 50, 100, 150, 200 μM for permethrin.

3.4. 7-Amino Actinomycin D (7-AAD) Staining Assay

7-AAD is a fluorescent DNA binding agent widely used for measuring apoptosis in cells. This dye intercalates between cytosine and guanine bases in the di-nucleotide strands and makes the cells fluoresce. It is readily excluded by live cells and has high DNA binding constant which makes the data reliable. The amount of binding depends on the extent of damage and permeability of the cell membrane. This dye fluoresces at 488 nm and the extent of fluorescence depicts the amount of bound DNA in the cells. The staining intensities in early apoptotic and late apoptotic/necrotic populations vary, as the former will have more membrane integrity than the later. Three degrees of staining intensities are measured via flow cytometry to distinguish between live, early apoptotic and late apoptotic/necrotic cell populations (Schmid et al., 1994a; 1994b).

Splenocytes were treated with 50, 100, 150 and 200 μM permethrin or 25, 50, 100 and 150 μM endosulfan separately, in order to obtain a dose dependent response regarding each pesticide exposure. A time-dependent exposure of cells to either 200 μM permethrin or 100 μM endosulfan was carried out for 4 or 8 or 12 hours. Of these three time points, 12 hours was chosen as appropriate time point to perform the next set of experiments. Treatments with the lowest toxic effects were chosen for mixture studies. Mixture study was performed with 25 and 50 μM endosulfan and 50 and 100 μM permethrin alone and in-combination.

After treatments, cells were rinsed with 37°C PBS and centrifuged at 1200 RPM, 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% BSA 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). The method described in Schmid et al., was used to identify and quantify the apoptotic populations. By using forward (for size) and side scatter (granularity) cells were gated. The cells were classified as $7AAD^{dull}$ (live cells), $7AAD^{moderate}$ (early apoptotic cells) and $7AAD^{bright}$ (late apoptotic/necrotic) based on their ability to take up the 7-AAD (Schmid et al., 1994a; 1994b).

3.5. DNA Ladder Assay

One million cells were treated with either 100 μM permethrin or 25 or 50 μM endosulfan alone and in-combination for 12 hours. After treatments, the wells were rinsed with PBS and the rinses were added to the microcentrifuge tube. Upon centrifugation, the pellet was resuspended in 200 μl cold PBS. For isolation of DNA, the procedure described in the apoptotic kit was followed (Boehringer Mannheim apoptotic DNA ladder kit). According to the procedure, cells were initially lysed with binding buffer that had EDTA and Triton – X, a detergent. Fragmented DNA is separated from intact chromatin by centrifugation. Residual impurities are removed by a wash step and subsequently DNA is eluted in elution buffer. The samples were stored at -20°C for up to two weeks. A 1% agarose gel (with ethidium bromide) was prepared with Tris-Borate-EDTA buffer, PH 8.0 and run at 45V for 90 minutes. The gel was viewed under UV light and photodyne pictures were taken and observed for the evidence of apoptosis and necrosis.

3.6. Cytospin Preparation

One million cells were exposed to 100 μM permethrin or 50 μM endosulfan or mixture for 12 hours. After treatments, a 150-200 μl aliquot of 1.5×10^5- 2×10^5 cells was diluted with 100 μl Hank's balanced salt solution and loaded in to a centrifugation chamber containing ~50 μl of 30% bovine serum albumin (BSA; Sigma Chemical Co.). The cells were spun at 600 rpm for 8 minutes at high acceleration. The slides were air-dried followed by fixing and staining with modified Wright–Giemsa stain (Jorvet dip quick stain, Jorgerson laboratories, CO). Slides were cover slipped and sealed using permount (Fisher Scientific). Minimum two slides were prepared for the treatment and the experiment was repeated twice. Cells were examined under oil immersion at 100X and photographed using Nikon magnafire software system.

3.7. Statistical Analysis

Results were expressed as arithmetic mean ± SEM. Data were evaluated using ANOVA analyses. Tukey-Kramer t test was used to establish differences between treatment groups. Groups demonstrating no statistical significance are represented with the same letter (e.g., a, b, or c), whereas groups demonstrating statistical significance at $p < 0.05$ are represented with different letters of the alphabet.

3.8. Results

3.8.1. Effect of pesticide exposures on apoptotic and necrotic cell death of murine splenocytes: 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). To assess the cytotoxic effects of endosulfan or permethrin exposure on splenocytes, a flow cytometric analysis was performed subsequent to 7-AAD staining of the cells. Controls included untreated cells and cells treated with acetone alone (solvent control). The final concentration of acetone in cell cultures did not exceed 0.1% and did not have any toxic effects.

Cells exposed to 25-150 μM endosulfan showed 19-62% decrease in viability over the control, which was highly significant ($p < 0.0001$) among different doses of endosulfan (Fig. 1). As shown in Figure 1, a dose-dependent increase (14-30%) in early apoptosis over control ($p < 0.005$) was observed in splenocytes exposed to endosulfan. Similarly, a 40-60% enhancement in late apoptosis or necrosis over control ($p < 0.0001$) was seen in a dose-related fashion with endosulfan exposure (Fig. 1). Cells exposed to permethrin (50-200 μM) had no significant effects on viability/early apoptosis/late apoptosis or necrosis (Fig. 2).

Murine splenocytes were treated with either permethrin (200 μM) or endosulfan (100 μM) for 4 or 8 or 12 hours to study the time-dependent exposure effects. Exposure to 100 μM endosulfan increased early apoptosis and late apoptosis/necrosis with a

corresponding decrease in viability (Table 1). However, exposure to permethrin (200 μM) was found to have little toxic effects up to 12 hours of studies (Table 1).

With mixtures of permethrin and endosulfan, depressed viability and enhanced early apoptosis and late apoptosis/necrosis were also observed (Figs. 3, 4 & 5). Thus, exposure to mixtures of 50 μM endosulfan with 50 or 100 μM permethrin increased the late apoptosis/necrosis compared to the results of exposure to individual chemicals (Fig. 5).

3.8.2. DNA ladder studies to confirm apoptotic cell death: Oligonucleosomal fragmentation characteristic of apoptotic cells can be viewed as a ladder pattern with agarose gel eletrophoresis and is considered as convincing evidence of apoptosis in cytotoxicity studies. The isolated DNA of the control and treated cells was electrophoresed and evaluated for laddering effects. As shown in Fig. 6 a & b, lane 1, DNA was intact in freshly isolated cells. However, after 12 hours even the controls exhibited some amount of laddering along with the treated cells (Fig. 6 a & b, lanes 2 & 3). The pronounced laddering observed in endosulfan (25 & 50 μM) and mixture treatments (Fig. 6 a & b, lanes 6 & 7) confirms the flow cytometry results. The presence of less genomic DNA (Fig. 6 a & b, lane 7) at the top of the gels, in mixture studies indicates DNA degradation via apoptotic process. The smearing observed in Figure. 6 a & b, lane 7 supports the contribution of necrotic cell death and confirms flow cytometric observation of cytotoxicity (Fig. 5).

3.8.3. Microscopic examination of pesticide exposure effects on splenocytes: Because flow cytometric analysis in-combination with 7-AAD fail to differentiate late apoptosis from necrosis, morphological evaluation using cytospin technique was conducted.

Microscopic examination of cytospins prepared from splenocytes revealed a normal discernible lymphocyte population with a few macrophages in freshly isolated cells (Fig. 7 a). After 12 hours the controls (both untreated and solvent treated cells) and permethrin exposed cells showed both apoptotic and necrotic cells in a similar manner (Fig. 7 b, c & d). Endosulfan and mixture treatments displayed more necrotic populations than apoptotic (Fig. 7 e & f), indicating that the late apoptotic/necrotic population observed in from flow cytometric analysis may actually be more necrotic than apoptotic in nature. However, flow cytometric data cannot be correlated with cytospin-generated data, as the identification in the later is based on morphology (Gorczyca, 1999) and is a qualitative method.

3.9. Discussion

Physiological removal of cells that are not required or that are functionally impaired, without eliciting an inflammatory response is essential for maintaining cellular homeostasis and health in normal vertebrate development. In many of the systems studied, this physiological removal is essentially regulated and occurs via apoptosis, hence the name "programmed cell death" (Wood et al., 1994). Along with participating in development of other systems, apoptosis has an essential role in the immune system. This process plays an important role in survival and selection of mature T and B-lymphocytes, CTL-induced cytotoxicity or homeostatic regulation after antigen stimulation (Arends et al., 1991; Kagi et al., 1994; Lenardo et al., 1999). But this programmed cell demise responds to both internal and external stimuli and can lead to pathological consequences in the later case.

Apoptosis is an active process, associated with profound changes in structural and biochemical events that lead to irreversible destruction of the cell (Lecoeur et al., 2002). Morphological characteristics of apoptosis include cell shrinkage because of disordered volume regulation (Maeno et al., 2000), loss of membrane integrity (Ormerod et al., 1993), alteration in mitochondrial structure, chromatin condensation, and release of apoptotic bodies (Darzynkiewicz et al., 1992). Functional and biochemical alterations include the loss of mitochondrial transmembrane potential (Petit et al., 1995; Kroemer et al., 1997), DNA fragmentation and phosphotidyl serine externalization (Koopman et al., 1994).

Chemicals such as TCDD (2,3,7,8- tetrachloro-p-benzo dioxin), DDT (1,1 bis (p-chlorophenyl) 2,2,2 trichloroethane) and deltamethrin are immunotoxicants that are known to cause immune dysfunction in non-target organisms (Dean et al., 1994; Kerkvliet et al., 1994; Lukowicz et al., 1992). Recent reports suggest that permethrin, may cause several impaired immune functions, including inhibition of mixed lymphocyte responses and splenic macrophage function and depressed humoral immunity, cytotoxic T lymphocyte and NK cell activity and thymocyte viability in mice (Blaylock et al., 1995; Punareewattana et al., 2000; Olgun et al., 2004). Likewise, endosulfan, an organochlorine causes loss of cortical thymocytes and depressed CMI in mice and depressed humoral immunity and CMI in rats, *in vivo* (Kannan, 1983; Banerjee et al., 1986; 1987). Collectively these reports raise questions about the cytotoxic mechanisms associated with the immune alterations. Further, triggering of unwanted apoptosis by some chemicals is suggested to cause immune dysfunction (Rhile et al., 1996; Camacho et al., 2001; Tebourbi et al., 1998; Enan et al., 1996). Our goal in the present study was to

investigate the two common pesticides, endosulfan and permethrin for their potential to induce apoptosis in C57BL/6 marine splenocytes, *in vitro*.

In this study we show that endosulfan singly causes both apoptosis and necrosis and in mixtures predominantly necrosis. Endosulfan upon exposure enhances cytotoxicity via apoptotic and necrotic cell death in a dose and time dependent manner in murine splenocytes indicating the involvement of both processes. Enhanced toxicity seen in isolated rat thymocytes with increasing doses of endosulfan supports our finding (Kannan, 1983). Further, Kannan et al., showed that endosulfan induces apoptosis also in Jurkat cells, a human T-cell leukemic line (2000). At high doses endosulfan appeared to be toxic by a pronounced enhancement of necrosis. Consistent with the reports which showed, that xenobiotics at low or moderate concentrations accelerate DNA fragmentation or apoptosis whereas at high concentrations cause necrosis (McConkey et al., 1988; Kannan et al., 2000). Additionally, cytotoxicity in case of endosulfan was a linear function of concentration and exposure duration starting as early as four hours, in agreement with the effects of endosulfan on Jurkat cells (Kannan et al., 2000).

No difference in viability or early apoptosis or late apoptosis/necrosis was seen between control and permethrin, upon dose and time dependent exposures. These results differ from testicular germ cell apoptosis seen in stressed rats with permethrin, DEET (N, N Diethyl m-Toluamide) and pyridostigmine bromide (Abou-Donia et al., 2003). This observation was also in contrast to decreased thymic weight and cellularity in C57Bl/6N mice due to apoptosis (Prater et al., 2002; Olgun et al., 2004). However, permethrin neither altered humoral immunity nor CMI (Institoris et al., 1999) nor the release of brain

mitochondrial cytochrome *c* (Abu-Qare et al., 2001) in rats by itself. These contrasting results compounded with dearth of *in vitro* studies that focus on mechanisms of permethrin mediated immune toxicity necessitate further investigation.

Viability or early apoptotic cell death in splenocytes exposed to mixture combinations was different from control and permethrin but not from endosulfan, asserting the latter's role in toxicity. In addition, exposure to mixtures of 50 μM endosulfan with 50 or 100 μM permethrin increased the late apoptosis/necrosis compared to the results of exposure to either chemical alone. This is in correlation with permethrin's enhanced release of rat brain mitochondrial *c* in mixtures rather than alone (Abu-Qare et al., 2001). In what may be a related observation, an increase in testicular germ cell apoptosis was noticed with permethrin in mixtures (Abou-Donia et al., 2003). The increase suggests the possibility of permethrin contribution to toxicity in mixtures.

Extensive DNA fragmentation detected by DNA gel electrophoresis is a known method to distinguish apoptosis. Absence of laddering in freshly isolated cells (0 hr) indicates the absence of artificial degradation during the DNA isolation or the background apoptosis and gives credibility to the method. Presence of oligonucleosomal fragmentation in controls and treatments confirms the presence of apoptotic cell death as evident from flow cytometry data. Presence of less DNA amount in endosulfan and mixture treated cells compared to controls may be an indication of intensity of the toxic insult. Although this method is not a quantitative index for apoptotic cell death, the laddering was more distinct in endosulfan and mixtures and might compare to the increase seen in flow

cytometry data. Smearing noticed in mixtures also is indicative of necrotic cell death involvement.

Microscopic observation of cytospins indicated both apoptotic and necrotic death processes in all treatments. Trend towards increased necrosis in endosulfan and mixture exposed splenocytes was noted.

Consistently, the three methods used indicated the involvement of both necrosis and apoptosis in permethrin and endosulfan mediated toxicity. In conclusion, results of this study suggest that exposure to endosulfan and to mixtures with permethrin cause both apoptotic and necrotic cell death in murine splenocytes, *in vitro*.

Table 1. Measurement of viability, early apoptosis and late apoptosis in murine splenocytes exposed to permethrin or endosulfan in a time dependent manner

	Time in Hours	Viability	Early Apoptotsis	Late Apoptotsis/ Necrosis
Untreated	4 h	80.24 ± 0.46	12.82 ± 0.93	7.01 ± 0.83
	8 h	69.99 ± 1.45	19.47 ± 1.58	10.72 ± 1.30
	12 h	59.14 ± 0.96	26.04 ± 0.67	15.06 ± 0.75
Solvent Control	4 h	80.72 ± 0.41	12.67 ± 0.71	6.68 ± 0.74
	8 h	73.22 ± 1.06	18.16 ± 1.38	8.82 ± 0.99
	12 h	53.61 ± 2.01	29.28 ± 1.56	17.29 ± 0.70
Permethrin 200 μM	4 h	80.29 ± 0.48	12.98 ± 0.60	6.84 ± 0.95
	8 h	68.54 ± 1.76	21.06 ± 2.20	10.65 ± 0.78
	12 h	56.14 ± 1.04	27.96 ± 1.23	16.10± 0.69
Endosulfan 100 μM	4 h	71.46 ± 1.06 *	15.22 ± 1.71*	13.53 ± 1.21*
	8 h	43.07 ± 1.75 *	29.07 ± 2.38*	27.97 ± 2.51*
	12 h	30.54 ± 3.40 *	33.41 ± 2.41*	36.42 ± 2.59*

The percent of live, early apoptotic or late apoptotic/necrotic cells following treatment with pesticides as assessed by 7-AAD flow cytometric assay. Cells were treated with solvent control (acetone 0.1%) or permethrin (200 μM) or endosulfan (100 μM) for 4 or 8 or 12 hours. The numbers represented were cell percentages. Results were shown as means ± SE, N = 3. Numbers with * were different from control, with significance at $p < 0.05$.

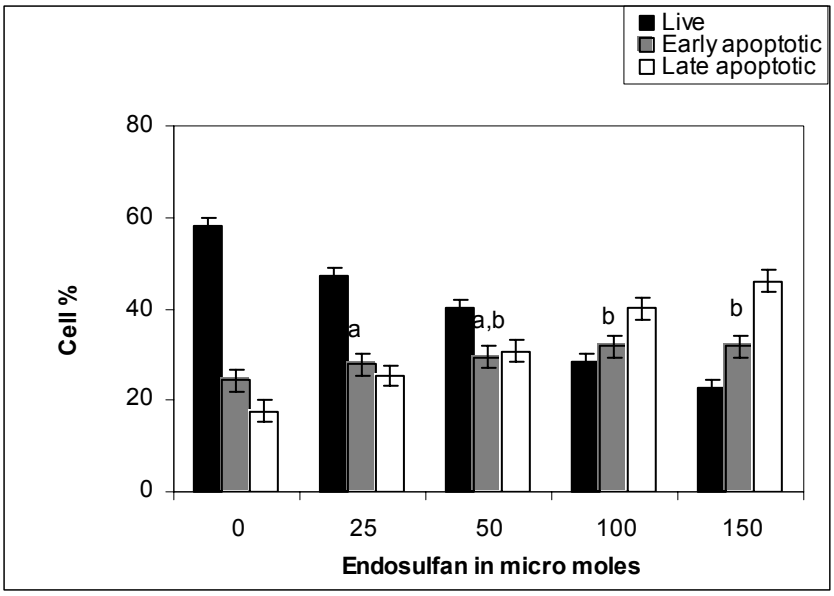


Figure 1: 7-AAD Staining of C57BL/6 splenocytes after 12 hour incubation with endosulfan: Murine splenocytes were exposed to endosulfan (25-150 μM) for a period of 12 hours. Endosulfan showed a dose-dependent decrease in viability and a corresponding increase in early apopotosis and late apoptosis/necrosis of the cells. Results are expressed as percentage means ± SEM. N = 9. All treatments were different from control and treatments indicated with same letters are not different from each other ($p < 0.05$).

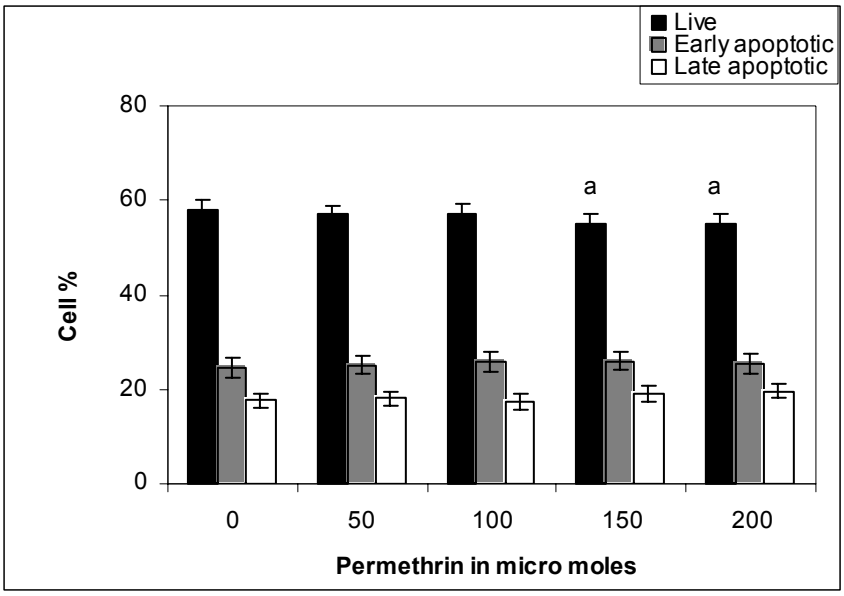


Figure 2: 7-AAD Staining of C57BL/6 splenocytes after 12 hour incubation with permethrin: Murine splenocytes were exposed to permethrin (50-200 μM) for a period of 12 hours. Permethrin at 150 & 200 μM decreased the viability of the cells with a corresponding increase in early apoptosis with the former and both early and late apoptosis/necrosis with the later. Results were expressed as percentage means ± SEM. N = 9. Treatments with different letters are significant at $p < 0.05$ from control.

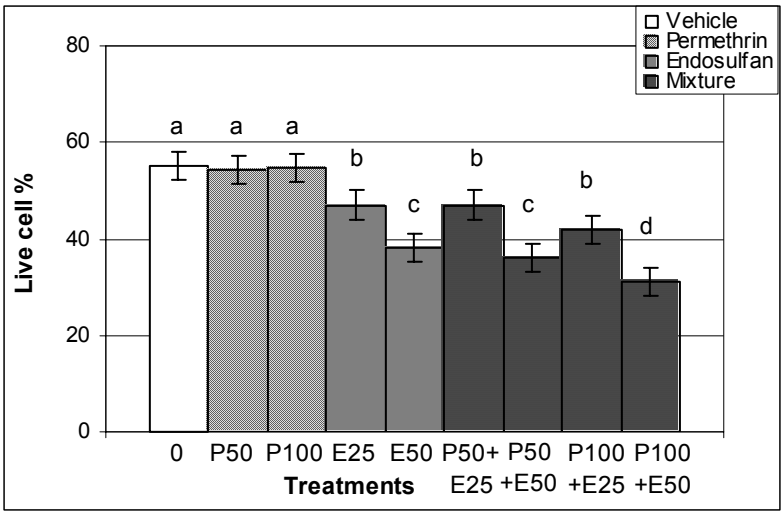


Figure 3: 7-AAD Staining of C57BL/6 splenocytes after 12 hour incubation with endosulfan and permethrin mixture: Murine splenocytes were exposed to either permethrin (50 & 100 μM) or endosulfan (25 & 50 μM) or mixtures for a period of 12 hours. Mixture elicited decrease in viability was different from that of permethrin but not from endosulfan except in case of 50 μM endosulfan and 100 μM permethrin combination. Results are expressed as percentage means ± SEM. N = 9. Treatments with same letters are not different from each other ($p < 0.05$).

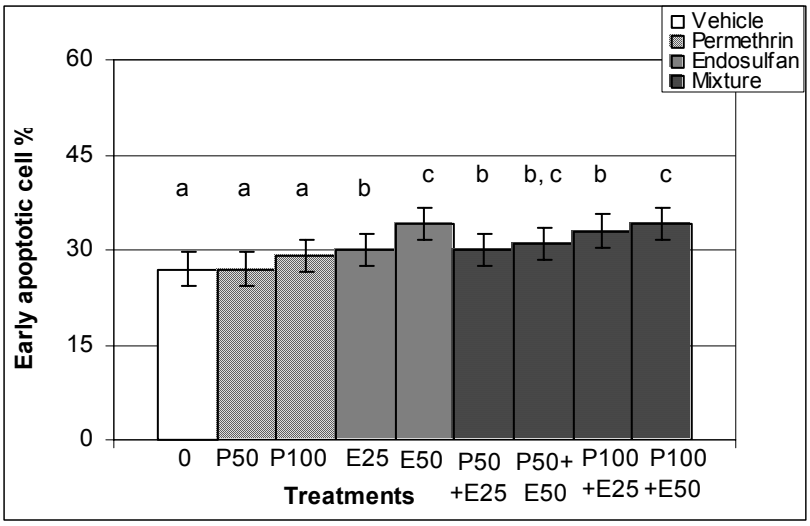


Figure 4: 7-AAD Staining of C57BL/6 splenocytes after 12 hour incubation with endosulfan and permethrin mixture: Murine splenocytes were exposed to either permethrin (50 & 100 μM) or endosulfan (25 & 50 μM) or mixtures for a period of 12 hours. Mixture elicited increase in early apoptosis was different from that of permethrin but not from endosulfan. Results are expressed as percentage means ± SEM. N = 9. Treatments with same letters are not different from each other ($p < 0.05$).

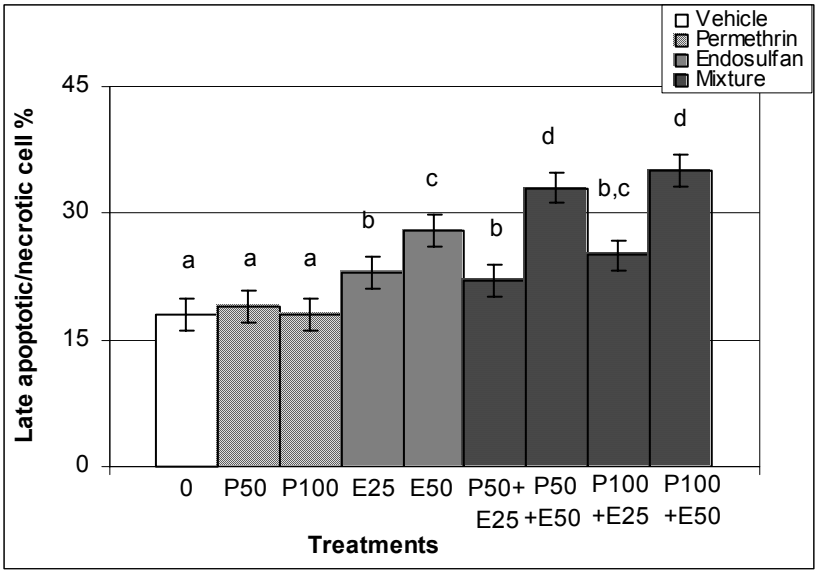
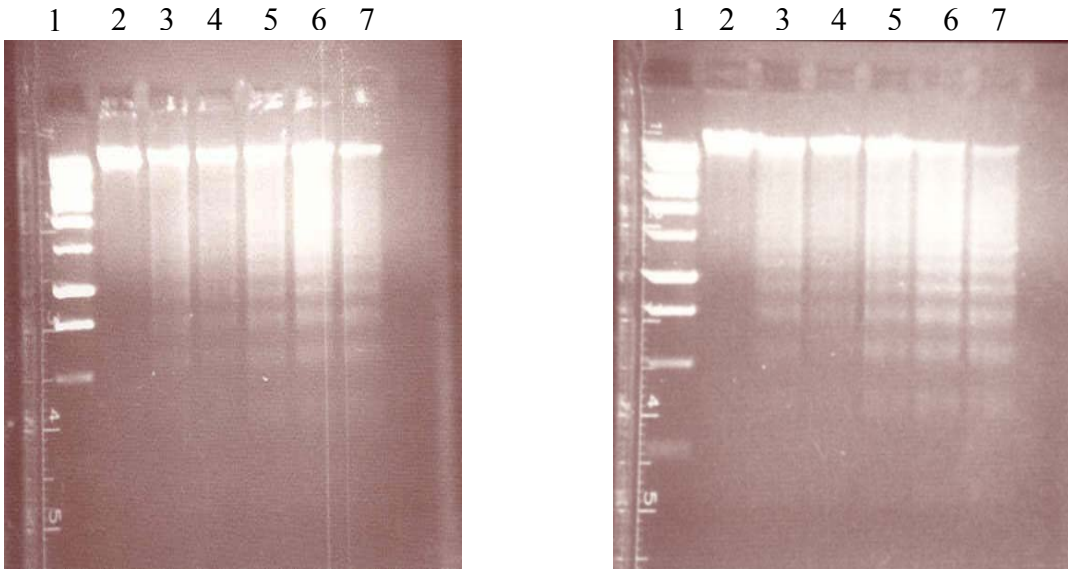


Figure 5: 7-AAD Staining of C57BL/6 splenocytes after 12 hour incubation with endosulfan and permethrin mixture: Murine splenocytes were exposed to either permethrin (50 & 100 μM) or endosulfan (25 & 50 μM) or mixtures for a period of 12 hours. Mixture elicited increase in late apoptosis/necrosis was different from that of permethrin and 50 μM endosulfan. Results are expressed as percentage means ± SEM. N = 9. Treatments with same letters are not different from each other ($p < 0.05$).



6 a **6 b**

Figure 6: DNA laddering of C57BL/6 splenocytes after 12 hour incubation with endosulfan, permethrin and their mixture: Agarose gel electrophoresis to detect DNA ladder pattern in endosulfan and permethrin induced apoptosis. Cells (1×10^6) were untreated or treated with either permethrin or endosulfan or mixtures for 12 hours and DNA was isolated. A 10 µl of isolated DNA per treatment was electrophoresed on a 1% agarose gel. The lanes represent: (a & b) 1 = 1kb DNA ladder; 2 = control, freshly isolated and untreated cells; 3 = control, untreated cells after 12 hour incubation; 4 = vehicle control (acetone); 5 = permethrin 100 µM; 6 = endosulfan 25 µM; 7 = mixture; These experiments were repeated 2 more times with the same results.

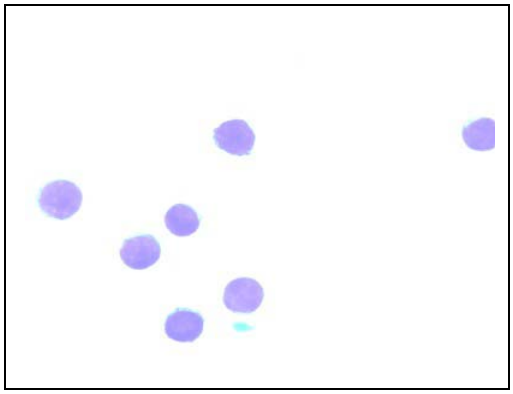
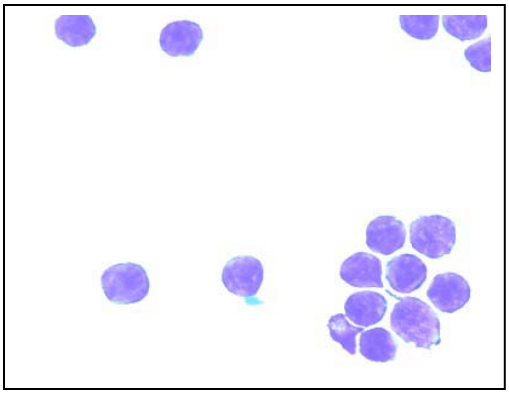


Figure 7a:



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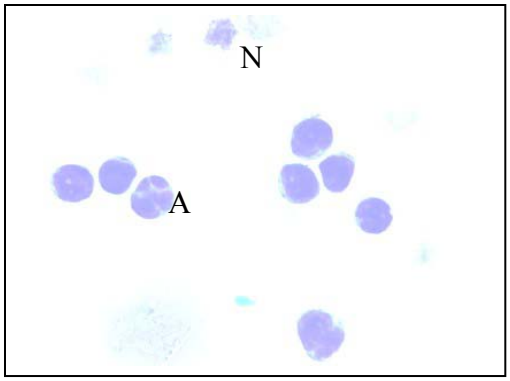

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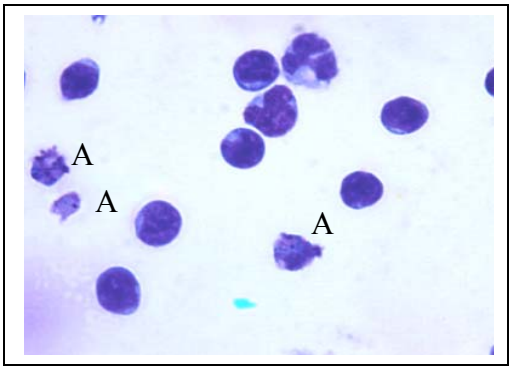
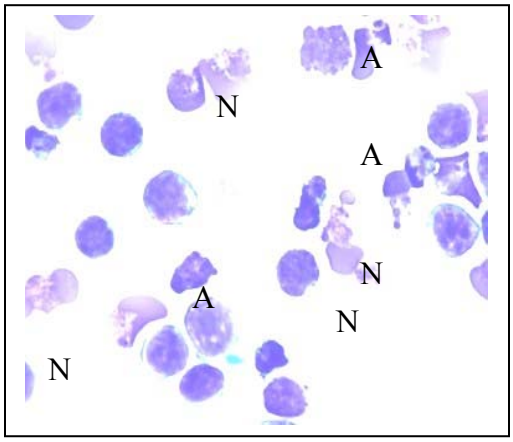


Figure 7d:



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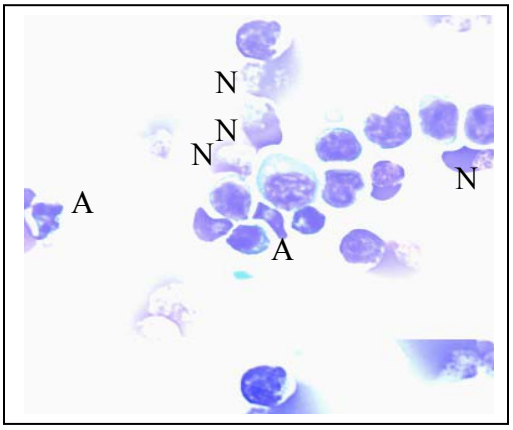

Figure 7f:

Figure 7: Cytospin preparations of C57BL/6 splenocytes after 12 hour incubation with endosulfan, permethrin and their mixture: Cytopathological identification of apoptosis and necrosis in C57Bl/6 male mouse splenocytes treated with endosulfan and peremthrin

for 12 hours. a = untreated cells, 0 hr; b = untreated cells, 12 hr; c = vehicle; d = permethrin 100 μM; e = endosulfan 50 μM; f = permethrin and endosulfan mixture. Magnification 100X. A = apoptosis; N = necrosis.

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4.1. Abstract

The insecticides endosulfan and permethrin cause immune cell dysfunctions and are known immunotoxic agents. We hypothesized that these chemicals induce immunotoxicity by modulating the oxidative status of the cells. In an attempt to test this hypothesis, we have investigated the insecticides' ability to act as pro-oxidants via generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) or superoxide anion (O_2^-) in splenocytes of C57BL/6 adult male mice, *in vitro*. Further their role in modulating the levels of intracellular antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPX) was investigated. Results of the analyses revealed that individual pesticides increased the production of H_2O_2 in a time and dose-dependent manner. Both time and dose-dependent increase in O_2^- anion production was caused by permethrin; whereas endosulfan exposure resulted in a dose-dependent increase in free radical production. However, exposure to mixtures of these pesticides had little or no effect on the generation of H_2O_2 and O_2^- radicals as compared to individual pesticides. In agreement, only individual pesticides altered the intracellular antioxidant enzyme levels rather than the mixtures. The levels of SOD and GPX in pesticide treated splenocytes were not different from vehicle. An increase in GR and CAT was noticed with permethrin exposure; however only the lower of the two doses of endosulfan altered the levels of the latter. These findings suggest that permethrin and endosulfan have the ability to affect the oxidative status of immune cells and this might play a role in immunotoxicity.

4.2. Introduction

Imbalance or perturbations in immune function might result upon exposure to various xenobiotics/chemicals, owing to altered physiological, biochemical or cellular parameters. These alterations may lead to increased susceptibility to infection, prolonged recovery periods, ineffective immune surveillance or decreased response to antibiotics or vaccines (Voccia et al., 1999). Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) or superoxide anion (O_2^-) or hydroxyl ($OH\cdot$) radicals are implicated with pesticide-mediated toxicity.

Free radical generation has been implicated behind the toxicity of several organochlorine (Bagchi et al., 1992, 1993) and organophosphate (Yang et al., 1996; Yang and Dettbarn, 1996) compounds. Organochlorine insecticides are extensively used around the world both in agriculture and in households because of their effectiveness for insect control. Endosulfan, an organochlorine insecticide has been shown to affect humoral and cell mediated immunity in albino rats (Banerjee and Hussain, 1987). Moreover, endosulfan induced immunotoxicity in human T- leukemic cell line is associated with apoptosis (Kannan et al., 2000). Due to their low environmental persistence pyrethroid insecticides are often used indiscriminately, resulting in detrimental exposure to humans and other non target organisms. Permethrin's ability to cause immunosuppression was implicated to gulf war syndrome. Topical permethrin exposure has been shown to affect antibody production and function of macrophages, spleen and thymus (Punareewattana et al., 2001; Prater et al., 2002). Permethrin also suppresses cellular immune responses in BALB/c mice via oral exposure (Blaylock et al., 1995). Inspite of the general belief that

these pesticides are immunotoxic, there has not been much focus on mechanisms involved in their toxicity.

Oxidative stress has been suggested as a contributor to pesticide-induced immune toxicity (Koner et al., 1997; 1998). Although the body maintains an effective balance between pro-oxidants and anti-oxidants, an imbalance favoring the former may lead to oxidative stress. Immune cells are particularly prone to oxidative stress because of the high polyunsaturated fatty acids percent in the plasma membrane and a higher production of reactive oxygen species (ROS) to carry out phagocytosis (Knight, 2000). It has been showed that endosulfan upon exposure causes oxidative stress and alteration of glutathione redox status in both the hepatic and nervous systems of male Wistar rats (Hincal et al., 1995). Free radical generation is proposed to be responsible for endosulfan-induced toxicity in Jurkat- T cells (Kannan and Jain, 2003). In addition, endosulfan also appears to cause lipid peroxidation and the induction of endogenous antioxidants in fresh water fish, *Channa punctatus* (Pandey et al., 2001).

Permethrin with pyridostigmine bromide and DEET (N, N-diethyl-m-toluamide) is shown to induce the urinary excretion of 3-nitrotyrosine a marker of oxidative stress upon dermal exposure in rats (Abu-Qare et al., 2001). Moreover, the resistance noticed in insects exposed to pyrethroids and permethrin was attributed to increase in esterase, glutathione s transferase (GST) and peroxidase activities (Vontas et al., 2001). We have therefore explored the possible involvement of oxy radicals and altered intracellular antioxidant enzyme levels in permethrin and endosulfan exposed murine splenocytes, *in vitro*.

The results from this study indicate that the toxicity of these pesticides to splenocytes is mediated at least in part through an oxyradical mechanism involving generation of ROS and that the induction of certain key antioxidant enzymes seen might be a consequence of oxidative stress caused by these reactive oxygen species.

4.3. Material and Methods

4.3.1. Animals: All animals in this experiment were treated as blocks. Eight to twelve week old C57BL/6 male mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were acclimatized for 1 week 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. Splenocyte isolation: Animals were euthanized by cervical dislocation and body weights were recorded. The average body weight of mice was 27 ± 0.41 g and average spleen weight was 250 ± 3.3 mg. The spleens were removed, weighed and placed individually in a 60 mm × 15 mm petri dish with phenol red-depleted RPMI-1640 media (Gibco BRL, Grand Island, NY). Splenocytes were isolated by dissociating spleens on 60 μm mesh Seives screens (Sigma, St. Louis, MO) and lysing of erythrocytes was carried out with ACK lysis buffer. Splenocytes were washed and diluted in supplemented RPMI-1640 media to obtain a final concentration of 4×10^6cells/ ml. A million cells treated with or without pesticides were incubated at 37°C, 5% CO_2 and 95% humidity in 24 well plates.

4.3.3. Preparation of pesticides: Permethrin and endosulfan were purchased from Chem Service (West Chester, PA) and stock solutions (200 mM) were prepared using 100 % Ethanol. These stock solutions were serially diluted with incomplete media to prepare 2X working solutions. Final concentrations were 25, 50, 100, 150 μM for endosulfan; 50, 100, 150, 200 μM for permethrin.

4.4. 2′, 7′- dichlorofluorescin diacetate (DCFH-DA) Assay

H_2O_2 generation in splenocytes was determined according to a modification of the method of Weissman et al., 1980 and Das et al., 1994. Intracellular H_2O_2 production converts the non-fluorescent compound DCFH-DA to a fluorescent compound called 2′, 7′- dichlorofluorescin or DCF (Boss et al., 1983). Prior to the treatments, 25 mM 3 – amino -2, 3, 4-triazole (AT) was added to a suspension of splenocytes (1×10^6cells in 500 μl) with or without pesticide (Murray et al., 1980). AT inhibits endogeneous catalase from scavenging H_2O_2. The cells were stained for 15 minutes with 5 μM DCFH-DA prior to the pesticide treatments. The production of H_2O_2 in splenocytes was monitored with a Coulter Epics XL/MXL flow cytometer (Hialeah, FL).

4.5. Hydroethidine Assay

Hydroethidine (HE), a sodium borohydride-reduced derivative of ethidium bromide, was used to detect O_2^-. Once O_2^- is generated, it converts hydroethidine to ethidium bromide and increases red fluorescence (Rothe and Valet, 1990). The generation of O_2^- in cells was evaluated by using Coulter Epics XL/MXL flow cytometer (Hialeah, FL).

Splenocytes were stained with 10 μM HE for 15 minutes before addition of pesticide treatments.

4.6. Antioxidant Enzyme Assays

For antioxidant enzyme assays, a million cells treated with or without pesticides or solvent control, were evaluated. Splenocytes were treated with either permethrin (100 μM or 25 μM) or endosulfan (50 μM) or combinations of both for 12 hours. The cells were then washed in PBS and aliquoted to a concentration of 20 million/ml and stored at -70°C until use. The cells were thawed and homogenates were used for various enzyme assays. Each sample was measured in triplicates. The protein content in cell homogenates was determined with Biorad protein assay (BD Biosciences, KY), using bovine serum albumin as standard (Weiner and Kaminski, 1990).

4.6.1 Superoxide dismutase (SOD): SOD is a metallo enzyme that catalyzes the dismutation of superoxide anion into hydrogen peroxide (H_2O_2) and oxygen (O_2). Total SOD amount in treated and untreated cells was measured by an Oxyscan™ automated analyzer using Oxyscan™ SOD-525™ Kit from OXIS International Inc., (Portland, OR). This assay method is based on the SOD mediated increase in autooxidation of 5, 6, 6a, 11b-tetrahydroxybenzo[c]flourene at alkaline pH that will result in accumulation of a chromophore that exhibits an absorbance maxima at 525 nm (Nebot et al., 1993). A 50 μl sample was used measuring the SOD activity (units/ ml of sample). Specific activity of SOD was measured as units/mg protein.

4.6.2. Catalase (CAT): The procedure for measuring CAT was adopted from Beers and Sizer (1952) and the Freehold (1972). The rate of decrease in absorbance at 240 nm was monitored on a Shimazu UV spectrophotometer at 25°C. One unit of catalase is defined as the quantity of CAT required to decompose 1 micromole of H_2O_2 under specified conditions at 25°C, using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$ for H_2O_2 at 240 nm. Specific activity of CAT was expressed in units/mg protein.

4.6.3. Glutathione peroxidase (GPX): Total GPX activity was measured with Oxyscan™ automated analyzer using Oxyscan™ GPX-340™ Kit. In this assay, the reduction of oxidized glutathione (GSSG) is coupled to the oxidation of NADPH by glutathione reductase that can be monitored at 340 nm. The GPX activity was measured in units/ml of sample and a sample volume of 50 µl was used for each measurement. Specific activity of GPX was measured in milli units/mg protein.

4.6.4 Glutathione reductase (GR): The measurement of GR was adopted from the Carlberg and Mannervik (1985) method with slight modifications (Sies and Akerboom, 1984; Anderson et al., 1983). The change in absorbance per minute was monitored on a Shimazu UV spectrophotometer at 340 nm at 25°C. A reference blank containing all components except samples was used for obtaining the background rate. This background rate was subtracted from the samples readings before calculating the specific activity. One unit of GR is defined as the amount of enzyme catalyzing the oxidation of one micromole of NADPH per minute at pH 7.6 and 25°C. The rate of oxidation of NADPH was monitored using its molar extinction coefficient, 6220-$M^{-1}cm^{-1}$.

4.7. Statistical Analysis

All experiments described were repeated at least three times. Fluorescence of solvent control was taken as baseline. In treatments, the cells showing fluorescence after baseline subtractions were considered as positive for H_2O_2 production. Results were expressed as arithmetic mean ± SEM. Data were evaluated using ANOVA analyses. Tukey-Kramer t test was used to establish differences between treatment groups. Groups demonstrating no statistical significance at $p \leq 0.05$ were represented with the same letter, whereas groups demonstrating statistical significance at $p \leq 0.05$ were represented with different(a, b, or c) letters of the alphabet.

4.8. Results

4.8.1. Effects of pesticides on H_2O_2 generation: Pesticide-treated splenocytes were analyzed flow cytometrically for their intracellular H_2O_2 production. Optimum concentration and time from individual pesticide exposures were used in mixture assays. Initially splenocytes were stained for 15 minutes with 5 μM DCFH-DA. Stained cells were exposed to various concentrations of endosulfan (25, 50, 100 & 150 μM) or permethrin (50, 100, 150 & 200 μM) or both. As shown in Figure 1 & 2, a dose-dependent increase in generation of H_2O_2 was noticed in splenocytes exposed to both compounds. In time-dependent exposures, cells were incubated for 15, 30 and 45 minutes. Cells exposed to 150 μM permethrin showed a linear increase in H_2O_2 production in a time-dependent manner that was significant (Table. 1). In contrast, 50 μM endosulfan exhibited a non-significant trend towards a linear decrease in H_2O_2 production

up to 45 minutes (Table 1). In mixture studies, cells were exposed to 25 μM endosulfan and 50 μM permethrin individually and in mixtures for 15 minutes. Mixture-induced H_2O_2 generation was not different from endosulfan indicating that the addition of permethrin did not have any effect on this oxy species production (Fig. 3).

4.8.2. Effects pesticides on O_2^- generation: Flow cytometric analyses of O_2^- radical production in pesticide-treated splenocytes were carried out by using HE dye. 15 minute incubation with 10 μM HE was carried out before the addition of pesticides. In time response, cells were exposed for 5, 15, 30 and 45 minutes. Permethrin enhanced the free radical production linearly in a time-dependent manner (Table 2). However, as shown in Table 2, there was no time-dependent response observed when cells were exposed to endosulfan for 15-45 minutes. Dose response was obtained by exposing HE treated cells to 25, 50, 100 and 150 μM endosulfan and to 50, 100, 150 and 200 μM permethrin. Splenocytes when treated with increasing doses of endosulfan showed a dose-dependent increase in O_2^- generation (Fig. 4). A similar increase in O_2^- generation was observed in cells exposed to increasing doses of permethrin (Fig. 5). In the mixture studies, increased doses of permethrin (50-150 μM) in the presence of 150 μM endosulfan caused significant ($p \leq 0.05$) enhancement in O_2^- radical generation compared to 0 or 50 μM endosulfan (Fig. 6).

4.8.3. Effects of pesticides on intracellular antioxidant enzyme levels: Intracellular levels of antioxidant enzymes such as GPX, SOD, CAT and GR in splenocytes treated with permethrin and/or endosulfan were measured spectrophotometrically. The cells were incubated for 12 hours in the presence and absence of pesticide treatments. The viability

in these cells after incubation ranged from ~60-40% (data not shown). No difference in GPX and SOD levels was observed between control and pesticide exposed splenocytes (Table 3). However, the levels of CAT were significantly increased in cells treated with 100 μM permethrin or 25 μM endosulfan. Cells treated with 50 μM endosulfan or the mixture treatments did not alter enzyme levels (Fig. 7). An increase in intracellular GR levels was observed in splenocytes exposed to 100 μM permethrin but was not different with endosulfan or chemical mixture exposures (Fig. 8).

4.9. Discussion

Oxidative stress in cell is a result of either an increase in ROS, an imbalance of intracellular antioxidant defense systems, or inability of the cell to repair oxidative damage. A number of recent studies reported the ability of organochlorine pesticides (e.g., endrin, lindane) to induce oxidative stress in different organ systems (Singh and Pandey, 1989; Bagchi et al., 1993ab, 1995; Hassoun and Stohs, 1996; Bachowski et al., 1998). Endosulfan caused oxidative stress in rat cerebral and hepatic tissues, as demonstrated by lipid peroxidation and altered redox status (Hincal et al., 1995). Pyrethroids, including permethrin, were shown to induce oxidative stress in rodents (Kale et al., 1999; Abu-Quare et al., 2001). Our recent studies indicate that both permethrin and endosulfan are immunotoxic (Chapter 1); however the role of these pesticides and their mixtures in causing oxidative stress has not been reported.

In this study we have shown that permethrin and endosulfan exposure increased the levels of intracellular ROS in murine splenocytes in a dose-related manner. Specifically, both pesticides when treated alone resulted in increased production of H_2O_2 and O_2^-. The

mixtures of pesticides (25 μM endosulfan + 50 μM permethrin) had no significant change in H_2O_2 production compared to endosulfan (25 μM) alone (Fig. 3). When cells were exposed to mixtures of permehtrin (50 or 100 μM) and endosulfan (150 μM), a significant ($p < 0.05$) increase in O_2^- production was observed. However, in the presence of high level of permethrin (150 μM) addition of endosulfan at 50 or 150 μM had no significant change in O_2^- production (Fig. 6). Thus it appears that cells could produce a certain level of O_2^- and once it is attained, no further increase could be achieved by addition of other pesticide. This observation of non-additive effect on ROS with the chemical mixtures may be supported by the additional observation that mixtures did not affect antioxidant enzyme levels beyond that seen with the individual pesticide exposures.

Enzymatic antioxidants are essential for the conversion of ROS to innocuous metabolites. We monitored the levels of endogenous enzymatic antioxidants GPX, SOD, CAT and GR in control and pesticide-exposed splenic leukocytes. Neither permethrin nor endosulfan, singly or in combination, affected the splenocyte GPX and SOD enzyme levels. These results are consistent with a recent report of unaffected liver GPX in endosulfan-exposed rats (Frederick et al., 2003). However, in the same report the authors noted increased plasma GPX and decreased lung GPX in the endosulfan-exposed rats. Reduced GPX activity has also been reported in fish adrenocortical cells exposed *in vitro* to endosulfan; whereas *in vivo* exposure caused significant induction of this enzyme in gill, kidney and liver tissue (Dorval et al., 2002; Pandey et al., 2001). These results suggest that different modes of exposure and/or different tissues may show different GPX enzyme induction or activity following endosulfan exposure. However, no previous reports were found that

examined GPX in immune cells following *in vivo* or *in vitro* endosulfan or permethrin exposure. Kale et al. (1999) suggested that aldehydes and lipophilic conjugates (metabolites of pyrethroids in rats, mice and other species) might play a role in pyrethroid induced oxidative stress. The absence or reduced production of these metabolites in, *in vitro* systems could also relate to unaffected GPX in cells cultured with permethrin, including the present immune cells.

The unaltered SOD levels observed in this study differ from previous reports of decreased SOD levels in rat liver and erythrocytes following whole-body topical exposure to low doses of endosulfan (Frederick et al., 2003) or increased rat erythrocyte SOD following oral pyrethroid exposure (Kale et al., 1999). These results again indicate that different cells react differently to endosulfan and permethrin to neutralize ROS. However, the antioxidant enzyme CAT was increased in splenocytes following either permethrin (100 μM) or endosulfan (25 μM) treatment. Higher dose of endosulfan or mixtures of endosulfan and permethrin had trivial effects on the levels of the CAT in cells compared to controls. Because both CAT and GPX are responsible for keeping H_2O_2 low within the cells, it appears that the small increase in H_2O_2 production observed with the pesticide exposures did not warrant the induction of these enzymes.

A significant increase in GR activity was present in permethrin (100 μM) exposed splenocytes. However, this effect was not present when permethrin (100 μM) was administered in combination with endosulfan (25 or 50 μM). Effects of these two pesticides on intracellular GR levels in mammals have not previously been reported. GR plays a role in keeping GSH, a substrate for GPX in a reduced state. Therefore, increased

GR levels might be an adaptive response to altered or possibly depleted GSH levels due to permethrin-induced oxidative stress. In this regard, pyrethroid exposure decreased reduced GSH in rat erythrocytes (Kale et al., 1999). Frederick et al. (2003) also reported decreased GSH levels in rat liver and lung with low-level topical exposure to endosulfan. The GR activity seen with the present *in vitro* exposure was not correlated with the diminishment of GSH levels seen in the above *in vivo* studies, which may relate to differences in the immune target cells, dose of chemical received, or variables between these study models.

In conclusion, results of this study suggest that exposure to the immunotoxicants endosulfan or permethrin leads to significant increase of ROS levels in splenic leukocytes. These altered pro-oxidant levels appear to have minimal effects on antioxidant enzyme status of the cells after pesticide exposures. It is uncertain at this time whether the increased ROS may be sufficient to cause oxidative stress, leading to reduced immune cell activity or increased cell death in the study cells or following *in vivo* exposures. Further investigations will be needed to determine if such oxidative stress may relate to the immunotoxic effects of these pesticides in splenocytes.

Table 1. Splenocyte generation of H_2O_2, after exposure to permethrin or endosulfan in a time-dependent manner

	Percentage Relative Fluorescence		
	15 min	30 min	45 min
Permethrin 150 μM	14.59 ± 0.81^a	18.84 ± 0.97^b	21.81 ± 1.81^c
Endosulfan 50 μM	15.39 ± 0.81^a	18.35 ± 0.58^a	16.53 ± 0.81^a

The percent increase in relative flourescence of H_2O_2 after baseline subtraction of vehicle control values was presented for cells exposed to permethrin (150 μM) or endosulfan (50 μM) as measured by DCFH-DA assay in combination with flow cytometry. Results were shown as means ± SE, N = 5. Within rows values with same letters were not different from each other, $p < 0.05$.

Table 2. Splenocyte generation of O_2^-, after exposure to permethrin or endosulfan

	Percentage Relative Fluorescence			
	5 min[X]	15 min[Y]	30 min[Y]	45 min[Y]
Permethrin 150 μM	5 ± 0.20^a	7.13 ± 0.27^b	9.88 ± 0.35^c	12.97 ± 0.10^d
Endosulfan 50 μM	2.88 ± 0.29^a	1.53 ± 0.24^a	1.87 ± 0.28^a	1.92 ± 0.25^a

[X] N = 4

[Y] N = 5

The percent increase in relative flourescence of O_2^- after baseline subtraction of vehicle control values was presented for cells exposed to permethrin (150 μM) or endosulfan (50 μM) as measured by HE assay in combination with flow cytometry. Results were shown

as means ± SE, N = 5. Within rows values with different letters (a, b, c) are different from each other, $p < 0.05$

Table 3. Permethrin and/or endosulfan effects on intracellular levels of GPX and SOD

Treatments	GPX mU/mg Protein	SOD U/mg Protein
Untreated	64.42 ± 6.5^{a}	46.99 ± 4.5^{b}
Solvent Control	65.35 ± 4.4^{a}	45.00 ± 2.8^{b}
Permethrin 100 μM	66.11 ± 2.2^{a}	50.99 ± 3.3^{b}
Endosulfan 25 μM	$59. 90 \pm 5.3^{a}$	40.03 ± 4.3^{b}
Endosulfan 50 μM	65.83 ± 6.0^{a}	49.10 ± 4.8^{b}
Permethrin 100 μM + Endosulfan 25 μM	68.12 ± 2.6^{a}	52.35 ± 5.6^{b}
Permethrin 100 μM + Endosulfan 50 μM	59.70 ± 3.0^{a}	45.08 ± 3.7^{b}

Glutathione peroxidase (GPX) and superoxide dismutase specific activity in splenocytes exposed to permethrin or endosulfan or their mixtures. Cells were exposed to solvent control (0.1% acetone) or permethrin (100 μM) or endosulfan (25, 50 μM) or mixtures. Results were expressed as the mean ± SEM. N = 5. Treatments with same letters within each column (a or b) were not different from each other, $p < 0.05$.

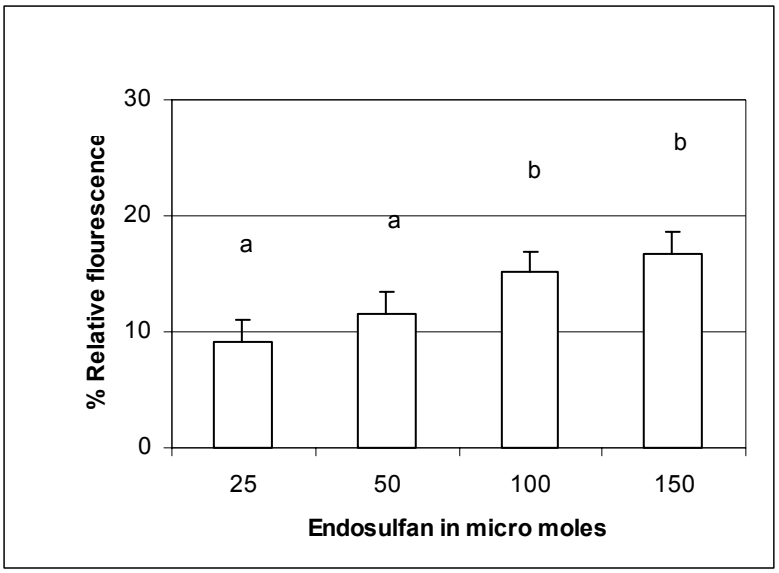

Figure 1: DCFH-DA staining of C57BL/6 splenocytes exposed to endosulfan: Effect of increased doses of endosulfan exposure on splenocyte H_2O_2 generation was monitored. A corresponding increase in H_2O_2 generation was seen with the increase of dose when exposed for 15 minutes. Baseline subtraction with controls was done and the results are expressed as the mean ± SEM. N = 6. Treatments with different letters were different from each other ($p < 0.05$).

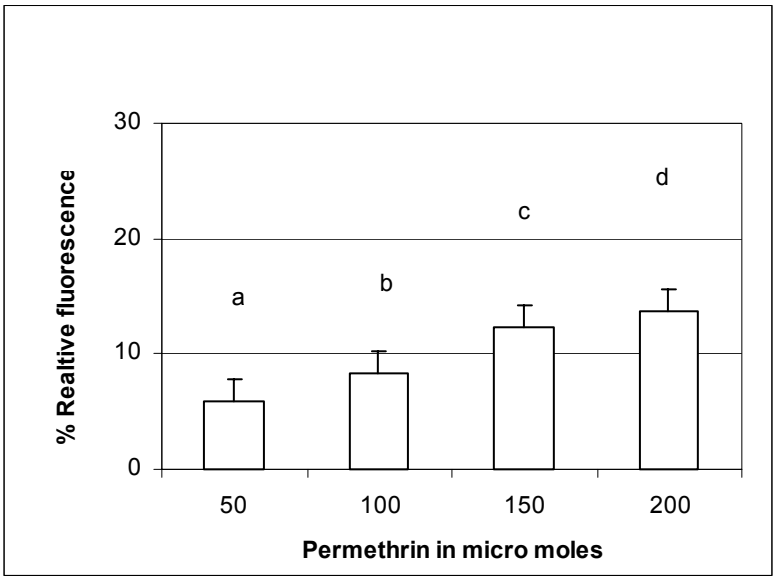

Figure 2: DCFH-DA staining of C57BL/6 splenocytes exposed to permethrin: The change in H_2O_2 generation in splenocytes caused by exposure to increased doses of permethrin for a period of 15 minutes was observed. Permethrin caused an increase in H_2O_2 levels in a dose-dependent manner. Baseline subtraction with controls was done and the results were expressed as the mean ± SEM. N = 6. Treatments with same letters were not different from each other ($p < 0.05$).

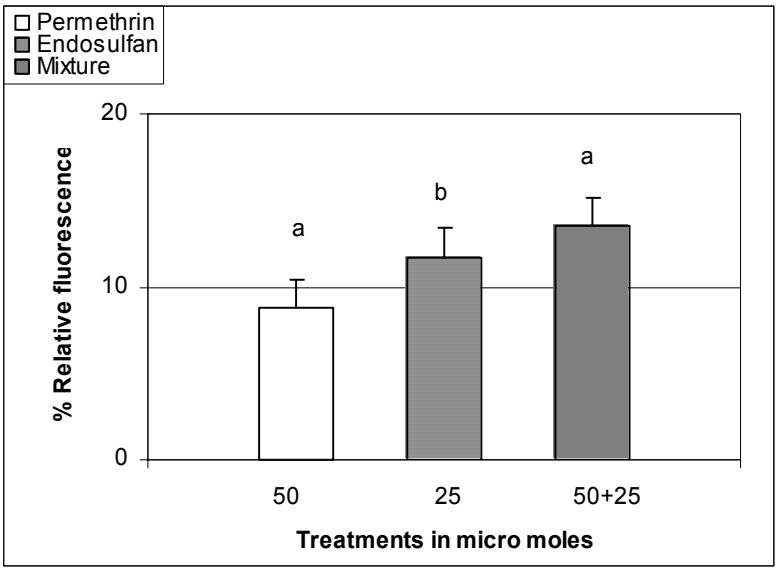

Figure 3: DCFH-DA staining of C57BL/6 splenocytes exposed to endosulfan, permethrin and their mixture: Murine splenocytes were exposed to a combination of 25 μM endosulfan and 50 μM permethrin for 15 minutes and the change in H_2O_2 was measured. Mixture exposure resulted in an increase in H_2O_2 generation compared to 50 μM permethrin alone. Baseline subtraction with controls was done and the results were expressed as the mean ± SEM. N = 5. Treatments with different letters were different from each other ($p < 0.05$).

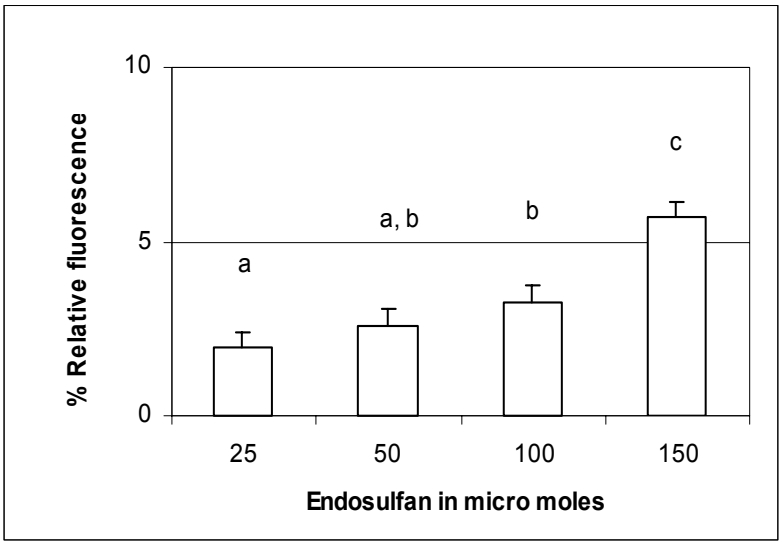

Figure 4: HE staining of C57BL/6 splenocytes exposed to endosulfan: The O_2^- radical production in splenocytes was measured upon exposure to 25-150 μM endosulfan for a period of 15 minutes. Control values were used as baseline and were subtracted from the treatments. Values were expressed as the mean ± SEM, N = 4. Treatments with same letter were not different from each other ($p < 0.05$).

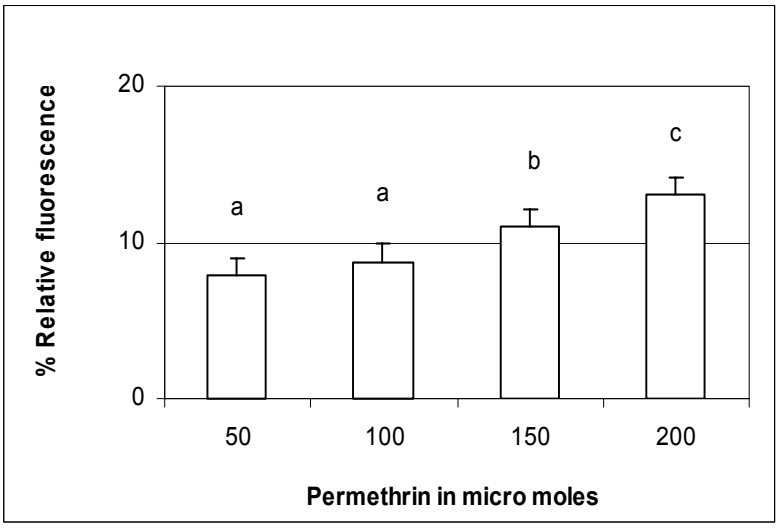

Figure 5: HE staining of C57BL/6 splenocytes exposed to permethrin: A change in intracellular O_2^- generation in relation with increased concentrations of permethrin was monitored upon exposure for 15 minutes. A dose-dependant increase in O_2^- generation was noticed with increased permethrin dosage. Control values were used as baseline and were subtracted from the treatments. The resulted values were expressed as the mean ± SEM. N = 3. Treatments with same letter were not different from each other ($p < 0.05$).

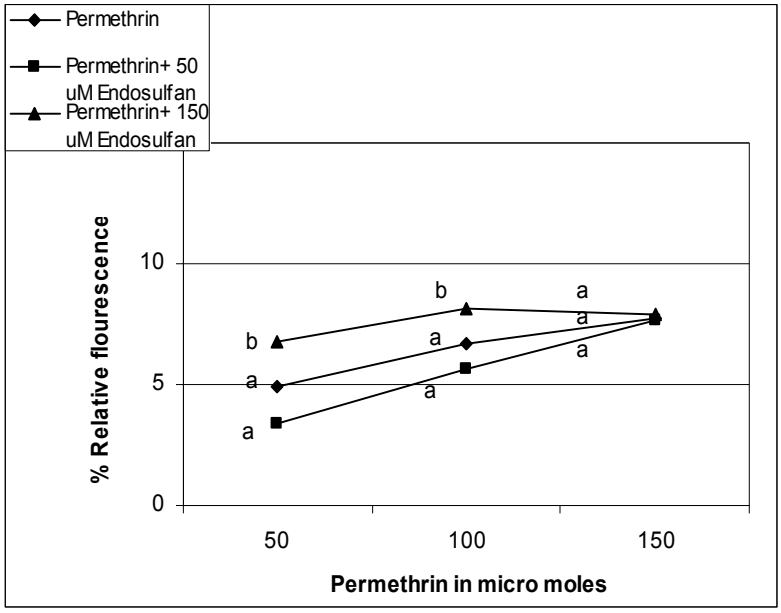

Figure 6: HE staining of C57BL/6 splenocytes exposed to endosulfan, permethrin and their mixture: Splenocytes were exposed to 50, 100 and 150μM permethrin individually and along with 50 and 150 μM endosulfan for 15 minutes. Mixture combinations with high dose of endosulfan enhanced the generation of O_2^- over the individual treatments. Control values were used as baseline and were subtracted from the treatments. Values were expressed as the mean ± SEM. N = 4. Treatments with same letter were not different from each other ($p < 0.05$).

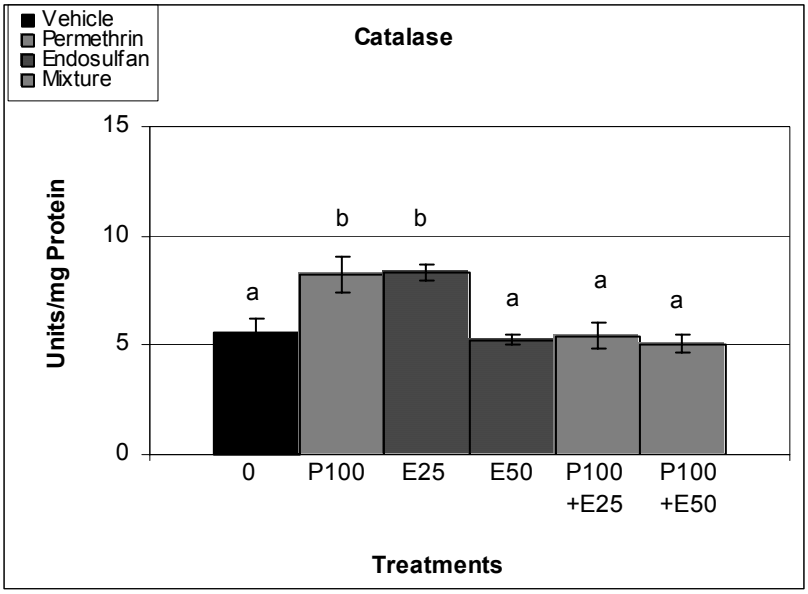

Figure 7: Measurement of Intracellular CAT levels in splenocytes exposed to permethrin, endosulfan and their mixtures: Murine splenocytes were treated with permethrin 100 μM and 25 and 50 μM endosulfan alone and in combination for 12 hours and were evaluated for intracellular CAT levels spectrophotometrically at 240 nm. Splenocytes treated with 100 μM permethrin and 25 μM endosulfan showed significant increase from vehicle control. Results were expressed as the mean ± SEM. N = 4.

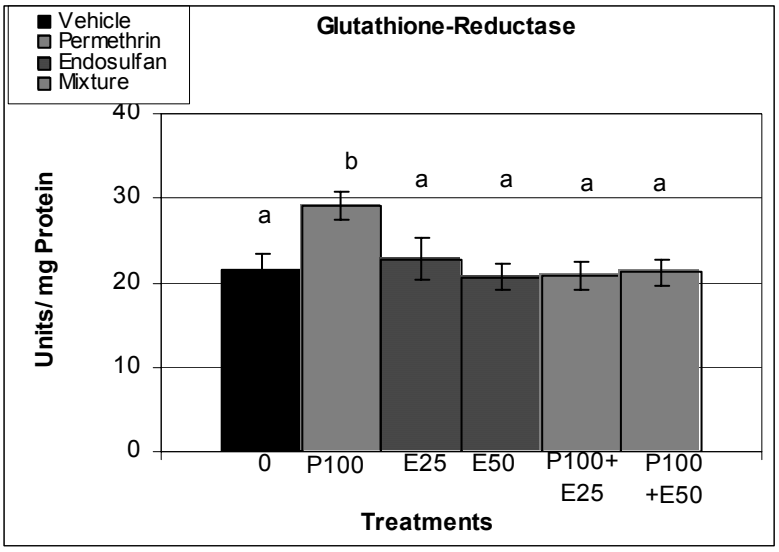

Figure 8: Measurement of Intracellular GR levels in splenocytes exposed to permethrin, endosulfan and their mixtures: Murine splenocytes were treated with permethrin 100 μM and endosulfan 25 μM alone and in combination for 12 hours and were evaluated for GR enzyme levels spectrophotometrically at 340 nm. Splenocytes treated with 100 μM permethrin alone showed significant increase from vehicle control. Results were expressed as the mean ± SEM. N = 6. Treatments with different letters (a & b) were different from each other, $p < 0.05$.

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APPENDIX

Oxidative stress is the state in which exposure to oxidative insult, often involving free radicals, presents a challenge to normal cellular function and even cellular survival (Halliwell and Gutteridge, 1989). It is demonstrated that oxidative stress can cause damage to macromolecules including proteins and DNA (Jain et al., 1997), inhibit cell survival and growth (Haddad et al., 2001), and accelerate apoptosis (Buttke et al., 2001; Kannan et al., 2000a; 2000b). Although initiation of apoptosis is complex and varies with different agents, ROS are thought to be one of the major players in this event (Bauer et al., 1998; Liu et al., 1999).

We have shown that permethrin and endosulfan are toxic to immune cells and also cause oxidative stress upon exposure, in separate experiments. In this study we have investigated to determine the relationship between these two events. The purpose of this study was to evaluate the protective properties of antioxidants in pesticide-induced cytotoxicity. The antioxidants used were reduced glutathione (GSH, 5 mM), catalase (10,000 U/ml), superoxide dismutase (1,000 U/ml), mannitol (5 mM) and uric acid (100 μM). Cells exposed to either endosulfan (50 μM) or permethrin (100 μM) or both, in presence and absence of antioxidants were evaluated for cytoxicity. The cytotoxicity of splenocytes was measured by 7-AAD flow cytometric assay.

As presented in Table 1, the antioxidants used were ineffective in protecting the murine splenocytes from permethrin or endosulfan-induced cytotoxicity, *in vitro*.

Antioxidants in general act as traps or physical quenchers of activated oxygen species and thus protect cells from the toxicity (Chaudiere et al., 1999). Apoptosis caused by many xenobiotics has been linked to oxidative stress and was shown to be inhibited by addition of antioxidants (McGowan et al., 1996). In previous studies we have observed the cytotoxic effects of these pesticides (Chapter 3). These pesticides were also shown to cause oxidative stress in splenocytes (Chapter 4). However, the results of the present study indicate that the antioxidants have little effects in protecting the splenocytes against pesticide-induced toxicity. The results of this study are in contrast to some recent works that have linked oxidative stress or ROS with endosulfan mediated toxicity (Kannan et al., 2000; 2003). These studies cited ROS production, glutathione depletion and drop in mitochondrial transmembrane potential as the primary reasons for endosulfan-induced apoptosis observed in Jurkat T-cells and have shown that GSH (100 μM) reduces apoptosis incidence. In contrast, ROS does not appear to contribute to Fas-mediated apoptosis in Jurkat T-cells (Creagh and Cotter, 1999). Further, GSH depletion is said to lower cell's reducing capacity and can cause oxidative stress without ROS involvement (Curtin et al., 2002). However, in our studies GSH addition did not protect the cells from pesticide-induced toxicity indicating the involvement of other possible mechanisms. Recently, role of reactive nitrogen intermediates (RNI) as both positive and negative regulators of cell death, especially apoptosis has been established. Moreover, anti-oxidants has been shown to be less effective in determining the involvement of RNI-induced apoptosis (Li et al., 1997). It will be prudent to consider the limitations of

antioxidants in determining the involvement of ROS, as changing the redox state of the cell may affect the signaling pathways of the cells and thus the outcome. Further, cell type, level of stress, redox state and the type and dosage of antioxidants used may also play a role. Therefore, pesticide-induced cytotoxicity and oxidative stress seen in our studies seem to be not related under the present experimental conditions. However, further understanding of the mechanisms behind pesticide-induced cytotoxicity might provide further insights.

Table 1: Measurement of viability in splenocytes exposed to pesticides with and without antioxidants

Treatments	Viable Cell Percentage						
	None	+ GSH 5 mM	+ SOD 1,000 U/ml	+ CAT 10,000 U/ml	+ CAT + SOD - 70 U/ml	+ Mannitol 5 mM	+ Uric Acid 100 μM
Solvent Control	63 ± 2	67 ± 1	26 ± 4	63 ± 1	41 ± 3	66 ± 2	60 ± 3
Permethrin 100 μM	62 ± 3	66 ± 1	29 ± 4	59 ± 2	N/A	50 ± 5	45 ± 0
Endosulfan 50 μM	45 ± 7	49 ± 9	21 ± 4	43 ± 3	21 ± 7	52 ± 1	48 ± 5

The percent of live cells following treatment with pesticides as assessed by 7-AAD flow cytometric assay. Cells were treated with solvent control (acetone 0.1%) or permethrin (100 μM) or endosulfan (50 μM) for 4 or 8 or 12 hours. The numbers represented were cell percentages as means ± SD. N/A- No data available

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CONCLUSION AND SIGNIFICANCE OF THE CURRENT STUDY

A large number of pesticides because of their indiscriminate use and poor bio-degradability have become environmental pollutants and consequently bear a potential risk of exposure to humans and animals. And often, these exposures are not limited to single, but to mixtures of pesticides. Most of these pesticides have shown to affect the immune system at doses where no other systemic toxicity was found. It is also suggested that increased ROS production might be responsible for some of the pesticide-induced toxic manifestations. Endosulfan and permethrin, are usually considered to be neurotoxic, have been shown to affect immune system *in vivo* and *in vitro* studies. Therefore, endosulfan and permethrin were evaluated individually and together, for their potential to be toxic and cause oxidative stress (by modulation of pro- and anti-oxidant levels) in immune cells, *in vitro*.

This study examined the cytotoxic and oxidative effects of endosulfan and permethrin and their mixtures on C57BL/6 mouse splencoytes. Splenocytes were exposed to either endosulfan or permethrin or combination for 4 or 8 or 12 hours for time-response studies and for 12 hours for dose-response and mixture studies. To measure the cytotoxicity we have used three techniques, 7-Aminoactinomycin D staining, cytospun slide prepartions (modes of cell death, apoptotic/necrotic), and DNA laddering technique (DNA fragmentation). To measure pro-oxidant levels, we have used flow cytometry in combination with 2′, 7′- Dichlorofluorescin diacetate (DCFH-DA), (for H_2O_2), and hydroethidine (HE), (for O_2^-). We have used spectrophotometry along with specific anti-oxidant enzyme assays to measure intracellular antioxidant enzyme levels (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase).

In this study, endosulfan induced apoptosis, necrosis and enhanced ROS production. Permethrin enhanced ROS production but appeared to have trivial effects on cell death. Potentiation of late apoptosis/necrosis was observed when cells were exposed to higher doses of pesticide mixtures compared to individual pesticides. A non-additive increase in O_2^- generation was seen with pesticide mixture. However, the increase in H_2O_2 production seen with mixture was not different from endosulfan. Individual pesticides minimally increased some of the intracellular antioxidant enzymes such as catalase (both) and glutathione reductase (permethrin). Pesticide mixtures had trivial effects on intracellular antioxidant enzyme levels. Further, antioxidants used in a preliminary study were found to be not effective in protecting the splenocytes from pesticide-induced cytotoxicity.

Endosulfan appeared to enhance the toxicity by causing necrosis at higher doses and in mixtures. This is supported by reports which showed that xenobiotics at low or moderate concentrations cause apoptosis whereas at high concentrations cause necrosis. Possible contribution of permethrin in potentiation of late apoptosis/necrosis seen with mixtures was supported by the reports of permethrin enhanced testicular germ cell apoptosis and rat brain mitochondrial *c* in mixtures. The absence of increase (H_2O_2) or minimal increase (O_2^-) seen in pesticide mixtures could be due to attainment of maximum level of ROS that can be generated by a cell. This may be supported by the additional observation that mixtures did not affect antioxidant enzyme levels beyond that seen with the individual pesticide exposures. Further, involvement of reactive species other than ROS (example: reactive nitrogen intermediates), or variables like cell type, level of stress, redox status,

type and dosage of antioxidants used might provide some explanation for our inability to establish a correlation between pesticide-induced toxicity and oxidative stress.

In conclusion, endosulfan is a potent immunotoxicant whereas permethrin showed trivial effects on immune cell toxicity. However, both pesticides enhanced the ROS generation along with a minimal modulation of some intracellular antioxidant enzyme levels. Therefore, pesticide-induced cytotoxicity and oxidative stress seems to be not related under the present experimental conditions. However, further understanding of the mechanisms behind pesticide-induced cytotoxicity might provide further insights.

FUTURE STUDIES

Future studies should involve investigation in to alternative mechanisms involved in apoptosis, for example, involvement of caspases or Bcl-2 or change in mitochondrial potential. Measuring the intracellular redox status (GSH/GSSG ratio) of the cells might provide some insights regarding the cell's oxidative status. Use of more cell permeable antioxidants and other indices of apoptosis might also be helpful in investigating the role of oxidative stress, in immunotoxicity of these pesticides.

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