

*Age Dependent Immunomodulation,
Genotoxicity and Oxidative Stress due to
Pesticide Mixture Exposure and
Protective Potential of Antioxidants in
Albino Rats*

Thesis

*Submitted in partial fulfillment for the MD degree in forensic
medicine and clinical toxicology*

By

Mustafa Mohsen Mustafa Afify

*Assistant Lecturer of Forensic Medicine
And Clinical Toxicology
Faculty of medicine - Beni Suef University*

Under supervision of

Prof. Dr. Aly Gamal Eldin Abd El-Aal

*Professor of Forensic Medicine
And Clinical Toxicology
Faculty of Medicine – Cairo University*

Prof. Dr. Dina Ali Shokry

*Professor and Head of Forensic Medicine
And Clinical Toxicology Department
Faculty of Medicine – Cairo University*

A. Prof. DR. Manal El -Sayed El-Halwagy

*Assistant Professor of Biochemistry
Mammalian toxicology Department
Central pesticide laboratory*

Dr. Mohamed Adly Mohamed

*Lecturer of Forensic Medicine
And Clinical Toxicology
Faculty of Medicine – Cairo University*

**Faculty of Medicine
Cairo University
2009**

Abstract

This thesis was designed to study dose-related and age-related effects of the mixture of four pesticide residues extract (chlorpyrifos, profenofos, fenitrothion and dicofol) on Oxidative Stress, Genotoxicity and immunotoxicity, and Protective Potential of natural Antioxidants (garlic 250 mg/kg and alpha lipoic acid 60mg/kg), 120 males of Albino rats will be divided into two main groups according to age; weaning group (2 months age) and Adult group (6 months age). Each age group of rats were divided into 6 subgroups (*10 rats in each*) the 1st group served as control, *the 2nd & 3rd* groups were orally treated with high & low level of pesticides residue mixture, respectively, the 4th group served as +ve control (antioxidants only), the 5th & 6th groups were orally treated with antioxidants 1h after administration of high & low level of pesticides residue mixture, respectively, All groups were force-fed by gastric intubations 5 days per week for 3 months. The oxidative stress status of treated animals has been evaluated by assessment of reduced glutathione (GSH), Glutathione-S-Transferase (GST), malondialdehyde (MDA). In addition, the acetylcholinesterase (AChE) activity was measured as a biomarker of toxicity. The mean *comet tail length* and *Comet DNA %* were used to measure DNA damage. We used IgG, IgM, rate of leucocyte phagocytosis and of lymphocyte transformation as immunotoxicological biomarkers to test the immune function as well as Histopathological studies in lymph node. Our result revealed that pesticide mixture induce inhibitory effect on AChE, depletion in GSH content, alteration in GST and elevation in lipid peroxidation (MDA). A significant increase in mean *comet tail length* and Comet DNA % indicating DNA damage was observed. The damage was dose related. The results showed that pesticides mixture produced a decrease in Both IgG and IgM, the rate of lymphocyte transformation and the rate of leucocyte phagocytosis also decrease in both age groups. In additions, our result revealed that natural antioxidants (ALA and garlic extracts) have more or less counteracting effect on Oxidative Stress, Genotoxicity and immunotoxicity caused by pesticides.

(Key words: pesticide residues, acetylcholinesterase, Oxidative Stress, lipid peroxidation, Genotoxicity, comet assay, immunotoxicity, lymphocyte transformation, phagocytosis)

ACKNOWLEDGEMENT

First of all greatest thanks and merciful to Allah, the great creator and the source of all man's knowledge.

I would like very much to express my deepest gratitude and sincere thanks to Prof. Dr. Aly Gamal El-Din Professor of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Cairo University, for his fatherly attitude, sincere encouragement, valuable guidance, kind supervision and scientific support.

Wording is not enough to express my sincere gratitude and deep appreciation to Prof. Dr. Dina Ali Shokry Professor and Head of Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Cairo University, for the idea and planning of this work, her efficient and indispensable help, faithful guidance and willing efforts to facilitate the difficulties during this work.

I offer my great appreciation and thanks to A. Prof. Dr. Manal El - Sayed El-Halwagy Assistant Professor of Biochemistry, Mammalian toxicology Department , Central pesticide laboratory for her meticulous supervisions, sincere guidance, significant input and kind help in every step of this work.

Thanks to Dr. Mohamed Adly Mohamed, Lecturer of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Cairo University for his supervision and encouragement.

I wish also to express my sincere thanks to Prof. Dr. Nevine Darwish Professor of pathology, Research institute of ophthalmology, for her support in the Histopathological part of this work, and for her valuable advices

Thanks to Dr. Sanaa El Sawi, Researcher in Central laboratory for analysis of pesticide residues and heavy metal in food, for her help.

Last, but not least, my deep thanks and gratitude to all my professors and my colleagues and to my family for their kind support and encouragement.

Mustafa M. Afify

This work is dedicated

TO SOUL OF MY FATHER

TO MY MOTHER

TO MY WIFE

AND

TO MY KID

TABLE OF CONTENTS

Title	Page
LIST OF ABBREVIATIONS	i
LIST OF FIGURES	iii
LIST OF PHOTOMICROGRAPH	v
LIST OF TABLES	viii
I- INTRODUCTION AND AIM OF WORK	1
II- REVIEW OF LITERATURE	
Chapter (1) <u>PESTICIDE</u>	6
• <i>WHAT IS A PESTICIDE?</i>	6
• <i>HISTORICAL REVIEW OF PESTICIDE</i>	6
• <i>CLASSIFICATION OF PESTICIDES</i>	8
• <i>PESTICIDE RESIDUES</i>	10
• <i>ORGANOPHOSPHATE PESTICIDE</i>	16
• <i>ORGANOCHOLRINE PESTICIDE</i>	33
Chapter (2)<u>PESTICIDE AND OXIDATIVE STRESS</u>	36
• <i>FREE RADICALS AND OXIDATIVE STRESS</i>	37
• <i>ANTIOXIDANT DEFENSES</i>	40
• <i>PESTICIDE-INDUCED OXIDATIVE STRESS</i>	44
• <i>ALPHA LIPOIC (ALA) AND GARLIC AS NATURAL ANTIOXIDANTS</i>	47
Chapter (3) <u>PESTICIDE AND GENOTOXICITY</u>	51
• <i>PESTICIDE-INDUCED GENOTOXICITY</i>	52
• <i>THE COMET ASSAY</i>	55

Chapter (4) <u>PESTICIDE AND IMMUNOTOXICITY</u>	60
• <i>THE IMMUNE SYSTEM</i>	60
• <i>IMMUNOTOXICITY</i>	64
• <i>PESTICIDE-INDUCED IMMUNOTOXICITY</i>	66
III- MATERIALS AND METHODS	72
IV- RESULTS	94
V- DISCUSSION	141
VI- SUMMERY AND CONCLUSION	162
VII- RECOMMENDATION	166
VIII- REFERENCES	167
IX- ARABIC SUMMARY	

LIST OF ABBREVIATIONS

ABTS	2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
AChE	Acetylcholinesterase
ADI	Acceptable Daily Intake
AGE	aged garlic extract
ALA	alpha lipoic acid
ALS	alkali-labile sites
AOX	Antioxidants
ARfD	Acute Reference Dose
CA	chromosome aberrations
CAT	catalase
CFU	colony forming units
CTL	cytotoxic T cells
DHLA	dihydrolipoic acid
DSB	double-strand breaks
EMRL	Extraneous Maximum Residues Limits
EPA	Environmental Protection Agency
GGT	gamma-glutamyl transpeptidase
GR	glutathione reductase
GPx	glutathione peroxidase
GSH	Reduced glutathione
GST	glutathione-S-transferase
HDPM	High Dose Pesticides Mixture
H₂O₂	hydrogen peroxide
LD₅₀	lethal dose in 50% of animals
LDPM	Low Dose Pesticides Mixture

LGL	large granular lymphocyte
MALT	mucosa-associated lymphoid tissues
MDA	malonyl aldehyde
MEM	Minimum Essential Medium (culture medium)
MHC	major histocompatibility complex
MRL	Maximum Residue Level
MTT	[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] The MTT assay has been used to test cytotoxicity of reagents and cell viability.
NPD	Nitrogen Phosphorous Detectors
O₂^{-•}	superoxide anion
¹O₂	singlet oxygen
OC	Organochlorines
OH	hydroperoxyl radicals
OFR	Oxygen free radicals
OP	Organophosphate
PBS	Phosphate Buffered Saline
PRC	Pesticide Residues Committee
PSD	Pesticide Safety Directorate
RDA	Recommended Dietary Allowance
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute, Media
SCE	sister chromatid exchanges
SOD	superoxide dismutase
SSB	single -strand breaks
TCR	T cell receptor
WHO	World Health Organization

LIST OF FIGURES

Fig.		Page
1	Key reactions occurring between organophosphates and AChE	21
2	Sources of ROS and other free radicals.	39
3	Cell injury by ROS or free radical-induced oxidative stress	40
4	Lipoic acid (a) and its reduced form – dihydrolipoic acid (b)	49
5	Schematic representation of critical steps in the alkaline (pH > 13) Comet assay	59
6	Images of comets (from lymphocytes)	59
7	The realm of pesticide immunotoxicology	66
8	Correlation between (AChE) and (GSH) Content in plasma of the weaning albino rats at the end of 1 st month.	107
9	Correlation between (AChE) and (GST& MDA) in plasma of the weaning albino rats at the end of 1 st month.	107
10	Correlation between (AChE) and (GST& MDA) in plasma of the weaning albino rats at the end of the 2 nd month.	108
11	Correlation between (AChE) and (Comet Tail, Comet DNA %) in blood of the weaning albino rats at the end of the 3 rd month	108
12	Correlation between (GSH) and (MDA) in plasma of the weaning albino rats at the end of the 1 st month.	109
13	Correlation between (GSH3) and (Comet Tail, Comet DNA %) in blood of the weaning albino rats at the end of the 3 rd month.	109
14	Correlation between (GSH3) and (Comet Tail, Comet DNA %) in blood of the weaning albino rats at the end of the 3 rd month	110
15	Correlation between (MDA3) and (Comet Tail, Comet DNA %) in blood of the weaning albino rats at the end of the 3 rd month.	110

16	Correlation between (AChE) and (GSH) Content in plasma of the adult albino rats at the end of 1 st month.	129
17	Correlation between (AChE) and (GSH) Content in plasma of the adult albino rats at the end of 3 rd month.	129
18	Correlation between (AChE) and (GST& MDA) in plasma of the adult albino rats at the end of 1 st month.	130
19	Correlation between (AChE) and (GST& MDA) in plasma of the adult albino rats at the end of 3 rd month	130
20	Correlation between (AChE) and (Comet Tail, Comet DNA %) in blood of the adult albino rats at the end of the 3 rd month	131
21	Correlation between (GSH) and (MDA) in plasma of the adult albino rats at the end of the 2 nd month.	131
22	Correlation between (GSH) and (Comet Tail, Comet DNA %) in blood of the adult albino rats at the end of the 3 rd month	132
23	Correlation between (MDA) and (Comet Tail, Comet DNA %) in blood of the adult albino rats at the end of the 3 rd month	132

LIST OF PHOTOMICROGRAPH

Photo		Page
1	Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of a control weaning albino rats. 2200x (40x obj).	111
2	Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of a +ve control (antioxidants treated) weaning albino rats. 2200x (40x obj).	111
3	Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of the HD pesticides mixture treated weaning albino rats. 2200x (40x obj).	112
4	Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of the HD pesticides mixture and antioxidant treated weaning albino rats. 2200x (40x obj).	112
5	Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of the LD pesticides mixture treated weaning albino rats. 2200x (40x obj).	113
6	Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of the LD pesticides mixture and antioxidant treated weaning albino rats. 2200x (40x obj).	113
7	Photomicrograph of a section in the lymph node from a control weaning albino rats showing the medullary cords (m) and the medullary sinuses (S) (H&E x40).	114
8	Photomicrograph of a section in the lymph node from a +ve control (antioxidants treated) weaning albino rats showing the medullary cords and the medullary sinuses (H&E x40)	114
9	Photomicrograph of a section in the lymph node from the HD pesticides	115

mixture treated weaning albino rats showing some hemorrhagic spots (H), decreased cellularity widening of the sinuses (S). Cells with condensed chromatin were noticed (>) (H&E x40).

- 10 Photomicrograph of a section in the lymph node from the HD pesticides mixture and antioxidant treated weaning albino rats showing widening of the medullary sinuses (S), few macrophages were seen (>) but cellularity was close to normal (H&E x40). 115
- 11 Photomicrograph of a section in the lymph node from the LD pesticides mixture treated weaning albino rats mild congestion (c), minimal widening of the sinuses (S). Cells with condensed chromatin were seen (>) (H&E x40). 116
- 12 Photomicrograph of a section in the lymph node from the LD pesticides mixture and antioxidant treated weaning albino rats showing mild congestion, normal cellularity (H&E x40). 116
- 13 Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of a control adult albino rats. 2200x (40x obj). 133
- 14 Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of a +ve control (antioxidants treated) adult albino rats. 2200x (40x obj). 133
- 15 Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of the HD pesticides mixture treated adult albino rats. 2200x (40x obj). 134
- 16 Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of the HD pesticides mixture and antioxidant treated adult albino rats. 2200x (40x obj). 134

17	Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of the LD pesticides mixture treated adult albino rats. 2200x (40x obj).	135
18	Images of comets (from lymphocytes), stained with ethidium bromide, showing DNA damage in single cells (COMET assay) of the LD pesticides mixture and antioxidant treated adult albino rats. 2200x (40x obj).	135
19	Photomicrograph of a section in the lymph node from a control adult rat showing normal architecture. The Cortex (C) containing the follicles with their germinal and the Medulla (M) (H&E x20)	136
20	Photomicrograph of a section in the lymph node from a +ve control (antioxidants treated) adult rat showing normal architecture. The Cortex containing the follicles with their germinal and the Medulla (H&E x40)	136
21	Photomicrograph of a section in the lymph node from the HD pesticides mixture treated adult rats showing areas of hemorrhage (H), decreased cellularity (*). Few macrophages could be seen between the lymphocytes (>) (H&E x40).	137
22	Photomicrograph of a section in the lymph node from the HD pesticides mixture and antioxidant treated adult rats showing better cellularity, few widened sinuses (S) and few macrophages between the lymphocytes (>) (H&E x40).	137
23	Photomicrograph of a section in the lymph node from the LD pesticides mixture treated adult rats showing areas of hemorrhage (H), widening of the medullary sinuses (S), cells with condensed chromatin were noticed (>) and decreased cellularity (H&E x40).	138
24	Photomicrograph of a section in the lymph node from the LD pesticides mixture and antioxidant treated adult rats showing mild congestion (c) better cellularity and few chromatin condensed cells (>) (H&E x40).	138

LIST OF TABLES

Fig.		Page
1	The WHO Recommended Classification of Pesticides by Hazard	9
2	Sources of ROS and other free radicals.	42
3	Cell injury by ROS or free radical-induced oxidative stress	43
4	Lipoic acid (a) and its reduced form – dihydrolipoic acid (b)	70
5	Schematic representation of critical steps in the alkaline (pH > 13) Comet assay	78
6	Pesticide residues monitored in vegetable samples collected from Great Cairo markets during February 2001 to February 2003.	94
7	Time dependant effect of pesticide mixture in presence or absence of combined antioxidants on Acetylcholinesterase (<i>AchE</i>) (U/L) in plasma of a weaning albino rats.	103
8	Time dependant effect of pesticide mixture in presence or absence of combined antioxidants on reduced glutathione (GSH) Content (Mg/dl) in plasma of a weaning albino rats.	103
9	Time dependant effect of pesticide mixture in presence or absence of combined antioxidants on glutathione-S-Transferase (GST) Activity (μmol/min/ml plasma) in plasma of a weaning albino rats.	104
10	Time dependant effect of pesticide mixture in presence or absence of combined antioxidants on malondialdehyde (MDA) (μmol/ml) in plasma of a weaning albino rats.	104
11	Effect of Chronic intoxication with pesticide mixture in presence or absence of combined antioxidants on (<i>Comet Tail, Comet DNA %</i>) in blood of <i>a weaning</i> albino rats.	105
12	Effect of Chronic intoxication with pesticide mixture in presence or absence of combined antioxidants on (<i>IGG, IGM</i>) in standard serum of <i>a weaning</i> albino rats.	105

- 13 Effect of Chronic intoxication with pesticide mixture in presence or 106
absence of combined antioxidants on (*Lymphocyte transformation, Phagocytosis*) in heparinized blood of **a weaning** albino rats.
- 14 Time dependant effect of pesticide mixture in presence or absence of 125
combined antioxidants on *Acetylcholinesterase (AChE) (U/L)* in plasma of **an adult** albino rats.
- 15 Time dependant effect of pesticide mixture in presence or absence of 125
combined antioxidants on *reduced glutathione (GSH) Content (Mg/dl)* in plasma of **an adult** albino rats.
- 16 Time dependant effect of pesticide mixture in presence or absence of 126
combined antioxidants on *Glutathione-S-Transferase (GST) Activity (μmol/min/ml plasma)* in plasma of **an adult** albino rats.
- 17 Time dependant effect of pesticide mixture in presence or absence of 126
combined antioxidants on *malondialdehyde (MDA) (μmol/ml)* in plasma of an **adult** albino rats.
- 18 Effect of Chronic intoxication with pesticide mixture in presence or 127
absence of combined antioxidants on (*Comet Tail, Comet DNA %*) in plasma of **an adult** albino rats.
- 19 Effect of Chronic intoxication with pesticide mixture in presence or 127
absence of combined antioxidants on (*IGG, IGM*) in standard serum of **an adult** albino rats.
- 20 Effect of Chronic intoxication with pesticide mixture in presence or 128
absence of combined antioxidants on (*Lymphocyte transformation, Phagocytosis*) in heparinized blood of **an adult** albino rats.
- 21 Statistical results from two-way ANOVA comparing the effect of pesticide 139
mixture and antioxidant supplementation on Dependent Variable at weaning and adult age group.