1-1-2015

Synergetic Effect Of Permethrin And Dmh On Anti-Oxidation And Damage Response Genes

Karim Mohamed

Wayne State University,

SYNERGISTIC EFFECT OF PERMETRIN AND DMH ON ANTI-OXIDATION AND DAMAGE RESPONSE GENES

by

KARIM MOHAMED

THESIS

Submitted to the Graduate School

of Wayne State University

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2016

MAJOR: NUTRITION AND FOOD SCIENCE

Approved by

_____

Advisor Date

DEDICATION

This thesis is dedicated to my family, mentors,

advisors, and friends for their constant support and guidance in the pursuit of knowledge.

ACKNOWLEDGEMENTS

It is an honor for me to express my gratitude

first and foremost to my supervisor, Dr. Ahmad Heydari whose continuous support and feedback has been critical in the development of this thesis.

Thanks to my lab mates and colleagues Michelle Jones, Ali Fardous, Dr. Safa Beydoun, Tom Prychitko, John Wittitaker,

and all the faculty and staff of the Department of Nutrition and Food Science for the opportunities, experiences, and assistance in the pursuit of my completing this thesis.

I would like to thank Dr. Heydari and Dr. Cabelof for taking the time to be on my committee and giving me their valuable input.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

ACF	Aberrant Crypt Foci
DMH	1,2-Dimethylhydrazine
RT PCR	Reverse Transcription Polymerase Chain Reaction
TRX	Thioredoxin
Combo	Combination of Permethrin and DMH
FA	Folate Adequate
FD	Folate Deficient
GPx	Glutathione Peroxidase
PER	Permethrin
PM	Permethrin
PRDX	Peroxiredoxin
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
TRXR	Thioredoxin Reductase

Chapter I. Background & Introduction

Past and current studies in the research lab attempt to study the impact of short and long term deficiency in dietary folate on a number of genes on the mTOR pathway. Female C57/blk6 mice were put on a Folate Adequate (FA) diet which was then followed by a Folate Deficient (FD) diet. During the study, a city-wide electrical outage occurred causing the animals to undergo heat exhaustion for two days. After the power returned, the mice were discovered to be infected with mites and were treated with cotton balls coated with permethrin. A week later, the mice were injected with 1,2-Dimethylhydrazine (DMH) and sacrificed. The mice were with FA diets had high levels of ACF formation, inflammation in the colon, and unusually high mortality rates.

The purpose of this study was to see the ultimate impact of anti-oxidation and damage response genes when the animal is exposed to controlled amounts of permethrin and DMH.

Pesticides: Permethrin and DMH

Pesticides are toxic substances used for increasing agricultural productivity by killing or deterring harmful pests. The use of pesticides has dramatically increased since the 1960's. As a result, human exposure to pesticides is unavoidable which of concern due to their potential cytotoxicity. The interactions between pesticides in the environment and potential of cytotoxic effects has been of growing public concern since the 1990's leading to the passage of the Food Quality Protection Act of 1996 (Vogt et al. 2012). Since the passage of the Act, the cytotoxic potential of pesticides has been conducted and databases have been developed. However, pesticides are commonly used in combination for increased, broad-range protection of foodstuffs (Morgan 2012).

In comparison to single pesticide exposure, pesticide mixtures have shown increased damaging effects on murine thymocytes (Olgun S et al. 2003), induced transgenerational inheritance of disease and sperm epimutations in mice (Manikkam et al. 2012), and increased diffuse neuronal cell death in rats (Abdel-Rahman A et al. 2001).

Permethrin is a synthetic, second generation pyrethroid which is commonly used in control of ticks (Roma et al. 2012). The toxicity to permethrin is due to prolonged opening of sodium channels which causes repetitive discharges after a single stimulus causing tremors, hyperactivity, ataxia, convulsions, paralysis and eventually death. Although permethrin is considered non-carcinogenic to human beings, the potential still exists (Dong 2007). In-vitro, permethrin has shown significant genotoxic and mutagenic potential in as little as 24 hours after treatment (Roma et al. 2012).

1,2-Dimethylhydrazine (DMH) is a potent pro-carcinogen which acts as a DNA methylating agent and is commonly used to induce colon tumors in experimental animals (Cruse JP et al. 1978). Pro-carcinogens are carcinogens which require activation by drug metabolism enzymes before becoming carcinogenic (Stralka D et al. 1991).

DMH's damage is caused by mast cells producing super oxide anions which cause inflammation. Mast cells then recruit neutrophils to the inflammatory site to further provoke an inflammatory reaction, generating excessive amounts of reactive oxygen species (ROS), proteolytic enzymes, and cytokines which could potentially yield tissue damage in the colon.

Anti-oxidation genes

ROS are necessary for intracellular signaling and redox regulation. However, an increase in ROS is hypothesized to be the cause of tissue damage in most human diseases (Whittemore

ER et al. 1995). The damage caused by ROS is counteracted by a large redox-balance maintenance system known as anti-oxidation enzymes. The major enzymes that are members of this antioxidant defense system include superoxide dismutase (SOD), catalase (CAT) (Arigesavan et al. 2015), glutathione peroxidase (GPx), and thioredoxin reductase (TRXR). Damage to DNA caused by ROS is the main culprit in the development of colon cancer. (Murawaki Y et al. 2008).

Damage Response Genes

Damage response genes ensure the integrity of the genome through cell cycle checkpoints and DNA repair pathways. Dysregulation of DNA repair factors can promote the accumulation of DNA errors and genomic instability which is implicated in several diseases such as cancer. Up regulation of damage response genes decreases the efficacy of anti-cancer drugs. Down regulating DNA damage response pathways causes genomic instability which is the hallmark of cancer (Broustas et al. 2014).

Chapter II. Materials and Methods

Animals

Experiments were performed on the liver of wildtype C57/blk 6 mice which were sacrificed at 8 weeks after being exposed to their respective pesticides for 24 hours. The mice were pathogen free and kept in accordance with the National Institute of Health guidelines for the use and care of Laboratory animals. All procedures for handling and sacrificing the mice were approved by the Department of Laboratory and Animal Research (DLAR) of Wayne State University (Detroit, MI). Permethrin is degraded quickly in the liver by and excreted in the urine as alcohols, phenols, or carboxylic acids and their glycine, sulfate, or glucuronide conjugates

(National Academies Press [US] 1994). The mouse livers were stored in liquid nitrogen after sacrifice until they were ready for homogenization.

Pesticides

The mice (n=12) were divided into four groups. Group 1 (n=3) were injected intraperitoneally injected with DMH. Group 2 (n=3) were injected intraperitoneally with permethrin. Group 3 (n=3) were injected intraperitoneally of a combination of both permethrin and DMH. Group 4 (n=3) were controls who were not exposed to any pesticides. The mice were all fed folate adequate semisynthetic diets purchased from Dyets Inc (Bethlehem, PA) and sacrificed 24 hours after injection with their respective pesticide(s).

Homogenization and RNA Isolation

RNA isolation from liver tissue was carried out at 4^{o}C. The RNA was isolated from the liver using the Trizol kit Invitrogen by following the manufacturers' protocol. Homogenization occurred using a polytron tissue homogenizer. Each sample was completely liquefied. Several beakers were filled with distilled water for washing the homogenizer tip between each liver sample homogenization. Following the cleaning with distilled water, the homogenizer tip was sterilized with 70% ethanol and the tip was inspected to assure no pieces of mouse liver sample remained in the tip. The ethanol was dried with Kim wipes. This procedure was repeated for all 12 samples. Homogenized samples were then transferred into labeled 1.5 mL centrifuge tubes and incubated with trizol for 5 minutes. The tubes were centrifuged at 12,000xg for 10 minutes at 4^{o}C. The supernatant was collected with a pipette and transferred into fresh 1.5 mL centrifuge tubes and the pellet discarded. To each sample, 200 µL of chloroform was pipetted, shaken vigorously for 15 seconds, and incubated at room temperature for 2-3 minutes. This

resulted in a phase separation consisting of a clear aqueous phase, white, semi-solid interphase, and a red organic phase. RNA is located in the in the aqueous phase.

The samples were then centrifuged at 12,000 xg for 15 minutes at 4^{o}C to get maximal phase separation. The aqueous phase was transferred to fresh 1.5 mL centrifuge tubes. 500 µL of Isopropanol was added and mixed with a pipette. This caused the precipitation of RNA. The samples were incubated at room temperature for 10 minutes and centrifuged for 10 minutes at 12,000 xg at 4^{o}C. Supernatant was discarded and the pellet contained the RNA. The pellet was washed with 1 mL of 75% ethanol and tubes were flicked and inverted. Centrifugation was done at 7,500 xg for 5 minutes at 4^{o}C. Ethanol was then discarded without disturbing the pellet and 50 µL of water was added.

The tube was incubated at 55-60^{o}C for 10 mnutes in a water bath. The RNA samples were stored in -80^{o}C until they were ready to be quantified in order to be ran on an RNA gel or synthesized into cDNA.

Quantification

RNA was quantified using an ND3000 Nanodrop. Blank was set using 1 µL. 1 µL of sample was used to measure the RNA concentration of each sample. The values were recorded and based on the concentration; the RNA was diluted to get a concentration of 1000 µg.

RNA Gel Electrophoresis

RNA integrity was confirmed by using RNA gel electrophoresis. The electorphoreiss tank buffer was prepared and stored at -20^{o}C. The castor, well plate, and comb were well-cleaned and dried. To prepare the gel, 1.5 g of agarose was weighed and added to an Erlenmeyer flask. 72 mL of water and 10 mL of 10x MOPS were added and mixed well.

The flask was microwaved for a total of a minute and 30 seconds and stopped at intervals to prevent the agar gel solution from overflowing. 19 mL of formaldehyde was added to the flask under the hood and well-shaken. The agar gel solution was then poured into the well plate and the comb was inserted to form wells. The gel was left at room temperature to polymerize.

5 µL of each RNA sample was aliquoted into fresh 15 mL centrifuge tubes. 25 µL of RNA loading dye and 1 µL of ethidium bromide were added to each of the tubes and and spun. The samples were kept at 65^{o}C water bath for 15 minutes. After the gel polymerized, the comb was removed without disturbing the wells in the electrophoresis tank. The samples were carefully loaded into the wells and allowed to electrophorese at 150V at 4^{o}C for one and a half hours. The electrophoresis chamber was kept on a magnetic stirrer to allow the movement of ions. After the dye migrated to 3/4th of the way through the gel, the gel was visualized using a UV imager.

cDNA Preparation

RNA was extracted from -80^{o}C and kept on thaw on ice. The isolated RNA was used for preparing complementary DNA (cDNA) using IM PROM II Reverse Transcriptase system kit from Promega. The manufacturer's protocol was followed to synthesize the cDNA.

Master mix preparation

To prepare the master mix, the components were assembled in 1.5 Ml micro centrifuge tubes on ice. The reagents for the master mix were added in the following order and amounts seen on Table 1. The master mix was vortexed gently and aliquoted 15 Ml of master mix into 0.2 Ml microcentrifuge tubes which were kept on ice

Reagent	Volume [7]	# of Samples	Total volume
Nuclease Free Water	5 µL	12	60 µL
5x Reaction Buffer	4 µL	12	48 µL
$MgCl_2$	4 µL	12	48 µL
dNTP	1 µL	12	12 µL
Reverse Transcriptase	1 µL	12	12 µL

TABLE 1. Composition of Mastermix for cDNA synthesis. To prepare the master mix, the components were assembled in 1.5 mL micro centrifuge tubes on ice and the reagents were added in the above amounts and order

RNA sample preparation and cDNA synthesis

Concentrations of RNA samples were measured using a ND3000 Nanodrop and measured in duplicates and averaged. From the concentration, the volume needed to obtain 1 µg of RNA was calculated. RNA sample was prepared in 15 appropriately labeled 0.5 mL microcentrifuge tubes. Each tube contained 1µL of reaction primer (also known as random primer), enough volume of RNA sample to obtain 1 µg of RNA, and enough nuclease free water to bring the total volume in each tube to 5 µL. The RNA tubes were put in a 70^{o}C heat block for 5 minutes (which denatures RNA secondary structure) then immediately chilled on ice for 5 minutes (to help the primer for annealing). Then the RNA sample was centrifuged by pulse.

The RNA sample was aliquoted into the master mix on ice and the two solutions were mixed by pipetting. The tubes were spun by pulse and placed in the Eppendorf thermocycler with the cycle parameters seen on Table 2. The thermocycler ran for one cycle and produced cDNA. cDNA was stored at -20^{o}C until the purification step.

Time	Temperature
5 minutes	25^{o}C
60 minutes	42^{o}C
15 minutes	70^{o}C
Hold	4^{o}C

TABLE 2. Thermocycler parameters for synthesis of cDNA. To synthesize cDNA from RNA, the thermocycler ran for one cycle with the parameters above

cDNA purification

The cDNA was purified using the QIAquick PCR Purification Kit Protocol from Qiagen Inc. (Valencia, CA). The column tubes and centrifuge tubes were labeled. cDNA wells were assembled on ice. As per the protocol, 5x the volume of Buffer PB was added to 1 volume of the PCR sample (i.e. 20 µL of PCR sample used 100 µL of Buffer PB) and mixed well. A QIAquick spin column tube was placed in a 2 mL collection tube. The samples were transferred to the column and centrifuged at 10,000xg for 30-60 seconds. The flow through was discarded and the column was put back onto the same tube. 0.75 mL of buffer PE (with ethanol) was added to the column and centrifuged at 10,000xg for 30-60 seconds. The flow through was discarded and the column was returned onto the same tube. The column was spun for one minute to remove any residual ethanol and the flow through was discarded. A fresh 1.5 mL centrifuge tube was placed on the column. 50 µL of buffer EB was added to the center of the membrane and allowed to stand for one minute. The column and tube were centrifuged for one minute. We reload the flow through into the column and spin again. As a final step, we quantify the concentration of the cDNA using the ND3000 Nanodrop. cDNA was normalized at 40 µg.

Real-Time Quantitative PCR

SYBR Green QRT-PCR master mix from Stratagene kit was used to run Real Time PCR (RT-PCR). Forward and Reverse Primers were obtained for the genes seen in Table 3.

glutathione peroxidase (GPx)	**thioredoxin reductase (TRXR).**
p53	**GADD45**
thioredoxin (TRX)	**Peroxiredoxin (PRDX)**
mTor	**PCNA**

Table 3. Panel of anti-oxidation and damage response genes observed. Damage to DNA caused by ROS is the main culprit in the development of colon cancer. (Murawaki Y et al. 2008). Hence, in this study, we looked at a panel of anti-oxidation and damage response genes. The damage caused by ROS is counteracted by a large redox-balance maintenance system known as anti-oxidation enzymes. Damage response genes ensure the integrity of the genome through cell cycle checkpoints and DNA repair pathways. Dysregulation of DNA repair factors can promote the accumulation of DNA errors and genomic instability which has also been implicated in cancers.

Purified cDNA samples were thawed gently and placed on ice. RT-PCR master mix was prepared in accordance with the SYBR Green dye protocol. Each Forward and Reverse primer pair for the genes listed in Table 3 had their own master mix because the genes differ in sequence. The reaction components for each gene were as seen on Table 4.

Reagent	Volume	# of sample	Total
RT-Master mix	12.5 µL	12	150 µL
Forward Primer	0.5 µL	12	12 µL
Reverse Primer	0.5 µL	12	12 µL
PCR grade water	8.5 µL	12	102 µL

Table 4. Master Mix SYBR green protocol. RT-PCR master mix was prepared in accordance with the SYBR Green dye protocol. Each Forward and Reverse primer pair for the genes listed in Table 3 had their own master mix.

While on ice, 22 µL of master mix were added into each well of a 96 well plate. 3 µL of cDNA sample were added into each well bringing the total volume in each well to 25 µL. The order of which gene was being amplified in each well was carefully kept track of. Following the filling of all the charted wells, the PCR optical caps were placed over the wells and centrifuged before placing in the RT-PCR.

RT PCR analysis was done using the program Mx Pro. The thermal profile was as seen in Figure 1.

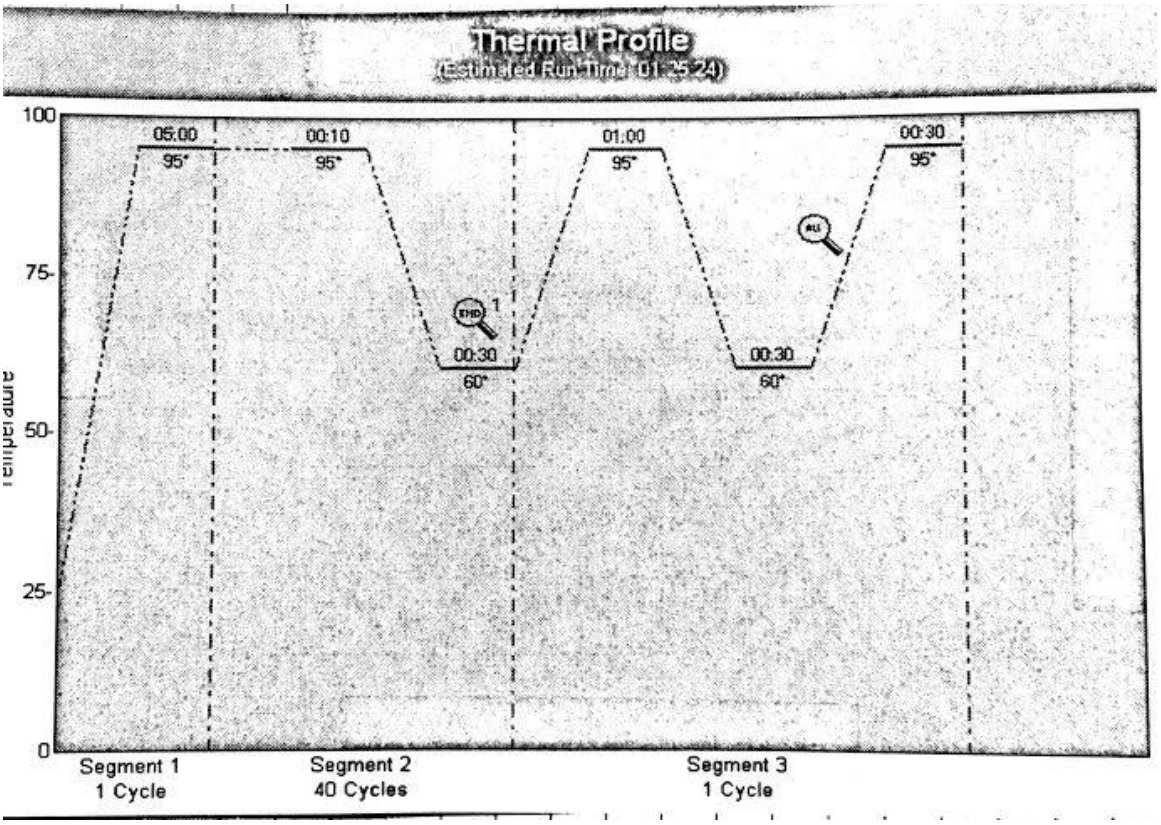

Figure 1. Thermal profile for amplifying cDNA

Chapter III. Results & Discussion

In this study, we have analyzed the transcription of anti-oxidation and damage response genes in the liver of wildtype C57/blk 6 mice in response to intraperitoneally injected DMH, permethrin (PER), and a combination of both pesticides. In a previous study, mice had uncontrolled contact with cotton balls containing an uncontrolled amount of permethrin. After a week, DMH was injected at 30 mg/kg body weight per mouse to induce ACF. As a result, unusual insults were seen in animals exposed to the combination of insecticides that weren't seen before or expected. These Insults included higher amounts of ACF, severe colonic inflammation, and an unusually high mortality in mice that were given a folate adequate diet.

Three 8-week old mice were injected intraperitoneally with a mix of cis and trans permethrin (75%:25% respectively) at 30% median lethal dose (96 mg/kg body weight). Permethrin was dissolved in corn oil and injected 24 hours before sacrifice. In three other mice, DMH was also injected intraperitoneally 24 hours before sacrifice at 30 mg/kg body weight per mouse. Three mice were also injected 24 hours before sacrifice with a combination of DMH and permethrin at the same dosage. The mice were sacrificed and the liver tissue was stored in liquid nitrogen.

Diet	Number of animals in each group at start of experiment	Deaths
FA	16	3 died
FA/FD	16	1 died
FD	16	2 died
FA/MF	6	1 died
FD/MF	6	2 died
Total	60	9 (15% mortality)

Table 5. Mice mortality rates. Power outage – June 9-10, 2011. Mice were exposed to PER due to infection with fleas and followed by a planned injection of DMH. This study shows huge variations within groups that differences between the FA and FD groups. We saw unusually high mortality in mice who had folate adequate diets when exposed to both permethrin and DMH. Higher amounts of ACF and severe colonic inflammation were also seen.

The RNA was isolated using the trizol method and the integrity was determined using RNA gel electrophoresis. Trizol method yields impure RNA at high concentrations. To confirm the integrity, the isolated RNA samples were run after quantification. Figure 2 shows the RNA is highly degraded but the 18S and 28S bands were still visible meaning there was still some integrity. Using nanodrop, high concentrations of RNA were measured. Nanodrop also showed the RNA had solvent contamination but no protein contamination which is to be expected when using the trizol method. Each reading was done in duplicates to obtain an average concentration.

cDNA was synthesized from the isolated RNA and purified. After purification, anti-oxidation and damage response genes were amplified with RT PCR and quantified using SYBR green. The null hypothesis was our combo and control are the same (i.e. $p<0.05$)

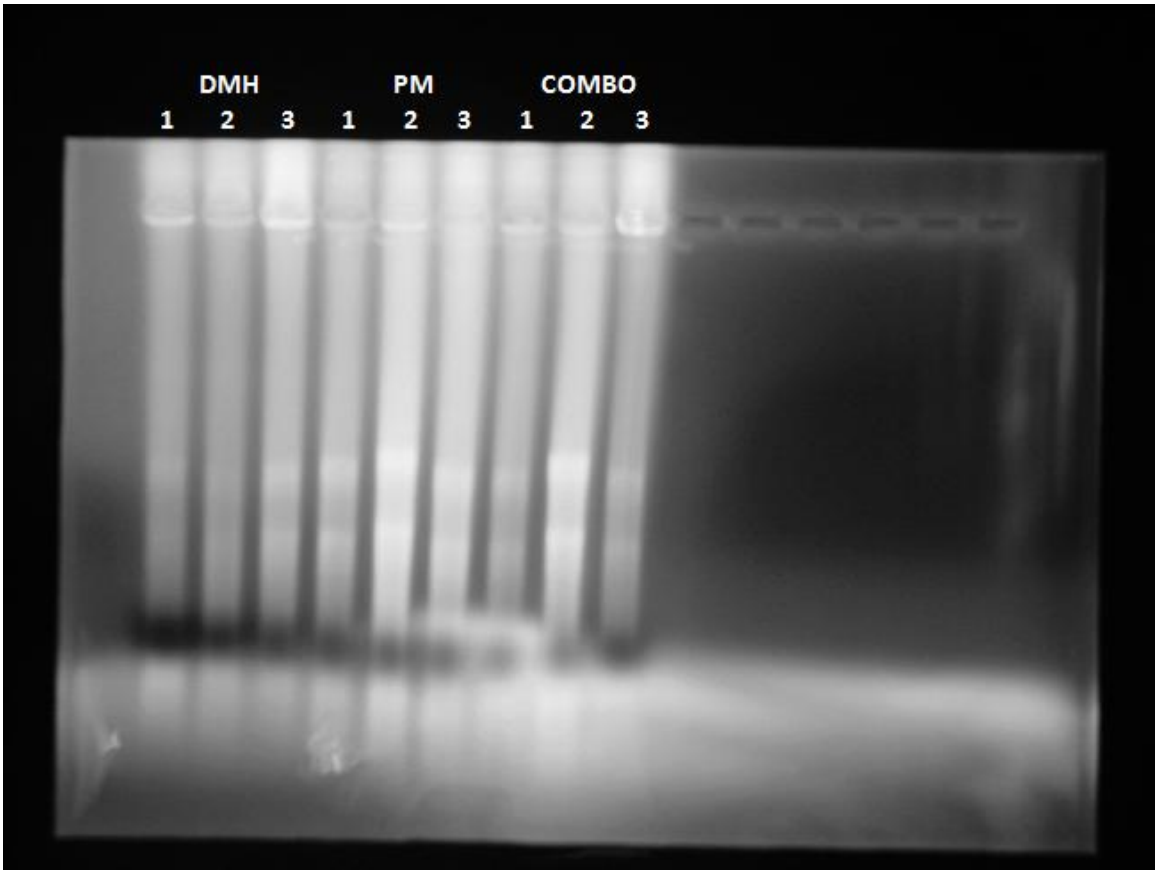

Figure 2. Image of the RNA gel. The RNA is highly degraded but the 18S and 28S bands were still visible meaning there was still some integrity.

Exposure type	av [RNA] in ng/uL
DMH 1	914 ng/uL
DMH 2	1301 ng/uL
DMH 3	2316 ng/uL
PER 1	1531 ng/uL
PER 2	2932 ng/uL
PER 3	2184 ng/uL
Combo 1	2229 ng/uL
Combo 2	2188 ng/uL
Combo 3	1209 ng/uL

Table 6. Average concentrations of RNA samples. Samples were measured in duplicates

Anti-oxidation genes

Glutathione peroxidase

Along with catalase enzymes, hydrogen peroxide is further decreased by the GPX family of enzymes. Like catalase, GPX proteins convert hydrogen peroxide to water. The most abundant form of GPX is GPX-1. GPX-1 knockout mice show no pathological issues when compared to wildtype mice other than early development of cataracts.

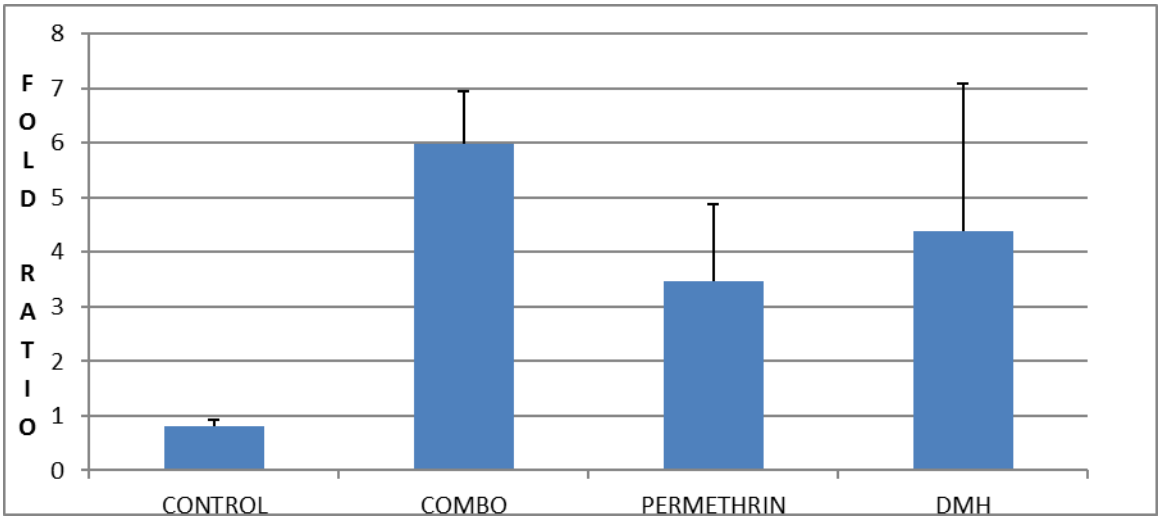

Figure 3. Impact of pesticides on GPx gene expression. The figure shows the fold ratios between Control, Combo, PER, and DMH exposed mice for GPx. The p values are as follows:

	P-value
DMH vs. Control	0.179445607
PER vs. Control	0.080647416
COMBO vs. Control	0.00276645

P-value (<0.05) is considered significant for the purposes of this study. There is a significant difference between control and combo groups but no statistical significance with DMH vs. Control or PER vs. Control

There was a clear increase when comparing the combination to control meaning GPX-1 gene is being expressed due to an increase in oxidation. This shows an additive effect of combo of the two pesticides.

Thioredoxin

Thioredoxins (TRX) are a class of redox proteins which is the best representative enzyme for a group of proteins which possess dithiol-disulfide oxidoreductase activity. TRX is a specific electron donor for many peroxiredoins. And is highly important for reduction of peroxides (details) TRX is also important for preventing apoptosis via an inhibitory binding to apoptosis signal-regulating kinase (ASK-1) whereas this binding is lost when TRX is oxidized.

TRX protects the lens from oxidative stress and cataract formation. Increased plasma levels of TRX are linked with hepatocellular carcinoma. TRX has a direct effect on the reduction of intracellular proteins as part of the anti-oxidation defense along with indirect anti-oxidation effects by modulating the signal transduction properties caused ROS thereby reducing the need for ROS in the cellular environment. As seen in Figure 4, permethrin and combo showed significant increase in expression when compared to the control. Increased oxidation in the mouse's liver increased expression of the thioredoxin gene. Figure 4 shows additive effect of the combination of pesticides.

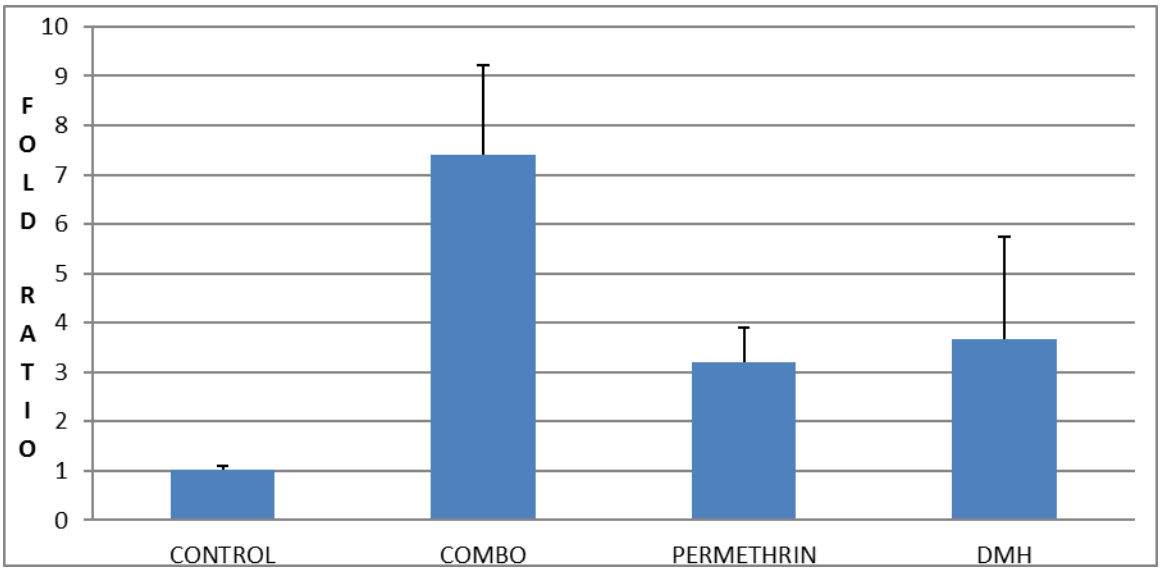

Figure 4. Impact of pesticides on TRX gene expression. The figure shows the fold ratios between Control, Combo, PER, and DMH exposed mice for TRX. The p values are as follows:

	P-value
DMH vs. Control	0.1922661
PER vs. Control	0.019706051
COMBO vs. Control	0.01296328

P-value (<0.05) is considered significant for the purposes of this study. There is a significant increase between control and combo groups and PER vs. Control group but no statistical significance with DMH vs. Control.

Thioredoxin reductase

TRX is reduced by thioredoxin reductase (TRXR) in mammals. It reduces the oxidized active site of TRX. TRXR also reduces other protein disulfides and a wide spectrum of oxidized low molecular compounds and has the ability to reduce hydrogen peroxide independently of TRX. As seen in Figure 5.2, there is no change in TRXR. TRXR reduces both GPX and TRX. TRXR also has independent reduction abilities.

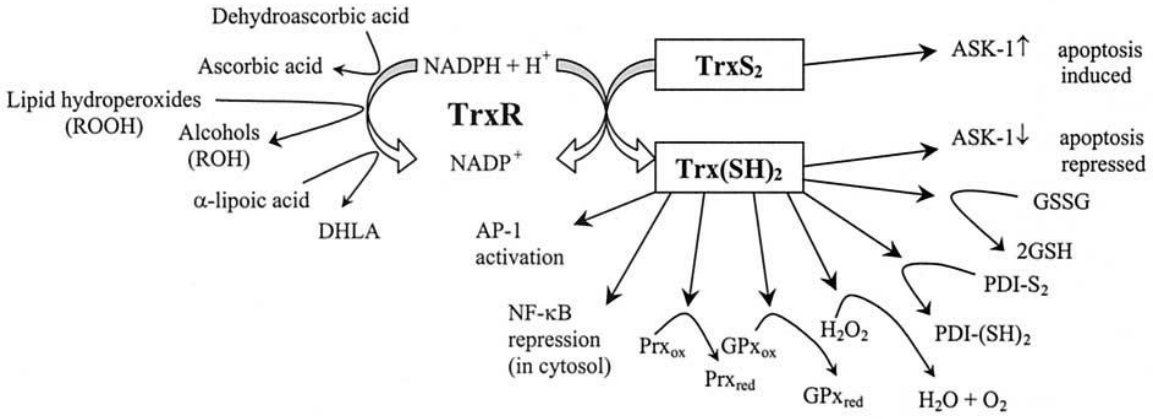

Figure 5.1: **Mechanism of mammalian thioredoxin system.** These are the major physiological functions of the mammalian thioredoxin system. Not only does TRXR reduce TRX, it also catalyzes the regeneration of the active site of GPX. Reduction of hydrogen peroxide and regeneration of the active site in glutathione peroxidase are reactions that can also be directly catalyzed by TrxR. (taken from: Nordberg et al. 2001)

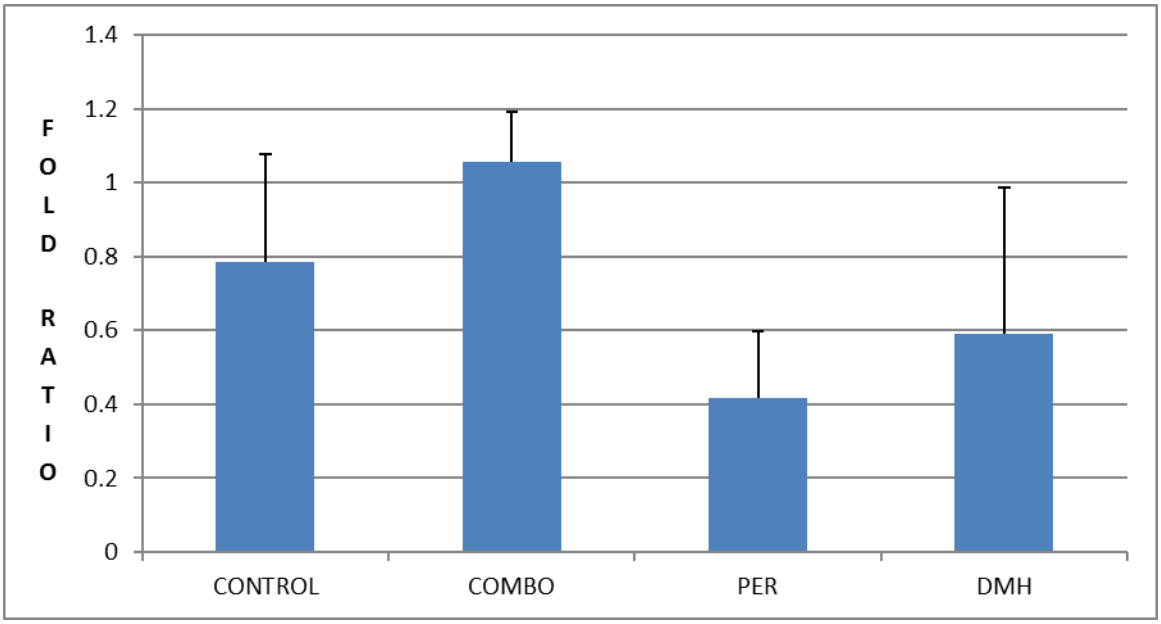

Figure 5.2 Impact of pesticides on TRXR1 gene expression. The figure shows the fold ratios between Control, Combo, PER, and DMH for TRXR. The p values are as follows:

P-value	
DMH vs. CONTROL	0.650459219
PER vs. CONTROL	0.256616391
COMBO vs. CONTROL	0.359453948

No statistical change in TRXR in DMH vs. Control, PER vs. Control, or Combo vs. Control.

Peroxiredoxin

Peroxiredoxin (PRDX) is a family of enzymes that are found in all kingdoms in at least six isoforms. Some of these isoforms are defensive against oxidative species and others participate in cell signaling mechanisms by controlling H_2O_2 concentration. In this study, we looked at PRDX-6. Upon exposure to H_2O_2, the NH2-terminal Cys-SH of the PRDX-6 becomes oxidized. Unlike other isoforms of PRDX, no disulfide bridge is formed due to unavailability of another Cys-SH within proximity. The physiological reducer of PRDX-6 has not been identified. (Rhee et al. 2001)

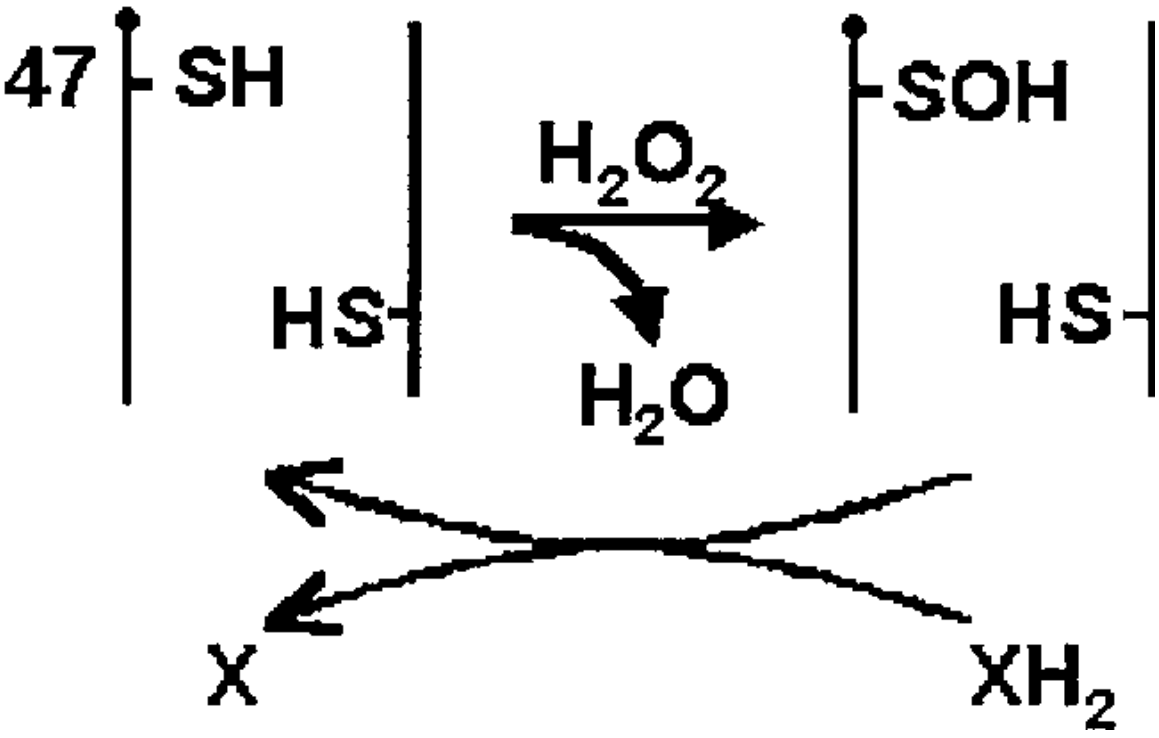

Figure 6.1: **Mechanism of PRDX.** H_2O_2, the NH2-terminal Cys-SH of the PRDX-6 becomes oxidized. Unlike other isoforms of PRDX, no disulfide bridge is formed due to unavailability of another Cys-SH within proximity. The physiological reducer of PRDX-6 has not been identified. *(taken from: Rhee et al. 2001)*

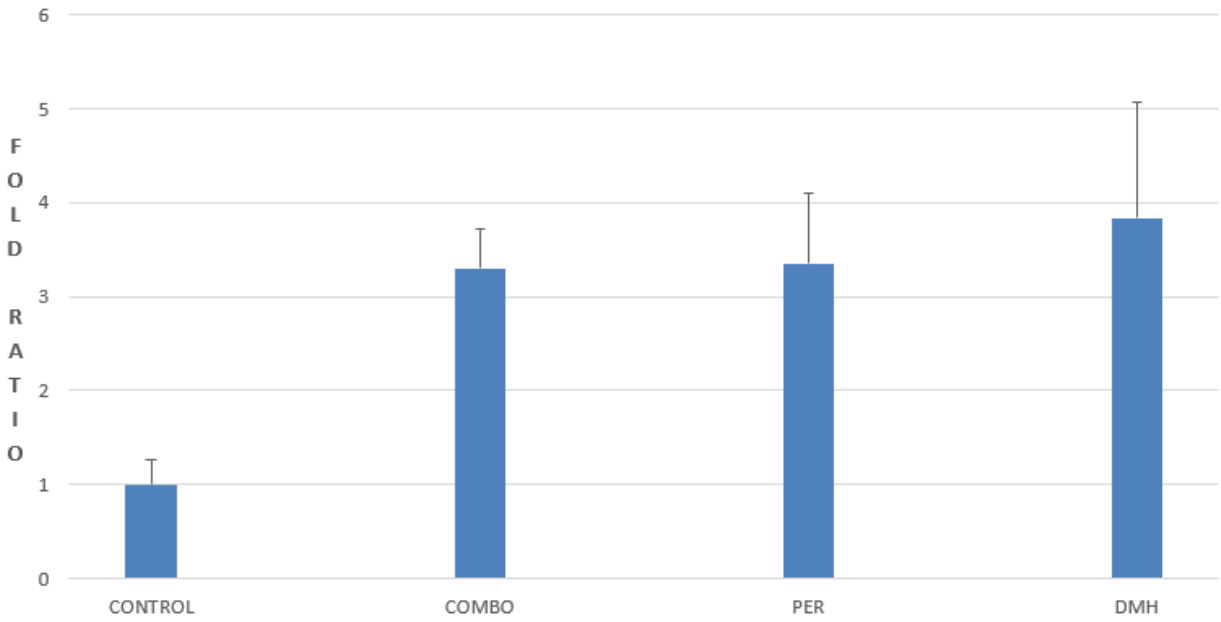

Figure 6.2. Impact of pesticides on PRDX6 gene expression. The figure shows the fold ratios between Control, Combo, PER, and DMH for PRDX-6. The p values are as follows:

	p-value
DMH vs. CONTROL	0.132077277
PER vs. CONTROL	0.049548391
COMBO vs. CONTROL	0.005507856

There is a significant increase in the expression of PRDX-6 in DMH vs. Control, PER vs. Control, and Combo vs. Control.

Damage response genes

P53

The tumor suppressor p53 plays a vital role in cell cycle checkpoints by repressing the expression of cyclin B, CDC25B and polo-like kinase 1 (Plk1) that are required for mitotic entry following exposure to genotoxic stressors such as pesticides. P53 is also the main factor that determines the choice between DNA damage repair, induction of senescence, or apoptosis. Mutation in the p53 gene is related to colorectal cancer (Broustas et al. 2014).

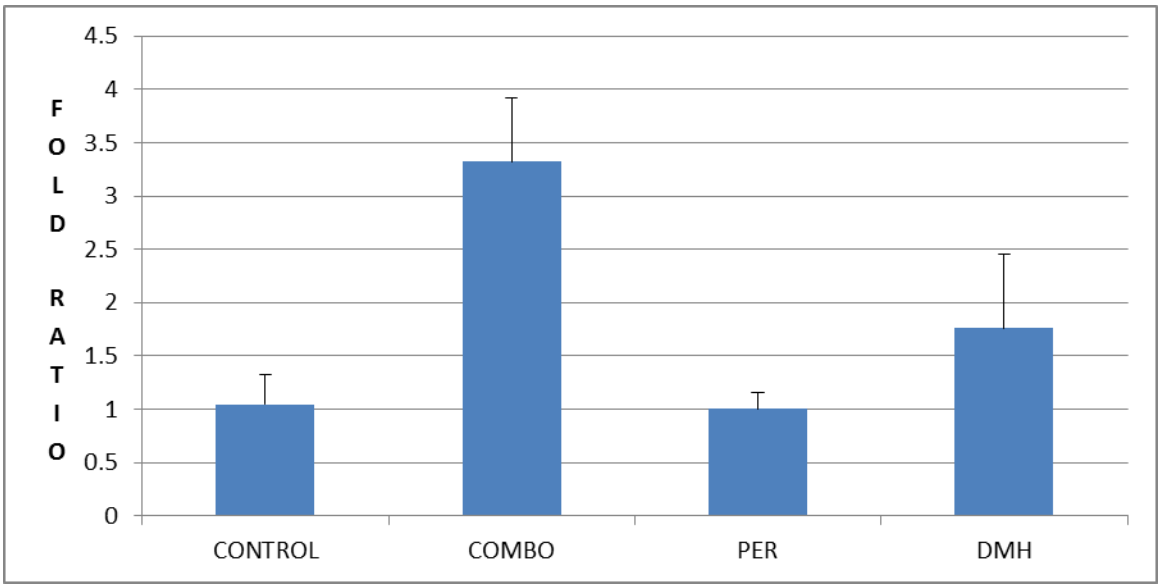

Figure 7. Impact of pesticides on p53 gene expression. The figure shows the fold ratios between Control, Combo, PER, and DMH for p53. The p values are as follows:

DMH vs. CONTROL	0.304923614
PER vs. CONTROL	0.879430214
COMBO vs. CONTROL	0.012924905

This graph shows significant increased expression of p53 due to damage in the cellular environment if permethrin and DMH are used in combination. However, Control vs. PER and Control vs. DMH alone see no significant increase

GADD45

GADD45 is a damage response gene that is important in the regulation of cell cycle checkpoints, DNA repair, and apoptosis. GADD45 knockout mice exhibit genomic instability, single oncogene-mediated transportation, loss of normal cellular senescence, increased cellular proliferation, centrosome amplification, and reduced DNA repair. Upregulation of GADD45 is associated with human pancreatic cancer. It's been inferred that GADD45 is involved in control of cell contact inhibition and cell-cell adhesion by enhancing β-catenin protein stability and translocation to the cell membrane. GADD45 also inhibits cell migration and extracellular matrix, cell communication, and cell adhesion proteins. I ran this gene twice and used the combined data to see if I could get any significance. This gene is not expressed more when exposed to a combination of DMH and permethrin.

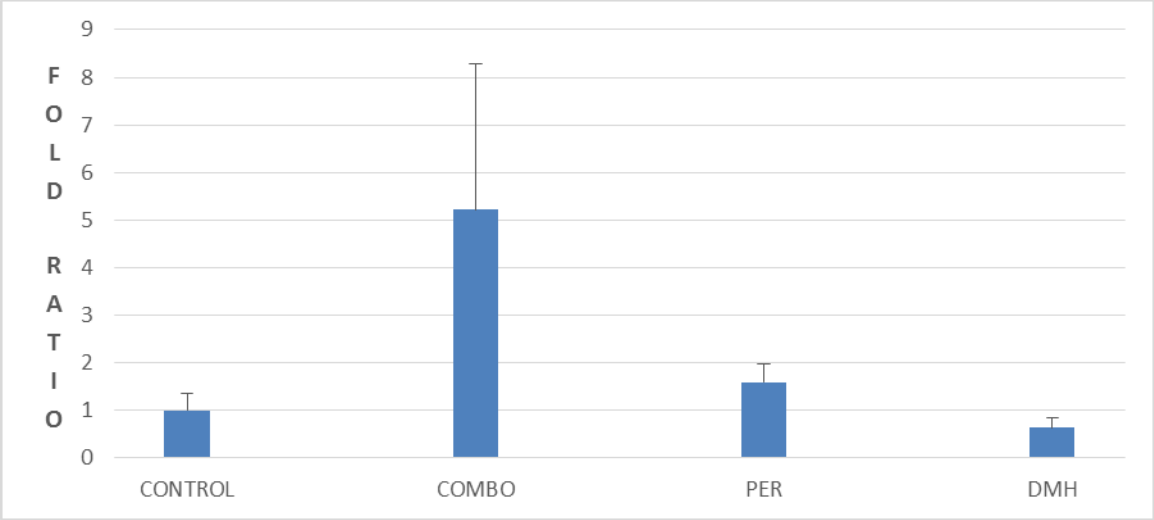

Figure 8. Impact of pesticides on GADD45 gene expression. The figure shows the fold ratios between Control, Combo, PER, and DMH for GADD45. The p values are as follows:

P-value	
DMH vs. CONTROL	0.307505231
PER vs. CONTROL	0.169216935
COMBO vs CONTROL	0.094602283

There is no significant change in the expression of GADD45

PCNA

Proliferating Cell Nuclear Antigen (PCNA) is a DNA clamp that functions as a processivity factor which provides a scaffold for DNA replication machinery. PCNA also functions as a platform for recruiting DNA damage response and replication surveillance machinery (Mailand et al. 2013).

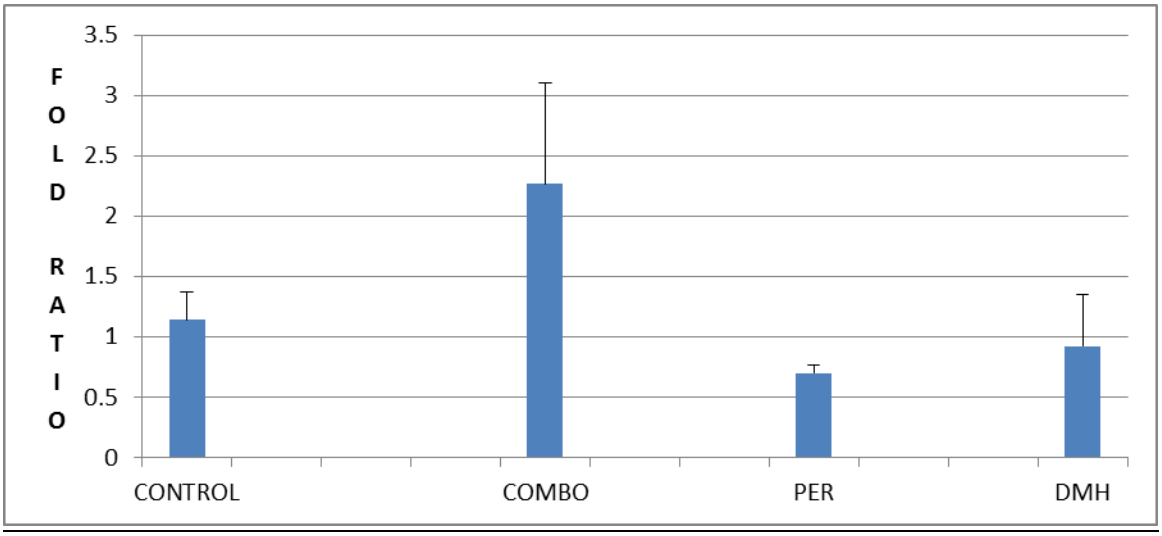

Figure 9. Impact of pesticides on PCNA gene expression. The figure shows the fold ratios between Control, Combo, PER, and DMH for PCNA. The p values are as follows:

p-value	
DMH vs. Control	0.610093891
PER vs. Control	0.085485261
COMBO vs. Control	0.185071312

No statistical change in PCNA in DMH vs. Control, PER vs. Control, or Combo vs. Control.

mTOR

Rapamycin (mTOR) has the ability to suppress cell proliferation and growth via inhibition of its complex, mTOR complex 1 (mTORC1). mTORC1 signaling with dysregulated translational control is frequently seen in colon cancer cells and causes alterations in the eIF4E complex which could yield hyperactive translational activity. This links abnormal mTORC1 signaling with dysregulated translational control in cancer (Dowling et al. 2004).

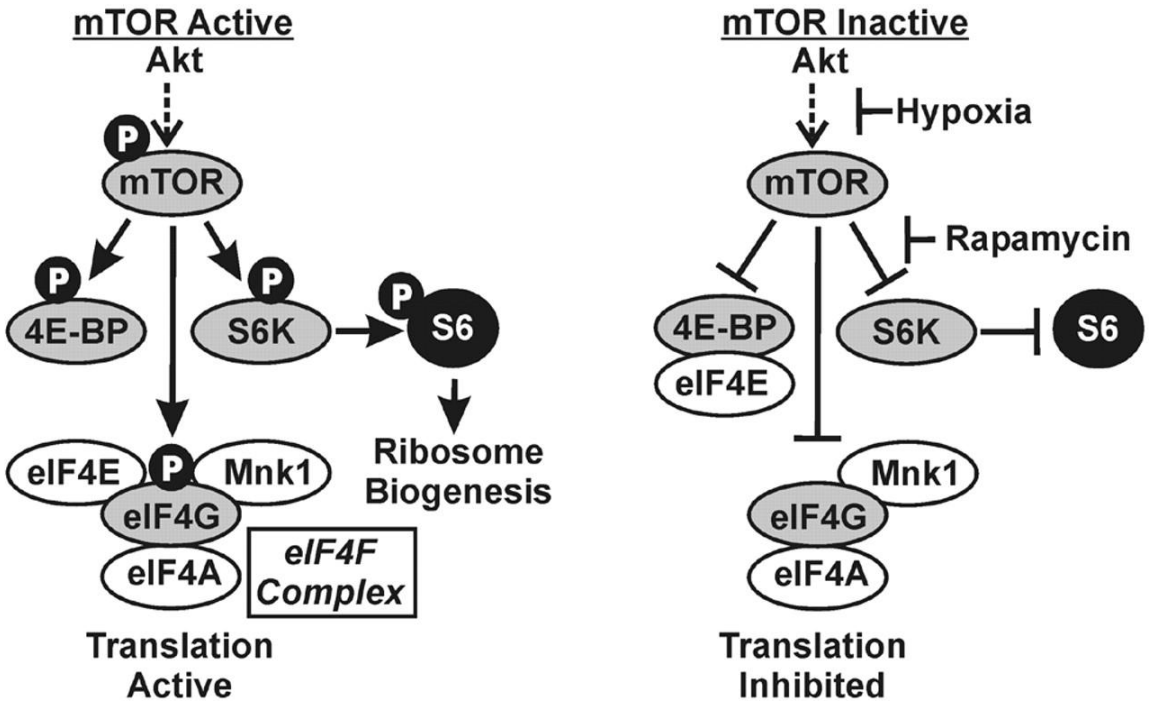

Figure 10.1. **Mechanism of mTOR signaling pathway.** mTOR causes the phosphorylation of eIF4F complex which activates translation. When mTor is inactive, translation by the eIF4F complex is inhibited. Dysregulation of the mTOR can cause hyperactivity of the eIF4F complex causing cell proliferation. (taken from: Kudchodkar et al. 2004)

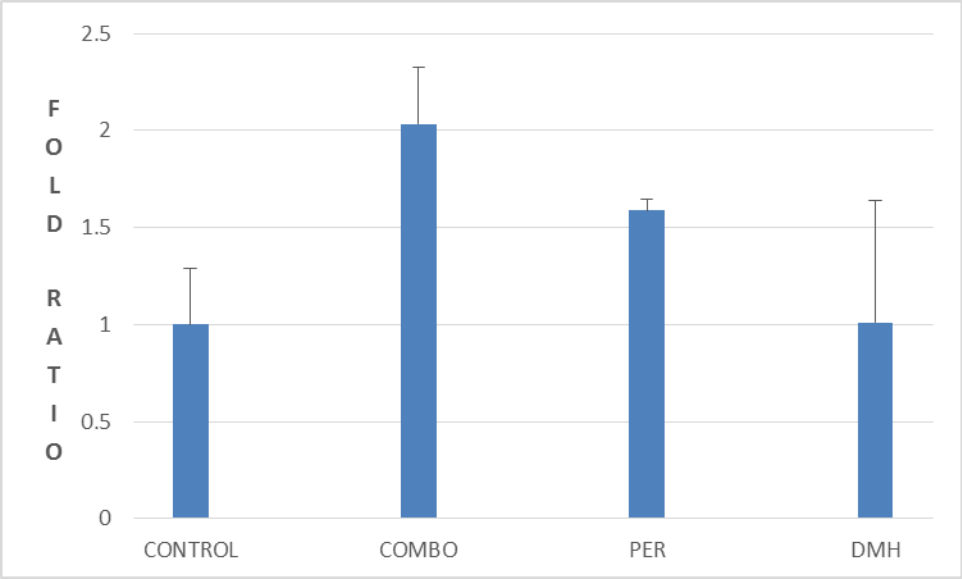

Figure 10.2. Impact of pesticides on mTOR. The figure shows the fold ratios between Control, Combo, PER, and DMH for mTOR. The p values are as follows:

p-value	
DMH vs. CONTROL	0.985348
PER vs. CONTROL	0.071317
COMBO vs. CONTROL	0.027008

No statistical change in mTOR in DMH vs. Control, PER vs. Control, or Combo vs. Control.

Chapter IV. Conclusion

Although permethrin is considered noncarcinogenic (Dong 2007), this study shows an additive effect when combined with a known carcinogen. Single-dose topical exposure to the permethrin in C57BL/6N mice has shown inhibitory effects of splenic T cell proliferation, diminished splenocyte proliferation, apoptosis in CD4 and CD8 thymocytes, and splenic hypocellularity (Prater, 2002). Topical absorption of permethrin is also considered to be rapid and can be detected in the blood after five minutes. These data suggest that absorption of permethrin (> 0.536 mmol/cm2) across the skin could result in systemic immune effects, similar to oral exposure where uptake from the gut is limited. (Shah et al. 1981)

Reports suggest that low levels of permethrin (34 mg/kg/day topically in treated military clothing) may contribute to the persistent local and systemic immunotoxicity referred to as the ‘‘Persian Gulf Syndrome’’ as the mechanism of action of permethrin and DDT are similar. (Plapp 1999)

Although results were not statistically significant across all tested genes, we do see a trend that our hypothesis is correct. All anti-oxidation genes tested with the exception of TRXR showed significant increased expression in combination comparison to control. With respect to damage response genes, only p53 showed a significant increase with respect to combination to control. P53 is the main factor that determines the choice between DNA damage repair, induction of senescence, or apoptosis. Although these genes are mostly regulated at the expression level, further study can be conducted to explore what’s happening at the protein level via kits to detect apoptosis and damage by oxidation in tissues.

Chapter V. References

1. Abdel-Rahman A, Shetty AK, Abou-Donia MB. Subchronic Dermal Application of N,N-Diethyl m-Toluamide (DEET) and Permethrin to Adult Rats, Alone or in Combination, Causes Diffuse Neuronal Cell Death and Cytoskeletal Abnormalities in the Cerebral Cortex and the Hippocampus, and Purkinje Neuron Loss in the Cerebellum. Experimental Neurology 2001;172: 153-171
2. Arigesavan K, Sudhandiran G. Carvacrol exhibits anti-oxidant and anti-inflammatory effects against 1, 2-dimethyl hydrazine plus dextran sodium sulfate induced inflammation associated carcinogenicity in the colon of Fischer 344 rat. Biochemical and Biophysical Research Communications 2015; 461: 314-320
3. Broustas CG, Lieberman HB. Review DNA Damage Response Genes and the Development of Cancer Metastasis. Radiation Research 2014; 181:111-130
4. Cruse JP, Lewin MR, Ferulano GP, Clark CG. Co-Carcinogenic Effects of Dietary Cholesterol in Experimental Colon Cancer. Nature 1978;276(5690):822-825
5. Dong K. Insect Sodium Channels and Insecticide Resistance. Inverteb Neuroci 2007; 7:17-30
6. Goodsell D. Catalase, Molecule of the Month. RCSB Protein Data Bank. September 2004
7. Kudchodkar SB, Yu Y, Maguire TG, Alwine JC. Human cytomegalovirus infection induces rapamycin-insensitive phosphorylation of downstream effectors of mTOR kinase. Journal of Virology 2004; 78:20 11030-11039

8. Mailand Niels, Gibbs-Seymour I, Bekker-Jensen S. Regulation of PCNA-Protein Interactions for Genome Stability. Nature Reviews Molecular Cell Biology 2013;14;269-282
9. National Research Council (US) Subcommittee to Review Permethrin Toxicity from Military Uniforms. Health Effects of Permethrin-Impregnated Army Battle-Dress Uniforms. Washington (DC): National Academies Press (US); 1994.
10. Nordberg J, Arn'er ESJ. Reactive Oxygen Species, and the Mammalian Thioredoxin Systems. Free Radical Biology and Medicine 2001;31;1287-1312
11. Plapp FW. Permethrin and the Gulf War Syndrome. Archives of Environmental Health 1999;54;5;312
12. Prater MR, Gogal RM, Blaylock BL, Longstreth J, Holladay SD. Single-dose topical exposure to the pyrethroid insecticide, permethrin in C57BL/6N mice: effects on thymus and spleen, Food and Chemical Toxicology 2002;40(12):1863-1873
13. Rhee SG, Kang SW, Chang TS, Jeong W, Kim K. Peroxiredoxin, a Novel Family of Peroxidases. IUBMB Life 2001;52(1-2);35-41
14. Roma GC, De Oliveira PR, Araujo AM, Bechara GH, Mathias MI. Genotoxic and Mutagenic Effects of Permethrin in Mice: Micronuclei Analysis in Peripheral Blood Erythrocytes. Microscopy Research and Technique 2012;75:1732-1736
15. Roma GC, De Oliveira PR, Bechara GH, Mathias MI. Cytotoxic Effects of Permethrin on Mouse Liver and Spleen Cells. Microscopy Research and Technique 2012;75:229-238

16. Stralka D, Strobel HW. Characterization of Cytochrome P450-Dependent Dimethylhydrazine Metabolism in Human Colon Microsomes. Cancer 1991;68:2363-2369

17. McCord JM, Fridovich I. Super Dismutase An Enzymic Function for Erythrocuprein (HemoCuprein). The Journal of Biological Chemistry 1969;244;49-55

18. Morgan MK. Children's Exposures to Pyrethroid Insecticides at Home: A Review of Data Collected in Published Exposure Measurement Studies Conducted in the United States. Int. J. Enviorm. Res. Public Health 2012;9;2964-2985

19. Murawaki Y, Tsuchiya H, Kanbe T, Harada K, Yashima K, Nozaka K, Tanida O , Kohno M, Mukoyama T, Nishimuki E, Kojo H, Matsura T, Takahashi K, Osaki M, Ito H , Yodoi J , Murawaki Y, Shiota G. Aberrant expression of selenoproteins in the progression of colorectal cancer. Cancer letters 2008; 259(2): 218-230

20. Shah, P. V., Monroe, R. J., and Guthrie, F. E. 1981. Comparative rates of dermal penetration of insecticide in mice. *Toxicol. Appl. Pharmacol*. 59:414-423.

21. Whittemore ER, Loo DT, Watt JA, Cotman CW. A Detailed Analysis of Hydrogen Peroxide Induced Cell Death in Primary Neuronal Culture. Neuroscience 1995;67(4):921-932

Abstract

SYNERGISTIC EFFECT OF PERMETRIN AND DMH ON ANTI-OXIDATION AND DAMAGE RESPONSE GENES

by

KARIM MOHAMED

MAY 2015

Advisor: Dr. Ahmad Heydari

Major: Nutrition and Food Science

Degree: Masters of Science

The objective of this study was to evaluate the Synergetic Effect of Permethrin and 1,2-Dimethylhydrazine (DMH) on Anti-oxidation and Damage Response genes**.** The animal models used for this study were 8 week old C57/blk6 female mice. The mice were fed a Folate Adequate (FA) diet. At 8 weeks, the mice were intraperitoneally injected with pesticides. Three 8-week old female mice were injected intraperitoneally with a mix of cis and trans permethrin (75%:25% respectively) at 30% median lethal dose (96 mg/kg body weight). Permethrin was dissolved in corn oil and injected 24 hours before sacrifice. In three other mice, DMH was also injected intraperitoneally 24 hours before sacrifice at 30 mg/kg body weight per mouse. Three mice were also injected 24 hours before sacrifice with a combination of DMH and permethrin at the same dosage. High levels of ACF formation and inflammation in the colon were seen in the mice injected with the combination of pesticides (combo) when compared to permethrin and DMH alone.

To determine synergism of the pesticides, levels of gene expression was measured using cDNA. Anti-oxidation gene expression studied was glutathione peroxidase (GPx), thioredoxin (TRX), thioredoxin reductase (TRXR), and Peroxiredoxin (PRDX). The damage response genes

studied were Tumor Protein p53 (p53), Growth Arrest and DNA Damage (GADD45), Mammalian Target of Rapamycin (mTor), and Proliferating cell nuclear antigen (PCNA).

As a general trend, anti-oxidation genes had increased expression in mice given the combination of pesticides. An exception was the anti-oxidation gene TRXR which saw no change in expression. Damage response genes had unchanged levels of expression. An exception to this was p53 gene which saw increased expression in mice exposed to combo conditions.

Autobiographical Statement

Karim Mohamed, BS

Wayne State University – Detroit, MI

- **Master of Science in Nutrition & Food Science** 2013- Present

Lebanese American University – Beirut, Lebanon

- **Bachelors of Sciences in Biology** 2010-2012