Toxic Effects of Allethrin on the Male Reproductive System

A thesis submitted to University of Hyderabad

for the award of Ph.D. degree in the

Department of Animal Biology

By

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NOVEMBER 2014

University of Hyderabad
(A Central University established in 1974 by an Act of Parliament)

Department of Animal Biology
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DECLARATION

I, **Madhu Babu Golla**, hereby declare that this thesis entitled "Toxic effects of allethrin on the male reproductive system" submitted by me under the guidance and supervision of Dr. Suresh Yenugu is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date: (Madhu Babu Golla)

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CERTIFICATE

This is to certify that this thesis entitled "Toxic effects of allethrin on the male reproductive system" is a record of bonafide work done by **Mr. Madhu Babu Golla,** a research scholar for Ph.D. programme in the Department of Animal Biology, University of Hyderabad under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

Dr. Suresh Yenugu
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Acknowledgements

My supervisor Dr. Suresh Yenugu: As a teacher, he was truly inspiring and thought provoking, which brought me in to the field of research. During my difficult times, he was so caring. As a guide, he gave me complete freedom to execute the experiments, along with his critical suggestions and discussions. He's a great human being. I express my deepest sense of gratitude towards him. His advices on research as well as on career have been priceless.

I'm grateful to my doctoral committee members Prof. Aparna Dutta Gupta and Dr. Naresh Babu V Sepuri for their valuable inputs which were instrumental in designing several experiments discussed in this thesis.

I sincerely acknowledge the Head, Prof. B. Senthilkumaran, Department of Animal Biology, and former Heads Prof. Manjula Sritharan and Prof. S. Dayananda for providing good equipment and facilities to carry out this work.

I thank present Dean, Prof. A. S. Raghavendra, School of Life Sciences, and former Deans Prof. R.P. Sharma, Prof. Aparna Dutta Gupta and Prof. M. Ramanadham for allowing me to use the school facilities.

I thank Dr. K. Arun Kumar and Dr. Niyaz Ahmed for allowing me to use real-time PCR machine; Prof. M. J. Swamy and Mr. Sudheer, School of Chemistry for fluorescence spectrophotometer. I thank all the faculty in the school of life sciences.

I express my sincere thanks to Dr. S. Shivaji, Dr. Archana Bharadwaj and Mr. Durgesh, CCMB, Hyderabad for their help in CASA analysis; and Prof. M. M. Aruldhas, Dr. Kathiresh kumar, Dr. A.L.M. PG Institute of Basic Medical Sciences, Chennai for their help in isolation of primary Leydig cells.

I thank University of Hyderabad and CSIR (JRF and SRF) for providing financial assistance to carry out this work. UGC-SAP, UPE, DST-FIST and DBT-CREBB and University of Hyderabad are accredited for providing necessary facilities in school. Financial assistance to supervisor's laboratory from DST, DBT, CSIR, ICMR and UGC are greatly acknowledged.

I thank UPE for sponsoring travel grant to participate in the 7th European conference on pesticides, 2012, Porto, Portugal.

My Special thanks to Dr. Roy Karnati, Dr. R.K. Chaitanya and Dr. B. Sunil for their valuable and friendly help during my course of the graduate and doctoral studies.

Acknowledgements

The immense help and moral support rendered by colleagues Dr. Barnali Biswas, Rajesh Angireddy, and G. Narmadha is highly appreciated in all respects during my work. I also thank my junior colleagues Reejuana Parveen, Lavanya P, Anandha Rao Ravuala, Suresh Munnepalli and Sangeetha for their help.

My gratitude goes to my friend Kishore and I would always cherish the beautiful moments I shared with some of my friends Surendra, Subbu, Eswar, Adi, Anil Ch, Sugith, Kamu, Sunil P, Srinivas Y, Venkat, Ashok, Mastan, Paparao, Maruthi, Jyothi, Chaitanya, Bimolatha and others. I also thank my childhood friends Anil Kumar, Venakt Rao and Naveen for their support.

I am eternally indebted to my teachers Mrs. Radha, Mr. Sreedhar, Dr. C. S. Srinivas Rao and S. Kiran Kumar for their guidence.

I thank laboratory caretaker Mr. Narasimha and non-teaching staff of the department (Mr. Ankineedu, Mr. Gopi, Mr. Jagan, Mrs. Jyothi, Mr. Babu Rao and Mr. Srinivas) for their prompt and speedy administrative service whenever required.

I thank Mrs. Leena Bashyam, Mrs. Monika and Mrs. Kalpana for their technical help in using genomics and proteomics facility.

I owe my gratitude towards my soulmate Lakshmi Pravallika for her eternal support and understanding of my goals and aspirations. Her infallible love and support has always been my strength.

I am indebted to my beloved sister Shanthi and brother-in-law Narasimha Naidu and my fabulous nephew Navaneeth and cute niece Nikhila. Lighter moments shared with them eased my work pressure.

Thank the almighty for everything.

Finally, With all due respect, I thank my ever-loving parents, (Late) Sri. Chandra Mouli and Smt. Ramadevi.

DEDICATED TO MY PARENTS

CONTENTS

Abbreviations

°C	: Degree Celsius
µg	: microgram
µM	: micromolar
3β-HSD	: 3beta-Hydroxy steroid dehydrogenase
17β-HSD	: 3beta-Hydroxy steroid dehydrogenase
2,4-D	: 2,4-Dichlorophenoxyacetic acid
AR	: Androgen receptor
ATP	: Adenosine triphosphate
cAMP	: Cyclic adenosine monophosphate
CDNB	: 1-Chloro-2,4-dinitrobenzene
CHAPS	: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
$CuSO_4$	: Copper sulfate
DCFH-DA	: 2, 7-dichloro dihydro fluorescein diacetate
DDE	: Dichlorodiphenyldichloroethylene
DDT	: Dichlorodiphenyltrichloroethane
DEVD-AFC	: Aspartate-Glutamate-Valine-Aspartate-7-amino-4-trifluoromethylcoumarin
DHT	: Dihydrotestosterone
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
DTT	: Dithiothreitol
EDTA	: Ethylenediaminetetraacetic acid
EMEM	: Eagles minimal essential media
FACS	: Fluorescence-activated cell sorting
FITC	: Fluorescein isothiocyanate
Fura-2AM	: Fura-2-acetoxymethyl ester
gm	: gram
H_2O_2	: Hydrogen peroxide
Hb	: Hemoglobin
HBSS	: Hanks balanced salt solution
HEPES	: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	: Half maximal inhibitory concentration
LD_{50}	: Lethal dose

LH : Luteinizing hormone

MALDI-TOF : Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer

MDA : Malondialdehyde

mg : milligram

min : minute

ml : millilitre

mM : millimolar

MMP : Mitochondrial membrane potential

MTT : 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NaCl : Sodium chloride

NADP : Nicotinamide adenine dinucleotide phosphate

NaOH : Sodium hydroxide

nM : nanomolar

PBO : Piperonyl butoxide

PBS : Phosphate buffer saline

PI : Propidium iodide

ppm : parts per million

RNA : Ribonucleic acid

ROS : Reactive oxygen species

rpm : Revolutions per minute

S.D : Standard deviation

SDS-PAGE : Sodium dodecyl sulphate- polyacrylamide gel electrophoresis

StAR : Steroidogenic acute regulatory protein

TBA : Thiobarbituric acid

TCA : Trichloroacetic acid

TEP : 1, 1, 3, 3-tetraethoxy-propane

V : Volts

VGCC : Voltage gated calcium channel

WHO : World Health organization

ABSTRACT

Over the past decades, there has been an increased concern about the impact of environmental contaminants like pesticides on human reproductive system. These pesticides are used for both agricultural and non-agricultural purposes throughout the world. Synthetic pyrethroids persist in the environment and can enter into the geological cycle which can influence reproductive health. Allethrin, the first synthetic pyrethroid, which knock down insects has neurological effects and no reports are available about the impact on male reproductive system. In the present study, we studied the toxic effects of allethrin on male reproductive system using rat as a model system.

We identified the molecular mechanism of allethrin cytotoxicity in rat Leydig cell carcinoma cells (LC540). Allethrin shows cytotoxicity in a concentration dependent manner. It increases lipid peroxidation and alters antioxidant enzyme status. Morphological analyses of LC540 cells treated with allethrin revealed the presence of apoptotic bodies. Using flow cytometry, we observed that the number of cells that displayed early apoptotic features and entering into G_0 phase of cell cycle increased significantly with loss of mitochondrial membrane potential. The levels of *p53* mRNA and cleaved PARP-1 protein were increased, whereas BCL-2, pro-Caspase-3 and PARP-1 were decreased. Allethrin induced apoptosis was associated with voltage gated calcium channel mediated intracellular calcium release. Besides cytotoxicity, the reduced expression of spermatogenic factors namely *Tgf-β1* and *Scf* suggests the potential of allethrin to affect spermatogenesis.

We studied the effect of continuous inhalation of allethrin-based mosquito repellents on male reproductive system. Rats were exposed to allethrin-based mosquito coil smoke for 15-180 days. When compared to unexposed controls, lipid peroxidation

was increased in the cauda epididymis and testis not in caput epididymis. The activities of antioxidant enzymes like catalase, glutathione-S-transferase, glutathione peroxidase and superoxide dismutase remained largely unchanged in all the tissues analysed. Histopathological analyses revealed loss of tubule architecture, epithelial cell disruption, increase in lumen size, interstitial edema, and presence of dead spermatozoa after prolonged exposure. *p53* gene expression was differentially altered in the epididymis and testes. The expression of spermatogenic factors, namely, stem cell factor *(Scf)* and its ligand *c-Kit* was unaltered though decreased levels of transforming growth factor (*Tgf-β1)* were observed.

The effect of oral ingestion of allethrin is not yet studied. Rats were treated orally with 25, 50, 100 and 150 mg/kg body weight allethrin every day for 60 days. Lipid peroxidation was significantly increased in the caput, cauda and testes. Nitric oxide production was increased in the caput, but unaltered in the cauda and testes. Alterations in the activities of antioxidant enzymes were observed in the male reproductive tract tissues of allethrin treated rats. The mRNA expression of genes involved in spermatogenesis (*Scf, c-Kit, Tgf-β1, Hsf2, Ovol1, Brdt, Kdm3a, Ybx-2 and Grth),* spem maturation (*Spag11e*) and sperm function (*Defb22*) were reduced in allethrin treated rats. Significant decrease in the expression and enzyme activities of 3β-HSD and 17β-HSD and serum testosterone levels was observed in allethrin rats. Though daily sperm production was decreased in allethrin rats, the spermatozoa from these rats showed normal motility. The expression of *p53* gene was decreased and increased phosphorylation of MAPK (p42/p44) expression was observed the male reproductive tract tissues of allethrin treated rats. Glucose-regulatory protein 78 (GRP78 - a stress induced protein) was altered in epididymides of allethrin treated rats.

To study the effect of allethrin-based mosquito repellents during prenatal and postnatal stage, pregnant females were exposed to allethrin-based mosquito smoke. Though no marked difference in litter size and reproductive organ weights were observed, lipid peroxidation and antioxidant enzyme activities were significantly affected in developing rats. Altered antioxidant status lead to oxidative stress. The expression of *p53,* which is a key regulator cell cycle was drastically reduced in mosquito coil smoke exposed rats. Expression spermatogenic, sperm maturation and capacitation factors were down regulated in developing rats. Reduced expression of steroidogenic enzymes and serum testosterone levels was observed in these rats. Spermatozoa obtained from mosquito coil smoke exposed rats showed decreased ability to undergo acrosome reaction when compared to control rats and thus may affect fertility.

In conclusion, we report that the molecular mechanism of allethrin toxicity involves apoptotic mechanisms. Nasal or oral exposure to this compound results in damage to the male reproductive tract and compromised sperm function.

The male reproductive tract is a vital organ system which functions to produce testosterone and germ cells. Any damage to this system by infectious agents or environmental toxicants may result in altered hormonal status and reduced fertility. It is well established that, exposure to environmental pollutants such as pesticides and heavy metals contribute to the development of cancer and reduction in reproductive functions. In the view of the increasing use of pesticides for a variety of purposes, the health hazards need through investigation. The effect of pesticides, especially, allethrin on the male reproductive function is not clear. Hence, we attempt to study the effect of allethrin on male reproductive function using rat as a model system. To accomplish this, the following objectives were framed.

Objectives of the study:

1. **To decipher the molecular mechanisms of allethrin toxicity *in vitro.***
2. **Effect of continuous exposure to allethrin-based mosquito coil smoke on the male reproductive tract.**
3. **Study the effect of oral administration of allethrin in adult rats.**
4. **Effect of exposure of prenatal rats to allethrin-based mosquito coil smoke.**

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Introduction

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Introduction

Pesticides

Pesticides are used globally for agricultural and non-agricultural purposes. According to Environmental Protection Agency (EPA), USA, a pesticide is a biocide which is intended to kill, reduce or repell, prevent and destroy insects, mites, nematodes, weeds, rats, fungi etc. They have both beneficial and harmful effects. Beneficial effects in agricultural purposes include crop protection, preservation of stored grains and prevention of vector-borne diseases. At the domestic front (non-agricultural), they are used to control household pests and insects that acts as vectors for various diseases. Disinfectants like pine oil cleaners, cleaning materials for swimming pools and bathroom cleaners also contain pesticides.

In the history of pesticides, sulfur was used to control pests and then followed by natural materials and inorganic compounds. Organic synthetic pesticides like dichloro-diphenyl- trichloroethane (DDT), 2,4-Dichlorophenoxyacetic acid (2, 4-D), dieldrin etc. were emerged in the 19^{th} century.

I. Classification of pesticides

Pesticides are classified in to three categories based on:

a. Mode of action
b. Target species and
c. Chemical composition.

a. Mode of Action: Pesticides are classified in to systemic and non-systemic basing on which organ they act to bring the desired effect. The non-systemic pesticides tough sprayed topically will not penetrate tissues and consequently not transported in to the system and they show effect only when they are in contact with the target pest, whereas systemic pesticides are taken up by plant and transported in to the tissues, which may kill bees and other pollinators. Examples of non-systemic include paraquat and diquat dibromide and systemic pesticides are neonicotinoids and fipronil (neonics).

b. Target species: In this classification, pesticides are named depending on the target pest species on which they act and the table 1.1 shows the features in detail.

Table 1.1. Types of pesticides based on target pest species.

S. No	Type of pesticide	Target	Examples
1	Herbicides	Herbs	Glyphosate, 2,4-D
2	Fungicides	Fungi	$CuSO_4$, Hexachlorobenzene
3	Rodenticides	Rodents	Chlorophacinone, Difethialone
4	Insecticides	Insects	Pyrethroids
5	Nematicides	Nematodes	Aldicarb
6	Acaricides and	Ticks and mites	Permethrin, Dicofol

c. Chemical composition: According to Buchel, 1983, pesticides are classified in to four types based on chemical composition namely organochlorines, organophosphates, carbamates, pyrethrins and pyrethroids.

Organochlorines are used in agriculture and they are the first synthetic pesticides which are known to act on insect nervous system resulting in convulsions, paralysis and finally death. They persist in environment because of resistance to biochemical and chemical degradations. Examples include DDT ($C_4H_9Cl_5$) (Figure 1.1), endosulfan and dieldrin.

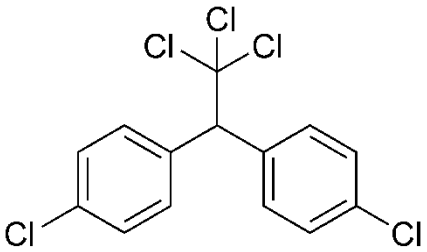


Figure 1.1. *Structure of dichlorodiphenyltrichloroethane (DDT).*

Organophosphorus insecticides contain phosphate group. They inhibit cholinesterase leading to accumulation of acetylcholine across synapse resulting in failure of nerve impulse and thus a rapid twitching of voluntary muscles leading to paralysis and death. These pesticides are easily decomposed by chemical and biochemical reactions which make them less persistent in the environment. Examples are parathion (Figure 1.2), malathion, diazinon and glyphosate.

Carbamates are derived from cabamic acid and are known to be cholinesterase inhibitors similar to organophosphates. But, carbamates are species specific and reversible. Examples are carbaryl, aminocarb and carbofuran.

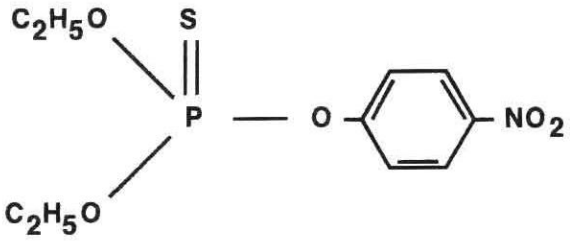


Figure 1.2. *Structure of diethyl-p-nitrophenyl monothiophospahte (parathion).*

Pyrethrins are extracted from flower heads of *Chrysanthemum cinerariaefolium* and are non-persistent. Pyrethroids are modified forms of natural pyrethrins. They are synthetically derived and are more potent and stable. Both pyrethrins and pyrethroids are immune and neurotoxic, but show low mammalian toxicity. Pyrethroids are used in agriculture for more than 70 years because of their high knockdown capacity of insects. On the other hand they are rapidly degraded with low mammalian toxicity. Pyrethrin I, rotenone and nicotine are some examples of pyrethrins, whereas allethrin, resmethrin and permethrin are examples of pyrethroids.

II. Classification based on human toxicity

The World Health Organization (WHO) identified four classes of pesticides (Table 1.2) according to the toxicity on humans.

Table 1.2. Classes of pesticides based on toxicity.

Class Ia	Extremely hazardous
Class Ib	Highly hazardous
Class II	Moderately hazardous
Class III	Slightly hazardous

There are 234 pesticides registered in India [The Central Insecticides Board and Registration Committee (CIBRC)]. Out of these 4 are class Ia, 15 are class Ib and 76 are class II. The remaining pesticides are yet to be analysed and classified.

III. Consumption and usage of pesticides

Insects and mites, weeds and plant pathogens cause extensive damage to the crops worldwide resulting in decreased crop yield. Pesticides are indiscriminately used to prevent crop damage. About one-third of the agricultural products are produced by using pesticides (Liu *et al.,* 2002)**.** Without pesticide usage the loss of fruits, vegetables and cereals from pest injury would reach 78%, 54% and 32% respectively (Dawang, 2008).

Crop loss from pests declines to 35% to 42% when pesticides are used (Liu, 1999; Pimentel, 1997).

Over the years, the use of pesticides has increased globally. As shown in figure 1.3, Asia and Europe are placed among the top consumers of pesticides. In India, insecticides are the most commonly used pesticides (Figure 1.4), which accounts for 65% of total pesticide usage. Uttara pradesh is the highest consumer of pesticides i.e. 39948 metric tonnes used from 2005-2010 (Directorate of Plant Protection, Quarantine and Storage, Govt. of India). India's pesticide industry is the largest in Asia and the twelfth largest in the world and it has grown by 7.6 per cent during the last 20 years. Sulfur, is the most consumed fungicide (16424 metric tonnes from 2005 -2010) pesticide in India followed by mancozeb, phorate and methyl parathion.

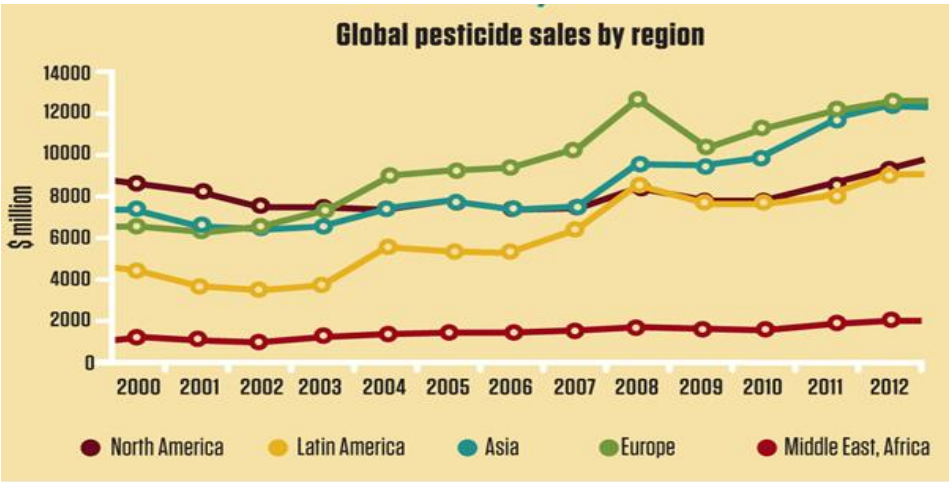
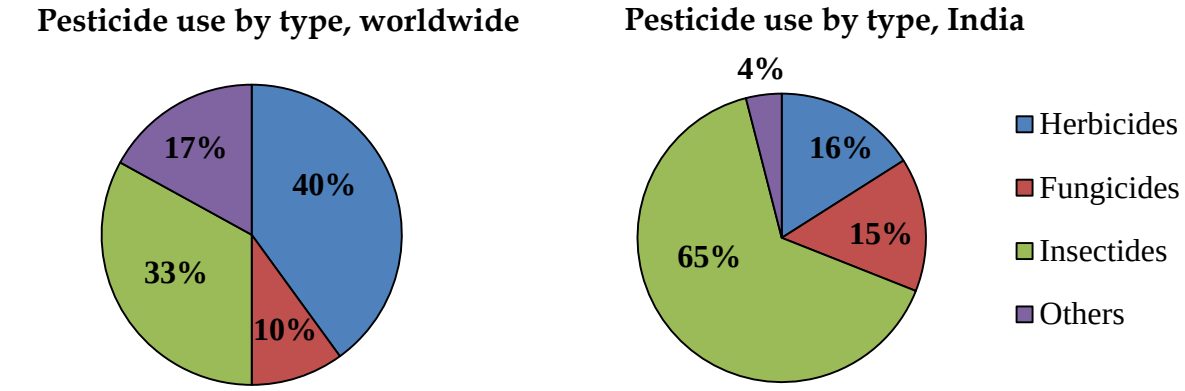


Figure 1.3. *Global sale of pesticides (Source: Science, 341, 730-731).*



(Source: FAO Statistical yearbook, 2013) (Source: Agropages annual reviews, 2014)

Figure 1 4. *Consumption pattern of pesticides. *Others include sulfur oil, moth control and insect repellents (FAO: Food and Agriculture Organization, USA and Agropages Business Magazine, March 2014).*

IV. Over use of pesticides

Pesticides are used worldwide and rank second after fertilizers in the amount applied and the extent of use. Usually they are toxic to pests but not to non-target species, but they are toxic for humans too. Each year in the United States, an estimated 17 pounds of pesticide per citizen will be incorporated into the environment for a total of 4.6 billion pounds (Aspelin, 1998). Of these 4.6 billion pounds, an estimated 85-90% will not reach their target organisms, thus, will enter the air, water and soil (Repetto and Sanjay, 1996). Pesticide ingestion is the leading global means of suicide, accounting for roughly one-third of the estimated 1 million cases annually (Hvistendahl, 2013). Pesticide-induced death of honeybees and wild bees results in loss of approximately 320 million dollars a year (Pimental *et.al,* 1992).

V. Toxicity of pesticides

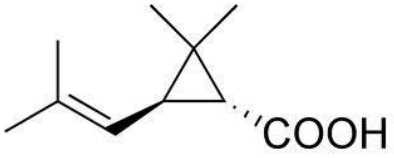
The widespread use of pesticides for agriculture and non-agricultural purposes has caused environmental pollution and severe human health effects. More than 800 pesticide active ingredients are present in a wide range of commercial products which are used in agriculture. Most of the sprayed insecticides and herbicides reach other than their natural targets like air, water and soil (Miller, 2004). These pesticides can cause toxic effects by interfering with reproductive system and foetal development and can also induce cancer and asthma (Gilden *et.al.*, 2010). They may act as endocrine disruptors and alter the hormonal homeostasis in both male and females leading to subfertility. DDT, endosulfan, methoxychlor and fenvalerate act as estrogen agonists. Other pesticides like vinclozolin, DDE and DDT possess anti-androgenic and or both estrogenic activity (Kelce *et.al.,* 1994; Kelce *et al.,* 1995; Kemppainen *et al.,* 1999). Sometimes pesticides remain in the body for longer durations which may induce oxidative stress, leading to generation of free radicals and alter the antioxidant enzyme system (Banerjee *et al.,* 1999).

Insecticides

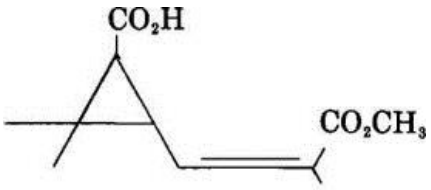
Among the pesticides classes, insecticides are mostly used. Both natural (plant derived) and synthetic insecticides are used to control pests and insects that affect human population directly and indirectly. Pyrethrin I (*Chrysanthemum cinerariifolium*), rotenone, nicotine (*Nicotiana sp.*), cevadine (*Veratrum sabadilla*), ryanodine (*Ryania*

speciosa) etc. are natural insecticides. Pyrethroids, organochlorines, organophosphates and carbamates are synthetic or man-made insecticides.

a. Pyrethrins: Pyrethrins are natural insecticides extracted by using solvents such as methanol or acetone from the flower heads of *Chrysanthemum*. They contain esters of two different alcohols with two different acids. Both acids contain a cyclopropane ring while one acid contain free carboxyl group and other one has free and one esterified carboxyl group.



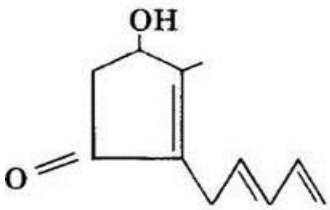
Chrysanthemic acid



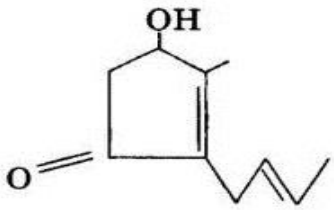
Pyrethric acid

The two alcohols contain a five membered ring to which a ketone oxygen and alcoholic OH is attached. Because of the presence of both keto and alcoholic groups, their names contain "-one-" and "-ol-" respectively.

A vinyl group is present in pyrethric acid, pyrethrolone and cinerolone and the double bond makes them to form cis and trans isomers. The esters containing pyrethrolones are called pyrethrin I if the acid is chrysanthemic acid and pyrethrin II if acid is pyrethric acid. Similar nomenclature is followed in the case of cinerolone.



Pyrethrolone



Cinerolone

b. Pyrethroids: Pyrethroids are synthetic forms of pyrethrins and contribute to 25% of the total pesticides used in the world. Their use is increasing from 1950 because of restricted use of organochlorines and phosphates. Pyrethroids show high toxicity to insects and low toxicity to mammals and birds. The toxicity involves delaying the closure of sodium channels in the nerve membrane. They are classified into type I and II (Table 1.3) based on chemical structure and symptoms of exposure. Besides sodium channels, they also acts on voltage sensitive calcium channels there by releasing the neurotransmitters to cause toxicity (Hildebrand *et al.*, 2004; Symington and Clark, 2005).

Table 1.3. Classification of pyrethroids based on structure.

	Type I pyrethroids	**Type II pyrethroids**
Cyano group	Absent	Present
Symptoms	Hyperexcitability and convulsions in insects and whole body tremors in mammals (T syndrome)	Ataxia and incoordination in insects and choreoathetosis (sinuous writhing) and salivation in mammals (CS syndrome)
Examples	Allethrin, tetramethrin, resmethrin and permethrin	Cypermethrin, cyfluthrin, deltamethrin and fenvalerate

The Type 1 group includes pyrethroids containing descyano-3-phenoxybenzyl or other alcohols and are unstable in the environment. Phenoxybenzyl in the type II pyrethroids makes them more stable and it increases insecticidal activity. Synergists like piperonyl butoxide (PBO), n-octyl bicycloheptene caboxymide (MGK 264) are included to increase the activity of insecticides.

Toxicity of pyrethroids

Pyrethroids affect nerve impulse generation in both central and peripheral nervous systems. Neurons at resting phase have -70V. Transmission of nerve impulses involves opening and closing of voltage sensitive sodium and potassium channels present on the nerve membrane. Pyrethroids bind to voltage sensitive sodium channels in the insect nerve membrane, thus preventing their closure to cause delayed depolarization and eventually paralysis of insect. The principal mechanism of pyrethroid toxicity involves the inhibition of voltage sensitive sodium channels besides inhibition of sodium-potassium ATPase in the neuronal membranes (Vijverberg and de Weille, 1985; Kakko *et al.,* 2003; Tan and Soderlund, 2010).

Allethrin

Allethrin is the first synthetic pyrethroid used to control pests like wasps and homets, roaches, ants, fleas and mosquitoes. It is considered as a type I pyrethroid because of lack of α-cyano group and thus structure (Figure 1.5) is very similar to cinerin I of naturally occurring pyrethrum.

The chemical composition of allethrin contains three asymmetric carbons and, thus, eight potential isomers are possible in the proportion of 1:1:1:1:1:1:1:1 (Figure 1.6). One of the stereoisomers, d-trans of d component (isomer), is recognized as being the most

insecticidally active and toxicologically significant of the four isomers. Bioallethrin, esbiol, esbiothrin, pynamin forte are common names of allethrin. Physical and chemical properties of allethrin are included in table 1.4

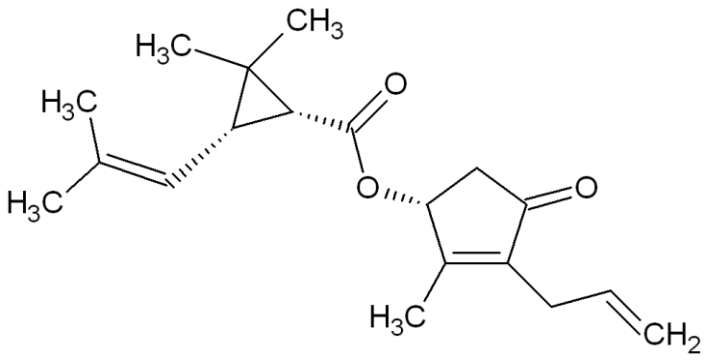


Figure 1.5. *Structure of allethrin (2-methyl-1-propenyl)-2-methyl-4-oxo-3-(2 propenyl)-2-cyclo-penten-1-yl este).*

Isomers composition in allethrin and its trades

1. **Bioallethrin:** Consists of [1R, trans; 1R] + [1R, trans;1S] in an approximate ratio of 1:1.
2. **Esbiol:** Consists of [1R, trans; 1R] + [1R, trans; 1S] in an approximate ratio of 1:1.
3. **Esbiothrin:** Consists of [1R, trans; R] + [1R, trans; S] in approximate ratio of 1:3.
4. **Pynamin forte:** Consists of [1R, trans; 1R] + [1R, trans; 1S] + [1R, cis; 1R] + [1R, cis; 1S] in an approximate ratio of 4:4:1:1.

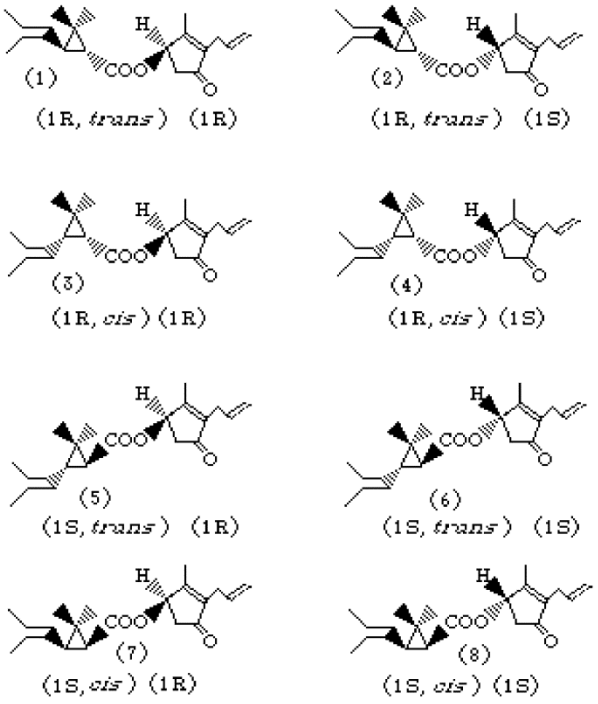


Figure 1.6. *Stereoisomers of allethrin (Shafer et al., 2005).*

Allethrin is an axonic poison that binds and inhibits the closing of sodium channels in the nerve membrane, which prolongs the membrane potential to its resting stage leading to hyperactivity of the nervous system and finally paralysis and/or death. This mode of action is similar to the insecticide dichloro diphenyl trichloroethane (DDT).

Table 1.4. Physical and chemical properties of allethrin.

S. No	Property	
1	Appearance	Yellow colour
2	Molecular weight	302.4
3	Density	0.997 g/cm^3 at 20°C
4	Emperical formula	$C_{19}H_{26}O_3$
5	Water solubility	Insoluble
6	Solubility	DMSO, ethanol

Allethrin applications

Allethrin is an active ingredient in insect repellents like mosquito coils, mats, vaporizers, aerosols and sprays. Annual world worldwide consumption of these repellents is in the billions of units. Allethrin when compared to other insecticide repellents, shows inhibition of insect landing and insect binding (Gibian *et al.,* 2005). Pyrethroids are rapidly metabolized by enzymes like cytochrome P450 and esterases in insects. To inhibit the insect enzymes, synergists like octachlorodipropyl ether (S-2, S-421), piperonyl butoxide (PBO) are used as ingredients in house hold pesticide products. These synergists bind to P450 enzymes and inhibit the degradation/metabolism of insecticides in insects, so that the insecticide will stay for longer time to show its action thus preventing recovery of insects. These synergists are in general found to be carcinogens.

Metabolism and toxicity of allethrin

Allethrin metabolism in mammalian cell and in environment is quite different. In mammals, allethrin degraded by oxidation at various sites resulting in ester metabolites (Figure 1.7). Major metabolites are those which are oxidized at methyl groups in the acid moiety or at allyl group in allethrolone alcohol with minor metabolites like chrysanthemic acid and allethrolone (Miyamoto, 1976). In mammals, allethrin is oxidatively degraded during which free radicals are generated that can cause DNA

damage and various health effects in exposed populations (Kumaraguruparan *et al.,* 2005).

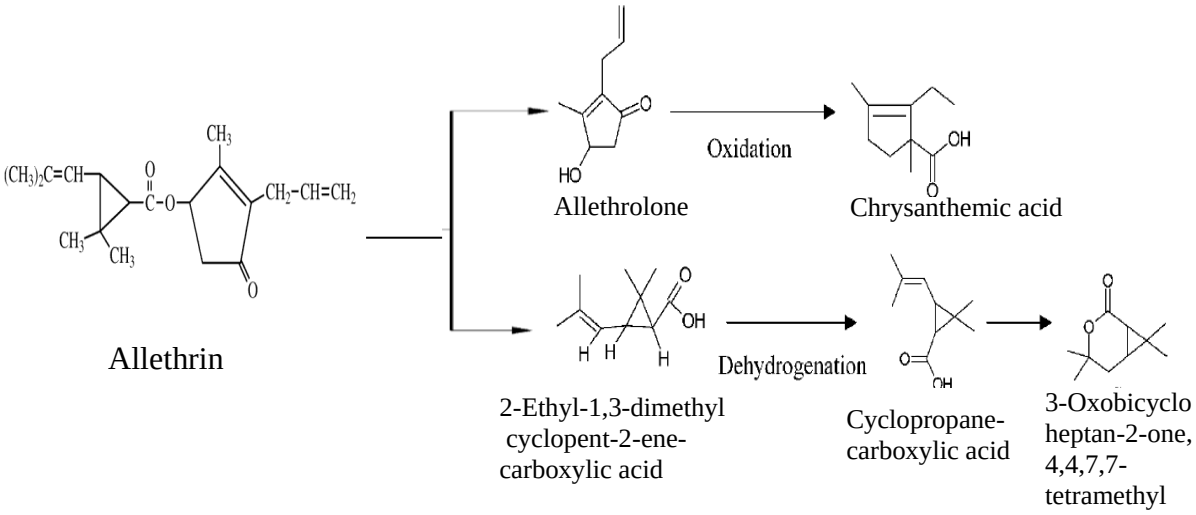


Figure 1.7. *Allethrin metabolism (Paingankar et al., 2005). Chrysanthemic acid and cyclopropane-carboxylic acid are major end products.*

In the environment, it is degraded by photoreactions like ester cleavage, di-pi-methane rearrangement, oxidation at the isobuteneyl double bond and cis/trans-isomerization. Allethrin persists in environment for longer periods which may induce toxicity in insects, mammals, birds, fishes. Toxicity differs in different species (WHO, 1989). It is severely toxic to fishes and aquatic invertebrates at very low concentrations 8.6-9.5 µg/L. Honey bees are also severely affected at low concentration (3-9 µg/bee). Below is the table showing toxicological information about allethrin.

Table 1.5. Lethal dose of allethrin on different organisms.

S. No	Organism	LD_{50}
1	Rat (Wistar)	395 (mg/kg)
2	Fish (*Brachydanio rerio*)	8.6 (µg/L)
3	Birds (*Colinus virginianus*)	5620 (mg/kg)
4	Honey bees	3-9 (µg/bee)

Allethrin accumulates on different household surfaces and persists in the environment for an extended period of time. Allethrin degradation via oxidative pathway (Miyamoto, 1976) leads to the generation of free radicals causing DNA damage and various adverse health effects in exposed populations (Kale *et al.,* 1999; Srivastava *et al.,* 2012). Earlier studies assessed allethrin's neurotoxicity in rodents (Bloomquist, 1996;

Hossain *et al.,* 2005) and genotoxicity in bacterial systems (Hour *et al.,* 1998). The usage of mosquito coil repellants release high concentrations of allethrin into the surrounding air within short period of time (0.0120 ppm/45 min), which is several folds greater than the level required to control mosquitoes (Ramesh and Vijayalakshmi, 2001). Allethrin can induce genotoxicity and oxidative stress in Swiss albino mice (Srivastava *et al.,* 2012). The toxicity of allethrin on reproductive system is not yet studied.

Mosquito coils

Mosquitoes transmit diseases to 700 million people annually (Fradin and Day, 2002). The use of insect repellents like mosquito coils are increasing because of ever increasing awareness about the spread of diseases like malaria, dengue and yellow fever. Mosquito coils are used by low income group people mostly in Asia, Africa, South America and China. Coils prevent adult mosquitoes from biting, particularly during the sleeping hours and these are the preferred mosquito repelling products in low income communities (Mulla *et al.,* 2001).

The ingredients in mosquito coil are starch (potato, corn, rice or wheat), coconut flour mix, sawdust, dye (for colour), burning acids (sodium and potassium nitrate), sodium benzoate and different types of pyrethroid insecticides (allethrin, permethrin, transfluthrin) with varying percentage. Chlorinated hydrocarbons (DDT, lindane), organophosphorus compounds like parathion and carbamates like propoxus were also used. But, these are not effective to repel mosquitoes. The insecticide evaporates with the smoke from the coil which repel the mosquitoes from entering in to room, or immobilize and paralyze and finally making them unconscious. The advantages of using coils are cost effectiveness, gives 70-80% protection for 8-10 hours and no need of special equipment. The disadvantages are odour and smoke which can cause asthma, release of volatile organic compounds like formaldehydes, polycyclic aromatic hydrocarbons (PAHs) and particulate matter which are hazardous. One coil can emit smoke equivalent to more than 75-150 cigarettes. Ignorance of toxicity of active ingredients, organic fillers, binders, particulate matter and other additives in the coil may cause toxicity to the consumers (Lin and Lee, 1997).

Mosquito repellent market in India is valued at ₹3200 crore, in which urban share is 70% and more and more people are using the repellents as a precautionary measure against diseases spread by mosquitoes. The market in India is dominated by Reckitt Benckiser with its brand “Mortein”, Jyothi Laboratories with “Maxo” and Godrej Sara

Lee with "Good knight" and Karamchand Appliances Private Ltd. with its "All Out". The relative share of coils is 50%, mats 10%, vaporizers 20% and aerosols and cream 5% (NIIR project consultancy services (NPCR), Pune). Allethrin concentration in mosquito repellents varies depending on the type of repellent. In coils it ranges from 0.1-0.5%, where as in liquid repellents it is 1.6- 3%.

Toxicity of mosquito coils

Analysis of air samples after mosquito coil usage in a room showed a maximum concentration of allethrin (0.0120 ppm) within 30- 45 min of use followed by a decline up to 6 h (Ramesh and Vijayalakshmi, 2001). When mosquito repellent mat containing 4% allethrin was burned in a windows closed and opened room, the concentration ($\mu g/m^3$) of allethrin in the room is significantly different (Figure 1.8). The concentration of allethrin is more in a windows closed room compared to windows open room (Dua *et al.*, 2005).

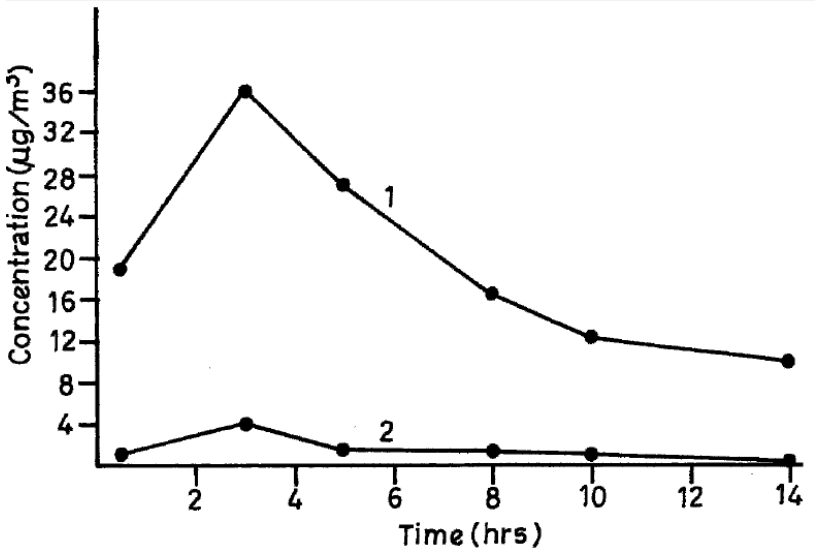


Figure 1.8. *Allethrin concentration profile in air samples taken at various time intervals during heating of mosquito repellent mat (4% allethrin) in a room (3 x 3 x 2.5m). Line 1- window closed; Line 2- window open. (Dua et al., 2005).*

Along with insect repellent (0.1 to 0.5%) in mosquito coils, other major constituents such as wood dust and coconut shell powder (Lin and Lee, 1997) are also present. The particulate matter (PM) released up on burning coils will be of the size $P_{2.5\text{-}10}$. Bigger the particle size, the easier it is for the particulate to bombard the insect's body. Smaller the particulate size, longer is the time it sustains in air (Wang, 1993). Smoke form certain mosquito coils contains less aliphatic aldehydes and more amounts of formaldehyde, acetaldehyde and acrolein which are carcinogens (Chang and Lin, 1998).

Exposure to environmental pollutants such as pesticides and heavy metals contribute to the development of cancer and reduction in reproductive functions (Issam *et al.,* 2009; Sharpe and Irvine, 2004; Sharpe and Skakkebaek, 1993; Tan and Soderlund, 2010; Wang *et al.,* 2009). Allethrin exposure via inhalation leads to moderate toxicity in rodent models (Tomlin, 1994). When rat pups were exposed to liquid repellents up to post natal day 9, body and brain weights were decreased; lipid peroxidation levels were decreased in brain, liver and kidney (Gupta *et al.,* 1999). Previous studies indicated that rats exposed to allethrin based liquid mosquito repellants (using a vapouriser) for 90 days do not show any adverse effect on the clinical enzyme profile and reproductive indices in weaning and adult rats (Anvita Srivastava *et al.,* 2006). Further, it is demonstrated that allethrin exhibits recombinogenic activity in the male germ cells of *Drosophila melanogaster* (Pontecorvo and Fantaccione, 2006). Significant increase in white bold cell count and basophil and lymphocyte percentage; severe congestion of venous sinusoids, hyperplasia and regression of both the red and white pulps in spleen, increased serum levels of urea and creatinine and may cause nephrotoxicity in rats exposed to coil smoke for 28 days (Garba *et al.,* 2007). In a case study in Taiwan, mosquito coil smoke was one of the factors contributing to lung cancer (Chen *et al.,* 2008). Epidemiological studies have shown that long-term exposure to mosquito coils smoke can induce asthma and persistent wheeze in children, especially in under the age group of five (Krieger *et al.,* 2003). Mutagenicity effect of mosquito coil smoke have been reported to cause chromosomal aberrations in metaphase and a significantly higher incidence of chromosomal aberration frequency in exposed rats and mice (Das *et al.,* 1994; Moorthy and Murthy, 1994). Adult male Wistar rats exposed to mosquito coil smoke for 6 weeks significantly decreased the drug metabolizing enzyme activities in lungs and liver (Okine *et al.,* 2006). Though information about allethrin toxicity on the nervous system is available and to some extent on other organ systems, its effect on the reproductive tract is not well understood.

The Male Reproductive System

The male reproductive system consists of a pair of testes, epididymides and accessory sex organs which include the seminal vesicles (Figure 1.9). The testes are encapsulated ovoid organs consisting of seminiferous tubules (produce germ cells-spermatozoa) separated by interstitial tissue consisting of Leydig cells (produce testosterone) and Sertoli cells (support and nourishing of germ cells). Epididymis is a

single highly convoluted duct, closely applied to the surface of the testes extending from the anterior to the posterior pole of the testis. It is delineated in to caput, corpus and cauda regions (Robaire and Hermo, 1988). The delineation is based on morphology, the physiological processes that occur within each region and the localization of discrete stages of sperm maturation (Hinton, 1995). The epididymis act as a storage place for the spermatozoa where they become motile and acquire the capacity to fertilize. The seminal vesicles secrete seminal fluid, which provides energy source for sperm and alkalinity to enhance sperm mobility. Prostate gland helps in the expulsion of semen from urethra.

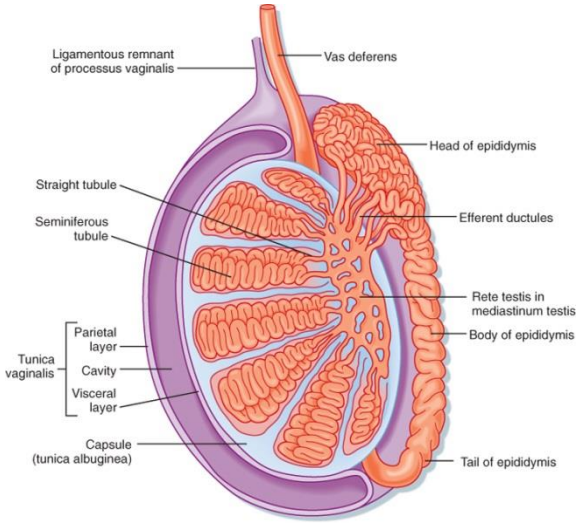


Figure1.9. *The male reproductive system* ***(****Gray's Anatomy for Students. Philadelphia, Churchill Livingstone, 2005).*

Steroidogenesis

Steroids are synthesized by testis and adrenal gland. In testis, Leydig cells in interstitium which possess abundant smooth endoplamic reticulam and numerous mitochondria, synthesize testosterone from cholesterol. The free cholesterol from plasma is transported from outer mitochondrial membrane to inner mitochondria with the help of steroidogenic acute regulatory protein (StAR) protein (Stocco, 1999) and is facilitated by luteinizing hormone (LH), testosterone and adrenocorticotropic hormone. This transfer of cholesterol is the rate limiting step in testosterone biosynthesis. Cytochrome P450 side chain cleavage enzyme (P450scc) converts cholesterol to pregnenolone (Arakane *et al.,* 1996) which is promoted by luteinizing hormone. Pregnenolone in smooth endoplasmic reticulum is converted to testosterone in a series of reactions catalyzed by 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase and 17, 20-lyase; and 17β-hydroxysteroid dehydrogenase (17β-HSD) (Huang *et al.,* 2002). Testosterone synthesis occurs in Leydig cells of testis (Figure 1.10) and is induced by Leydig cell LH receptor

signaling. Steroidogenesis is influenced by drugs and environmental agents. Rats exposed to synthetic pyrethroids cypermethrin and fenvalerate decreases serum testosterone concentrations (Mani *et al.,* 2002; Wang *et al.,* 2010). In addition, herbicide, atrazine decreases the expression of 3β-HSD (Victor-costa *et al.,* 2010).

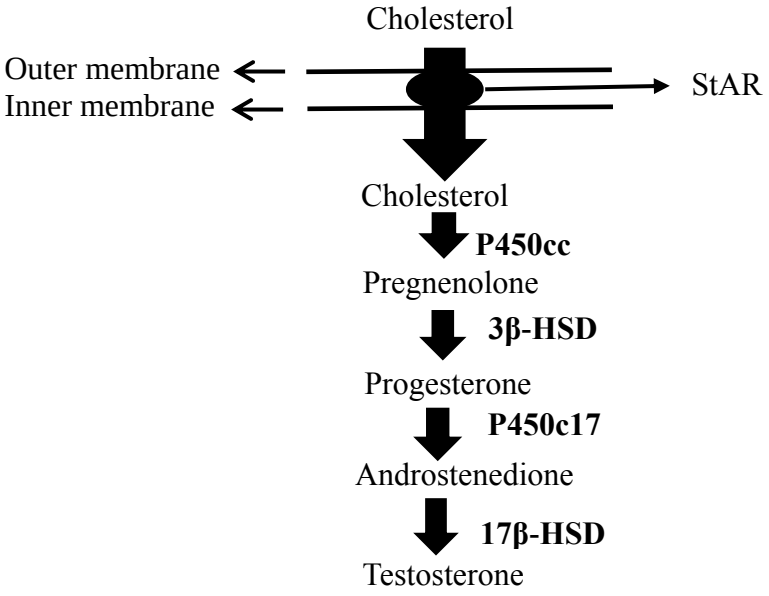


Figure 1.10. *Testosterone biosynthetic pathway (Stocco and Clark, 1997).*

Androgens and androgen receptor

Androgens play important roles in development of male phenotype, maintenance of secondary sexual characteristics and initiation and maintenance of spermatogenesis (Cunha *et al.,* 1992; Sinisi *et al.,* 2003). Androgens are produced by testis, ovary and adrenal gland. Earlier studies indicate that androgens are important to the development and physiology of the prostate and seminal vesicle (Kinghorn *et al.,* 1987; Martikainen *et al.,* 1987). Androgens acts through androgen receptor (AR or NR3C4 (nuclear receptor subfamily 3, group C, member 4)) encoded by single gene located on long arm of X chromosome to regulate the expression of genes important for reproductive functions like male development and fertility (Collins *et al.,* 2003; Lubahn *et al.,* 1988). Androgen receptor expression is seen in Sertoli, myoid and Leydig cells of testis and epithelial and stromal cells of the epididymis (Sar *et al.,* 1990; Takeda *et al.,* 1990), spermatogonia (Warikoo *et al.,* 1986) and they belong to nuclear receptor superfamily and it functions as ligand-activated transcription factor. Androgens regulate the expression of androgen receptor in a tissue- and cell-type-specific fashion (Dai *et al.,* 1996; González-Cadavid *et al.,* 1993; Nastiuk *et al.,* 1994; Shan *et.al.,* 1990). Testosterone or its metabolite 5α-

dihydroxytestosterone (DHT) binds to androgen receptor in cytoplasm, translocate in to nucleus and subsequently dimerizes to bind androgen response element (ARE) sequence in the DNA. In non-genomic action androgens binds to AR in cytoplasm which interacts with signal transduction proteins causes modulation of intracellular calcium levels (Heinlein and Chang, 2002). A decline in semen quality and quantity is observed when androgen receptor and its signaling axis is interrupted (Fisher, 2004; Gray *et al.*, 2001).

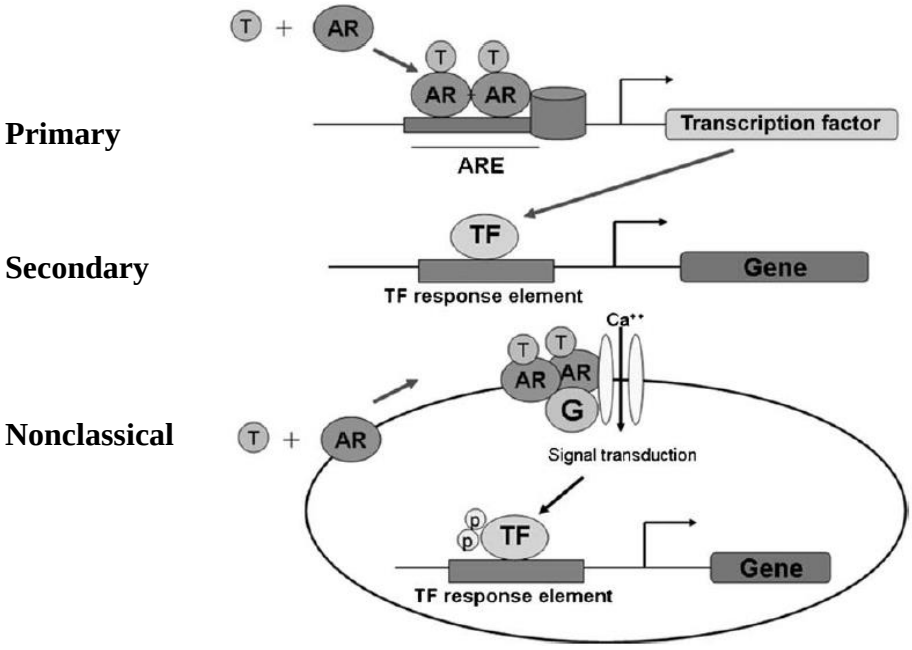


Figure 1.11. *Mode of action of testosterone (Maclean and Wilkinson, 2005).*

Spermatogenesis

Spermatogenesis is a process of generating mature sperm with half the number of chromosomes (haploid) produced from germ cell precursors (Figure 1.12). It can be divided in to three phases: proliferation, reduction-division and differentiation. During early embryogenesis, primordial germ cells from embryo migrate to testis with the help of SCF-C-kit to form gonocytes. Gonocytes differentiate in to spermatogonia and then to spermatids in a series of stages that involve undergoing repeated mitotic divisions, chromosomal duplication and recombination and meiosis. Spermatogenesis (formation of spermatid) happens in seminiferous tubules of testis. Spermatids in the stage I-V, VI-VIII and IX-XIX are referred to as early, middle and late stage spermatids respectively. In rats and mice, spermatogonia are divided in to four classes: undifferentiated type A spermatogonia (A); differentiated type A spermatogonia (A1, A2, A3, A4); intermediate spermatogonia (In); and type B spermatogonia (B) (Russell *et al.*, 1993).

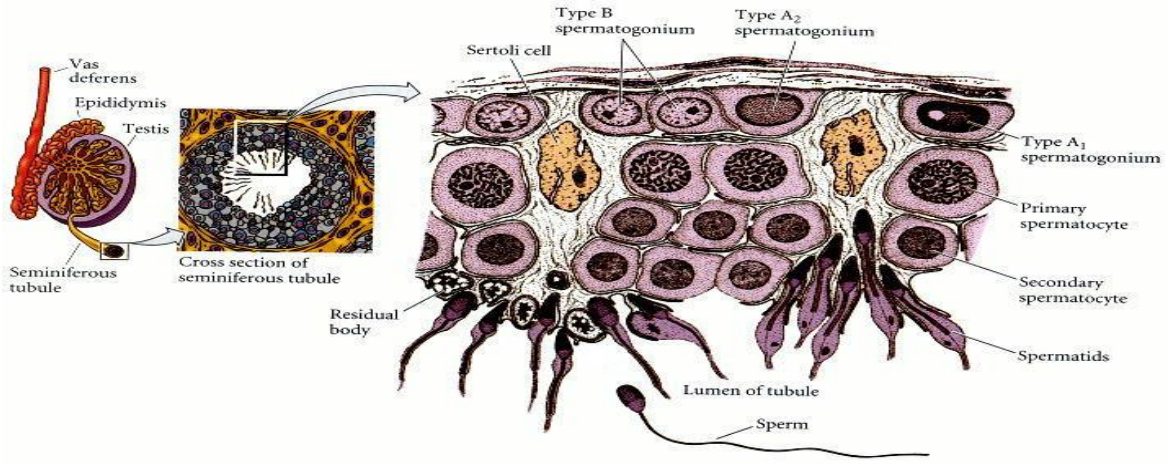


Figure 1.12. *Section of the seminiferous tubule showing the relationship between Sertoli cells and the developing sperm (Dym, 1977).*

Type B spermatogonia undergo repeated mitotic divisions to form spermatocytes, representing the beginning of meiosis (Figure 1.12). Up to this mitotic proliferation, which takes around 16 days, testosterone is not required. These spermatocytes undergo first meiosis which takes a round 24 days to produce secondary spermatocytes that are round and spherical and this requires low levels of testosterone. Secondary spermatocytes undergo second meiotic division to transform to haploid spermatids in a series of transformations like nuclear shrinkage, losing of water and packing of DNA, formation of acrosome from golgi complex, axial filament formation from distal centriole and mitochondrial ring. This transformation of secondary spermatocytes to immature spermatids is called spermiogenesis (Figure 1.13).

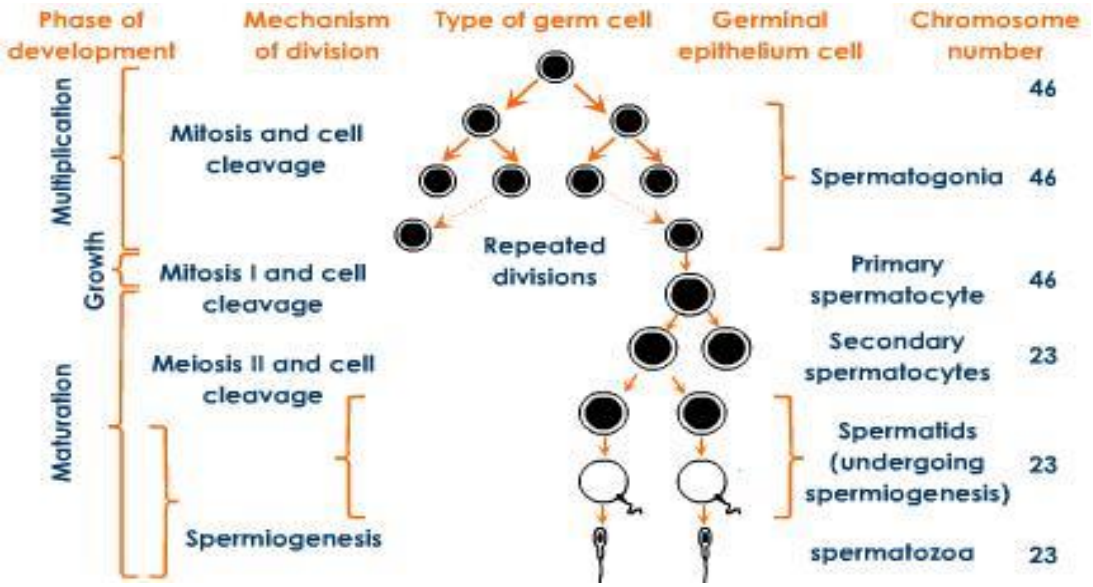


Figure 1.13. *Spermatogenesis and spermiogenesis in rat.*

Gene regulation in spermatogenesis

Mammalian spermatogenesis is tightly regulated by androgens and estrogens. A variety of germ cell differentiation factors such as the tyrosine kinase receptor, c-KIT, or its ligand, stem cell factor (SCF) and transforming growth factor- β-1 (TGF-β1) have been implicated during spermatogenesis (Besmer *et al.*, 1993; Meehan *et al.*, 2000; Olaso *et al.*, 1998). Several transcription factors belongs to heat-shock, zinc-finger, homeobox and cAMP family are involved in spermatogenesis. DNA-binding proteins like heat shock factor 2 (HSF2), OVOL1 and WT1 (Wilms' tumor) are critical for all stages of male gametogenesis (Bettegowda and Wilkinson, 2010). Chromatin-associated factors like bromodomain testis-specific protein (BRDT), Jumonji-C-domain-containing histone demethylase-2A (JHDM2A or KDM3A) and RNA-binding proteins like Gonadotrophin-regulated testicular RNA helicase (GRTH), deleted in azooseprmia-associated protein-1 (DAZAP1) and Y box binding protein-2 (Ybx-2) are important key regulators in spermatogenesis (Figure 1.14).

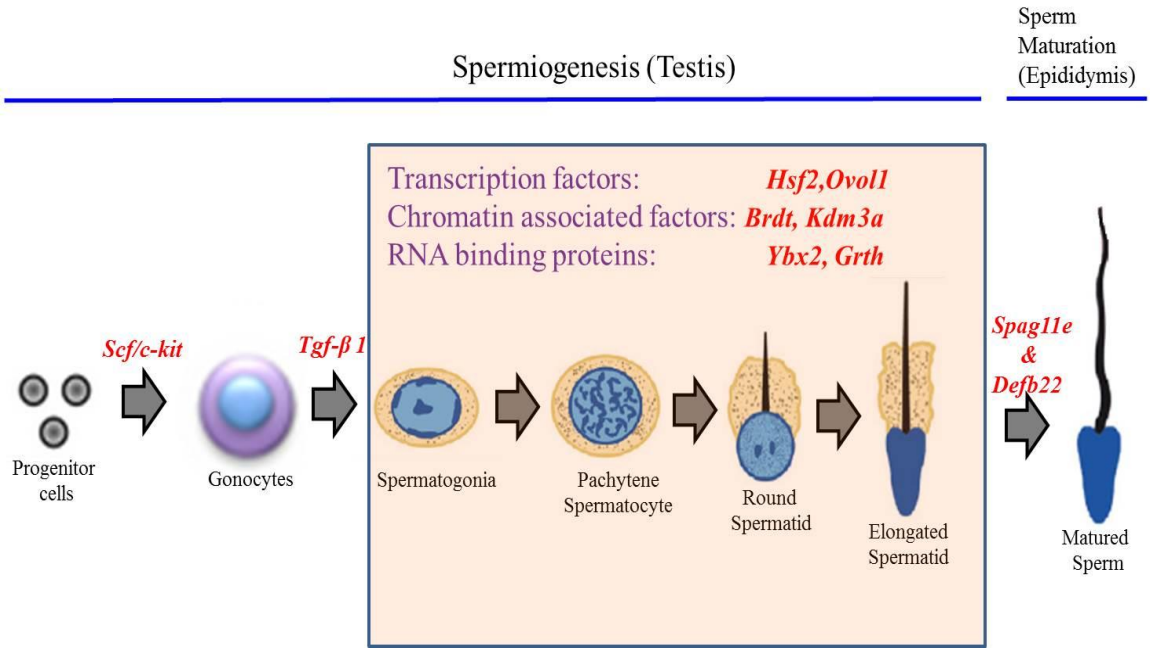


Figure 1.14. *Germ cell regulatory factors involved in spermatogenesis. (Modified from Bettegowda and Wilkinson, 2010).*

Sperm maturation

Spermatozoa formed in testis are immotile and lack fertilizing ability. These immature spermatozoa undergo maturation in epididymis, which also serves as storage site sperms. SPAG11E, a member of the sperm Associated Antigen 11 (SPAG11) family,

plays an important role in spermatid maturation (Zhao *et al.,* 2011; Zhou *et al.,* 2004). Further, the macaque Defensin 126 (DEFB126) and its the rat homologue, Defensin 22 (DEFB22), are known to be involved in sperm capacitation (Yudin *et al.,* 2003) mediate attachment of sperm to the epithelium of oviduct (Tollner *et al.,* 2008) and protect the sperm from immuno-recognition (Yudin *et al.,* 2005). The rat Defb22 is expressed in the epididymis and its protein product is localized on the sperm implying roles similar to its macaque counterpart (Rao *et al.,* 2002; Zanich *et al.,* 2003).

Toxicity of pesticides on reproduction

Pesticides are used in agricultural production to control pests and increase in usage of pesticides has increased globally (Farag *et al.,* 2011). Humans are exposed to pesticides by nasally, oral and dermal routes. Other risk of exposure is reuse of pesticide containers for food and water (Damalas and Eleftherohorinos, 2011; Ecobichon, 2001). Pesticide residues were found in agricultural soils (Al-Wabel *et al.,* 2011) and vegetables. In a study conducted by Armah, 2011, on cabbage samples in cape coast, Ghana, allethrin is found to be above maximum residue limits where permitted is 0.5 mg/kg (Table 1.6). Pesticides have adverse effects on reproductive functions. Benomyl, a fungicide extremely toxic to testis causing sloughing of germ cells (Nakai *et al.,* 1992). Permethrin exposure increases lipid peroxidation, inhibits splenocyte proliferation in fishes like juvenile red drum (*Sciaenops ocellatus*) and adult mummichog (*Fundulus heteroclitus*) (Parent *et al.,* 2011). Pesticides like atrazine, cabofuran, endosulfan, 2,4-D induces testicular cell disorganization, oedema, congestion, damage to Sertoli cells, increase in the number of Leydig cells, germ cells and sperm head abnormalities (Amer and Aly, 2001; Pant *et al.,* 1995; Rao *et al.,* 2005).

Table 1.6. Pesticide residues found in cabbage (Armah, 2011).

S. No	Pesticide	Mean of 9 samples	Standard deviation	Minimum residue (mg/kg)	Maximum residue (mg/kg)
1	Allethrin	1.52	± 3.02	0.27	9.56
2	Deltamethrin	4.74	± 1.69	2.71	7.05
3	Cypermethrin	0.31	± 0.36	0.02	1.16
4	Bifenthrin	0.01	± 0.009	0.004	0.036

REFERENCES

Al-Wabel MI, El-Saeid MH, Al-Turki AM, and AbdelNasser G. (2011). Monitoring of Pesticide Residues in Saudi Arabia Agricultural Soils.pdf. *Research Journal of Environmental Sciences*, *5*(3), 269–278.

Amer SM, and Aly FA. (2001). Genotoxic effect of 2,4-dichlorophenoxy acetic acid and its metabolite 2,4-dichlorophenol in mouse. *Mutation Research*, *494*(1-2), 1–12.

Anvita Srivastava, Mithilesh Kumar Srivastava, and Rajendra Behari Raizada. (2006). Ninety-day toxicity and one-generation reproduction study in rats exposed to allethrin-based liquid mosquito repellent. *The Journal of Toxicological Sciences*, *31*(1), 1–7.

Arakane F, Sugawara T, Nishino H, Liu Z, Holt JA, Pain D, Stocco DM, Miller WL, and Strauss JF, III. (1996). Steroidogenic acute regulatory protein (StAR) retains activity in the absence of its mitochondrial import sequence: implications for the mechanism of StAR action. *PNAS*, *93*(24), 13731–6.

Armah FA. (2011). Assessment of Pesticide Residues in Vegetables at the Farm Gate: Cabbage (Brassica oleracea) Cultivation in Cape Coast, Ghana. *Research Journal of Environmental Toxicology*, *5*(3), 180–202.

Aspelin AL. (1998). US EPA Office of Pesticide Programs. Pesticide Industry Sales and Usage 1996 and 1997 Market Estimates.

Banerjee BD, Seth V, Bhattacharya A, and Pasha ST. (1999). Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers, *107*, 33–47.

Besmer P, Manova K, Duttlinger R, Huang EJ, Packer A, Gyssler C, and Bachvarova RF. (1993). The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. *Development. Supplement*, 125–37.

Bettegowda A and Wilkinson MF. (2010). Transcription and post-transcriptional regulation of spermatogenesis. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *365*(1546), 1637–51.

Bloomquist JR. (1996). Ion channels as targets for insecticides. *Annual Review of Entomology*, *41*, 163–90.

Buchel KH. (1983). Chemistry of pesticides. *John Wiley and Sons Inc., New York*.

Chang JY and Lin JM. (1998). Aliphatic aldehydes and allethrin in mosquito-coil smoke. *Chemosphere*, *36*(3), 617–24.

Chen SC, Wong RH, Shiu LJ, Chiou MC, and Lee H. (2008). Exposure to mosquito coil smoke may be a risk factor for lung cancer in Taiwan. *Journal of Epidemiology / Japan Epidemiological Association*, *18*(1), 19–25.

Collins LL, Lee HJ, Chen YT, Chang M, Hsu HY, Yeh S, and Chang C. (2003). The androgen receptor in spermatogenesis. *Cytogenetic and Genome Research*, *103*(3-4), 299–301.

Cunha GR, Alarid ET, Turner T, Donjacour AA, Boutin EL, and Foster BA. (1992). Normal and abnormal development of the male Urogenital Tract. *Journal of Andrology*, *13*(6), 465–475.

Dai JL, Maiorino CA, Gkonos PJ, and Burnstein KL. (1996). Androgenic up-regulation of androgen receptor cDNA expression in androgen-independent prostate cancer cells. *Steroids*, *61*(9), 531–9.

Damalas CA and Eleftherohorinos IG. (2011). Pesticide exposure, safety issues, and risk assessment indicators. *International Journal of Environmental Research and Public Health*, *8*(5), 1402–19.

Das RK, Sahu K, and Dash BC. (1994). Induction of chromosome aberrations and micronuclei in pulmonary alveolar macrophages of rats following inhalation of mosquito coil smoke. *Mutation Research*, *320*(4), 285–92.

Dawang C. (2008). Understand the role of chemical pesticides and prevent misuses of pesticides. *Bulletin of Agricultural Science and Technology*, *1*, 36–38.

Dua VK, Gurwara R, Sinha SN, and Dash AP. (2005). Allethrin in the air during the use of a heated mosquito repellent mat. *Bulletin of Environmental Contamination and Toxicology*, *75*(4), 747–51.

Dym M. (1977). The male reproductive system. In L. Weiss and R. O. Greep (eds.), Histology, 4th Ed. McGraw-Hill, New York, pp. 979–1038.

Ecobichon DJ. (2001). Pesticide use in developing countries. *Toxicology*, *160*(1-3), 27–33.

Farag RS, Latif A, and El-gawad A. (2011). Monitoring of pesticide residues in some Egyptian herbs , fruits and vegetables. *International Food Research Journal*, *665*(18), 659–665.

Fisher JS. (2004). Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. *Reproduction, 127(3),* 305-15.

Fradin MS and Day JF. (2002). Comparative efficacy of insect repellents against mosquito bites. *The New England Journal of Medicine*, *347*(1), 13–8.

Garba SH, Adelaiye AB, and Mshelia, LY. (2007). Histopathological and Biochemical Changes in the Rats Kidney Following Exposure to a Pyrethroid Based Mosquito Coil, *3*(12), 1788–1793.

Gibian M, Benjamin David Hindle, Sangita Devi K, Andrew Martin Scrivener, and Graeme Bruce Smith KGW. (2005). Combustible Mosquito coil or Stick. Patent EP 1686851 A1.

Gilden RC, Huffling K, and Sattler B. (2010). Pesticides and health risks. *Journal of Obstetric, Gynecologic, and Neonatal Nursing*, *39*(1), 103–10.

González-Cadavid N, Vernet D, Fuentes Navarro A, Rodríguez JA, Swerdloff RS, and Rajfer J. (1993). Up-regulation of the levels of androgen receptor and its mRNA by androgens in smooth-muscle cells from rat penis. *Molecular and Cellular Endocrinology*, *90*(2), 219–29.

Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A, Orlando E, and Guillette L (2001). Effects of environmental antiandrogens on reproductive development in experimental animals. *Human Reproduction Update*, *7*(3), 248–64.

Gupta A, Nigam D, Shukla GS, and Agarwal AK. (1999). Effect of pyrethroid-based liquid mosquito repellent inhalation on the blood-brain barrier function and oxidative damage in selected organs of developing rats. *Journal of Applied Toxicology,19*(1), 67–72.

Heinlein CA and Chang C. (2002). The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. *Molecular Endocrinology*, *16*(10), 2181–7.

Hildebrand ME, Mcrory JE., Snutch TP, and Stea A. (2004). Mammalian Voltage-Gated Calcium Channels Are Potently Blocked by the Pyrethroid Insecticide Allethrin. *The Jouranl of Pharmacology and Experimental Therapeutics*, *308*(3), 805–813.

Hinton BT. (1995). What does the epididymis do and how does it do it? In: Robaire B, Pryor JL, et al, eds. *Handbook of Andrology*. Lawrence, KS. Allen Press, 18-20.

Hossain MM, Suzuki T, Sato I, Takewaki T, Suzuki K, and Kobayashi H. (2005). Neuromechanical effects of pyrethroids, allethrin, cyhalothrin and deltamethrin on the cholinergic processes in rat brain. *Life Sciences*, *77*(7), 795–807.

Hour TC, Chen L, and Lin JK. (1998). Comparative investigation on the mutagenicities of organophosphate, phthalimide, pyrethroid and carbamate insecticides by the Ames and lactam tests. *Mutagenesis*, *13*(2), 157–66.

Huang BM, Lai HY, and Liu MY. (2002). Concentration dependency in lead-inhibited steroidogenesis in MA-10 mouse Leydig tumor cells. *Journal of Toxicology and Environmental Health. Part A*, *65*(7), 557–67.

Hvistendahl M. (2013). In rural Asia, locking up poisons to prevent suicides. *Science*, *341*, 738–739.

Issam C, Samir H, Zohra H, Monia Z, and Hassen BC. (2009). Toxic responses to deltamethrin (DM) low doses on gonads , sex hormones and lipoperoxidation in male rats following subcutaneous treatments. *The Journal of Toxicological Sciences*, *34*(6), 663–670.

Kakko I, Toimela T, and Tähti H. (2003). The synaptosomal membrane bound ATPase as a target for the neurotoxic effects of pyrethroids, permethrin and cypermethrin. *Chemosphere*, *51*(6), 475–80.

Kale M, Rathore N, John S, and Bhatnagar D. (1999). Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes : a possible involvement of reactive oxygen species. *Toxicology letters*, *105(3)*, 197–205.

Kelce WR, Monosson E, Gamcsik MP, Laws SC, and Gray LE. (1994). Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. *Toxicology and Applied Pharmacology*, *126*(2), 276–85.

Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, and Wilson EM. (1995). Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. *Nature*, *375*(6532), 581–5.

Kemppainen JA, Langley E, Wong CI, Bobseine K, Kelce WR, and Wilson EM. (1999). Distinguishing androgen receptor agonists and antagonists: distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone. *Molecular Endocrinology (Baltimore, Md.)*, *13*(3), 440–54.

Kinghorn EM, Bate AS, and Higgins SJ. (1987). Growth of rat seminal vesicle epithelial cells in culture: neurotransmitters are required for androgen-regulated synthesis of tissue-specific secretory proteins. *Endocrinology*, *121*(5), 1678–88.

Krieger RI, Dinoff TM, and Zhang X. (2003). Octachlorodipropyl Ether (S-2) Mosquito Coils Are Inadequately Studied for Residential Use in Asia and Illegal in the United States. *Environmental Health Perspectives*, *111*(12), 1439–1442.

Kumaraguruparan R, Chandra Mohan KVP, Abraham SK, and Nagini S. (2005). Attenuation of N-methyl-N'-nitro-N-nitrosoguanidine induced genotoxicity and oxidative stress by tomato and garlic combination. *Life Sciences*, *76*(19), 2247–55.

Lin JM and Lee JK. (1997). Vapor phase and particulate bound polycyclic aromatic hydrocarbons in the smoke of mosquito coils. *Bulletin of Environmental Contamination and Toxicology*, *59*(6), 868–74.

Liu CJ, Men WJ, and Liu YJ. (2002). The pollution of pesticides in soils and its bioremediation. *System Sciences and Comprehensive Studies in Agriculture*, *18*(4), 295–297.

Liu ZJ and Liu ZB. (1999). The Status of the world agrochemicals. *Chemical Technology Market*, *22*(12), 14–17.

Lubahn D, Joseph D, Sullivan P, Willard H, French F, and Wilson, E. (1988). Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science*, *240*(4850), 327–330.

Maclean JA and Wilkinson MF. (2005). Gene regulation in spermatogenesis. *Current Topics in Developmental Biology*, *71*(i), 131–97.

Mani U, Islam F, Prasad AK, Kumar P, Suresh Kumar V, Maji BK, and Dutta KK. (2002). Steroidogenic alterations in testes and sera of rats exposed to formulated Fenvalerate by inhalation. *Human & Experimental Toxicology*, *21*(11), 593–7.

Martikainen P, Härkönen P, Vanhala T, Mäkelä S, Viljanen M, and Suominen J. (1987). Multihormonal control of synthesis and secretion of prostatein in cultured rat ventral prostate. *Endocrinology*, *121*(2), 604–11.

Meehan T, Schlatt S, O'Bryan MK, de Kretser DM, and Loveland KL. (2000). Regulation of germ cell and Sertoli cell development by activin, follistatin, and FSH. *Developmental Biology*, *220*(2), 225–37.

Miller GT. (2004). Sustaining the Earth. *6th Edition, Thompson Learning, Inc. Pacific Grove, California*.

Miyamoto J. (1976). Degradation, metabolism and toxicity of synthetic pyrethroids. *Environmental Health Perspectives*, *14*(April), 15–28.

Moorthy MV and Murthy PB. (1994). Analysis of sister chromatid exchange, micronucleus and chromosomal aberration frequencies in rodents exposed to mosquito coil smoke by inhalation route. *Toxicology Letters*, *70*(3), 357–62.

Mulla MS, Thavara U, Tawatsin A, Kong-Ngamsuk W, and Chompoosri J. (2001). Mosquito burden and impact on the poor: measures and costs for personal protection in some communities in Thailand. *Journal of the American Mosquito Control Association*, *17*(3), 153–9.

Nakai M, Hess RA, Moore BJ, Guttroff RF, Strader LF, and Linder RE. (1992). Acute and long-term effects of a single dose of the fungicide carbendazim (methyl 2-benzimidazole carbamate) on the male reproductive system in the rat. *Journal of Andrology*, *13*(6), 507–18.

Nastiuk KL and Clayton DF. (1994). Seasonal and tissue-specific regulation of canary androgen receptor messenger ribonucleic acid. *Endocrinology,* 134 (2). 640-9.

Okine L, Nyarko A, Armah G, Awumbila B, Owusu K, Set-Soafia S, and Ofosuhene M. (2006). Adverse effects of mosquito coil smoke on lung, liver and certain drug metabilishing enzymes in male wistar albino rats. *Ghana Medical Journal*, *38*(3), 89–95.

Olaso R, Pairault C, Boulogne B, Durand P, and Habert R. (1998). Transforming growth factor beta1 and beta2 reduce the number of gonocytes by increasing apoptosis. *Endocrinology*, *139*(2), 733–40.

Paingankar M, Jain M, and Deobagkar D. (2005). Biodegradation of allethrin, a pyrethroid insecticide, by an acidomonas sp. *Biotechnology Letters*, *27*(23-24), 1909–13.

Pant N, Prasad AK, Srivastava SC, Shankar R, and Srivastava SP. (1995). Effect of oral administration of carbofuran on male reproductive system of rat. *Human & Experimental Toxicology*, *14*(11), 889–94.

Parent LM, Delorenzo ME, and Fulton MH. (2011). Effects of the synthetic pyrethroid insecticide, permethrin, on two estuarine fish species. *Journal of Environmental Science and Health. Part. B, Pesticides, Food Contaminants, and Agricultural Wastes*, *46*(7), 615–22.

Pimental D, Acquay H, M Biltonen, P Rice, M Silva, J Nelson, V Lipnor, S Giordano, A Horowitz, and M. D'Amore (1992). Environmental and Economic Impacts of Pesticide Use. *The Pesticide Question*, 47-84.

Pimentel D. (1997). Techniques for Reducing Pesticide Use: Economic and Environmental Benefits. *John Wiley and Sons, Chichester, USA.*

Pontecorvo G and Fantaccione S. (2006). Recombinogenic activity of 10 chemical compounds in male germ cells of Drosophila melanogaster. *Ecotoxicology and Environmental Safety*, *65*(1), 93–101.

Ramesh A and Vijayalakshmi A. (2001). Monitoring of allethrin, deltamethrin, esbiothrin, prallethrin and transfluthrin in air during the use of household mosquito repellents. *Journal of Environmental Monitoring*, *3*(2), 191–193.

Rao J, Herr JC, Reddi PP, Wolkowicz MJ, Bush LA, Sherman NE, Black M, and Flickinger CJ (2002). Cloning and Characterization of a novel sperm-sssociated isoantigen (E-3) with defensin- and lectin-like motifs expressed in rat epididymis. *Biology of Reproduction*, *68*(1), 290–301.

Rao M, Narayana K, Benjamin S, and Bairy KL. (2005). L-ascorbic acid ameliorates postnatal endosulfan induced testicular damage in rats. *Indian Journal of Physiology and Pharmacology*, *49*(3), 331–6.

Robaire B and Hermo L. (1988). Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. in: *The Physiology of Reproduction,* E.Knobil, and J.Neill, et al, eds. *Raven Press, New York*, 999–1080.

Repetto R and Sanjay S. Baliga. (1996). Pesticides and the Immune System: The Public health Risk. *World Resources Institute.*

Russell LD, Ettlin RA, Hikim APS, and Clegg ED. (1993). Histological and Histopathological Evaluation of the Testis. *International Journal of Andrology*, *16*(1), 83–83.

Sar M, Lubahn DB, French FS, and Wilson E M. (1990). Immunohistochemical localization of the androgen receptor in rat and human tissues. *Endocrinology*, *127*(6), 3180–6.

Shafer TJ, Meyer DA, and Crofton KM. (2005). Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. *Environmental Health Perspectives*, *113*(2), 123–36.

Shan LX, Rodriguez MC, and Jänne OA. (1990). Regulation of androgen receptor protein and mRNA concentrations by androgens in rat ventral prostate and seminal vesicles and in human hepatoma cells. *Molecular Endocrinology (Baltimore, Md.)*, *4*(11), 1636–46.

Sharpe RM and Irvine DS. (2004). How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health? *BMJ (Clinical Research Ed.)*, *328*(7437), 447–51.

Sharpe RM and Skakkebaek NE. (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *The Lancet*, *341*(8857), 1392–1396.

Sinisi AA, Pasquali D, Notaro A, and Bellastella A. (2003). Sexual differentiation. *Journal of Endocrinological Investigation*, *26*(3 Suppl), 23–8.

Srivastava AK, Srivastava PK, Al-Khedhairy AA, Musarrat J, and Shukla Y. (2012). Allethrin-induced genotoxicity and oxidative stress in Swiss albino mice. *Mutation Research*, *747*(1), 22–8.

Stocco DM. (1999). An update on the mechanism of action of the Steroidogenic Acute Regulatory (StAR) protein. *Experimental and Clinical Endocrinology & Diabetes.*, *107*(4), 229–35.

Stocco DM and Clark BJ. (1997). The role of the steroidogenic acute regulatory protein in steroidogenesis. *Steroids*, *62*, 29–36.

Symington SB and Clark JM. (2005). Action of deltamethrin on N-type (Cav2.2) voltage-sensitive calcium channels in rat brain. *Pesticide Biochemistry and Physiology*, *82*(1), 1–15.

Takeda H, Chodak G, Mutchnik S, Nakamoto T, and Chang C. (1990). Immunohistochemical localization of androgen receptors with mono- and polyclonal antibodies to androgen receptor. *The Journal of Endocrinology*, *126*(1), 17–25.

Tan J and Soderlund DM. (2010). Divergent actions of the pyrethroid insecticides S-bioallethrin, tefluthrin, and deltamethrin on rat Na(v)1.6 sodium channels. *Toxicology and Applied Pharmacology*, *247*(3), 229–37.

Tollner TL, Yudin AI, Tarantal AF, Treece CA, Overstreet JW, and Cherr GN. (2008). Beta-defensin 126 on the surface of macaque sperm mediates attachment of sperm to oviductal epithelia. *Biology of Reproduction*, *78*(3), 400–12.

Tomlin C. (1994). *A world compendium: The pesticide manual. British Crop Protection Council*. Farnham Surrey ; Cambridge.

Victor-Costa AB, Bandeira SM, Oliveira AG, Mahecha GA, and Oliveira CA. (2010). Changes in testicular morphology and steroidogenesis in adult rats exposed to Atrazine. *Reproductive Toxicology*, *29*, 323–331.

Vijverberg HP and de Weille JR. (1985). The interaction of pyrethroids with voltage-dependent Na channels. *Neurotoxicology*, *6*(2), 23–34.

Wang CH. (1993). Application of dosage technology on insecticides for household use. *The Bulletin of Environmental Health*, *1*, 23–37.

Wang H, Wang Q, Ping XZ, Tao XM, Ji YY, Zhang H, Zhang C, Zhang Y, and Xu DX. (2010). Cypermethrin exposure during puberty disrupts testosterone synthesis via downregulating StAR in mouse testes. *Archives of Toxicology*, *84*, 53–61.

Wang XZ, Liu SS, Sun Y, Wu JY, Zhou YL, and Zhang JH. (2009). Beta-cypermethrin impairs reproductive function in male mice by inducing oxidative stress. *Theriogenology*, *72*(5), 599–611.

Warikoo PK, Majumdar SS, Allag IS, Das RP, and Roy S. (1986). Interactions among motility, fertilizing ability, and testosterone binding on spermatozoa of bonnet monkey (Macaca radiata). *Archives of Andrology*, *16*(2), 135–41.

WHO. (1989). Allethrins: Allethrin, D-allethrin, Bioallethrin, S-bioallethrin-Environmental Health Criteria. *Internal Programme on Chemical Safety, Geneva, Switzerland*, *87*.

Yudin AI, Tollner TL, Li MW, Treece CA, Overstreet JW, and Cherr GN. (2003). ESP13.2, a member of the beta-defensin family, is a macaque sperm surface-coating protein involved in the capacitation process. *Biology of Reproduction*, *69*(4), 1118–28.

Yudin AI, Generao SE, Tollner TL, Treece CA, Overstreet JW, and Cherr GN. (2005). Beta-defensin 126 on the cell surface protects sperm from immunorecognition and binding of anti-sperm antibodies. *Biology of Reproduction*, *73*(6), 1243–52.

Zanich A, Pascall JC, and Jones R. (2003). Secreted epididymal glycoprotein 2D6 that binds to the sperm's plasma membrane is a member of the beta-defensin superfamily of pore-forming glycopeptides. *Biology of Reproduction*, *69*(6), 1831–42.

Zhao Y, Diao H, Ni Z, Hu S, Yu H, and Zhang Y. (2011). The epididymis-specific antimicrobial peptide β-defensin 15 is required for sperm motility and male fertility in the rat (Rattus norvegicus). *Cellular and Molecular Life Sciences*, *68*(4), 697–708.

Zhou CX, Zhang YL, Xiao L, Zheng M, Leung KM, Chan MY., Lo PS, Tsang LL, Wong Hy, Ho LS, Chung YW, and Chan HC. (2004). An epididymis-specific beta-defensin is important for the initiation of sperm maturation. *Nature Cell Biology*, *6*(5), 458–64.

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Chapter 1

Decipher the molecular mechanisms of allethrin toxicity *in vitro*.

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Introduction

Among a variety of insecticides used for agricultural and domestic purposes, pyrethroid based are the most common. Pyrethroids are mainly classified as type I and type II depending on the presence of a cyano group. They are known to cause insecticidal effects and the mechanism involves prolonged activation of voltage-gated sodium channels (VGSC) and delayed channel activation in neuronal cells (Narahashi, 1986; Neal *et al.,* 2010; Ray and Fry, 2006). Symptoms of pyrethroid poisoning in insects range from body tremors to convulsions resulting in death. Besides their neurotoxic effects, they are known to be carcinogenic, hepatotoxic, immunosuppressive, estrogenic and antiprogestagenic in a variety of species (Institoris et al., 1999; Nasuti *et al.,* 2007; Shukla *et al.,* 2002; Yokohira et al., 2011).

The type I pyrethroid, D-allethrin (mixture of eight stereoisomers) is one of the preferred household pest control insecticide throughout the world. It is used in mosquito repellants (mats, coils and vaporizers) and human exposure occurs due to inhalation of allethrin emitted along with the smoke. In many animals and humans, it is moderately toxic and primarily acts on the nervous system (Tomlin, 1994). Though pyrethroids are used at low concentrations in domestic set ups, prolonged or recurrent use at these levels is known to cause locomotory defects in rats (Abou-Donia *et al.,* 2002) We previously demonstrated alterations in antioxidant enzyme profile and reproductive parameters in male rats exposed to allethrin nasally or orally (Madhubabu and Yenugu, 2012, 2014). On the contrary, the clinical enzyme profile and reproductive indices in rats nasally exposed to allethrin were found to be unaltered (Anvita Srivastava, 2006). Further, pyrethroids are known to contribute to recombinogenic activity in the male germ cells of *Drosophila melanogaster* (Pontecorvo and Fantaccione, 2006), estrogenic and antiprogestagenic activity in endometrial and breast cancer cells respectively (Garey and Wolff, 1998; Go *et al.,* 1999; Kasat *et al.,* 2002), gynecomastia (Eil and Nisula, 1990; Sattin *et al.,* 1984) and oxidative stress in the male reproductive system (Issam *et al.,* 2009; Issam *et al.,* 2011; Madhubabu and Yenugu, 2012, 2014; Wang *et al.,* 2009).

Pyrethroids are actively metabolized *in vivo* to generate free radicals that can damage the integrity of cellular membranes and initiate a variety of events leading to cell death. Free radical generation during pyrethroid metabolism causes oxidative stress in a (Banerjee *et al.,* 1999; El-Demerdash, 2011; Giray *et al.,* 2001; Romero *et al.,* 2012; Sadowska-Woda *et al.,* 2010) and alter antioxidant status in different organ systems of

rats (Giray *et al.,* 2001; Kale *et al.,* 1999; Li *et al.,* 2005; Maiti *et al.,* 1997). Further, studies including ours demonstrated that pyrethroid compounds cause oxidative stress in the male reproductive system and disturbs the antioxidant status (Issam *et al.,* 2009, 2011; Madhubabu and Yenugu, 2012, 2014; Wang *et al.,* 2009). It is well established that sperm production is seriously compromised due to oxidative stress (Bal *et al.,* 2012; Naziroglu *et al.,* 2011). Free radical generation during oxidative stress promotes apoptosis; affected by a variety of signaling events that involve alteration in the mitochondrial membrane potential, release of cytochrome C from mitochondria and changes in the levels of apoptotic effectors such as p53, BCL-2, PARP-1 and Caspase-3 (Booth *et al.,* 2014). Though *in vivo* studies have demonstrated the ability of pyrethroids to induce oxidative stress, alter antioxidant enzyme activities and loss of germ cell production, limited information is available on the molecular mechanisms of pyrethroid toxicity (Chi *et al.,* 2014; Romero *et al.,* 2012; Yokohira *et al.,* 2011). Further, the molecular mechanism of allethrin induced cell death has not been reported till date. Hence, in this study, we attempt to decipher the toxic actions of allethrin at the cellular and molecular level with emphasis on the role of apoptotic factors.

Intracellular calcium plays a key role in factor in many cellular functions including apoptosis (Bootman *et al.,* 2002). Alterations in intracellular calcium concentrations are due to release of calcium by store operated calcium entry from endoplasmic reticulum stores or by influx through the plasma membrane. Loss of calcium from endoplasmic reticulum leads to apoptotic cell death (Yoshida *et al.,* 2006). Similarly, high calcium influx also favors release of apoptotic factors resulting in cell death (Paschen, 2000). It is reported that oxidative stress caused by toxic chemicals including pyrethroids induces apoptosis that is associated with calcium dynamics. Deltamethrin causes apoptosis in human oral cancer cells by altering intracellular calcium levels (Chi *et al.,* 2014). However, the role of calcium in allethrin induced cytotoxicity is not reported. Voltage gated calcium channels (VGCCs) are multimeric proteins with a common pore forming motif and are involved in a variety of physiological events. The ten members of the VGCC family are divided into L-, N-, P/Q-, R- and T-types depending on the calcium current. Pyrethroids interact with VGCCs to facilitate neurotransmitter release and increased miniature excitatory postsynaptic current (Clark and Symington, 2008; Meyer and Shafer, 2006). It is demonstrated that allethrin modulates VGCCs in rat PC12 cells (Neal *et al.,* 2010). The involvement of VGCCs in allethrin induced apoptosis remains to be investigated.

Though the effects of allethrin at the organ level are documented, the mechanism of its toxicity at the cellular levels remains largely unknown. The primary goal of this study is to understand the mechanism of allethrin toxicity in cells related to the male reproductive system. Analyzing the cellular toxicity of allethrin at the testicular level requires treatment of animals with the compound and the isolation of the specific cell type (Leydig or Sertoli). The yields of these cells isolated from testis are low and non-homogenous and makes it difficult to analyze a variety of parameters. To circumvent this, as an initial step, the mechanisms of allethrin induced cell death were analyzed using rat Leydig cell tumor cell line (LC540 cells). In this study, we evaluated the cytotoxicity of allethrin in rat Leydig carcinoma cells with emphasis on oxidant-antioxidant dynamics, molecular events of apoptotic cell death and the expression of factors that govern germ cell production. We observed that allethrin is cytotoxic to Leydig cells and initiates a cascade of events that involves generation of reactive oxygen species (ROS), lipid peroxidation and altered antioxidant enzyme profile. Mitochondrial membrane potential dependent apoptotic cell death mediated by BCL-2, PARP-1, Caspase-3 and *p53* was evident. Allethrin induced apoptotic cell death seems to involve VGCC mediated release of intracellular calcium.

Materials and Methods

1. Materials

Allethrin, phosphate buffered saline (PBS), Eagle's Minimal Essential medium (EMEM) was from Sigma-Aldrich (St. Louis MO, USA). Fetal bovine serum (FBS) were purchased from GIBCO Ltd (Life Technologies^{TM}., Grand Island, NY). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], Proteinase K, RNase A, Propidium iodide were from Sigma Chemical Co (St Louis, MO). Polyvinylidene fluoride membrane (PVDF) from Millipore (Merck KGaA, Darmstadt, Germany); Monoclonal antibodies against Bcl-2, caspase-3 and PARP antibodies were from Santa Cruz, CA, USA.

2. Maintenance of cell lines (LC540)

The LC540 cells are derived from a rat Leydig cell tumor. They (adherent cell line) were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. These cells were cultured in Eagle's Minimal Essential Media with 10% heat-inactivated Fetal-Bovine Serum, 1.2 g/L $NaHCO_3$ and antibiotic antimycotic solution (10,000 units of Penicillin, 10 mg Streptomycin and 25µg amphotericin / ml). They were maintained at 37ºC in a humidified incubator containing 95% O_2 and 5% CO_2.

Cells were revived from the freezed vials and plated in cultured flasks. Cells were observed regularly under the inverted microscope to check for contamination and cell density. After reaching conlfluency, subculturing was performed. Old (spent) media was removed and Trypsin-EDTA (0.25% trypsin and 0.02% EDTA in Dulbecco's Phosphate buffered saline without Calcium and Magnesium) solution (pre-warmed to 37ºC) was added. The flask was then tilted gently for 3 min to separate the monolayer from the substratum of the flask. To stop the activity of trypsin, MEM was added. Cell suspension was transferred to 15 ml falcon tube and spun the suspension at 2,000 rpm for 2 min and the supernatant was discarded. To the pellet MEM was added and retro-pipetting was done so that the entire pellet is completely dissolved and the cells were seeded for experiments.

3. Isolation of primary Leydig cells

Leydig cells were isolated as described earlier (Abarikwu *et al.*, 2011). We used 27-30 day old rats since the yield of Leydig cells were higher in these animals when

compared to adult rats. Briefly, testes dissected out were decapsulated and subjected to enzymatic digestion in Hank's balanced salt solution (HBSS) containing 0.25 mg / mL collagenase type IV at 37°C in a shaking water bath (80 cycles / min) for 45 min. Following digestion, the debris in the sample was allowed to settle and the supernatant was aspirated carefully and filtered through a nylon mesh. The suspension was centrifuged at 800 g for 5 min and the pellet resuspended in DMEM-F12 and plated in culture flasks or plates. Cell count was determined by a hemocytometer and the viability by Trypan blue staning. The purity of the cells obtained was assessed by 3β-hydroxysteroid dehydrogenase (3β- HSD) staining (Aldred and Cooke, 1983) and was found to be more than 95%.

4. Measurement of cytotoxicity

MTT assay was used to assess the cytotoxicity of allethrin (Mosmann, 1983). In brief, LC540 cells or primary Leydig cells (0.5×10^6 cells / well) were seeded into 24 well microtiter plates and kept in CO_2 incubator for 18-24 h to adhere. The medium was replaced with fresh medium containing varying different concentrations (0.001 µM to 250 µM) of allethrin. The plates were then incubated for 24 h in a humidified CO_2 incubator. At the end of treatment, fresh medium containing MTT (40 µl / mL of 5 mg / mL) was added and incubated for 3 h. The medium was removed and 500 µl of 40 mM HCl / Isopropanol was added to each well to stop the MTT reduction and to dissolve the formazan crystals. The contents were collected and absorbance was measured at 570 nm in a spectrophotometer. The IC_{50} value for LC540 cells was found to be 125 µM and further experiments were carried out with the same dose.

5. Morphological differentiation

LC540 cells were treated with 125 µM allethrin for 3h, 6h, 9h, 12h and 24h. Cells were observed and photographs were taken using phase contrast inverted microscope (Olympus).

6. Estimation of reactive oxygen species (ROS)

The ability of allethrin to generate ROS in testicular cells was estimated as described earlier (Eruslanov and Kusmartsev, 2010). Briefly, 2×10^6 cells after treatment with 125 µM allethrin for 0-5 h were incubated for 15 min with 10 µM 2,7-dichloro dihydro fluorescein diacetate (DCFH-DA), a non-fluorescent cell-permeable dye. DCFH-DA upon oxidation by ROS generated in the cell is converted to 2,7-dichloro-

fluorescein, which exhibits fluorescence at 525 nm when excited with light of 488 nm. Cells were then collected, washed with phosphate buffer saline (PBS) and subjected to Flow cytometer analysis using a FACS Calibur flow cytometer (BD Biosciences) with detection settings of FITC range.

7. Estimation of lipid peroxidation (LPO) products

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA), the product of lipid peroxidation, under acidic conditions and high temperature (95°C) to form a pink colored complex with absorbance maxima at 532 nm. 6 x 10^6 cells were treated with 125 µM allethrin for 0-48 h. After trypsinization, pellet was washed with PBS and the extent of lipid peroxidation was estimated using the TBA method as described earlier (Bernheim, 1964). 1,1,3,3-tetraethoxy-propane (TEP) was used as the standard. Levels of lipid peroxidation were expressed as nano moles TBARS formed/ mg protein.

Reagents

1. 0.33% TBA: Prepared freshly in an amber colored bottle to protect from light. 200 mg of TBA is dissolved in 30 ml of distilled water and 30 ml of glacial acetic acid and mixed well to completely dissolve the TBA.
2. 10% Trichloroacetic acid (TCA) in distilled water (freshly prepared).
3. Standard: 1,1,3,3-tetraethoxy-propane (TEP).

Procedure

Preparation of TCA precipitates: To 0.5 ml of sample, 0.5 ml of normal saline (0.9% NaCl) and 1 ml of 10% TCA was added and mixed well and kept on ice for 30 min with intermittent shaking. Samples were centrifuged at 5000 rpm for 10 min at 4°C. Supernatant was separated carefully and used for the estimation of lipid peroxides.

Assay: To 1 ml of supernatant, 250 µl of 0.33% TBA was added and mixed well in a tight capped tube and boiled for 60 min at 95°C. The tubes were then cooled immediately under running water. The pink color developed was measured at 532 nm in a UV-visible spectrophotometer.

8. Enzyme assays

6 x 10^6 cells plated in 100 mm cell culture plate were treated with 125 µM allethrin for 0-48 h were collected and sonicated in phosphate buffer saline, pH 7.4. Catalase, superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione

peroxidase (GPx) activities were determined by using standard protocols described earlier (Aebi, 1984; Jakoby, 1978; McCord and Fridovich, 1969; Paglia and Valentine, 1967). Solvent controls were maintained for each time point tested. Enzyme activity of the control at each time point is taken as 100% and the activity in allethrin treated cells is expressed as percent of the respective control (without allethrin) at each time point.

8.1. Catalase assay

The activity of catalase present in the sample was assayed by standard method (Aebi, 1984).

Reagents

1. 50 mM mixed phosphate buffer, pH 7.0: The buffer is prepared by mixing 50 mM potassium dihydrogen phosphate and 50 mM disodium hydrogen phosphate in the ratio of 1:1.5.
2. 30 mM H_2O_2 in mixed phosphate buffer.

Briefly, assay mixture containing 1 ml of 30 mM H_2O_2 in 50 mM mixed phosphate buffer, pH 7.0 and 10 µl of sample. The change in the OD was monitored for three minutes at 30 secs intervals. The activity of catalase in the diluted sample is calculated using the first order reaction.

$$K_{30} = (2.303/30)* \log (A_1/A_2)$$

A_1: highest OD value, A_2: lowest OD value

The activity of catalase was expressed as K_{30}/mg protein (one unit is the amount of enzyme that utilizes 1 µmole of hydrogen peroxide/min).

8.2. Glutathione S-transferase activity

Glutathione S-transferase (GSTs) is an important enzyme in detoxification process. These enzymes conjugate the toxicants in the cells to glutathione and making the product water soluble. In this method, 1-chloro-2, 4-dinitrobenzene (CDNB) conjugate with reduced glutathione results in increase in absorbance at 340 nm. The rate of increase is proportional to GST activity in the sample (Jakoby, 1978).

Reagents

1. 0.1 M phosphate buffer, pH 6.5: Prepared by mixing potassium dihydrogen orthophosphate (KH_2PO_4**)** and di potassium monohydrogen phosphate (K_2HPO_4).
2. 10 mM CDNB in 30% ethanol.
3. 10 mM reduced glutathione in distilled water (kept on ice).

Assay procedure: The reaction mixture contains 924 µl of phosphate buffer, 33.3 µl of CDNB solution and 10 µl of sample and the reaction was initiated by adding 33.3 µl of GSH solution to the reaction mixture and the change in the OD was monitored at 340 nm for 5 min. Phosphate buffer serves as a blank. The activity of the enzyme in the sample is calculated using the following formula.

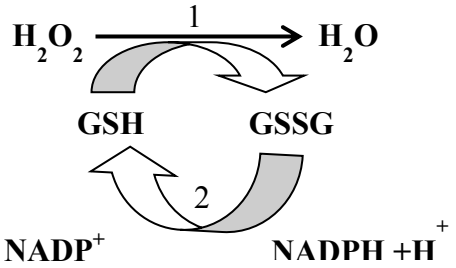
$$\text{GST activity} = \frac{\text{Absorbance difference } (\Delta OD/min) \text{ x V x 100 (dilution factor)}}{\text{9.6 x Conc. of protein in mg}}$$

V: Volume of reaction mixture; 9.6: Molar extinction coefficient of CDNB.

Activity of GST was expressed as µmoles of CDNB-GSH conjugate formed/min/mg protein.

8.3. Glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) catalyzes the reduction of hydroperoxides (R-O-O-H, H_2O_2) by reduced glutathione. The activity of GPx was determined standard method (Paglia and Valentine, 1967). It catalyzes the reduction of hydrogen peroxide. The oxidized glutathione is reduced by glutathione reductase in the presence of NADPH + H^+ (Figure 2.1).



1. Glutathione peroxidase (GPx)
2. Glutathione reductase (GR)

Figure 2.1. Scheme of Glutathione peroxidase assay

The extent of oxidation of NADPH + H^+ to $NADP^+$ is proportional to decrease in the absorbance which is proportional to GPx activity in the sample.

Reagents

1. 50 mM phosphate buffer, pH 7.0 containing 2.5 mM EDTA and 2.5 mM Sodium azide. (Phosphate buffer is prepared by mixing K_2HPO_4 and KH_2PO_4)
2. 10 mM reduced glutathione (GSH) in distilled water (kept on ice).
3. 2.5 mM NADPH in 0.1 % $NaHCO_3$ solution (kept on ice).
4. 1U/assay Glutathione Reductase (GR) in phosphate buffer.
5. 5mM H_2O_2 in phosphate buffer

Assay procedure: The reaction mixture contains 660 µl of phosphate buffer, 1U of glutathione reductase, 100 µl of GSH solution, 100 µl of NADPH solution and the mixture was incubated for 5 min to allow H_2O_2 free oxidation of NADPH to obtain a base line at 340 nm. The reaction was started by adding 10 µl of the sample and 100 µl of H_2O_2 and change in the absorbance was monitored at 340 nm for 5 min at one min intervals. The activity of the enzyme in the sample is calculated using the following formula.

$$\text{GPx activity} = \frac{\text{Absorbance difference } (\Delta OD/min) \times 1}{(6.2 \times \text{volume of sample})}$$

6.2: Extinction coefficient of NADPH at 340 nm. Activity of GPx was expressed as µmoles NADPH oxidized/min/mg protein.

8.4. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radicals to molecular oxygen and hydrogen peroxide.

$$2O_2^{\bullet-} + 2H^+ + SOD \rightarrow H_2O_2 + O_2$$

The activity was determined by measuring the amount of enzyme required to cause 50% inhibition in the reduction of cytochrome C (McCord and Fridovich, 1969). The superoxide required for the reduction was generated by the xanthine-xanthine oxidase system (Figure 2.2).

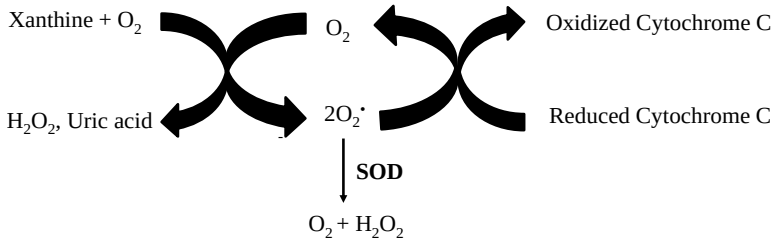


Figure 2.2. Scheme of superoxide dismutase assay

Reagents

1. Reagent 1: 50 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA
2. Reagent 2: Xanthine: 0.76 mg of xanthine is dissolved in 10 ml of 1 mM NaOH.
3. Reagent 3: 24.8 mg of cytochrome C in 100 ml of potassium phosphate buffer.
4. Reagent 4: 10 mM sodium azide in distilled water.
5. Solution A: Prepared freshly by mixing 100 ml of reagent 3, 10 ml of reagent 2 and 1 ml of reagent 4 in amber colored bottle (kept at 25°C).
6. Solution B: Xanthine oxidase is mixed with potassium phosphate buffer (4°C), such that the solution gives an activity of 0.2 U/ml, equals to a rate of reduction of cytochrome C of 0.025 absorbance units/min without SOD or sample.

Assay procedure

To 984 µl of solution A, 16.6 µl of solution B was added, mixed well and the change in the absorbance was measured at 550 nm for 5min. Water is used as a blank. If the change in the absorbance is 0.02 U/min, then the reaction is working.

Activity of SOD in the samples was determined by calculating the percent inhibition of cytochrome C reduction. The percent inhibition of cytochrome C was expressed in terms of the SOD activity using known amounts of SOD standards. One unit of SOD is defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome C by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 in a 1 ml reaction volume. Activity of SOD in the sample was expressed as U SOD/ gm Hb (or) U SOD/mg protein.

9. Cell cycle analyses

Cells were seeded at a density of 2 x 10^6 per well in 6-well plates and treated with 125 µM allethrin for 24 h. The cells were harvested, washed with PBS and centrifuged. Pellet was resuspended in 300 µl of PBS and absolute ethanol was added drop by drop taking care not to form clumps and stored at -20°C overnight. The suspension was centrifuged and to the pellet, PI solution (final concentration 50 mg / ml Propidium iodide, 0.1 mg/ ml RNase, and 1% Triton-X 100) was added and resuspended and incubated in dark for 45 min., and centrifuged and pellet was resuspended in sheath fluid. The DNA content was measured using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) (Hadnagy *et al., 1*999).

10. Measurement of mitochondrial membrane potential

LC540 cells (2 x 10^6) were plated in a 60 mm cell culture plate and treated with 125 µM allethrin for 24 h. To measure mitochondrial membrane potential, the cells were incubated with rhodamine (10 µg/ mL) for 20 min followed by measuring fluorescence intensity in a FACS Calibur flow cytometer (Scaduto and Grotyohann, 1999). As a positive control, 10 µM doxorubicin was also used. Data were collected using the Cell Quest software (Becton Dickinson, San Jose, CA, USA) using a FL-1 detector.

11. Detection of early apoptosis

The early apoptotic events initiated by allethrin treatment was detected using Annexin-V staining kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Following treatment with 125 µM allethrin for 24 h, 2 x 10^6 cells were collected and washed with PBS. The cells were then resuspended in 300 µl of 1X binding buffer and 5 µl of annexin V and incubated in dark for 15 min. The suspension was centrifuged at 4000 rpm for 4 min and washed with 200 µl of 1X binding buffer. The samples resuspended in 195 µl of binding buffer and 10 µl of propidium iodide were analyzed on FACS Calibur flow cytometer (BD Biosciences) to detect cells displaying features of early apoptosis.

12. Measurement of intracellular calcium release

The effect of allethrin on the dynamics of intracellular calcium was measured as described earlier (Chi *et al.*, 2014). 3 x 10^6 cells suspended in PBS were incubated with 2 µg/ mL FURA-2-AM (Molecular Probes, Eugene, USA) for 45 min in dark. After incubation, the cells were washed and resuspended in 2.5 mL of PBS and the fluorescence intensity measured using a multidimensional fluorescence spectrophotometer (ISS VINCI, Champaign, IL, USA). Fluorescence measurements were made in slow kinetics mode and data acquired as ratio metric measurement since FURA has dual excitation wavelengths at 340 and 380 nm with a single emission at 505 nm. Fluorescence intensity at 340 and 380 nm indicate the amount of calcium bound FURA and unbound FURA respectively. Baseline readings were obtained for 130 sec, after which the cells were treated with varying concentrations of allethrin. As a positive control, 10 µM H_2O_2 was used in the assays. Data were recorded until there was no appreciable change in fluorescence intensity. To test the validity of the assay, readings were obtained by adding 20 µl of Triton X-100 (to release the intracellular calcium) were

followed by 50 µl 0.5M EGTA (to chelate calcium). To determine whether the changes in calcium concentration during allethrin toxicity involves VGCCs, LC540 cells were pre treated with 5 and 10 µM Nimodipine (NME; L-type VGCC blocker) or Ruthenium Red (RTR; P/Q type VGCC blocker), prior to allethrin treatment.

13. Real time polymerase chain reaction (RT-PCR)

LC540 cells (2 x 10^6 in a 60 mm culture plate) after treatment with allethrin were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA extracted. 2 µg of total RNA was reverse transcribed using 50 U Stratascript (Invitrogen, USA) and 0.5 µg of oligodT (Invitrogen, Eugene, USA) to generate cDNA. 2 µl of cDNA was used for real time PCR analysis using gene specific primers and SYBR master mix kit (Applied Biosystems, Warrington, UK). As an internal control, the expression of Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) expression was determined. Real-time PCR was performed under the following conditions: initial denaturation at 94°C for 10 min; 40 cycles with 94°C for 15 s and 60°C for 1 min. To detect non-specific amplification, negative controls (no template control and minus RT control (only with RNA) were included in the assays. The amplicons were sequenced to confirm their identity.

14. Immunoblotting

Total protein was obtained from control and allethrin treated LC540 cells (6 x 10^6) by harvesting in radioimmuno precipitation assay (RIPA) buffer (25 mM Tris-HCl pH 7.6, containing 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) supplemented with proteinase cocktail inhibitor. The lysates were then centrifuged at 10,000 rpm and the concentration of the total protein in the supernatant was determined by Lowry's method (Lowry *et al.,* 1951) using bovine serum albumin as a standard. Each protein sample (100 µg) was separated on a 10% SDS-PAGE and transferred on to a 0.2 µm pore size nitrocellulose membrane (HybondTM ECLTM, GE Healthcare, UK). The non-specific binding sites were blocked by incubating the membranes in 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% Tween (TBS-T) for 1 h at room temperature. The membranes were then incubated with specific antibodies (Cell Signalling Technology, Danvers, USA) directed against rat BCL-2, pro-Caspase 3 and PARP-1 for 3 h at room temperature. The membrane was then washed and incubated with secondary antibody against rabbit IgG conjugated with horse radish peroxidase (Cell Signaling

Technology). Immunoreactive proteins were detected using an ECL prime Western blot detection Kit (GE Healthcare life sciences, MA, USA), according to the manufacturer's instructions.

15. Measurement of caspase-3 activity

Caspase-3 activity assay was measured in the allethrin treated LC540 cell lysates using DEVD-AFC substrate at wavelengths: λ_{exc}= 400 nm and λ_{emi}= 505 nm. Briefly, LC540 cells were treated with allethrin for 3, 6 and 9 hours. The cells were collected by trypsinization and cell suspension was washed with PBS and lysed on ice in 501 mM HEPES pH-7.4, 0.1% 3-[(3-cholamidopropyl) dimethylamminio]-1-propanesulfonate (CHAPS), 1 mM dithiothreitol (DTT), 0.1 mM EDTA and centrifuged at 10,000 rpm for 15 min. Protein content in the supernatant was determined by Lowry's method. The reaction mixture contains 100 µg of protein, 5 µl of DEVD-AFC substrate and assay buffer (20 mM HEPES pH-7.4, 100 mM NaCl, 10 mM DTT, 0.1% CHAPS and 10% sucrose). The reaction mixture was incubated at 37°C for 1 h in dark and the fluorescent intensity was monitored by wavelength scan at 400 nm – 580 nm.

16. Statistical analyses

Changes in the parameters analyzed in this study were compared with the DMSO control. Statistical analyses were performed using one way ANOVA and Holm-Sidak test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are Mean ± S.D. a, b and c indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Results

1. Evaluation of cytotoxicity using MTT Assay

The cytotoxicity of allethrin on LC540 cells was evaluated by MTT assay. Allethrin at low concentrations did not display appreciable cell killing activity up to 50 µM when incubated for 24 h (Figure 2.3A). At concentrations above 100 µM, cell killing was observed. Based on the results obtained, the IC_{50} of allethrin was found to be 125 µM (Figure 2.3A). All further experiments in this study were carried with this dose.

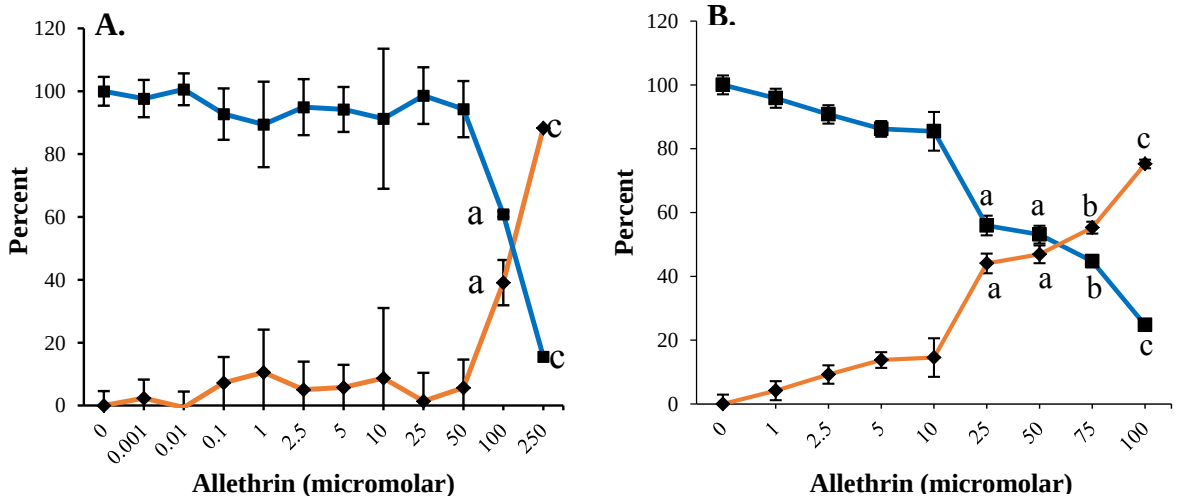


Figure 2.3. Allethrin induced cytotoxicity. (A) *LC540 cells and* ***(B)*** *Isolated primary Leydig cells were treated with 0 – 250 µM allethrin for 24 hours. The viability of the cells was measured using MTT assay and represented as percent survival (■) or death (♦). Values shown are Mean ± S.D. a, b and c indicates p <0.05, p <0.01 and p <0.001 respectively.*

In order to find out whether allethrin can also display similar cytotoxic effects on primary testicular cells, MTT assay was carried out with Leydig cells isolated from 27-30 day old rats. Allethrin was found to be cytotoxic to primary Leydig cells in a dose-dependent manner (Figure 2.3B) with an IC_{50} value of 59 µM. In all the assays conducted, the cells were incubated with DMSO equivalent to the amount present in 125 µM allethrin. DMSO alone did not contribute to any cell killing when incubated up to 48 h (data not shown). To study the mechanism of allethrin toxicity, LC540 cells were preferred over primary Leydig cells because of the difficulty in obtaining high number of primary cells that required sacrificing many animals.

2. Effect of allethrin on ROS generation

To determine whether allethrin induced cytotoxicity involves ROS generation, the oxidation of DCFH-DA to 2,7-dichloro-fluorescein was measured. The mean

fluorescence intensity increased significantly when compared to control within 1 h after allethrin treatment (Figure 2.4) indicating generation of ROS. However, a time dependent increase in the generation of ROS was not observed.

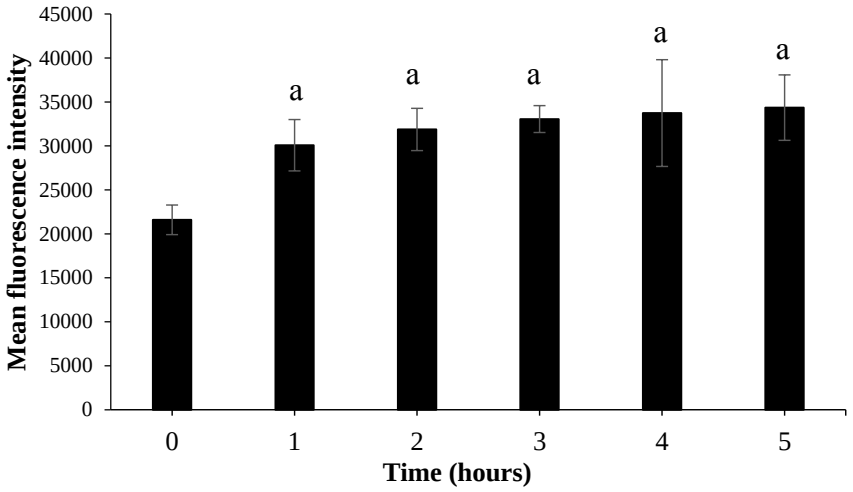


Figure 2.4. Allethrin induced oxidative stress*. LC540 cells treated with allethrin (125 µM) for 0-5 h were incubated with 10 µM 2,7-dichloro dihydro fluorescein diacetate (DCFH-DA). The oxidation of DCFH-DA by the ROS generated in the cells was analyzed in a flow cytometer by measuring the fluorescence intensity of oxidized DCFH-DA. Values shown are Mean ± S.D. a indicates p <0.05.*

3. Lipid peroxidation

Since allethrin induces reactive oxygen species, lipid peroxidation products were measured in allethrin treated cells. A time dependent increase in the concentration of lipid peroxidation products was observed (Figure 2.5) when LC540 cells were treated with allethrin.

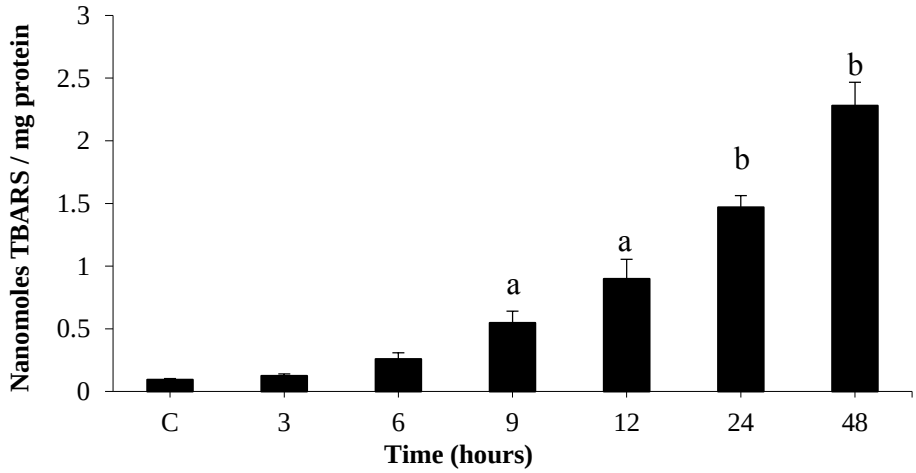


Figure 2.5. Allethrin induced oxidative stress. *The TCA precipitates of LC540 cells treated with 125 µM allethrin for 0-48 h were mixed with thiobarbituric acid and incubated for 1 h at 95°C. The pink color formed was estimated at 532 nm in a spectrophotometer. The concentration of lipid peroxidation products formed was calculated using 1,1,3,3-tetraethoxy-propane (TEP) as the standard. Values shown are Mean ± S.D. a and b indicates p <0.05 and p <0.01 respectively.*

4. Antioxidant enzymes

Catalase, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase activities were measured in LC540 cells treated with or without allethrin for 0 - 48 h. Cells treated with the solvent control for each time point were maintained and the enzyme activity observed in the allethrin treated cells was expressed as percent activity relative to the solvent control at each time point. When compared to the respective controls, no significant change in catalase activity was found up to 12 h after allethrin treatment. A significant increase at 24 h followed by sharp decline at the 48 h time point (Figure 2.6A) was evident. A significant increase at 24 h followed by sharp decline at the 48 h time point (Figure 2.6A) was evident. Superoxide dismutase activity increased significantly within 3 h after allethrin treatment and remained elevated until 9 h (Figure 2.6B). At the later time points, a time dependent decline up to 24 h followed by a sharp increase at the 48 h time point was observed (Figure 2.6B). Glutathione-S-transferase was decreased significantly in a time dependent manner (Figure 2.6C). Glutathione peroxidase activity increased significantly at 9 h time point followed by a sharp decline at the later time points (Figure 2.6D).

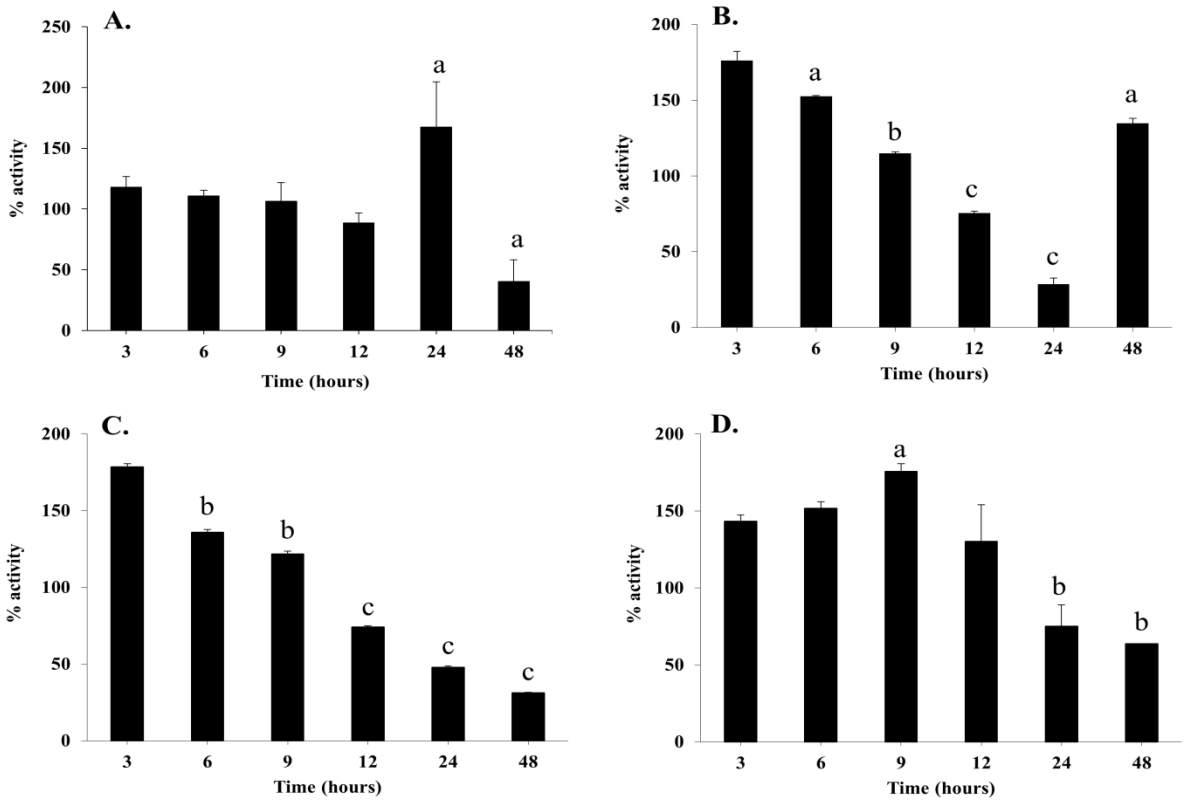


Figure 2.6. Antioxidant enzyme activity. *6 x 10^6 cells were treated with 125 µM allethrin for 0-48 h. Catalase* ***(A)****, superoxide dismutase* ***(B)****, glutathione-s-transferase* ***(C)*** *and glutathione peroxidase* ***(D)*** *enzyme activity was measured in the cell lysates. Values shown are Mean ± S.D. a, b and c indicates p <0.05, p <0.01 and p <0.001 respectively.*

5. Effect of allethrin on LC540 cell morphology

Morphological examination of LC540 cells treated with allethrin revealed the presence of apoptotic bodies (Figure 2.7A-F); and their frequency increased with the duration of treatment.

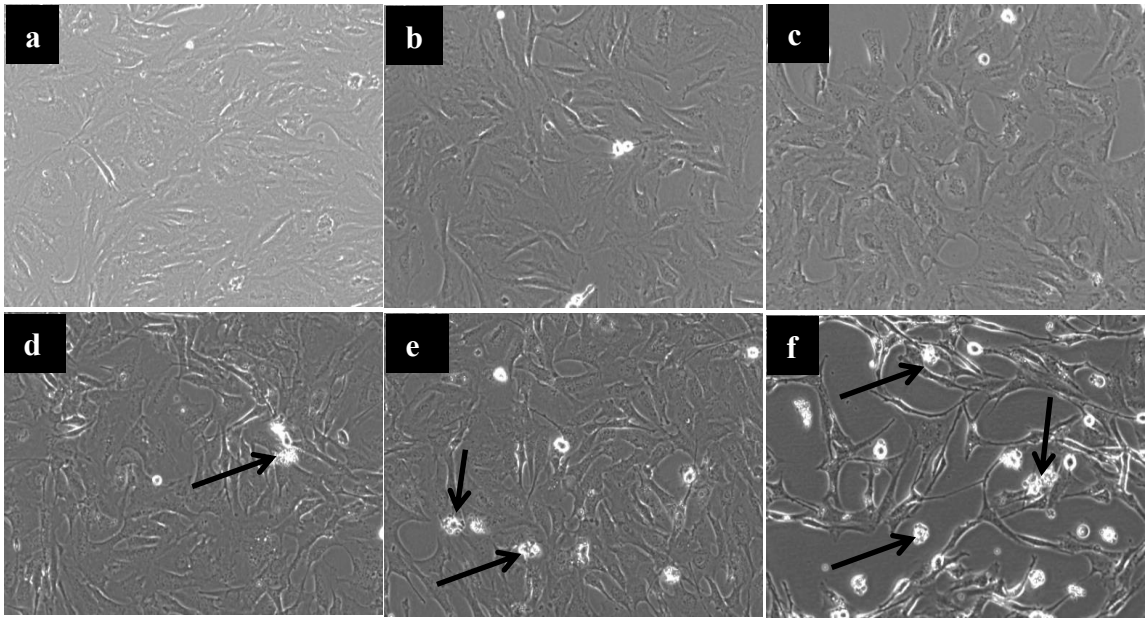


Figure 2.7. Morphological features of allethrin treated cells*. LC540 cells treated for 0-24 h with 125 µM allethrin were examined under a phase contrast microscope. Arrows indicate location of apoptotic bodies.*

6. Detection of apoptosis

Annexin-V staining and FACS analyses indicated the distribution of cells at different stages namely the necrotic, late apoptotic, live and early apoptotic cells in the Q1, Q2, Q3 and Q4 quadrants of the cytogram (Figure 2.8A). The percentage of cells displaying early apoptotic features increased significantly after allethrin treatment (Figure 2.8A and B). On the same lines, cells that are in late apoptosis are also increased, though not significant.

7. Effect of allethrin on cell cycle and mitochondrial membrane potential of LC540 cells

Cell cycle arrest is one of the key indicators that drive the fate of the cell. Propidium iodide staining followed by FACS analyses indicated the distribution of cells in different phases of cell cycle such as G_0, G_1, S and G_2/M in the M1, M2, M3 and M4 regions of the cytogram (Figure 2.9A). We observed that cells entering into G_0 phase increased significantly in a time dependent manner after allethrin treatment (Figure 2.9B).

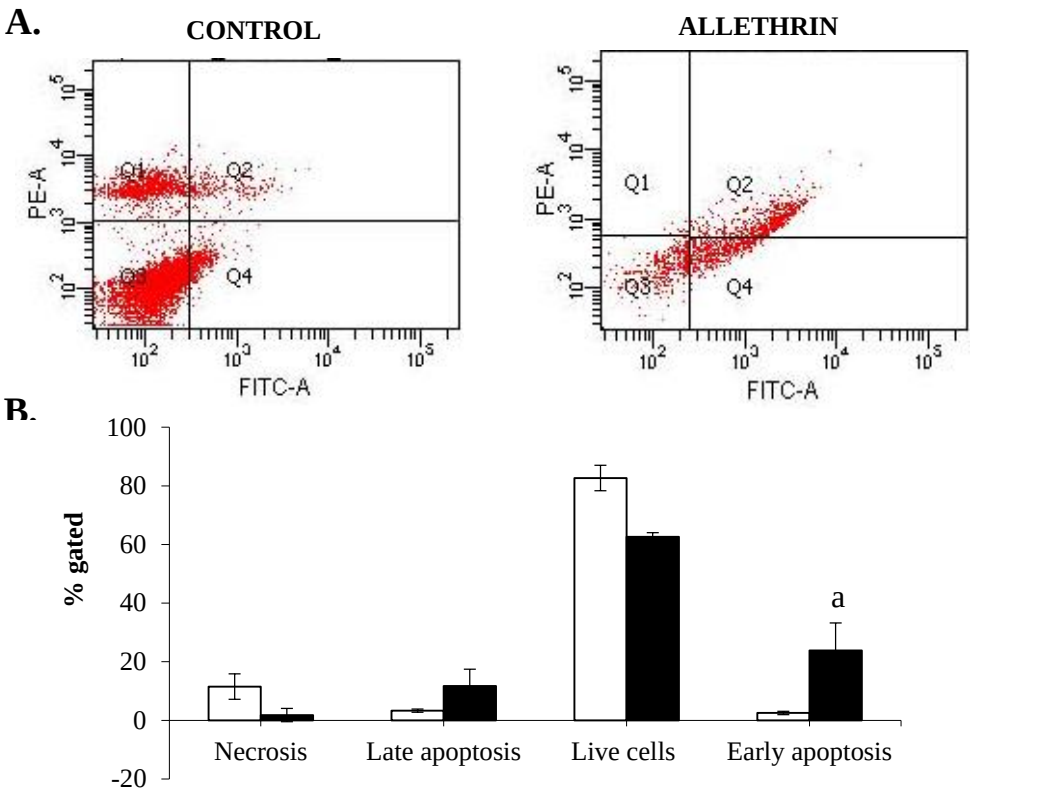


Figure 2.8. Apoptotic features in allethrin treated cells. (A) *Histogram of annexin-V assay. Events in the Q1, Q2, Q3 and Q4 quadrants of the cytogram indicate necrotic, late apoptotic, live and early apoptotic cells respectively.* ***(B)*** *Graphical representation of histogram. Control (□) and allethrin treated cells (■). Values shown are Mean ± S.D. a indicates p <0.05*

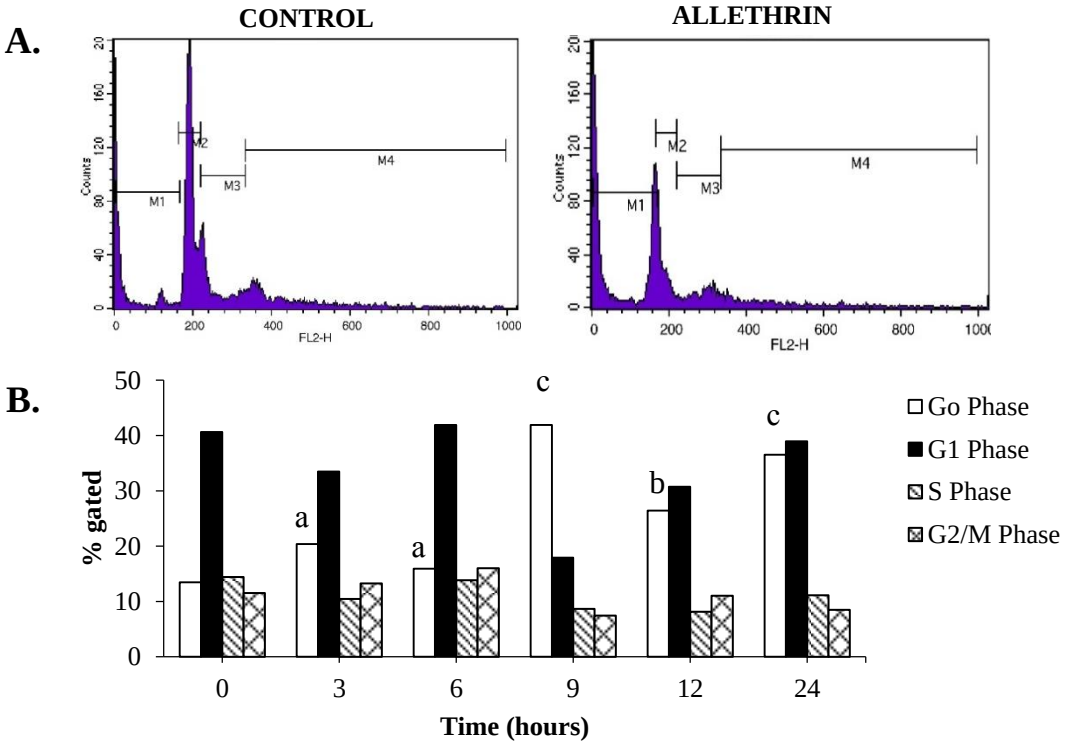


Figure 2.9. Apoptotic features in allethrin treated cells. (A) *Cytograms of control and allethrin treated cells (24 h).* ***(B)*** *Cell cycle analyses in control and allethrin treated cells using propidium iodide staining. M1, M2, M3 and M4 in the cytogram indicate cells that are in G_0, G_1, S and G_2/M phases of cell cycle respectively. Cells in the G_0 phase are indicated in (□).Values shown are Mean ± S.D. a, b and c indicates p <0.05, p <0.01 and p <0.001 respectively.*

The changes in MMP in cells treated with allethrin were assessed by Rhodamine staining followed by FACS analyses. The fluorescence intensity decreased significantly in cells treated with 125 μM allethrin indicating loss of MMP (Figure 2.10A and B); and the decrease was more than that induced by the MMP lowering drug doxorubicin. From the above results, it is clear that allethrin induces apoptosis in LC540 cells.

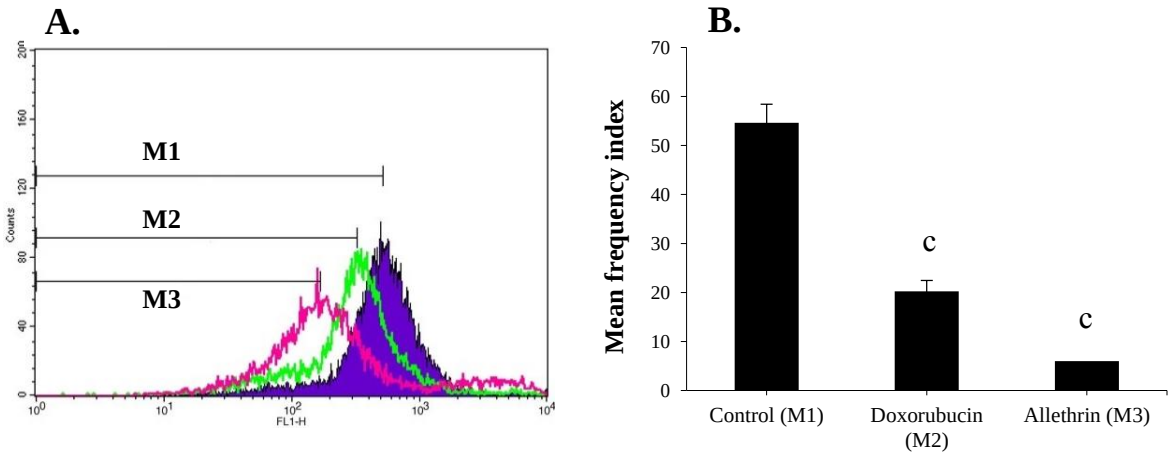


Figure 2.10. Apoptotic indicators in allethrin treated cells. *Mitochondrial membrane potential measurement in cells treated with allethrin or doxorubicin (10 μM). M1, M2 and M3 in the cytogram* ***(A)*** *indicate mitochondrial membrane dependent in fluorescence in control, doxorubicin and allethrin treated cells. Graphical* ***(B)*** *representation of cytogram.*

8. Effect of allethrin on cell cycle regulators and apoptotic proteins

A number of molecular factors such as p53, BCL-2, Caspase-3 and PARP-1 play a key role in the execution apoptosis. We analyzed their expression pattern n LC540 cells treated with allethrin. The mRNA levels of *p53* increased significantly at all the time points 6 h after allethrin treatment (Figure 2.11).

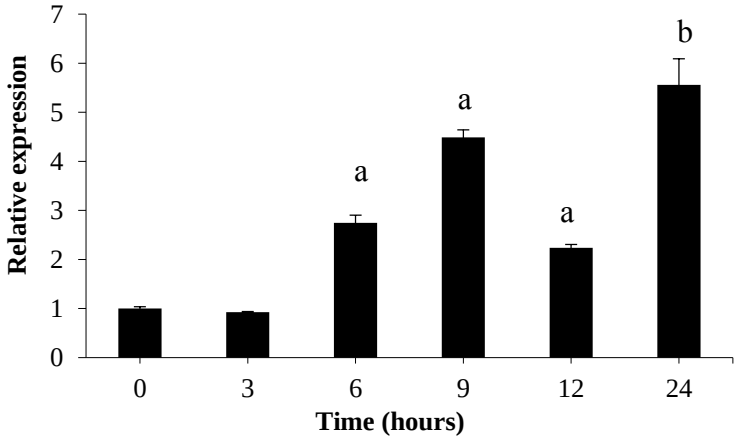


Figure 2.11. Apoptotic indicators in allethrin treated cells. *p53 gene expression. RNA isolated from allethrin treated cells was reverse transcribed and the expression of p53 analyzed using real time PCR. Values shown are Mean ± S.D. a, b and c indicates p <0.05, p <0.01 and p <0.001 respectively.*

To gather further evidence that allethrin induces apoptosis, Western blotting was performed to analyze BCL-2, pro-caspase-3 and PARP protein expression, the key indicators of apoptotic death. We observed decreased levels of BCL-2, pro-caspase-3 and cleaved PARP indicating the involvement of apoptosis in allethrin induced cell death (Figure 2.12A and B). Further, we observed that caspase-3-activity was also increased in a time dependent manner (Figure 2.12C).

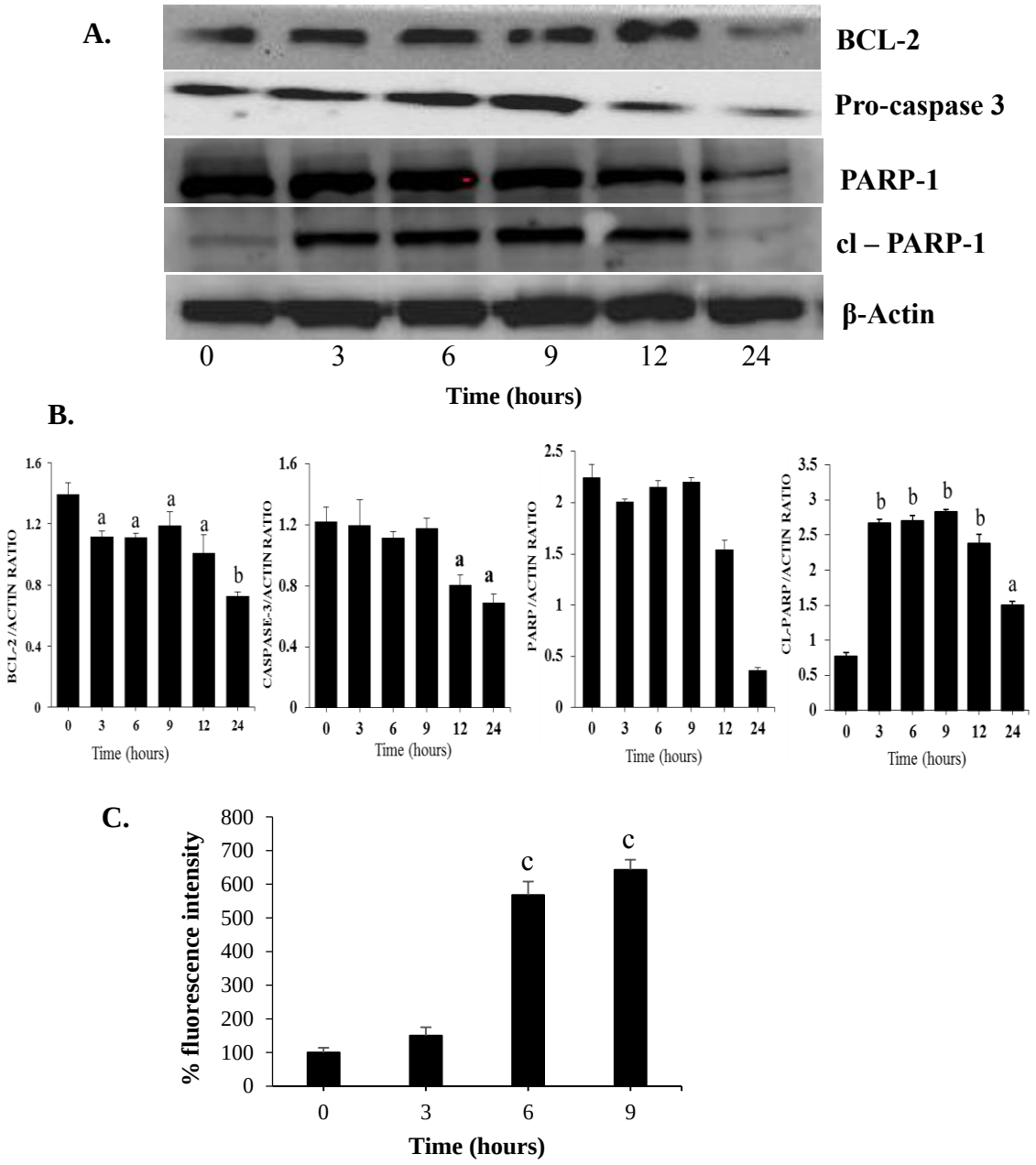


Figure 2.12. Expression pattern of apoptotic effectors. (A) *Western blotting analyses for BCL-2, pro-Caspase-3, PARP-1 and cleaved (cl) PARP-1. Result shown is a representative of three independent experiments.* ***(B)*** *Densitometric analyses to demonstrate the differential expression levels.* ***(C)*** *Caspase-3 activity in allethrin treated cells. Values shown are Mean ± S.D. a, b and c indicates p <0.05, p <0.01 and p <0.001 respectively.*

9. Effect of allethrin on calcium dynamics

The ability of allethrin to induce calcium release from intracellular sources was evaluated. Increased fluorescence (emission at 340 nm) exhibited by calcium bound FURA was observed immediately after allethrin addition in a dose-dependent fashion (Figure 2.13A). Surprisingly, a dose-dependent increase in the fluorescence intensity (emission at 380 nm) of free FURA 2-AM also increased in allethrin treated cells (Figure 2.13B). The increase in the intensity of fluorescence exhibited by free FURA was such that when the ratio of fluorescence exhibited at 340 and 380 nm was plotted, the net effect seems to appear that there is an overall decrease in fluorescence (data not shown). Hence, the changes in the fluorescence emitted at 340 and 380 nm were plotted separately (Figure 2.13A and B). Pretreatment of LC540 cells with L- and P- type VGCC blockers inhibited allethrin induced release of intracellular calcium (Figure 2.14A and B).

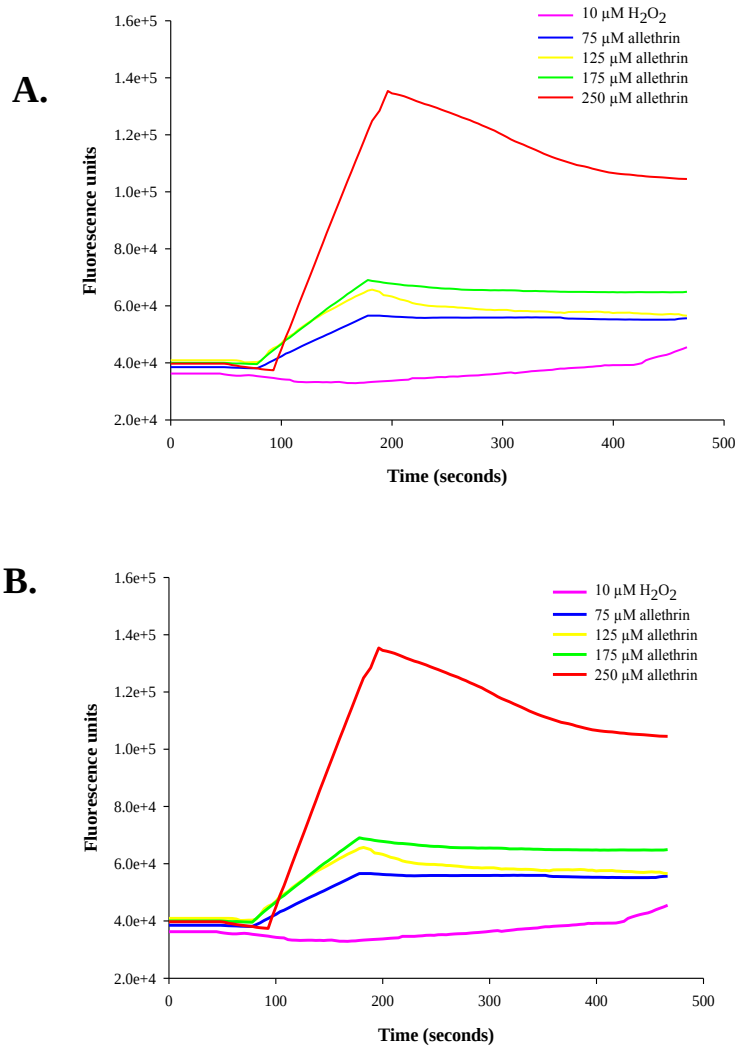


Figure 2.13. Dynamics of calcium concentration*. LC540 cells were incubated with FURA-2AM and treated with 75 – 250 µM allethrin and fluorescence levels measured at 340* ***(A)*** *and 380 nm* ***(B)*** *to detect calcium bound FURA and free FURA. As a positive control hydrogen peroxide was used. Result shown is a representative of three independent experiments.*

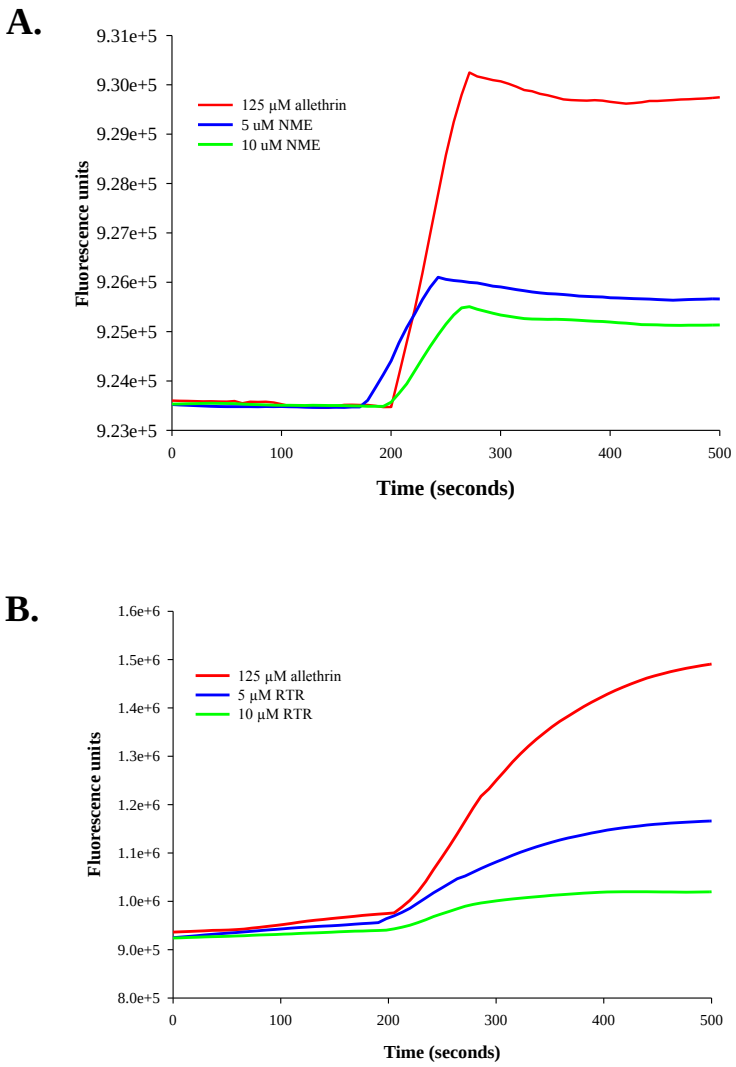


Figure 2.14: Voltage gated calcium channel mediated calcium release*. Prior to treatment with 125 µM allethrin, LC540 cells were preincubated with 5 and 10 µM* ***(A)*** *Nimodipine (NME) or* ***(B)*** *Ruthenium Red (RTR) for 15 min. Fluorescence was measured at 340 nm to detect calcium bound FURA.*

Discussion

Understanding the molecular mechanisms of allethrin cytotoxicity in a variety of *in vitro* models has been an active area of investigation because of its widespread domestic use. However, there is little information on the molecular mechanisms of toxicity in testicular cells, despite the fact that allethrin is demonstrated to be toxic to the male reproductive system (Madhubabu and Yenugu, 2012, 2014). The central focus of this study was to determine the mechanism by which allethrin causes cell death in testicular carcinoma cells. A dose-dependent cell death was observed when LC540 cells were treated with allethrin with an IC_{50} value of 125 µM. The structural, functional and molecular features of normal and cancer cells differ to a great extent and could result in variation in their susceptibility to toxic compounds. We observed that isolated primary Leydig cells were susceptible to the cytotoxic effects of allethrin with an IC_{50} of 59 µM. The variation in the IC_{50} values could be due to the differences in membrane composition, proliferation ability, endogenous expression of cellular proteins and overall survival mechanisms of LC540 cells and primary Leydig cells. It would be interesting to study the physiological, biochemical and molecular factors that contribute to the resistance of LC540 cells towards allethrin induced toxicity. However, the cytotoxicity observed in this study reaffirms our earlier observation that allethrin is toxic to the male reproductive system (Madhubabu and Yenugu, 2014) and the Leydig cells could be one of the target cell types.

The primary objective of this study was to evaluate the molecular mechanisms of allethrin toxicity at the cellular level. Hence, we used rat Leydig tumor cell lines as our model system, since they are easy to propagate to conduct a series of experiments. It is possible that allethrin may have deleterious effects on germ cells or Sertoli cells or other cells of the male reproductive tract. Further, allethrin dose used in this study is not precisely related to our previous *in vivo* studies (Madhubabu and Yenugu, 2012, 2014). Despite many on the toxic effects of pyrethroids on male reproductive function, their exact concentrations at the organ (testicular / epididymal) level during the experimental period are not documented. In this study, we focused on the molecular mechanisms of allethrin toxicity using IC_{50} concentration, though the best possible approach would be to treat the cells with the doses that exist at the organ level after allethrin exposure. Hence, our results are only an indicative of the possible toxic effects of allethrin *in vivo*.

It is well established that environmental factors including pyrethroid toxicity induces oxidative stress in many organ systems. Oxidative stress results in the generation of ROS, which in turn attack cellular membranes and DNA to induce repair mechanisms or initiate programmed cell death. ROS interaction with cellular membranes and DNA leads to the formation of lipid peroxidation products and strand breaks respectively. Oxidative stress during pyrethroid metabolism has been demonstrated both *in vitro* and *in vivo* (El-Demerdash, 2011; Giray *et al.*, 2001). In this study, ROS generated in LC540 cells could be due to the intermediate products of allethrin metabolism. Lipid peroxidation products were also found to be increased suggesting that the free radicals generated during allethrin treatment contribute to the peroxidation of membrane lipids and subsequently damage to cellular membranes. It is interesting to note that ROS generation increased within 1 h after allethrin treatment without any further increase in a time dependent manner. However, formation of lipid peroxidation products increased in a time dependent manner. It is possible that allethrin could have been metabolized immediately after addition and the free radicals generated could have long term actions on membrane lipids, thus generating lipid peroxidation products for longer durations. Increased oxidative stress leads to antioxidant responses that involve modulation of the activity of antioxidant enzymes.

Antioxidant enzymes such as catalase, glutathione peroxidase and superoxide disumutase, in general, aid in scavenging free radical generation, whereas glutathione-S-transferase plays a crucial role in xenobiotic metabolism to conjugate toxic compounds and facilitating clearance from the cell. Pyrethroid induced oxidative stress and the consequent lipid peroxidation is reported earlier (Sadowska-Woda *et al.*, 2010). Disruption in the antioxidant enzyme profile by pyrethroids was reported both *in vitro* and *in vivo* (Madhubabu and Yenugu, 2012, 2014). In general, we observed increased antioxidant enzyme activity at the initial time points after allethrin treatment followed by a decline. It appears that there is a protective effect conferred by the antioxidant enzymes immediately after allethrin exposure to scavenge free radicals. However, prolonged free radical presence could have led to failure of antioxidant defense mechanisms which is evident as a decrease of antioxidant enzyme activity. The marked induction of glutathione peroxidase and catalase at 9 and 24 h respectively could be one of the many protective mechanisms initiated by cells to combat allethrin induced toxicity. Decreased glutathione-S-transferase activity after allethrin treatment suggests failure of xenobiotic metabolism machinery.

Oxidative stress initiates of a number of biochemical and molecular events that direct the cells to either mount repair mechanisms or undergo self destruction. It is well known that apoptosis is the ubiquitous form of cell death under stress conditions induced by a variety of environmental toxicants including pyrethroids. The shift in dynamics of pro and anti-apoptotic gene expression during cellular stress determines the fate of the cell to either undergo apoptotic cell death or survive by initiating defense mechanisms. Transfluthrin and its metabolite tetrafluorobenzoic acid cytotoxicity on urothelial cells of the rat involved oxidative stress mediated apoptosis (Yokohira *et al.,* 2011). Deltamethrin induces apoptosis in the hippocampal neurons (Grosse *et al.,* 2002) and brain cells of the rat (Wu *et al.,* 2003). Similarly, pyrethroids induce apoptotic cell death in various organ systems including the male reproductive system (El-Gohary *et al.,* 1999; Hossain and Richardson, 2011). However, the molecular changes that occur during allethrin induced apoptosis are not reported. We observed the formation of apoptotic bodies, increased number of cells with cycle arrest and signs of early apoptosis, loss of membrane potential, decreased levels of BCL-2, pro-Caspase-3 and PARP-1 and increased levels of *p53* mRNA and cleaved PARP-1, all of which are the classical events that occur during apoptotic cell death. During apoptotic cell death, pro-caspase-3 is cleaved to form active caspase-3, which in turn acts on PARP-1. The decrease in the levels of pro-Caspase-3 after allethrin treatment could be due to its cleavage and subsequent activation. PARP-1 is a substrate for activated caspase-3. Increased levels of cleaved PARP-1 observed in this study may be due to cleavage of full length PARP-1 by the activated caspase-3. The dynamics of gene expression during oxidative stress determines the fate of the cell to either undergo apoptosis or mount defense mechanisms. The expression of *p53* gene during stress is vital for the cell to undergo apoptosis. Alterations in the expression of apoptosis related genes during oxidative stress in the testes were reported (Hsu *et al.,* 2007). We observed decreased *p53* expression after allethrin treatment and this could be the signal for the cells to undergo apoptosis. To the best of our knowledge, these results for the first time suggest that allethrin mediated cell death involves apoptotic mechanisms. It would be interesting to conduct further studies to determine whether allethrin induced apoptosis could be due to the direct modulation of pro apoptotic genes at the promoter level, besides the classical pathways.

Intracellular calcium levels are crucial in regulating a variety of cellular functions. Increase in intracellular calcium level is a key signal for apoptosis and necrosis, whereas lower calcium levels compromise cellular processes (Clapham, 1995).

We observed oxidative stress induced apoptosis in LC540 cells treated with allethrin. The correlation between oxidative stress induced apoptosis with the calcium dynamics during allethrin toxicity, especially in the male reproductive cells are not reported. Increased calcium levels as indicated by enhanced fluorescence emitted by calcium bound FURA, indicated that during allethrin toxicity, release of calcium could be due to the direct of allethrin so as to facilitate the opening of calcium channels located on the membranes of calcium storage organelles; or it could be due to the secondary effects of oxidative stress induced by allethrin. These changes in calcium dynamics are strengthened by the fact that the assays were performed in cells suspended in calcium free medium. It is demonstrated in oral cancer cells that deltamethrin affects calcium release in a PLC independent mechanism and its uptake is mediated by nifedipine sensitive calcium channels (Chi *et al.*, 2014). Further studies that involves pretreatment of LC540 cells with antioxidant molecules such as reduced glutathione will help to pin point whether induction of apoptosis observed in this study is due to allethrin itself (by modulating the voltage gated calcium channels) or due to the resultant oxidative stress. Surprisingly, we also found that the fluorescence emitted by calcium free FURA is also increased following allethrin treatment, indicating that allethrin may affect calcium efflux from the cells or compete with FURA for its binding and subsequently chelating it. Sudden decrease in intracellular calcium is also detrimental to the cells and in the event of allethrin chelating the calcium, the cellular death could also be due to this physiological change. Decreased calcium release in LC540 cells pretreated with L- and P/Q- type VGCC blockers indicate that allethrin causes release of calcium from intracellular stores. It will be worthwhile to determine the role of specific VGCC subtypes involved in calcium ion dynamics (release from intracellular stores and / or uptake from the extra cellular medium) during allethrin toxicity. More importantly, the possible calcium chelating or calcium effluxing ability of allethrin needs attention.

REFERENCES

Abarikwu SO, Farombi EO, Kashyap MP, and Pant AB. (2011). Atrazine induces transcriptional changes in marker genes associated with steroidogenesis in primary cultures of rat Leydig cells. *Toxicology in Vitro. 25*(8), 1588–95.

Aebi H. (1984). Catalase. *Methods in Enzymology*, *105*(1947), 121–126.

Abou-Donia MB, Goldstein LB, Jones KH, Abdel-Rahman AA, Damodaran TV, Dechkovskaia AM, Dechkovskaia AM, Bullman SL, Amir BE, and Khan WA. (2002). Locomotor and sensorimotor performance deficit in rats following exposure to pyridostigmine bromide, DEET, and permethrin. Alone and in combination. *Toxicological Sciences*, *68*(2), 516–7.

Aldred LF and Cooke BA. (1983). The effect of cell damage on the density and steroidogenic capacity of rat testis Leydig cells, using an NADH exclusion test for determination of viability. *Journal of Steroid Biochemistry*, *18*(4), 411–4.

Anvita Srivastava, Srivastava MK, and Raizada RB. (2006). Ninety-day toxicity and one-generation reproduction study in rats exposed to allethrin-based liquid mosquito repellent. *The Journal of Toxicological Sciences*, *31*(1), 1–7.

Bal R, Naziroglu M, Turk G, Yilmaz O, Kuloglu T, Etem E, and Baydas G. (2012). Insecticide imidacloprid induces morphological and DNA damage through oxidative toxicity on the reproductive organs of developing male rats. *Cell Biochemistry and Function*, *30*(6), 492–9.

Banerjee BD, Seth V, Bhattacharya A, and Pasha ST. (1999). Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. *Toxicology Letters*, *107(1-3)*, 33–47.

Bernheim F. (1964). The oxidative desulfuration of thio-acids. *Biochim. Biophys. Acta*, *90*, 426–428.

Booth LA, Tavallai S, Hamed HA, Cruickshanks N, and Dent P. (2014). The role of cell signalling in the crosstalk between autophagy and apoptosis. *Cellular Signalling*, *26*(3), 549–55.

Bootman MD, Berridge MJ, and Roderick HL. (2002). Calcium signalling : more messengers, more channels, more complexity. *Current Biology*, *12*(02), 563–565.

Chi CC, Chou CT, Liang WZ, and Jan CR. (2014). Effect of the pesticide, deltamethrin, on Ca2+ signaling and apoptosis in OC2 human oral cancer cells. *Drug and Chemical Toxicology*, *37*(1), 25–31.

Clapham DE. (1995). Intracellular calcium. Replenishing the stores. *Nature*, *375*(6533), 634–5.

Clark JM, and Symington SB. (2008). Neurotoxic implications of the agonistic action of CS-syndrome pyrethroids on the N-type Ca v 2 . 2 calcium channel. *Pest Management Science*, *638*(August 2007), 628–638.

Eil C, and Nisula BC. (1990). The binding properties of pyrethroids to human skin fibroblast androgen receptors and to sex hormone binding globulin. *Journal of Steroid Biochemistry*, *35*(3-4), 409–14.

El-Demerdash FM. (2011). Lipid peroxidation, oxidative stress and acetylcholinesterase in rat brain exposed to organophosphate and pyrethroid insecticides. *Food and Chemical Toxicology*, *49*(6), 1346–52.

El-Gohary M, Awarab WM, Nassarc S, and Hawas S. (1999). Deltamethrin-induced testicular apoptosis in rats: the protective effect of nitric oxide synthase inhibitor. *Toxicology*, *132*(1), 1–8.

Eruslanov E, and Kusmartsev S. (2010). Identification of ROS using oxidized DCFDA and flow-cytometry. *Methods in Molecular Biology*, *594*, 57–72.

Garey J, and Wolff MS. (1998). Estrogenic and antiprogestagenic activities of pyrethroid insecticides. *Biochemical and Biophysical Research Communications*, *251*(3), 855–9.

Giray B, Gürbay A, and Hincal F. (2001). Cypermethrin-induced oxidative stress in rat brain and liver is prevented by Vitamin E or allopurinol. *Toxicology Letters*, *118*(3), 139–146.

Go V, Garey J, Wolff MS, and Pogo BGT. (1999). Estrogenic potential of certain pyrethroid compounds in the MCF-7 human breast carcinoma cell line. *Environmental Health Perspectives*, *107*(3), 173–177.

Grosse G, Thiele T, Heuckendorf E, Schopp E, Merder S, Pickert G, and Ahnert-Hilger G. (2002). Deltamethrin differentially affects neuronal subtypes in hippocampal primary culture. *Neuroscience*, *112*(1), 233–241.

Hadnagy W, Seemayer NH, Kühn KH, Leng G, and Idel H. (1999). Induction of mitotic cell division distrubances and mitotic arrest by pyrethroids in V79 cell cultures. *Toxicology Letters*, *107*(1-3), 81–7.

Hsu PC, Pan MH, Li LA, Chen CJ, Tsai SS, and Guo YL. (2007). Exposure in utero to 2,2',3,3',4,6'-hexachlorobiphenyl (PCB 132) impairs sperm function and alters testicular apoptosis-related gene expression in rat offspring. *Toxicology and Applied Pharmacology*, *221*(1), 68–75.

Hossain MM, and Richardson JR. (2011). Mechanism of pyrethroid pesticide-induced apoptosis: role of calpain and the ER stress pathway. *Toxicological Sciences*, *122*(2), 512–25.

Institoris L, Ndeger L, Siroki O, Nehez M, and Desi I. (1999). Comparison of detection sensitivity of immuno- and genotoxicological effects of subacute cypermethrin and permethrin exposure in rats. *Toxicology*, *137*(1), 47–55.

Issam C, Samir H, Zohra H, Monia Z, and Hassen BC. (2009). Toxic responses to deltamethrin (DM) low doses on gonads, sex hormones and lipoperoxidation in male rats following subcutaneous treatments. *The Journal of Toxicological Sciences*, *34*(6), 663–670.

Issam C, Zohra H, Monia Z, and Hassen BC. (2011). Effects of dermal sub-chronic exposure of pubescent male rats to permethrin (PRMT) on the histological structures of genital tract, testosterone and lipoperoxidation. *Experimental and Toxicologic Pathology*. *63*(4), 393–400.

Jakoby WB. (1978). The glutathione S-transferases: a group of multifunctional detoxification proteins. *Advances in Enzymology and Related Areas of Molecular Biology*, *46*, 383–414.

Kale M, Rathore N, John S, and Bhatnagar D. (1999). Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes : a possible involvement of reactive oxygen species. *Toxicology Letters, 105*, 197–205.

Kasat K, Go V, and Pogo BG. (2002). Effects of pyrethroid insecticides and estrogen on WNT10B proto-oncogene expression. *Environmenta Internationals, 28*, 429–432.

Li H, Shi N, Chen D, Dai Z, Lu W, Wang B, and Li Y. (2005). [Oxidative stress of deltamethrin on rat nervous system]. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi*, *23*(2), 97–101.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*(1), 265–75.

Madhubabu G, and Yenugu S. (2012). Effect of continuous inhalation of allethrin-based mosquito coil smoke in the male reproductive tract of rats. *Inhalation Toxicology*, *24*(3), 143–52.

Madhubabu G, and Yenugu S. (2014). Allethrin induced toxicity in the male reproductive tract of rats contributes to disruption in the transcription of genes involved in germ cell production. *Environmental Toxicology*, *29*(11), 1330–45.

Maiti PK, and Kar A. (1997). Dual role of testosterone in fenvalerate- treated mice with respect to thyroid function and lipid peroxidation. *Journal of Applied Toxicology, 17(2)*, 127–131.

Maiti PK, Kar A, Gupta P, and Chaurasia SS. (1995). Loss of membrane integrity and inhibition of type-I iodothyronine 5'-monodeiodinase activity by fenvalerate in female mouse. *Biochemical and Biophysical Research Communications*, *214*(3), 905–9.

McCord JM, and Fridovich I. (1969). Superoxide Dismutase. An enzymic function for Erythrocuprein (Hemocuprein). *The Journal of Bological Chemistry*, *244*(22), 6049–6055

Meyer DA, and Shafer TJ. (2006). Permethrin, but not deltamethrin, increases spontaneous glutamate release from hippocampal neurons in culture. *Neurotoxicology*, *27*(4), 594–603.

Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1-2), 55–63.

Narahashi T. (1986). Toxins that modulate the sodium channel gating mechanism. *Annals of the New York Academy of Sciences, 479*, 133–151.

Nasuti C, Gabbianelli R, Falcioni ML, Di Stefano A, Sozio P, and Cantalamessa F. (2007). Dopaminergic system modulation, behavioral changes, and oxidative stress after neonatal administration of pyrethroids. *Toxicology*, *229*(3), 194–205.

Naziroglu M, Butterworth PJ, and Sonmez TT. (2011). Dietary vitamin C and E modulates antioxidant levels in blood, brain, liver, muscle, and testes in diabetic aged rats. *International Journal for Vitamin and Nutrition Research.*, *81*(6), 347–57.

Neal AP, Yuan Y, and Atchison WD. (2010). Allethrin differentially modulates voltage-gated calcium channel subtypes in rat PC12 cells. *Toxicological Sciences*, *116*(2), 604–613.

Paglia DE, and Valentine WN. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of Laboratory and Clinical Medicine*, *70*(1), 158–69.

Paschen W. (2000). Role of calcium in neuronal cell injury: which subcellular compartment is involved? *Brain Research Bulletin*, *53*(4), 409–413.

Pontecorvo G, and Fantaccione S. (2006). Recombinogenic activity of 10 chemical compounds in male germ cells of Drosophila melanogaster. *Ecotoxicology and Environmental Safety*, *65*(1), 93–101.

Ray DE, and Fry JR. (2006). A reassessment of the neurotoxicity of pyrethroid insecticides. *Pharmacology & Therapeutics*, *111*(1), 174–93.

Romero A, Ramos E, Castellano V, Martínez MA, Ares I, Martinez M., Martínez-Larrañaga MR, and Anadon A. (2012). Cytotoxicity induced by deltamethrin and its metabolites in SH-SY5Y cells can be differentially prevented by selected antioxidants. *Toxicology in Vitro*, *26*(6), 823–30.

Sadowska-Woda I, Wójcik N, Karowicz-Bilińska A, and Bieszczad-Bedrejczuk E. (2010). Effect of selected antioxidants in beta-cyfluthrin-induced oxidative stress in human erythrocytes in vitro. *Toxicology in Vitro*, *24*(3), 879–84.

Sattin RW, Roisin A, Kafrissen ME, Dugan JB, and Farer LS. (1984). An epidemic of gynecomastia among illegal Haitian entrants. *Public Health Reports*, *99*(5), 504–510.

Scaduto RC, and Grotyohann LW. (1999). Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophysical Journal*, *76* (January), 469–477.

Shukla Y, Yadav A, and Arora A. (2002). Carcinogenic and cocarcinogenic potential of cypermethrin on mouse skin. *Cancer Letters*, *182*(1), 33–41.

Tomlin C. (1994). A world compendium: The pesticide manual. *British Crop Protection Council*. Farnham Surrey; Cambridge.

Wang XZ, Liu SS, Sun Y, Wu JY, Zhou YL, and Zhang JH. (2009). Beta-cypermethrin impairs reproductive function in male mice by inducing oxidative stress. *Theriogenology*, *72*(5), 599–611.

Wu A, Li L, and Liu Y. (2003). Deltamethrin induces apoptotic cell death in cultured cerebral cortical neurons. *Toxicology and Applied Pharmacology*, *187*(1), 50–57.

Yokohira M, Arnold LL, Lautraite S, Sheets L, Wason S, Stahl B, Eigenberg D, Pennington KL, Kakiuchi-Kiyota S, and Cohen SM. (2011). The effects of oral treatment with transfluthrin on the urothelium of rats and its metabolite, tetrafluorobenzoic acid on urothelial cells in vitro. *Food and Chemical Toxicology*, *49*(6), 1215–1223.

Yoshida I, Monji A, Tashiro K, Nakamura K, Inoue R, and Kanba S. (2006). Depletion of intracellular Ca2+ store itself may be a major factor in thapsigargin-induced ER stress and apoptosis in PC12 cells. *Neurochemistry International*, *48*(8), 696–702.

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Chapter 2

Effect of continuous exposure to allethrin-based mosquito coil smoke on the male reproductive tract.

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Introduction

Humans are exposed to a variety of insecticides which are used for domestic and agricultural purposes. These insecticides primarily contain organochlorines, organophophates, carbamates and pyrethroids such as allethrin, resmethrin, tetramethrin, cypermethrin, deltamethrin, etc. Pyrethrins exhibit low levels of toxicity and are rapidly metabolized (Casida, 1995). Basing on their structure and toxicity, they are classified into type I and II (Verschoyle and Aldridge, 1980) and are known to mainly act on the nervous system (Soderlund *et al.,* 2002). The principal mechanism of pyrethroid toxicity involves the inhibition of voltage sensitive sodium channels besides inhibition of sodium-potassium ATPase in the neuronal membranes (Kakko *et al.,* 2003; Tan and Soderlund, 2010; Vijverberg and de Weille, 1985). The effects of pyrethroids on other organ systems including the reproductive system are also reported. Pyrethroids are shown to induce oxidative stress and alter antioxidant system in different organ systems of rats (Giray *et al.,* 2001; Kale *et al.,* 1999; Li *et al.,* 2005; Maiti *et al.,* 1995). They are known to be estrogenic and antiprogestagenic in endometrial and breast cancer cell lines (Garey and Wolff, 1998; Go *et al.,*1999; Kasat *et al.,* 2002), inhibit testosterone binding to the androgen receptor and sex hormone binding globulin at high concentrations (Eil and Nisula, 1990) and cause gynecomastia (Sattin *et al.,* 1984). Further, it is reported that pyrethroid compounds cause oxdative stress in the male reproductive tract (Issam *et al.,* 2009; Issam *et al.,* 2011; Wang *et al.,* 2009).

Mosquito repellants (mats, coils and vaporizers) contain d-allethrin, a mixture of eight stereoisomers that belong to the type I pyrethroids. In developing countries, mosquito coils are widely used in households (in closed door settings) and human exposure occurs due to inhalation of allethrin emitted along with the smoke. Analysis of air samples after mosquito coil usage in a room showed a maximum concentration of allethrin (0.0120 ppm) within 30-45 min of use followed by a decline up to 6 h (Ramesh and Vijayalakshmi, 2001). Allethrin exposure via inhalation leads to moderate toxicity in rodent models (Tomlin, 1994). Though information about allethrin toxicity on the nervous system is available and to some extent on other organ systems, its effect on the reproductive tract is not well understood. Exposure to environmental pollutants such as pesticides and heavy metals contribute to the development of cancer and reduction in reproductive functions (Issam et al., 2009; Sharpe and Skakkebaek, 1993; Sharpe and Irvine, 2004; Tan and Soderlund, 2010; Wang *et al.,* 2009). Previous studies indicated that rats exposed to allethrin based liquid mosquito repellants (using a vapouriser) for 90

days do not show any adverse effect on the clinical enzyme profile and reproductive indices in weaning and adult rats (Anvita Srivastava, 2006). Further, it is demonstrated that allethrin exhibits recombinogenic activity in the male germ cells of *Drosophila melanogaster* (Pontecorvo and Fantaccione, 2006).

Because of the wide spread use of allethrin based mosquito repellent coils, adults and children are exposed and their direct effects on the functioning of the reproductive tract needs investigation. Previous studies on the effect of mosquito repellents on reproductive tract used vapourisers, which creates a milder toxic environment when compared to mosquito coils. Studies focusing on harsher environments created by mosquito coils in different organ systems are not available. Hence, in this study, we attempt to determine the effect of mosquito coil smoke exposure (similar to that is experienced in a household setting) in the male reproductive tract of rat. Though fertility studies were undertaken to determine the effect of vapourisers, the effect of exposure to mosquito coil smoke on the pathological changes that occur in the reproductive tract is not reported till now. These morphological, cellular and hormonal effects may involve prior disturbance in molecular mechanisms and there are scanty reports in the literature citing the effect of exposure to mosquito coil smoke on disturbances in oxidant and anti-oxidant status, induction of apoptosis, inflammation and expression of male reproductive tract genes involved in spermatogenesis. Free radical generation in the male reproductive tract affects fertility (Ochsendorf, 1999). Apoptosis is a consequence of free radical generation and p53 being the major effector of apoptosis, the expression pattern p53 was along with free radical generation is analyzed in the male reproductive tract. A variety of germ cell differentiation factors such as the tyrosine kinase receptor, c-KIT, or its ligand, stem cell factor (SCF) and TGF-beta1 have been implicated during spermatogenesis (Besmer *et al.,* 1993; Meehan *et al.,* 2000; Olaso *et al.,* 1998). Hence in this study, the expression pattern of spermatogenic factors was analyzed in the male reproductive tract of rats exposed to allethrin based mosquito coil smoke.

We demonstrate that rats exposed to allethrin based mosquito coil smoke for 15-180 days display pathomorpholocial changes in the male reproductive tract tissues. Prolonged exposure leads to increased oxidant stress in the epididymis and testes without a marked change in the antioxidant status. Further, the stress response gene p53 expression was altered suggesting a loss of cell cycle regulation. Though the expression pattern of sperm maturation factors *c-Kit* and *Scf* were unaltered, a significant decrease in *Tgf-beta1* was observed in the testes.

Materials and Methods

1. Mosquito coil

Mosquito repellent coils were obtained from the local markets. The composition (in terms of w/w) of a typical mosquito coil as per the information given with the product is as follows: 0.1% d-trans allethrin, 40% coconut shell powder, 10% starch binder, 0.1% Lo88 emulsifier, 0.1% red dye, 0.5% fragrance, 0.3% sodium benzoate, 0.1% potassium nitrate and 6% jiggat (joss). Each mosquito coil is expected to burn for 8 hours.

2. Animals and tissue specimens

Adult male Wistar rats (90-day-old) were obtained from National Center for Laboratory Animals (NCLAS), National Institute of Nutrition (NIN), Hyderabad. They were maintained on a 12L:12D lighting schedule, at 22–25°C, with food and water *ad libitum*. After acclimatization, rats were divided into two groups (n = 5) and were housed in separate rooms. To simulate the human exposure settings, in a closed room mosquito coils were lit (one coil per night) and the animals were allowed to inhale coil smoke. Control animals were maintained under normal animal house conditions in a separate room. Animals were sacrificed by cervical dislocation and tissues were collected after 15–180 days from control and mosquito coil smoke exposed rats. Testis, caput and cauda epididymis were identified, stripped off the connective tissues, noted the weight, frozen in liquid nitrogen and kept at −70°C until use. Experiments were conducted using the guidelines for the care and use of laboratory animals, approved by the Institutional Animal Ethics Committee of University of Hyderabad (LS/IAEC/YS/2010/02).

3. Estimation of lipid peroxidation (LPO) products

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA), the product of lipid peroxidation, under acidic conditions and high temperature (95°C) to form a pink colored complex with absorbance maxima at 532 nm. 10% (w/v) homogenates of caput, cauda and testes obtained from the vehicle control and experimental animals were prepared in ice cold phosphate buffer saline, pH 7.4 and the extent of lipid peroxidation was estimated using the TBA method as described earlier (Bernheim, 1964). 1,1,3,3-tetraethoxy-propane (TEP) was used as the standard. Levels of lipid peroxidation was expressed as nano moles MDA / mg protein.

4. Antioxidant enzyme assays

Antioxidant enzyme activity was measured using spectrophotometric methods. Caput, cauda and testes collected from vehicle control and allethrin treated rats. 10% (w/v) homogenates were prepared in ice cod phosphate buffer saline, pH 7.4 and centrifuged at 3000 rpm to remove the debris. The supernatant was collected and the activities of catalase, glutathione-s-transferase (GST) and glutathione peroxidase (GPx) were performed using standard protocols described earlier (Aebi, 1984; Jakoby, 1978; Paglia and Valentine, 1967). The activity of the antioxidant enzymes were calculated from the change in optical density measured on a spectrophotometer. Detailed methodology for estimation of antioxidant enzyme activity are described in chapter 1.

5. Histopathology

Epididymides and testes fixed in Bouin's fluid for 24 h and then into 70% ethanol, which was changed 3–4 times every day until the yellow color of Bouin's fluid completely disappeared. Tissues were hydrated serially in 80, 90 and 100% ethanol and stored in isopropanol overnight at 60^0C before embedding in paraffin. Five micron sections were made for further analysis. Sections were deparaffinized by xylene and were re-hydrated serially in 100, 90, 80, 70 and 50% ethanol and then rinsed briefly in distilled water. Slides were immersed into Harris hematoxylin solution for 10 min, subsequently washed in distilled water for a while and differentiated in 1% hydrochloric acid for 30 s and then immersed in 0.2% ammonia water for 30 s to 1 min. After washing in running tap water for 10 min, sections were counterstained with 0.2% eosin Y solution for 1 min. Sections were washed in distilled water and followed by serial dehydration using 50, 70, 80, 90 and 100% alcohol. Slides were cleaned with three changes of xylene and then mounted with xylene based mounting medium. Photographs were taken on a Leica microscope.

6. Real time polymerase chain reaction (RT-PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from the epididymides and testis obtained from control and mosquito coil smoke exposed rats. Total RNA (2 μg) was reverse transcribed using 50 U Stratascript (Invitrogen, USA) and 0.5 μg of oligodT (Invitrogen) according to the manufacturer's instructions. Two microliter of the resultant cDNA was used for real-time polymerase chain reaction (PCR) analysis using SYBR master mix kit (Applied Biosystems, Warrington, UK). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) expression was

used as the internal control. A typical real-time PCR reaction was carried out with the following conditions: initial denaturation at 94°C for 10 min; 40 cycles with 94°C for 15 s and 60°C for 1 min. Negative controls (no template control and minus RT control only with RNA) were included in the assays to detect nonspecific amplification. The amplicons were sequenced to confirm their identity. Gene specific primers used to amplify cell cycle regulators and spermatogenic factors were listed in table 3.1.

Table 3.1. Gene Specific primers used to amplify cell cycle regulators and spermatogenic factors

Primer	**Sequence (5'-3')**
c-kit F	GGCCTCAGGAGCTCTATTTAC
c-kit R	GAGAGATTTCCCATCACACTC
Gapdh F	CCAATGTATCCGTTGTGGATCTG
Gapdh R	GAGTTGCTGTTGAAGTCACAG
p53 F	CAAGGCAACTATGGCTTCCAC
p53 R	GACTTCTTGTAGATGGCCATG
Scf F	GATGACCTCGTGGCATGTATG
Scf R	CAGGACCTCATGTTGAAGAGAG
Spag11e F	CACATCTGCTTTCCTGCACAG
Spag11e R	GCACCCCACATCTCAGATCTTC

7. Estimation of Serum testosterone

Blood from mosquito coil exposed and unexposed rats were collected and serum was separated by centrifugation at 5000 rpm for 15 min at 4°C. Serum was stored at -20°C until the experiment. Testosterone in the serum was estimated by using ELISA kit according to manufacturer's protocol (Omega diagnostics, UK).

8. Statistical analyses

Changes in the parameters analyzed in this study were compared with the respective control at each time point. Statistical analyses were performed using oneway ANOVA and student's t-test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are mean ± SD.

Results

1. Body and Organ weights

No significant changes in the body and organ weights were observed between the control and mosquito coil smoke exposed animals at all the time points tested (Table 3.2 and 3.3). Further, no mortality was observed during the course of study.

Table 3.2. Body weights (in grams) in mosquito coil smoke exposed rats.

	15 days	30 days	60 days	90 days	120 days	180 days
Control	252.3 ± 15.01	265.0 ± 8.12	321.0 ± 21.5	357.3 ± 11.01	326.3 ± 21.3	328.3 ± 20.2
Treated	248.3 ± 8.96	260.0 ± 12.1	345.0 ± 5.2	355.3 ± 11.03	321.6 ± 10.4	350.1 ± 10.7

Table 3.3. Organ weights (in grams) in mosquito coil smoke exposed rats.

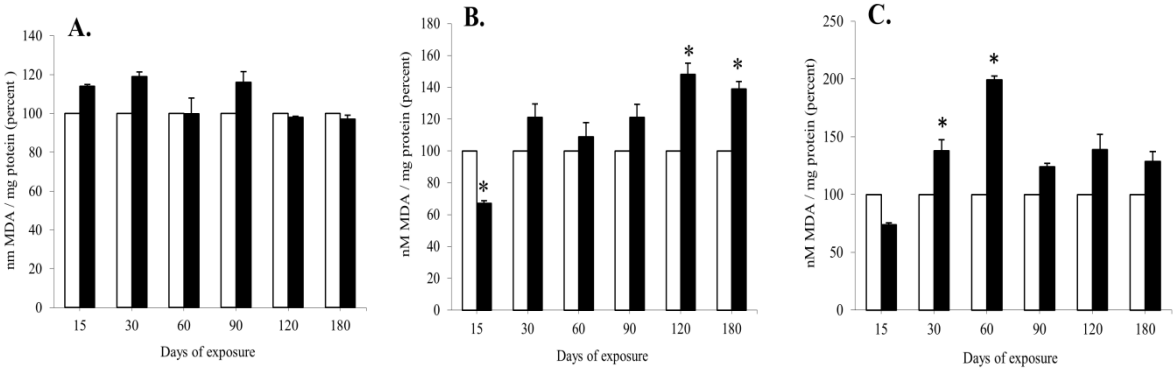
	15 days		30 days		60 days		90 days		120 days		180 days	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Caput	0.35 ± 0.1	0.36 ± 0.14	0.5 ± 0.1	0.45 ± 0.15	0.59 ± 0.3	0.56 ± 0.2	0.55 ± 0.17	0.5 ± 0.2	0.52 ± 0.13	0.56 ± 0.19	0.49 ± 0.12	0.48 ± 0.19
Cauda	0.29 ± 0.01	0.28 ± 0.02	0.46 ± 0.01	0.4 ± 0.02	0.44 ± 0.23	0.42 ± 0.18	0.44 ± 0.02	0.42 ± 0.05	0.43 ± 0.07	0.46 ± 0.09	0.37 ± 0.06	0.35 ± 0.03
Testis	2.58 ± 0.4	2.74 ± 0.7	2.9 ± 0.19	2.9 ± 0.9	3.1 ± 0.6	2.9 ± 0.4	2.92 ± 0.9	2.8 ± 0.3	2.78 ± 0.2	3.01 ± 0.2	2.53 ± 0.8	2.94 ± 0.5

2. Lipid peroxidation

Since pyrethroids are known to induce free radical generation, we measured end products of lipid peroxidation *i.e* MDA in the caput, cauda, and testes obtained from mosquito coil exposed rats. The levels of lipid peroxidation products in the control animals at different time points were considered to be 100%. Changes in lipid peroxidation in the treated groups were compared to their respective controls for each time point. In the caput, no significant changes in the lipid peroxidation status was evident (Figure 3.1A) at the time points analyzed. Significant increase in lipid peroxidation was observed in the cauda obtained from rats exposed to 120 and 180 days (Figure 3.1B). In the testes, significant increase in lipid peroxidation was observed at the

earlier time points (30 and 60 days) (Figure 3.1C). Surprisingly, decreased levels of lipid peroxidation were observed in the cauda obtained from rats exposed to mosquito coil smoke for 15 days (Figure 3.1B).

*Figure 3.1. Lipid peroxidation in the male reproductive tract of rats exposed to mosquito coil smoke. (**A**) Caput; (**B**) Cauda; (**C**) Testis. (□) Untreated control (■) Mosquito coil smoke exposed. * denotes p < 0.05.*



3. Antioxidant enzymes

Changes in oxidant status affect antioxidant defense mechanisms in many organ systems. To determine whether mosquito coil smoke-induced lipid peroxidation may have an affect on antioxidant status, the activity of catalase, GPx, and GST was measured in the caput, cauda, and testes. When compared to their respective controls, catalase activity was not altered in the caput, cauda, and testes of mosquito coil exposed animals, except for a significant increase in catalase activity in cauda obtained from 120 and 180 time point (Figure 3.2A, B, and C). No significant changes in GPx activity were observed in the caput, cauda, and testes obtained from mosquito coil smoke exposed rats, except for a decrease at the 15 day time point in the caput and testis (Figure 3.3A, B, and C). Similarly, GST activity was largely unaltered at all the time points analyzed (Figure 3.4A, B, and C). A decrease was observed in the cauda obtained at the 180 day time point and in the testes obtained at 30 day time point.

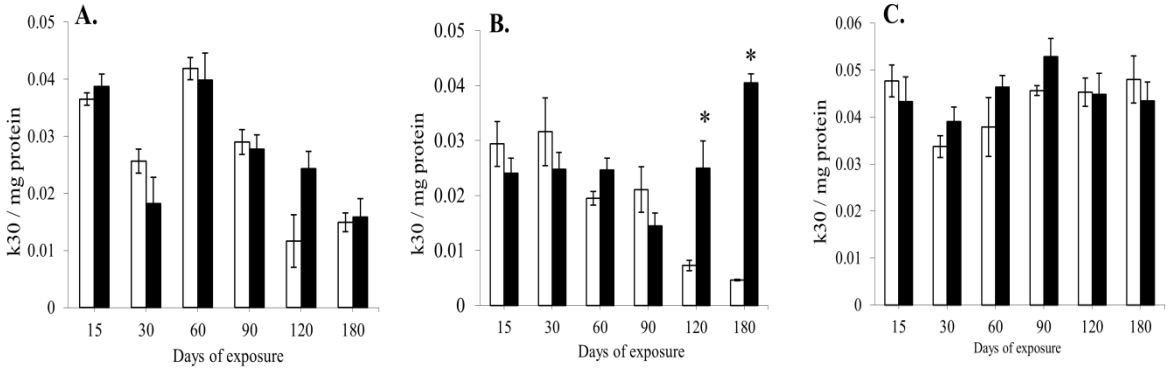


***Figure 3.2**. Catalase activity in the male reproductive tract of rats exposed to mosquito coil smoke. (**A**) Caput; (**B**) Cauda; (**C**) Testis. (□) Untreated control (■) Mosquito coil smoke exposed. * denotes p < 0.05.*

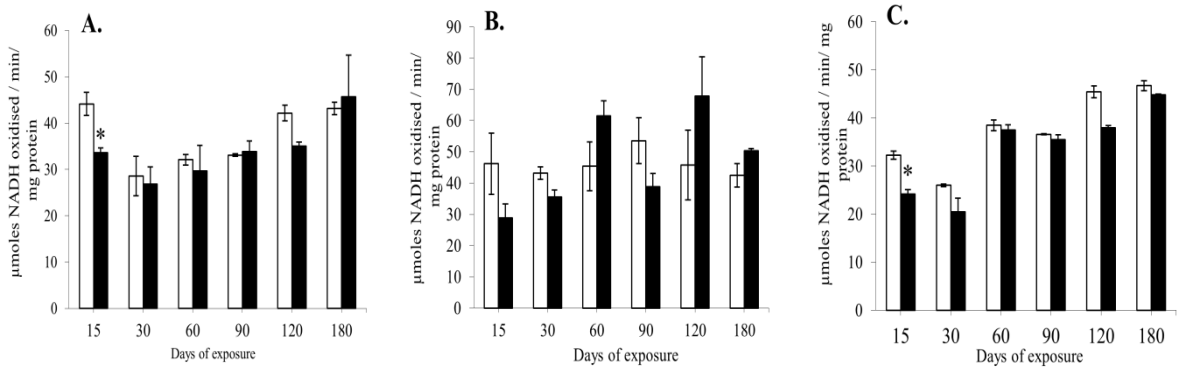


Figure 3.3*. Glutathione peroxidase activity in the male reproductive tract of rats exposed to mosquito coil smoke.* ***(A)*** *Caput;* ***(B)*** *Cauda;* ***(C)*** *Testis. (□) Untreated control (■) Mosquito coil smoke exposed. * denotes p < 0.05.*

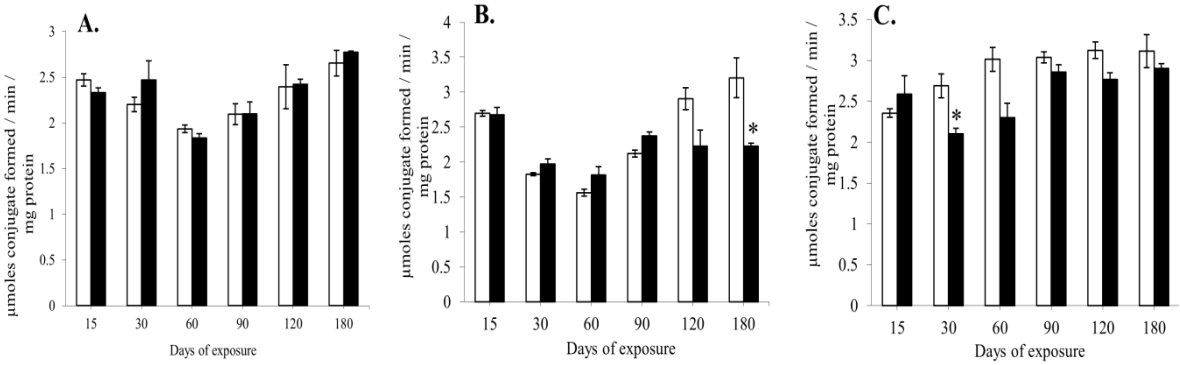


Figure 3.4*. Glutathione-s-transferase activity in the male reproductive tract of rats exposed to mosquito coil smoke.* ***(A)*** *Caput;* ***(B)*** *Cauda;* ***(C)*** *Testis. (□) Untreated control (■) Mosquito coil smoke exposed. * denotes p < 0.05.*

4. Histopathological analyses

Microscopic examination of caput, cauda, and testes obtained from control and mosquito coil smoke exposed rats for 15–180 days was performed to determine pathomorphological changes. Caput and cauda appeared apparently normal except for an occasional finding of pyknotic nuclei of the lining epithelial cells. Cells undergoing vacuolar changes were found in animals that were exposed to longer durations (Figures 3.5 and 3.6). The lesions may be categorized as mild changes only. Though no significant changes in morphology of testes were observed in rats exposed to mosquito coil smoke for 15–60 days, interstitial edema, loss of tubule architecture, epithelial cell disruption, and increase in lumen size were observed in the testes of rats exposed to 90–180 days (Figure 3.7). Similarly, dead spermatozoa, germ cell layer damage, and nuclear integrity of the gametes were observed in rats exposed to mosquito coil smoke for 90–180 days. The histopathology scores obtained are given in Table 3.4.

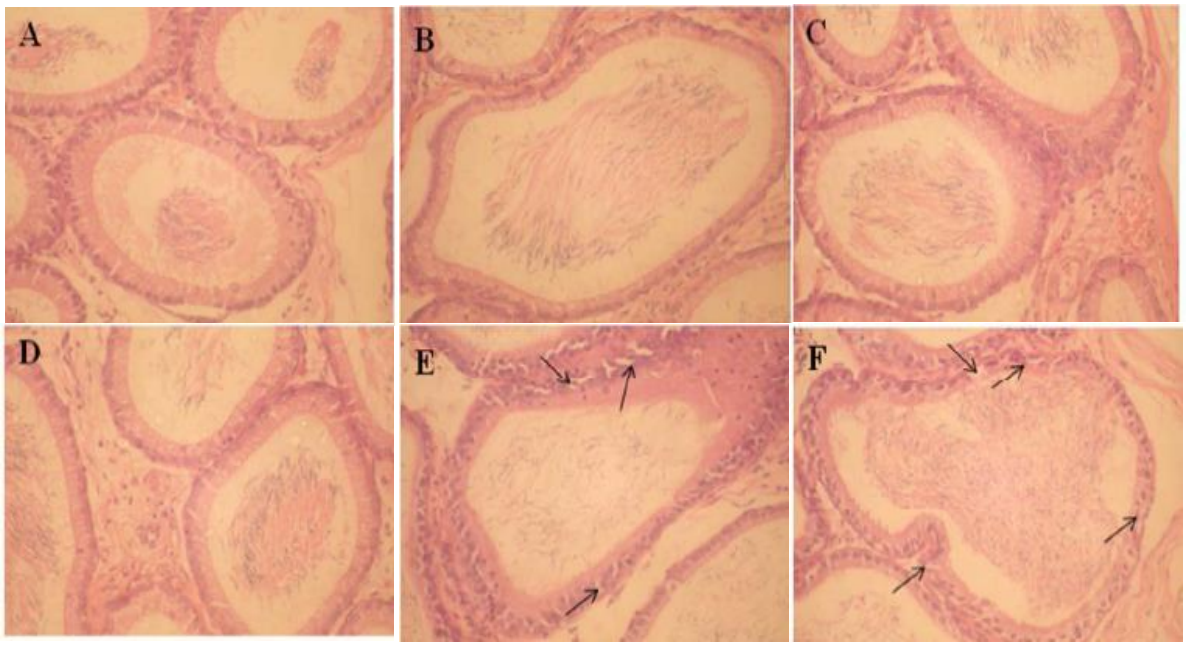


***Figure 3.5**. Histopathology of caput in the rats subjected to inhalation of mosquito coil smoke. **(A)** 15 d; **(B)** 30 d; **(C)** 60 d; **(D)** 90 d; **(E)** 120 d; **(F)** 180 d. Magnification: 40X. Arrows indicate epithelial cell damage.*

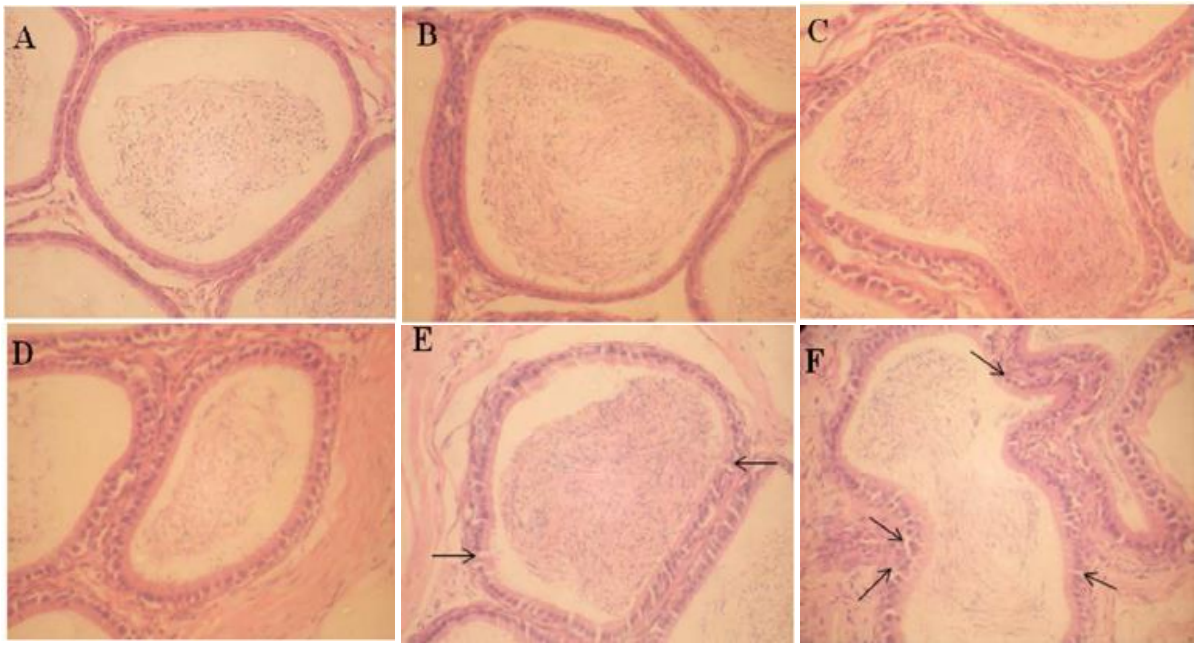


***Figure 3.6**. Histopathology of cauda in the rats subjected to inhalation of mosquito coil smoke. **(A)** 15 d; **(B)** 30 d; **(C)** 60 d; **(D)** 90 d; **(E)** 120 d; **(F)** 180 d. Magnification: 40X. Arrows indicate epithelial cell damage.*

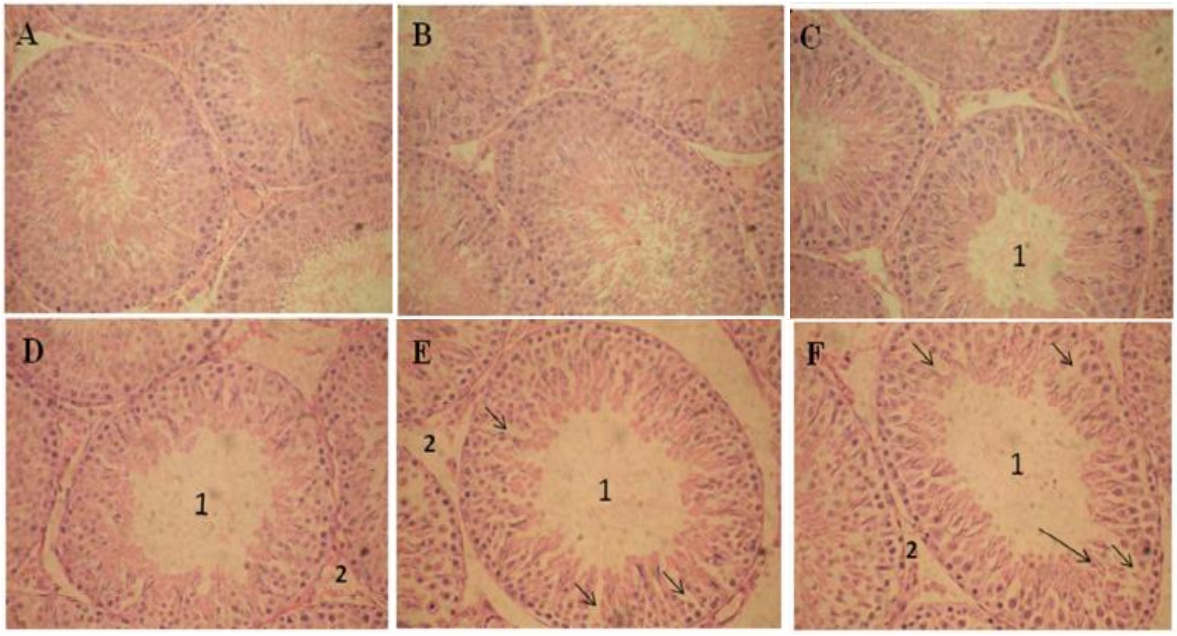


Figure 3.7*. Histopathology of testes in the rats subjected to inhalation of mosquito coil smoke.* ***(A)*** *15 d;* ***(B)*** *30 d;* ***(C)*** *60 d;* ***(D)*** *90 d;* ***(E)*** *120 d;* ***(F)*** *180 d. Magnification: 40X. "1" indicates lumen size and "2" indicates interstitial edema.*

Table 3.4. Histopathological score * of caput, cauda and testes.

S. No	Damage	15days	30days	60days	90days	120days	180days
A. Caput							
1	Dead/dry spermatozoa	-	-	-	-	-	+
2	Epithelial cell disruption	-	-	-	+	+	++
B. Cauda							
1	Dead/dry spermatozoa	-	-	-	-	+	++
2	Epithelial cell disruption	-	-	-	+	++	+++
C. Testis							
1	Interstitial odema	-	-	+	+	++	++
2	Tubule architecture disarray	-	±	+±	+±	++±	+++
3	Nuclear integrity damage	-	-	±	±	+±	++
4	Dead/dry spermatozoa	-	±	±	+	+	++
5	Epithelial cell disruption	-	-	-	-	-	+
6	Germ cell layer damage	-	±	+	++	++	+++
7	Lumen	-	±	++	++	+++	+++

On a score of 5 points:- +++++ : maximum damage; ++++ : high damage; +++ : Moderately damage; ++ : Low/moderate; + : Low; ± : more than zero, less than one; - : No lesions.

5. Gene expression

Oxidative stress leads to altered gene expression of the stress response genes. Increased free radical production contributes to DNA damage thereby initiating repair mechanisms at the cellular level. During DNA damage, p53 is known to activate a number of DNA repair proteins, thereby inducing cell-cycle arrest or allowing the cells

to undergo apoptosis. In order to determine whether increased lipid peroxidation in the reproductive system due to short-term and long-term mosquito coil exposure could initiate cell death, real-time PCR was carried out to analyze the expression of p53. In the epididymides obtained from mosquito coil exposed animals, p53 gene expression increased significantly until 90 days of exposure after which the levels reduced below the controls (Figure 3.8A). In contrast, p53 mRNA levels in the testes of mosquito coil exposed animals were decreased when compared to their respective controls throughout the experimental period (Figure 3.8B). These results suggest differential stress responses that can occur in the organ systems of the male reproductive tract.

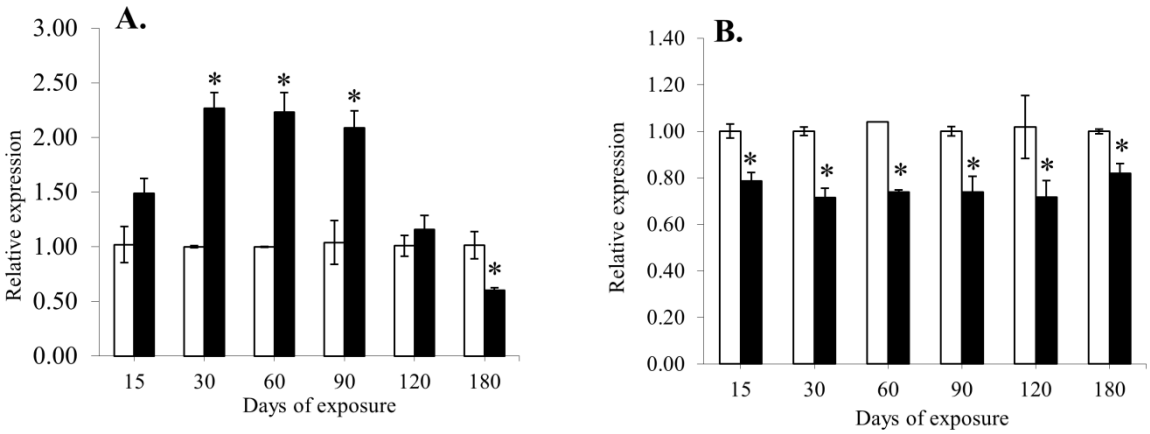


Figure 3.8*. mRNA expression of p53 in the epididymides and testes. (**A**) Epididymides **(B**) Testis. Untreated control (□); Mosquito coil smoke exposed (■).* denotes p < 0.05.*

Spermatogenesis, a complex process that occurs in the testes, is the major function of this organ system. Morphological, biochemical, and molecular changes due to a variety of factors including oxidative stress can affect spermatogenesis. To the best of our knowledge, there are no reports on the effect of short- and long-term mosquito coil smoke exposure on the molecular changes that can occur in the testes and thereby affecting spermatogenesis. A variety of spermatogenic factors such as TGF-β1, SCF, and c-KIT play a key role in spermatogenesis. To determine whether stress induced by mosquito coil smoke may have an effect on the expression profile of spermatogenic factors, real-time PCR was carried out for *Tgf-β1*, *Scf*, and *c-Kit*. *C-Kit* mRNA levels remained unchanged in the testes of mosquito coil smoke exposed rats at all the time points analyzed (Figure 3.9A). Similarly, *Scf* mRNA levels also remained unchanged during the entire experimental period (Figure 3.9B). The *Tgf-β1* mRNA levels were reduced significantly at all the time points in the testes obtained from mosquito coil smoke exposed animals when compared to their respective controls (Figure 3.9C).

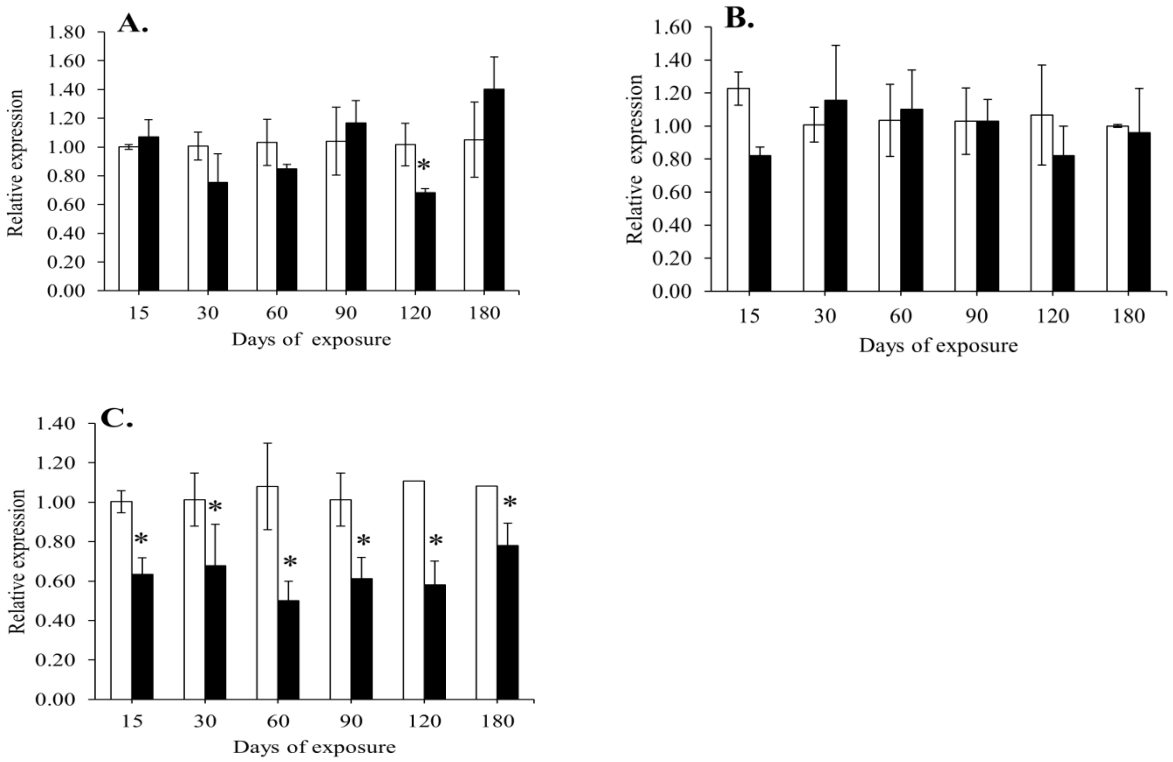


***Figure 3.9**. mRNA expression of sperm maturation factors in the testes of mosquito coil smoke exposed rats. **(A)** C-Kit; **(B)** Scf; **(C)** Transforming growth factor-β-1 (Tgf-β1). Untreated control (□); Mosquito coil smoke exposed (■).* denotes $p < 0.05$.*

Discussion

Environmental toxicants are known to have significant effects on male reproductive function (Cheng *et al.,* 2011; Wong and Cheng, 2011). Use of allethrin-based mosquito repellants for longer durations is a common practice because of which majority of the population are exposed to the active components (Huailu *et al.,* 1995). Though a large number of studies described the toxic effects of mosquito repellants on various organ systems, their effects on the reproductive system have received little attention. Because of the cost factor, mosquito coils are more preferred than the liquid vaporizers especially among the lower income strata population. To the best of our knowledge, there are no reports describing the toxic effects of short- and long-term mosquito coil smoke on the functionality of the male reproductive tract.

Oxidative stress due to environmental contaminants initiates of a variety of pathways leading to pathological conditions (Reuter *et al.,* 2010). Our results demonstrate that increased lipid peroxidation was observed in the testis and the cauda. This is in agreement with previous reports wherein increased lipid peroxidation was observed in various organs of animals exposed to pesticides (Gabbianelli *et al.,* 2002; Giray *et al.,* 2001; Gupta *et al.,* 1999; Kale *et al.,* 1999; Vontas *et al.,* 2001). For example, increased oxidative stress was observed in the brain and liver of rats exposed to cypermethrin (El-Demerdash, 2011a, 2011b; Giray *et al.,* 2001; Tuzmen *et al.,* 2008). Increased lipid peroxidation was observed in the brain, liver, and kidney of rats subjected to mosquito repellant inhalation (Gupta *et al.,* 1999). In the male reproductive tract, pyrethroid compounds were shown to induce lipid peroxidation (Issam *et al.,* 2009; Wang *et al.,* 2009). However, there are no reports on oxidant stress due to mosquito coil exposure in the male reproductive tract. In this study, we demonstrated for the first time that increased lipid peroxidation is observed in the testes and cauda of rats subjected to prolonged inhalation of allethrin-based mosquito coil smoke, which may lead to high levels of free radicals. It is well-known that free radicals interact with membrane lipids and alter membrane integrity. In this study, we observed damage of sperm nuclear and membrane integrity, testicular tubule and epithelial cell damage besides interstitial edema, which could be due to harmful effects of free radicals generated as a result of increased lipid peroxidation.

Alteration in antioxidant status is a consequence of increased oxidative stress and the same is also demonstrated during pyrethroid toxicity in different organ systems. Decreased levels of reduced glutathione (Gupta *et al.,* 1999), catalase, superoxide dismutase, and GPx were observed during pyrethroid toxicity. The changes in antioxidant status in the male reproductive tract in rats exposed to allethrin-based mosquito coil smoke were not reported till now. Increased lipid peroxidation observed in our study may lead to oxidative stress thereby affecting antioxidant status. Surprisingly, in general, antioxidant status was not altered in the caput, cauda, and testes except for a significant increase of catalase activity in the cauda obtained from 120 and 180 day exposed rats. It was reported earlier that a 90-day exposure to liquid mosquito repellants did not affect the gonadal enzymes such as alkaline phosphatase, sorbitol dehydrogenase, lactate dehydrogenase, and glucose-6-phosphate dehydrogenase (Anvita Srivastava, 2006). In this study, a general lack of changes in antioxidant status in the caput, cauda, and testes and the increased catalase activity in the cauda obtained from 120 and 180 day exposed rats could be due to the protective mechanisms adopted by the male reproductive tract to prevent loss of fertility under stress conditions.

The effects of inhalation of allethrin-based mosquito repellants on cellular architecture of reproductive organs and sperm parameters are reported earlier. Pathomorphological changes in the testes, sperm number, and motility were not observed in rats that were subjected to inhalation of liquid mosquito repellants using vaporizers for 90 days (Anvita Srivastava, 2006). Similarly, no decrease in spermatogenesis was observed in rats exposed to a single pesticide; however, sperm motility was decreased in rats exposed to a mixture of pesticides (Perobelli *et al.,* 2010). Fenvalerate, a pyrethroid insecticide, adversely affected sperm production and storage in male rats (Arena *et al.,* 2008). Our histopathological analyses revealed increased germ cell damage, loss of germ cell nuclear integrity, and increased number of dead spermatozoa in the testes of rats exposed to allethrin-based mosquito coil smoke. It is possible that prolonged exposure to mosquito coil smoke (>90 days) is required to cause a loss of germ cell integrity; since no changes in pathomorphological features of the caput, cauda, and testes and sperm parameters are reported for 15–90 day samples in this study as well as in a previous 90-day toxicity report (Anvita Srivastava, 2006). These pathomorphological changes observed could be due to increased free radical generation.

Of the many responses that occur during stress, the role of p53 is regarded as a key factor, since its dynamics dictates the fate of the cell to either undergo apoptosis or initiate repair mechanisms by temporarily halting the cell cycle. In our study, we observed increased *p53* mRNA expression in the epididymides obtained from rats exposed to mosquito coil smoke for 15–90 days and the levels gradually decreased until 180 days. In the testes, lower levels of *p53* were observed when compared to the controls at all the time points. The initial increase of *p53* expression in the epididymis may be a stress response to allow cells to initiate repair mechanisms. Loss of *p53* expression results in genomic instability and severely reduces tumor suppression (Han *et al.,* 2008; Reuter *et al.,* 2010). Reduced expression of *p53* in the testes and at later time points in the epididymides could lead to development of tumors, though no such features were observed in our histopathological analyses. However, further studies are required to determine whether tumor development due to loss of *p53* can occur in the male reproductive tract of rats exposed to mosquito coil smoke for more than 180 days.

Spermatozoa are produced in the male reproductive tract by a series of complex mechanisms that are broadly classified in to spermatogenesis and sperm maturation, the former occurring in the testes and latter in the epididymis respectively. A wide variety of molecules such as TGF-β1, stem cell factor, and its ligand, c-kit play a key role in spermatogenesis. Expression of spermatogenic factors depends on the microenvironment and can be influenced by various factors such as hormonal status, stress, etc. In our study, we demonstrate that the expression of *Scf* and its ligand *c-Kit* are not altered, whereas decreased levels of *Tgf-β1* are observed. Though *Tgf-β-1* levels were decreased, there was no marked change in the density of spermatids in the testes as revealed by our histopathological analyses. It is possible that the decreased levels of *Tgf-β1* alone may not be functionally significant.

Manufacturers of leading brands of mosquito coils available in the Indian market do not disclose the complete composition of these coils. As per the information provided in the leaflet along with the product, the composition of the coils used in our study contained 0.1% w/w d-tansallethrin, 40% w/w wood floor, 40% w/w coconut shell powder, 10% w/w starch and 9.9% other ingredients. The composition of mosquito coils available in retail outlets of Asian countries and those available in the Asian markets of United States is reported (Krieger *et al.,* 2003; Li *et al.,* 2005; Liu *et al.,* 1987). They are

known to contain octadichlorodipropyl ester (S-2) as a synergist. Further, use of mosquito coils increases the risk of exposure to bis (chloromethyl) ether and submicron particles (<1 μm) coated with heavy metals, allethrin, and phenol O-cresol. Though we observe biochemical and toxicogenomic effects in the male reproductive tract of rats exposed to allethrin-based mosquito coil smoke, caution is exercised to attribute all of the observed effects to be result of inhalation of allethrin released during burning of mosquito coils. It is possible that the effects observed could also be due to the other toxic materials released during mosquito coil combustion. Further, burning one mosquito coil smoke can emit particulate matter equivalent to that of 75–137 cigarettes (Liu *et al.,* 2003). It is well-known that cigarette (tobacco) smoking causes severe impairment to the reproductive function (Dechanet *et al.,* 2010). Hence, it can be expected that the impairment induced by mosquito coil smoke is far greater than cigarette smoke.

In conclusion, we report that in the male reproductive tract of rats exposed to mosquito coil smoke for 15–180 days, there are no significant changes in the organ weight. Further, increased lipid peroxidation was observed in the cauda and testes without any major changes in the antioxidant status, except for an increase in the catalase activity in the cauda obtained from 120 and 180 day mosquito coil smoke exposed rats. Pathomorphological changes were predominantly observed in the cauda and testes at the later time points. The expression pattern of stress response gene, *p53*, was altered in the epididymides and testes, whereas the mRNA expression of sperm maturation factors *C-Kit* and *Scf* remained largely unaltered, though decreased levels of *Tgf-β1* was observed. Results of this study indicate that exposure to mosquito coil smoke for prolonged periods of time increases oxidative stress with concomitant changes in tumor suppressor response gene, thereby increasing the susceptibility to develop cancers of the male reproductive tract. Further, severe damage to male reproductive tract function may occur due to loss of organ and spermatozoa architectural integrity.

REFERENCES

Aebi H. (1984). Catalase. *Methods in Enzymology*, *105*(1947), 121–126.

Anvita Srivastava, Srivastava MK, and Raizada RB. (2006). Ninety-day toxicity and one-generation reproduction study in rats exposed to allethrin-based liquid mosquito repellent. *The Journal of Toxicological Sciences*, *31*(1), 1–7.

Arena AC, Fernandez CD, Porto EM, Bissacot DZ, Pereira OC, and Kempinas WG. (2008). Fenvalerate, A Pyrethroid Insecticide, Adversely Affects Sperm Production and Storage in Male Rats. *Journal of Toxicology and Environmental Health, Part A*, *71*(23), 1550–1558.

Bernheim F. (1964). The oxidative desulfuration of thio-acids. *Biochim. Biophys. Acta*, *90*, 426–428.

Besmer P, Manova K, Duttlinger R, Huang EJ, Packer A, Gyssler C, and Bachvarova RF. (1993). The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. *Development. Supplement*, 125–37.

Casida JE. 1995. Metabolism and synergism of pyrethrins. In: Casida JE, Quistad GB, editors. Pyrethrum flowers production, chemistry, toxicology, and Uses. New York: Oxford University Press. p 258–276.

Cheng CY, Wong Elissa WP, Lie Pearl PY, Li Michelle MW, Su L, Siu Erica R, Yan Helen HN, Mannu J, Mathur PP, Bonanomi M, Silvestrini B, and Dolores D Mruk (2011). Environmental toxicants and male reproductive function. *Spermatogenesis*, *1*(1), 2–13.

Dechanet C, Anahory T, Mathieu Daude JC, Quantin X, Reyftmann L, Hamamah S, Hedon B, and Dechaud H. (2010). Effects of cigarette smoking on reproduction. *Human Reproduction Update*, *17*(1), 76–95.

Eil C, and Nisula BC. (1990). The binding properties of pyrethroids to human skin fibroblast androgen receptors and to sex hormone binding globulin. *Journal of Steroid Biochemistry*, *35*(3-4), 409–14.

El-Demerdash FM. (2011a). Lipid peroxidation, oxidative stress and acetylcholinesterase in rat brain exposed to organophosphate and pyrethroid insecticides. *Food and Chemical Toxicology*, *49*(6), 1346–52.

El-Demerdash FM. (2011b). Oxidative stress and hepatotoxicity induced by synthetic pyrethroids-organophosphate insecticides mixture in rat. *Journal of Environmental Science and Health. Part C, Environmental Carcinogenesis and Ecotoxicology Reviews*, *29*(2), 145–58.

Gabbianelli R, Falcioni G, Nasuti C, and Cantalamessa F. (2002). Cypermethrin-induced plasma membrane perturbation on erythrocytes from rats: reduction of fluidity in the hydrophobic core and in glutathione peroxidase activity. *Toxicology*, *175*(1-3), 91–101.

Garey J, and Wolff MS. (1998). Estrogenic and antiprogestagenic activities of pyrethroid insecticides. *Biochemical and Biophysical Research Communications*, *251*(3), 855–9.

Giray B, Gürbay A, and Hincal F. (2001). Cypermethrin-induced oxidative stress in rat brain and liver is prevented by Vitamin E or allopurinol. *Toxicology Letters*, *118*(3), 139–146.

Gupta A, Nigam D, Shukla GS, and Agarwal AK. (1999). Effect of pyrethroid-based liquid mosquito repellent inhalation on the blood-brain barrier function and oxidative damage in selected organs of developing rats. *Journal of Applied Toxicology*, *19*(1), 67–72.

Go V, Garey J, Wolff MS, and Pogo BG. (1999). Estrogenic potential of certain pyrethroid compounds in the MCF-7 human breast carcinoma cell line. *Environmental Health Perspectives*, *107*(3), 173–177.

Han ES, Muller FL, Pérez VI, Qi W, Liang H, Xi L, Fu C, Doyle E, Hickey M, Cornell J, Epstein CJ, Roberts LJ, Van Remmen H, and Richardson A. (2008). The in vivo gene expression signature of oxidative stress. *Physiological Genomics*, *34*(1), 112–26.

Huailu C, Wen Y, Kang W, and Liu C. (1995). Large-scale spraying of bednets to control mosquito vectors and malaria in Sichuan, China. *Bulletin WHO*, *73*, 321–328.

Issam C, Samir H, Zohra H, Monia Z, and Hassen BC. (2009). Toxic responses to deltamethrin (DM) low doses on gonads, sex hormones and lipoperoxidation in male rats following subcutaneous treatments. *The Journal of Toxicological Sciences*, *34*(6), 663–670.

Issam C, Zohra H, Monia Z, and Hassen BC. (2011). Effects of dermal sub-chronic exposure of pubescent male rats to permethrin (PRMT) on the histological structures of genital tract, testosterone and lipoperoxidation. *Experimental and Toxicologic Pathology*, *63*(4), 393–400.

Jakoby WB. (1978). The glutathione S-transferases: a group of multifunctional detoxification proteins. *Advances in Enzymology and Related Areas of Molecular Biology*, *46*, 383–414.

Kakko I, Toimela T, and Tähti H. (2003). The synaptosomal membrane bound ATPase as a target for the neurotoxic effects of pyrethroids, permethrin and cypermethrin. *Chemosphere*, *51*(6), 475–80.

Kale M, Rathore N, John S, and Bhatnagar D. (1999). Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes : a possible involvement of reactive oxygen species. *Toxicology Letters*, *105*, 197–205.

Kasat K, Go V, and Pogo BG. (2002). Effects of pyrethroid insecticides and estrogen on WNT10B proto-oncogene expression. *Envoronmenat International*, *28*, 429–432.

Krieger RI, Dinoff TM, and Zhang X. (2003). Octachlorodipropyl ether (S-2) mosquito coils are inadequately studied for residential use in Asia and Illegal in the United States. *Environmental Health Perspectives*, *111*(12), 1439–1442.

Li HY, Shi N, Chen D, Dai Z, Lu W, Wang B, and Li Y. (2005). Oxidative stress of deltamethrin on rat nervous system. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi*, *23*(2), 97–101.

Liu WK, Wong MH, and Mui YL. (1987). Toxic effects of mosquito coil (a mosquito repellent) smoke on rats. I. Properties of the mosquito coil and its smoke. *Toxicology Letters*, *39*(2-3), 223–30.

Liu W, Zhang J, Hashim JH, Jalaludin J, Hashim Z, and Goldstein BD. (2003). Mosquito coil emissions and health implications. *Environmental Health Perspectives*, *111*(12), 1454–60.

Maiti PK, Kar A, Gupta P, and Chaurasia SS. (1995). Loss of membrane integrity and inhibition of type-I iodothyronine 5'-monodeiodinase activity by fenvalerate in female mouse. *Biochemical and Biophysical Research Communications*, *214*(3), 905–9.

McCord JM and Fridovich I. (1969). Superoxide Dismutase. An enzymic function for Erythrocuprein (Hemocuprein). *The Journal of Bological Chemistry*, *244*(22), 6049–6055.

Meehan T, Schlatt S, O'Bryan MK, de Kretser DM, and Loveland KL. (2000). Regulation of germ cell and Sertoli cell development by activin, follistatin, and FSH. *Developmental Biology*, *220*(2), 225–37.

Ochsendorf FR. (1999). Infections in the male genital tract and reactive oxygen species. *Human Reproduction Update*, *5*(5), 399–420.

Olaso R, Pairault C, Boulogne B, Durand P, and Habert R. (1998). Transforming growth factor beta1 and beta2 reduce the number of gonocytes by increasing apoptosis. *Endocrinology*, *139*(2), 733–40.

Paglia DE, and Valentine WN. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of Laboratory and Clinical Medicine*, *70*(1), 158–69.

Perobelli JE, Martinez MF, da Silva Franchi CA, Fernandez CD, de Camargo JL, and Kempinas Wde G. (2010). Decreased sperm motility in rats orally exposed to single or mixed pesticides. *Journal of Toxicology and Environmental Health. Part A*, *73*(13-14), 991–1002.

Pontecorvo G, and Fantaccione S. (2006). Recombinogenic activity of 10 chemical compounds in male germ cells of Drosophila melanogaster. *Ecotoxicology and Environmental Safety*, *65*(1), 93–101.

Ramesh A, and Vijayalakshmi A. (2001). Monitoring of allethrin, deltamethrin, esbiothrin, prallethrin and transfluthrin in air during the use of household mosquito repellents. *Journal of Environmental Monitoring*, *3*(2), 191–193.

Reuter S, Gupta SC, Chaturvedi MM, and Aggarwal BB. (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radical Biology and Medicine*, *49*(11), 1603–16.

Sattin RW, Roisin A, Kafrissen ME, Dugan JB, and Farer LS. (1984). An Epidemic of Gynecomastia Among Illegal Haitian Entrants. *Public Health Reports*, *99*(5), 504–510.

Sharpe RM, and Irvine DS. (2004).How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health?*BMJ*, *328*(February),447–451.

Sharpe RM, and Skakkebaek NE. (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *The Lancet*, *341*(8857), 1392–1396.

Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, and Weiner ML. (2002). Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology*, *171*(1), 3–59.

Tan J, and Soderlund DM. (2010). Divergent actions of the pyrethroid insecticides S-bioallethrin, tefluthrin, and deltamethrin on rat Na(v)1.6 sodium channels. *Toxicology and Applied Pharmacology*, *247*(3), 229–37.

Tomlin C. (1994). *A world compendium: The pesticide manual. British Crop Protection Council*. Farnham Surrey; Cambridge.

Tuzmen N, Candan N, Kaya E, and Demiryas N. (2008). Biochemical effects of chlorpyrifos and deltamethrin on altered antioxidative defense mechanisms and lipid peroxidation in rat liver. *Cell Biochemistry and Function*, *26*(1), 119–24.

Verschoyle RD, and Aldridge WN. (1980). Structure-activity relationships of some pyrethroids in rats. *Archives of Toxicology*, *45*(4), 325–9.

Vijverberg HP, and de Weille JR. (1985). The interaction of pyrethroids with voltage-dependent Na channels. *Neurotoxicology*, *6*(2), 23–34.

Vontas JG, Small GJ, and Hemingway J. (2001). Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens. *Biochemical Journal*, *357*, 65–72.

Wang XZ, Liu SS, Sun Y, Wu JY, Zhou YL, and Zhang JH. (2009). Beta-cypermethrin impairs reproductive function in male mice by inducing oxidative stress. *Theriogenology*, *72*(5), 599–611.

Wong EW, and Cheng CY. (2011). Impacts of environmental toxicants on male reproductive dysfunction. *Trends in Pharmacological Sciences*, *32*(5), 290–9.

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Chapter *3*

Study the effect of oral administration of allethrin in adult rats.

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Introduction

Synthetic pyrethroids are used for pest control in crop production and households and exposure to pyrethrin based pesticides by humans occurs during such usage. Though, pyrethroids are known to exhibit low levels of toxicity (Casida, 1995), they primarily act on the nervous system (Soderlund *et al.*, 2002) to inhibit voltage sensitive sodium channels and sodium-potassium ATPase (Kakko *et al.*, 2003; Tan and Soderlund, 2010; Vijverberg and de Weille, 1985). Further, pyrethroids are known to be hepatotoxic, carcinogenic, immunosuppressive, estrogenic and antiprogestagenic in mammals (Institoris *et al.*, 1999; Nasuti *et al.*, 2007; Shukla *et al.*, 2002; Yokohira *et al.*, 2011). Pyrethroids induce oxidative stress and alter antioxidant system in different organ systems of rats (Giray *et al.*, 2001; Kale *et al.*, 1999; Li *et al.*, 2005). Their effects on the reproductive system include gynecomastia, inhibition of testosterone binding to the androgen receptor and sex hormone binding globulin at high concentrations (Eil and Nisula, 1990; Sattin *et al.*,1984). Further, it is reported that pyrethroid compounds cause oxidative stress in the male reproductive tract (Issam *et al.*, 2009; Issam *et al.*, 2011; Wang *et al.*, 2009). Endocrine disruption and oxidative stress caused by pyrethroid based pesticides (Jin *et al.*, 2011) is an important area of investigation in toxicology.

D-allethrin, a mixture of eight stereoisomers belongs to the type I pyrethroids and is used widely in developing countries for household pest control. It was found to be moderately toxic in rodent models (Tomlin, 1994). Though the effects of allethrin based pesticides are well studied in the nervous system, information about allethrin toxicity in the reproductive tract is limited. Rats exposed nasally to allethrin based mosquito repellents for 90 days did not show any change in their clinical enzyme profile and reproductive indices (Anvita Srivastava, 2006). Further, it is demonstrated that allethrin exhibits recombinogenic activity in the male germ cells of *Drosophila melanogaster* (Pontecorvo and Fantaccione, 2006).

Sperm production is seriously compromised due to oxidative stress (Bal *et al.*, 2012a; Naziroglu *et al.*, 2011). Nitric oxide production is reported in the reproductive tract (Shum *et al.*, 2009) and is considered to be a free radical scavenger during oxidative stress. A result of increased oxidative stress is the initiation of apoptosis, a process tightly under the control of p53 gene product. Production of male germ cells in the testis is controlled by spermatogenic factors such as *Tgf-β1* (Olaso *et al.*, 1998). Immature spermatids undergo maturation in the epididymis and it is demonstrated that SPAG11E

plays an important role in spermatid maturation (Zhao *et al.*, 2011b; Zhou *et al.*, 2004). Further, the macaque *Defensin 126* (*DEFB126*), the rat homologue of *Defensin 22* (*Defb22)*, is a key factor involved in sperm capacitation (Yudin *et al.*, 2003), mediates attachment of sperm to the epithelium of oviduct (Tollner *et al.*, 2008) and protects the sperm from immuno recognition (Yudin *et al.*, 2005). The rat *Defb22* is expressed in the epididymis and its protein product is localized on the sperm implying roles similar to its macaque counterpart (Rao *et al.*, 2003; Zanich *et al.*, 2003). The effects of allethrin based pesticides on the possible changes at the architectural and molecular levels besides alterations in the expression of genes that regulate spermatogenesis, sperm maturation, capacitation and fertilization is not yet investigated.

Environmental pollutants such as pesticides and heavy metals contribute to the development of neoplasia and reduction in reproductive functions (Aly *et al.*, 2012; Issam *et al.*, 2009; Sharpe and Irvine, 2004; Tan and Soderlund, 2010; Wang *et al.*, 2009). Basing on the available information, it can be hypothesized that prolonged exposure to pyrethroid based pesticides such as allethrin could cause oxidative stress and alteration in the antioxidant status, thereby contribute to the initiation of cancer in the male reproductive tract. Further, it is also possible that allethrin exposure could lead to disturbances in the morphological and cellular architecture of the male reproductive tract thereby affecting fertility i.e. spermatogenesis, sperm maturation and capacitation. Hence, in this study we attempt to study whether oral exposure of allethrin to rats could cause initiation of cancer via the induction of oxidative stress in the male reproductive tract and whether spermatogenesis and sperm maturation are compromised.

We observed increased oxidative stress in the testes and epididymides of rats orally exposed to allethrin for 60 days. Antioxidant enzyme status was altered due to increased lipid peroxidation. Significant pathomorphological changes in the male reproductive tract tissues were observed. *p53* gene expression was altered suggesting a loss of cell cycle regulation. The expression of *Tgf-β1, Spag11e and Defb22* were reduced indicating a loss of sperm function.

Materials and Methods

1. Animal treatments

Male Wistar rats aged 90 days were obtained from National Center for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad and were maintained on a 12L: 12D lighting schedule, at 22-25°C, with food and water *ad libitum*. After acclimatization, rats were divided into different groups (n=5) and were orally administered every day with 25, 50, 100 and 150 mg / kg body weight allethrin (mixture of stereo isomers; Sigma Aldrich, USA) dissolved in corn oil for 60 days. Treatment doses chosen were based on previous reports (Soderlund *et al.,* 2002). Control animals were treated with the vehicle. Animals were sacrificed by cervical dislocation and tissues were collected after 60 days from vehicle treated control and allethrin treated rats. Testis, caput and cauda epididymides were identified, stripped off the connective tissues, noted the weight, frozen in liquid nitrogen and kept at -70°C until use. Experiments were conducted using the guidelines for the care and use of laboratory animals, approved by the Institutional Animal Ethics Committee of University of Hyderabad (UH/IAEC/2012/YS/13).

2. Estimation of lipid peroxidation (LPO) products

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA), the product of lipid peroxidation, under acidic conditions and high temperature (95°C) to form a pink colored complex with absorbance maxima at 532 nm. 10% (w/v) homogenates of caput, cauda and testes obtained from the vehicle control and experimental animals were prepared in ice cold phosphate buffer saline, pH 7.4 and the extent of lipid peroxidation was estimated using the TBA method as described earlier (Bernheim, 1964). 1,1,3,3-tetraethoxy-propane (TEP) was used as the standard. Levels of lipid peroxidation was expressed as nano moles MDA/ mg protein.

3. Estimation of Nitric oxide as its stable metabolite nitrite

NO being a free radical is highly unstable and gets converted to an equimolar ratio of its stable metabolites, nitrite and nitrate. The estimation of NO is therefore done by estimating its stable metabolite. The amount of nitrite/ nitrate formed is an index of the amount of NO generated in the samples. Nitrite was measured by the method of Griess as described earlier (Lepoivre *et al.*, 1990).

Reagents

1. 70% 5-Sulfosalicylic acid (w/v) in distilled water
2. 50 mM Tris-Hcl buffer, pH 9.0
3. 10% sodium hydroxide solution
4. 0.3% NED (Naphthylethylenediamine dihydrochloride) in distilled water. Prepared freshly.
5. 3% Sulfanilamide in 1N HCl. Prepared freshly.
6. **Griess reagent:** Prepared by mixing reagents 4 and 5 in the ratio of 1:1 (v/v).

Briefly, 0.5 mL of caput, cauda and testicular homogenates obtained from allethrin treated rats, were mixed with 0.2 mL of 70% 5-sulfosalicylic acid and vortexed well for 30 min to precipitate the protein. After centrifugation at 15,000 rpm for 30 min, the supernatant was mixed with 10% NaOH, Tris-HCl buffer and Griess reagent and incubated in dark for 30 min at room temperature. The intensity of the purple color formed was measured at 540 nm using a spectrophotometer. Sodium nitrite was used as the standard and results were expressed as µmoles nitrite/mg protein.

4. Antioxidant enzyme activity

Antioxidant enzyme activity was measured using spectrophotometric methods. Caput, cauda and testes collected from vehicle control and allethrin treated rats. 10% (w/v) homogenates were prepared in ice cod phosphate buffer saline, pH 7.4 and centrifuged at 3000 rpm to remove the debris. The supernatant was collected and the activities of catalase, glutathione-s-transferase (GST), glutathione peroxidase (GPx) and superoxide dismutase (SOD) were performed using standard protocols described earlier (Aebi, 1984; Jakoby, 1978; McCord and Fridovich, 1969; Paglia and Valentine, 1967). The activity of the antioxidant enzymes were calculated from the change in optical density measured on a spectrophotometer. Detailed methodology for estimation of antioxidant enzyme activity are described in chapter 1.

5. Real time polymerase chain reaction (RT-PCR)

Tissues obtained from vehicle control and allethrin treated rats were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted. Total RNA (2 µg) was reverse transcribed using 50 U Stratascript (Invitrogen, USA) and 0.5 µg of oligodT (Invitrogen) according to the manufacturer's instructions.

The resultant cDNA (2 µl) was used for real time PCR analysis using SYBR master mix kit (Applied Biosystems, Warrington, UK) in a real time thermal cycler (Applied Biosystems, Warrington, UK). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression was used as the internal control. A typical real time PCR reaction consisted of the following conditions: initial denaturation at 94°C for 10 min; 40 cycles with 94°C for 15 secs and 60°C for 1 min. Negative controls (no template control and minus RT control (only with RNA) were included in the assays to detect non-specific amplification. The amplicons were sequenced to confirm their identity. Gene specific primers used were listed in table 4.1.

Table 4.1. Gene Specific primers

Primer	**Sequence (5'-3')**
17-β HSD F	CACTATCAGCGTGGTTATGAGCAAGC
17-β HSD R	GTGTCTGGATCCCCTGAAACTTGG
3-β HSD F	GGGCATCTCTGTTGTCATCCACAC
3-β HSD R	GATGGTCTTCTTGTAGGAGTTGGGC
Ar F	CACTACGGAGCTCTCACTTGTG
Ar R	CCAGAGTCATCCCTGCTTCATAAC
Brdt F	CAGTTCCTGCAGAGAGTCGT
Brdt R	CGTAGTACCTGTTTTCCAGCC
c-kit F	GGCCTCAGGAGCTCTATTTAC
c-kit R	GAGAGATTTCCCATCACACTC
Defb 22	CACTTGGTCACAGGTAACTG
Defb 22	CAGCAGCTTGGTTTCCATCTG
Gapdh F	CCAATGTATCCGTTGTGGATCTG
Gapdh R	GAGTTGCTGTTGAAGTCACAG
Grth/Ddx25 F	CCAGAACCTCATAGCACAGAG
Grth/Ddx25 F	CAGAGCCAGTTCATAGGTG
Hsf-2 F	TAAATGGCTCCTCCAACCTG
Hsf-2 R	CCAACAGCTCAACCTTTCCTA
Kdm3a F	CAAGTCAACTGTGAGGAG
Kdm3a R	GACTTGCGTTTCACAGCTC
Ovol1 F	GACCTCAAGAGACATGTCC
Ovol1 R	GGCGCTCCTTATATGCATAC
p53 F	CAAGGCAACTATGGCTTCCAC
p53 R	GACTTCTTGTAGATGGCCATG
Scf F	GATGACCTCGTGGCATGTATG
Scf R	CAGGACCTCATGTTGAAGAGAG
Spag11e F	CACATCTGCTTTCCTGCACAG
Spag11e R	GCACCCCACATCTCAGATCTTC
StAR F	CTCCTACAGACATATGCGGAAC
StAR R	CTGGTCACTGTAGAGTGTTG
Tgf-β1 F	GCAACAATTCCTGGCGTTAC
Tgf-β1 R	CCACGTGGAGTACATTATCTTTGC

6. Histopathology

Epididymides and testes fixed in Bouin's fluid for 24 h and then into 70% ethanol, which was changed 3–4 times every day until the yellow color of Bouin's fluid completely disappeared. Tissues were hydrated serially in 80, 90 and 100% ethanol and stored in isopropanol overnight at 60°C before embedding in paraffin. Five micron sections were made for further analysis. Sections were deparaffinized by xylene and were re-hydrated serially in 100, 90, 80, 70 and 50% ethanol and then rinsed briefly in distilled water. Slides were immersed into Harris hematoxylin solution for 10 min, subsequently washed in distilled water for a while and differentiated in 1% hydrochloric acid for 30 s and then immersed in 0.2% ammonia water for 30 s to 1 min. After washing in running tap water for 10 min, sections were counterstained with 0.2% eosin Y solution for 1 min. Sections were washed in distilled water and followed by serial dehydration using 50, 70, 80, 90 and 100% alcohol. Slides were cleaned with three changes of xylene and then mounted with xylene based mounting medium. Photographs were taken on a Leica microscope.

7. Steroidogenic enzymes activity

7.1. 3β-hydroxysteroid dehydrogenase (3β-HSD): It is a crucial enzyme which converts pregnenolone to progesterone in the testosterone synthesis in Leydig cells of testis. The activity of 3β-HSD in the testis homogenates was estimated as described earlier (Jana *et al.,* 2006). The testis from the vehicle control and allethrin treated rats were homogenized in 20% glycerol containing 5 mM potassium phosphate and 1 mM EDTA followed by centrifugation at 16,000g for 10 min at 4°C. The supernatant was collected and the concentration of the total protein was determined by Lowry's method using bovine serum albumin as a standard. The volume of reaction mixture is 2.5 ml which contains 500 µl pyrophosphate buffer (100 µM), 500 µl NAD (0.5 µM), 500 µl of 0.1 µM dehydroisoandrosterone (DHEA) and 750 µl of distilled water. The reaction was initiated by adding 250 µl of testicular supernatant and absorbance was measured at 340 nm for 5 min with 1 min intervals. The enzyme activity was expressed as nmole of NAD reduced/min/mg protein.

$$\text{3β-HSD activity} = \frac{\text{Absorbance difference } (\Delta OD/min) \times V}{(6.22 \times 10^{-5})}$$

(V: Volume of reaction mixture)

7.2. 17β-hydroxysteroid dehydrogenase (17β-HSD): It catalyses the last step in the synthesis of testosterone from androstenedione in Leydig cells. The activity of 17β-HSD in the testis homogenates was estimated as described earlier (Jana *et al.,* 2006). The volume of reaction mixture is 2.5 ml which contains 500 µl pyrophosphate buffer (100 µM), 500 µl NADPH (0.5 µM), 500 µl of 0.8 µM 4-androstene-3,17-dione (ASD) and 750 µl of distilled water. The reaction was initiated by adding 250 µl of testicular supernatant and absorbance was measured at 340 nm for 5 min with 1 min intervals. The enzyme activity was expressed as nmole of NADPH oxidized/min/mg protein.

$$17\beta\text{-HSD activity} = \frac{\text{Absorbance difference } (\Delta OD/min) \times V}{(6.22 \times 10^{-5})}$$

(V: Volume of reaction mixture)

8. Estimation of serum testosterone

Blood from vehicle control and allethrin treated rats was collected and serum was separated by centrifugation at 5000 rpm for 15 min at 4°C. Serum was stored at -20°C until further analysis. Testosterone was estimated by using ELISA kit according to manufacturer's protocol (Omega diagnostics, UK).

9. Sperm parameters

9.1. Sperm sample preparation: Left cauda epididymides from vehicle control and allethrin treated rats was excised. Gentle pressure was applied on the epididymides to exude epididymal contents in to DMEM-F12 medium with 5% BSA and kept in 5% CO_2 incubator maintained at 37°C for 10-15 min to allow sperm to swim out in to medium (liquefaction). The contents were transferred in to falcon tube and centrifuged at 500 rpm for 3 min. Sperm suspension in the middle layer (top-fat, bottom-debris) was carefully taken out, diluted with medium and used for total sperm count and dead/live count using tryphan blue staining (Seed *et al.,* 1996).

9.1.1. Sperm counting: 20 µl of diluted sperm suspension was transferred to Neubauer's chamber and sperm heads were counted under light microscope. Sperm counts were expressed as millions/ml.

9.1.2. Tryphan blue counting: This method was used to analyze percentage of live and dead sperms in the suspension. To 100 µl of sperm suspension equal volume of

0.05% tryphan blue was added and kept in 37°C for 15 min. Stained (dead) and unstained (live) sperms were counted.

9.2. Daily sperm production in testis

The right testis from vehicle control and allethrin treated rats was decapsulated, weighed and homogenized in 5 ml of homogenization buffer containing 0.9% NaCl and 0.05% Triton X-100. The sample was diluted 10 times with 0.9% NaCl. Stage 19 spermatids of spermiogenesis which are homogenization resistant was counted on Neubauer's chamber and the number of spermatozoa produced per gram of testicular tissue per day was calculated according to the following formula: (AxBxCxD/E) / F (Robb *et al.*, 1978), where

- A is the average count of sperm heads from chambers
- B is the square factor (5.0)
- C is the hemocytometer factor (10^4)
- D is the dilution factor (50)
- E is the testis weight (g) and
- F is the time (days) where stage 19 spermatids are present in seminiferous epithelium.

9.3. Computer Assisted Sperm Analysis (CASA)

The cauda epididymides of vehicle control and allethrin treated rats were slightly cut with surgical blade and squeezed the spermatozoa in to M2 medium (Sigma) containing 4 g/L bovine serum albumin and allowed to liquefy for 15 min at 37° C in 5% CO_2 incubator. Sperm counting was done on a Makler's chamber. Sperm suspension diluted with M2 medium to approximately $1x10^6$ spermatozoa/ml were taken for each sample in fresh medium. Sperm motility parameters on CASA system were measured according to instruction manual. 20 µl of sample was pipetted on to prewarmed (37°C), disposable count chamber and recordings were made from three different fields of the chamber at 20X magnification using Hamilton Thorne CASA. The recordings were carried at one hour interval for 3.5 hours. Motility parameters measured by CASA include linearity (LIN), straight-line velocity (VSL), straightness (STR), curvilinear velocity (VCL), average path velocity (VAP) and beat cross frequency (BCF).

10. Proteome analysis by two dimensional (2D) electrophoresis.

Caput epididymides from vehicle control and allethrin treated rats were chopped into pieces and homogenized in cell lysis buffer containing 50 mM HEPES, 10% glycerol, 1% triton X-100, 150 mM NaCl, 100 mM NaF. Homogenate was sonicated at 40% amplitude with 10 sec intervals and centrifuged at 13,000 rpm for 15 min at 4°C. Equal concentration of protein was taken and 1:2 ratio of 10% TCA was added and vortexed at 4°C for 4 hours or at -80°C overnight. The samples were centrifuged at 13,000 rpm for 15 min at 4°C and pellet was washed in ice-cold methanol and acetone for 10 min each. Pellet was partially air-dried and dissolved in rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris base and 65 mM DTT. Samples were vortexed and centrifuged. The protein content in the supernatant was estimated by Amido black method. To the protein sample, 4 µl of IPG buffer, 0.1% bromophenol solution was added and mixed. The sample was loaded on to rehydration tray well and 7 cm Immobiline dryStrip of pH 3-10 was placed facing the gel side to sample taking care no air bubble forms. 1 ml of mineral oil was added to prevent the sample from drying and kept at room temperature overnight undisturbed. The strip was kept for first dimension with the following focusing conditions.

S. No	Step	Conditions	
1	Rehydration	No	
2	Focus temperature	20°C	
3	Step 1	Linear method	250V – 20 min (Volt hours)
4	Step 2	Linear	4000V- 2 hours (Volt hours)
5	Step 3	Rapid	4000V – 12000V (Volt hours)

After IEF focusing, the immobiline strips were equilibrated in SDS-containing equilibration buffer 1 and 2 (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol) for 10 min. Buffer 1 contains 2% w/v DTT and buffer 2 contains 2% w/v iodoacetamide. Second dimension separation was done on 12% SDS-PAGE. The equilibrated immobiline DryStrip gel once dipped in SDS electrophoresis buffer was placed on SDS longer plate sealed with 0.5% agarose with bromophenol as tracking dye. Electrophoresis was carried out at 75V and gel was kept in 0.1% Coomassie R240 overnight. The gel was scanned using Image Scanner and spots were analyzed using

MALDI-TOF. The spot volumes were calculated using Image master 2D platinum software.

11. Immunoblotting

Total protein from tissues obtained from allethrin treated and vehicle control rats was harvested in radioimmuno precipitation assay (RIPA) buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate and 0.1% SDS) supplemented with proteinase cocktail inhibitor. Tissues were then homogenized and centrifuged at 10,000 rpm. The supernatant was collected and the concentration of the total protein was determined by Lowry's method using bovine serum albumin as a standard. Hundred microgram of each protein sample was separated on a 10% SDS-PAGE and transferred on to a 0.2 µm pore size nitrocellulose membrane (HybondTM ECLTM, GE Healthcare, UK). The nonspecific binding sites were blocked by incubating the membranes in 5% nonfat milk in Tris-buffered saline (TBS) with 0.1% Tween (TBS-T) for 1 h at room temperature. The membranes were then incubated with specific antibodies (Cell Signaling Technology, Danvers, MA) directed against rat phospho p44/42 MAPK for 3 h at room temperature. The membrane was then washed and incubated with secondary antibody against rabbit IgG conjugated with horse radish peroxidase (Cell Signaling Technology). Immunoreactive proteins were detected using an ECL prime Western blot detection Kit (GE Healthcare life sciences, MA), according to the manufacturer's instructions.

12. Statistical analyses

Changes in the parameters analyzed in this study were compared with the vehicle control. Statistical analyses were performed using one way ANOVA and Student's t-test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are Mean ± S.D. * and # denotes $p < 0.05$ and $p < 0.01$ respectively.

Results

1. Organ weights

When compared to vehicle treated control, no significant changes in the weights of caput, cauda and testes were observed in the animals treated with varying doses of allethrin (Table 3.2).

Table 3.2. Organ weights in allethrin treated rats.

Organ (weight in gms)	Allethrin dose (mg/kg body weight)				
	0	**25**	**50**	**100**	**150**
Caput	0.489 ± 0.4	0.48 ± 0.2	0.52± 0.3	0.56± 0.4	0.42± 0.3
Cauda	0.46 ± 0.4	0.42 ± 0.5	0.43± 0.7	0.46± 0.9	0.44± 0.6
Testis	2.9 ± 0.7	2.8 ± 0.3	3.07± 0.18	3.2± 0.2	2.53±0.8

2. Lipid peroxidation

Pyrethroids being potent inducers of free radical generation, lipid peroxidation products were measured in the caput, cauda and testes obtained from control and allethrin treated rats. Increased levels of lipid peroxidation products were observed in the caput, cauda and testes of allethrin treated rats (Figure 3.1A, B and C). In the testes, the increase in lipid peroxidation seems to be dose dependent (Figure 3.1C).

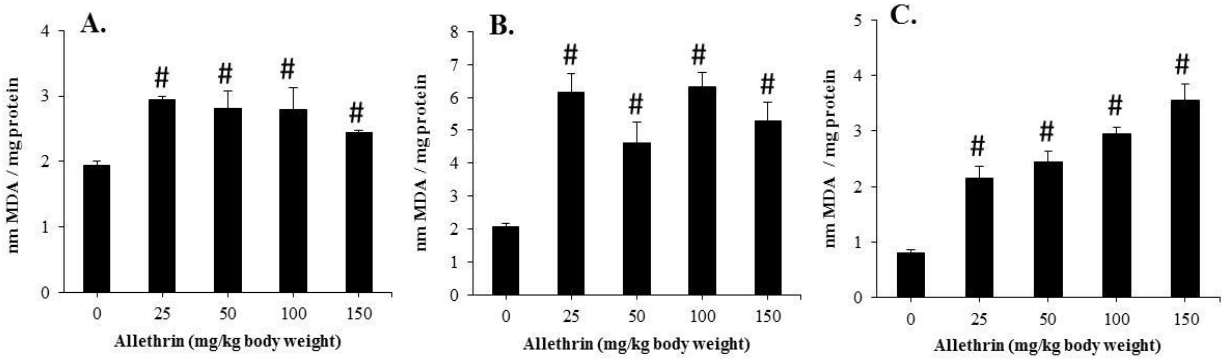

Figure 3.1. *Lipid peroxidation in the male reproductive tract of allethrin treated rats.* ***(A)*** *Caput;* ***(B)*** *Cauda;* ***(C)*** *Testis. Values shown are Mean ± S.D. # denotes p < 0.001.*

3. Nitric oxide

Nitric oxide plays an important role in the maintaining the oxidant and pro-oxidant mileu in the tissues. In the caput, increased levels of nitric oxide was observed at all the doses tested (Figure 3.2A), when compared to the vehicle treated control.

However, no changes in the levels of nitric oxide were observed in the cauda (Figure 3.2B) and testes (Figure 3.2C) of allethrin treated rats.

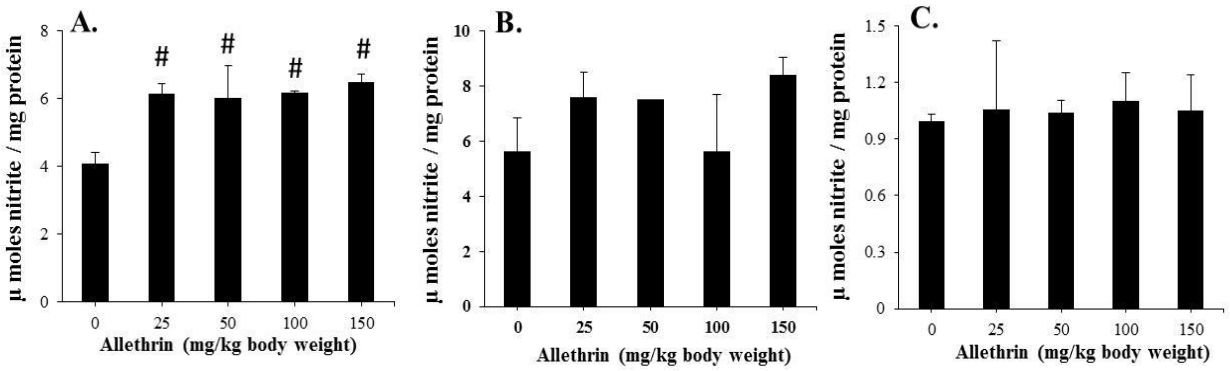

Figure 3.2. *Nitric oxide production in the male reproductive tract of allethrin treated rats.* ***(A)*** *Caput;* ***(B)*** *Cauda;* ***(C)*** *Testis. Values shown are Mean ± S.D. # denotes p < 0.001.*

4. NO/MDA ratio

Nitric oxide being highly reactive, couples with free radicals to maintain the oxidant to antioxidant status in the tissues. Hence, calculating the ratio of nitric oxide and lipid peroxidation (NO/MDA) provides information on the role of nitric oxide in preventing oxidative stress. In the caput, no significant changes were observed in the NO/MDA ratio (Figure 3.3A), suggesting that the increased lipid peroxidation (oxidative stress) is being quenched by nitric oxide. In the cauda and testes obtained from allethrin treated rats, the NO/MDA ratio was significantly lower when compared to the vehicle treated control (Figure 3.3B and C), indicating loss of the protective role of nitric oxide in these tissues.

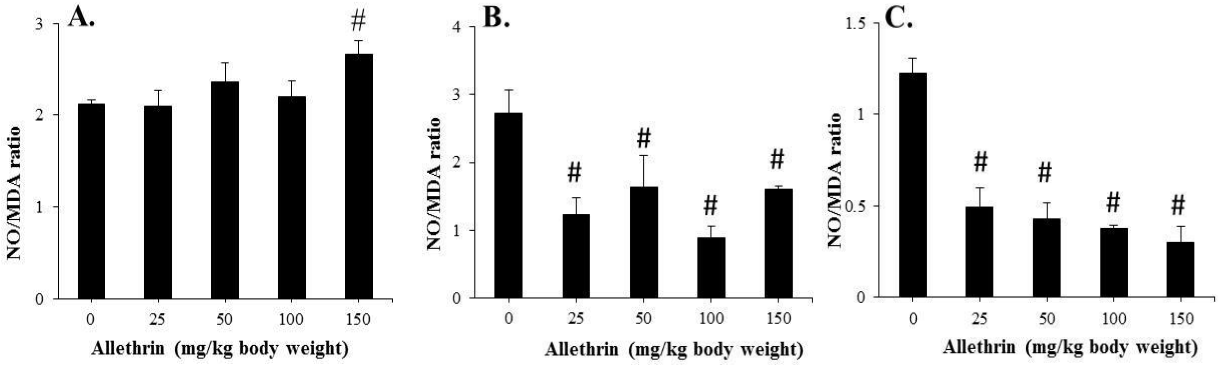

Figure 3.3. *NO/MDA ratio in the male reproductive tract tissues of allethrin treated rats.* ***(A)*** *Caput;* ***(B)*** *Cauda;* ***(C)*** *Testis. Values shown are Mean ± S.D. # denotes p < 0.001.*

5. Antioxidant enzymes

The defense against oxidative stress is provided by the antioxidant systems that primarily consist of the antioxidant enzymes. The activity of such enzymes namely,

catalase, glutathione peroxidase, glutathione-s-transferase and superoxide dismutase were measured in the caput, cauda and testes.

When compared to the vehicle treated control, catalase activity in the caput of allethrin treated rats remained largely unchanged except for a significant increase in the 150 mg/kg body weight treated animals (Figure 3.4A). Significant increase in catalase activity was observed in the cauda obtained from 100 and 150 mg / kg body weight treated rats (Figure 3.4B). In contrast, there was a dose dependent decrease in catalase activity in the testes of allethrin treated rats (Figure 3.4C). Glutathione peroxidase activity was significantly increased in the caput obtained from 150 mg / kg body weight allethrin treated rats (Figure 3.5A). In the cauda, it was found to be increased significantly in the 50 and 100 mg/kg allethrin treated groups (Figure 3.5B). In contrast, the activity of glutathione peroxidase activity was significantly decreased in the testes of rats treated with 150 mg/kg body weight allethrin (Figure 3.5C). Glutathione-S-transferase activity was found to be increased significantly in a dose dependent manner in the caput and cauda of allethrin treated rats (Figure 3.6A and B). However, in the testes, a dose dependent decrease was observed (Figure 3.6C), but this decrease was significant only in the testes of rats treated with the highest dose (150 mg/kg body weight). In the caput, significant increase in the activity of superoxide dismutase was observed in the 150 mg/kg body weight allethrin treated rats (Figure 3.7A). In the cauda and testes, allethrin treatment resulted in increase of superoxide dismutase activity at all the doses tested (Figure 3.7B and C).

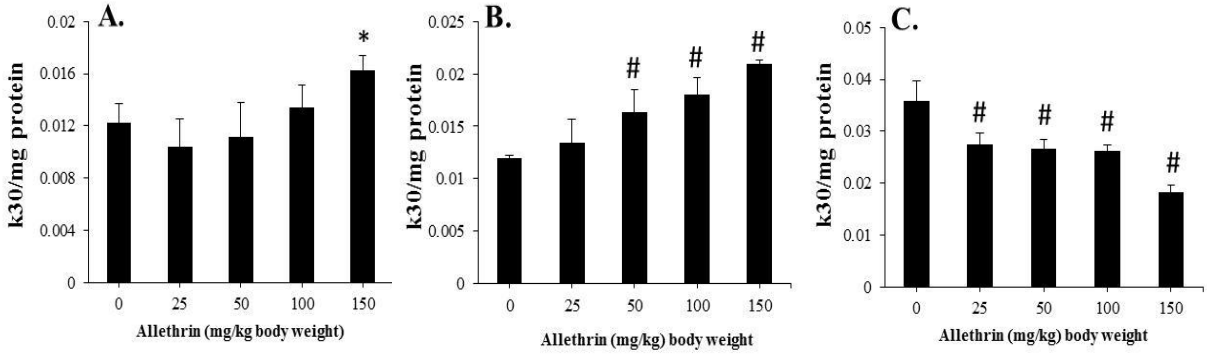

Figure 3.4. *Catalase activity in the male reproductive tract of allethrin treated rats.* ***(A)*** *Caput;* ***(B)*** *Cauda****; (C)*** *Testis. Values shown are Mean ± S.D. * and # denotes p <0.01 and 0.001, respectively.*

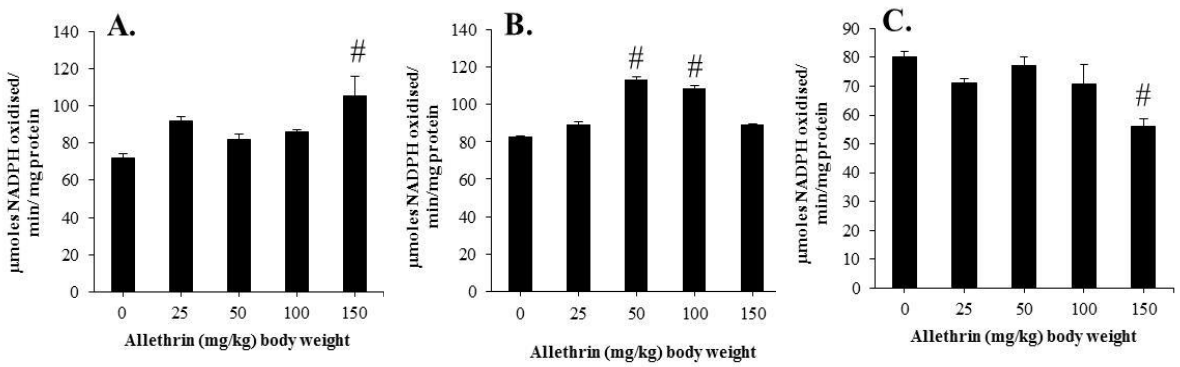

Figure 3.5. *Glutathione peroxidase activity in the male reproductive tract of allethrin treated.* ***(A)*** *Caput;* ***(B)*** *Cauda;* ***(C)*** *Testis. Values shown are Mean ± S.D. # denotes p< 0.001.*

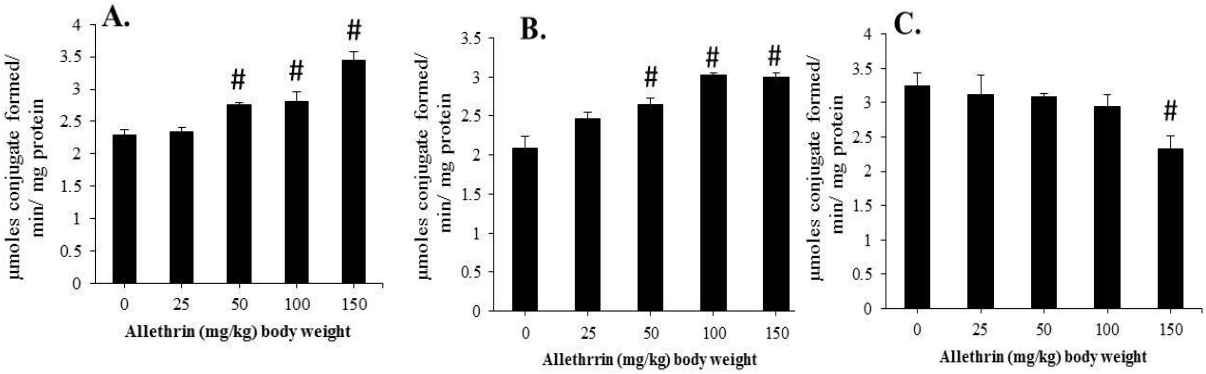

Figure 3.6. *Glutathione -S-transferase activity in the male reproductive tract of allethrin treated rats.* ***(A)*** *Caput;* ***(B)*** *Cauda;* ***(C)*** *Testis. Values shown are Mean ± S.D. # denotes p < 0.001.*

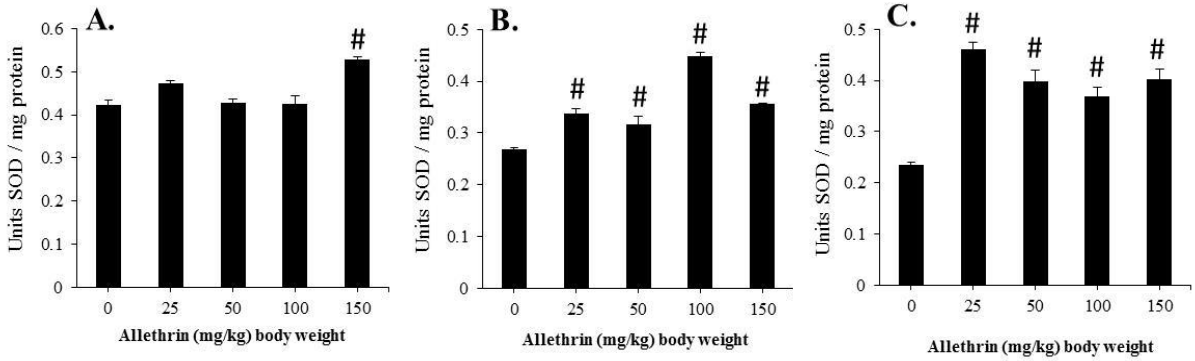

Figure 3.7. *Superoxide dismutase activity in the male reproductive tract of allethrin treated rats.* ***(A)*** *Caput;* ***(B)*** *Cauda;* ***(C)*** *Testis. Values shown are Mean ± S.D. # denotes p < 0.001.*

6. Histopathological analyses

The possible damages to the caput, cauda and testes obtained in the allethrin treated rats was analyzed by studying the pathomorphological changes. The effects observed were assessed as histological scores (Table 3.3). In the caput, presence of dead spermatozoa, damage to tubular architecture disarray, congestion (accumulation of blood or other fluid in the body part) and degeneration of epithelial cell lining were observed (Figure 3.8).

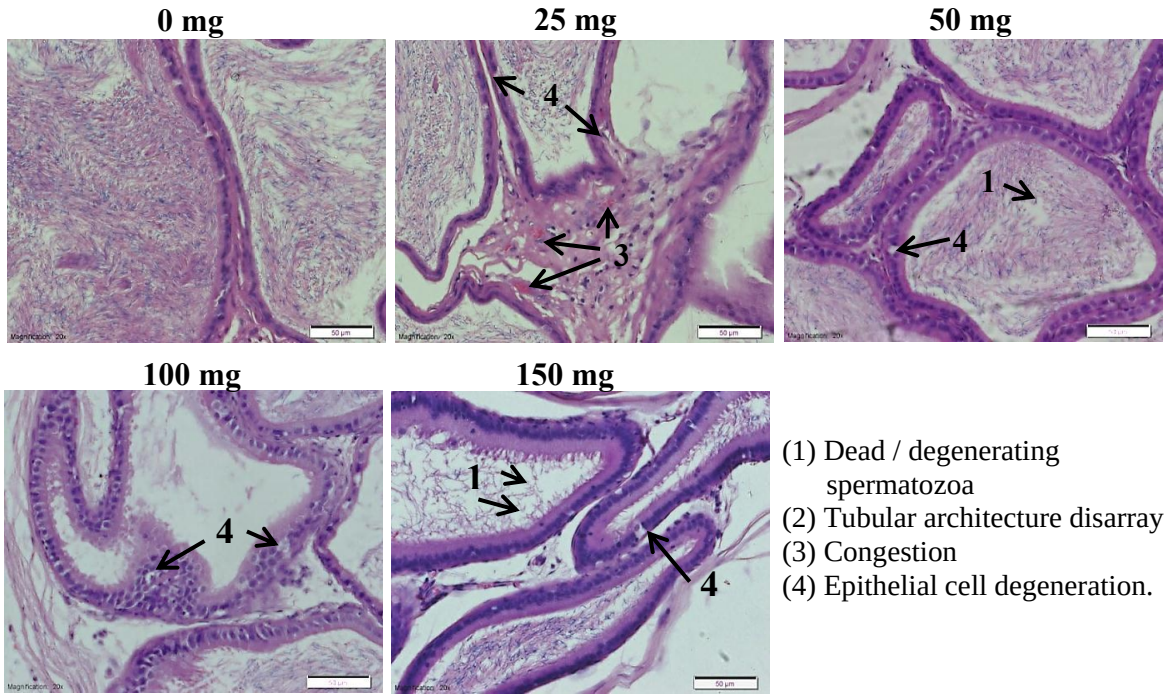

Figure 3.8. *Histopathology of caput in the allethrin treated rats, Arrows indicate site of lesion. Number indicates the type of lesion. Magnification: 40X.*

In the cauda, similar pathomorphological changes were observed and seem to be dose dependent (Figure 3.9).

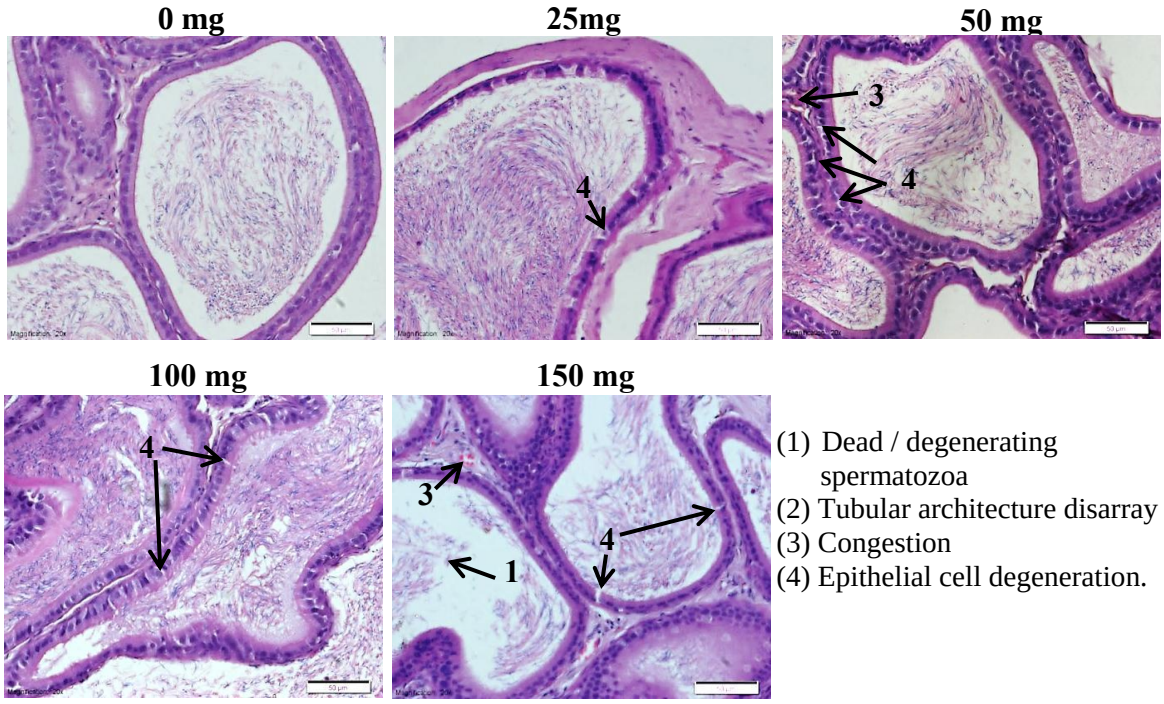

Figure 3.9. *Histopathology of cauda in the allethrin treated rats, Arrows indicate site of lesion. Number indicates the type of lesion. Magnification: 40X.*

In the testis, intestinal edema, tubule architecture disarray, changes in nuclear integrity, presence of dead or degenerating spermatids, epithelial cell disruption, germ cell layer damage and anomalies in lumen morphology were evident (Figure 3.10); and the effects observed were dose dependent.

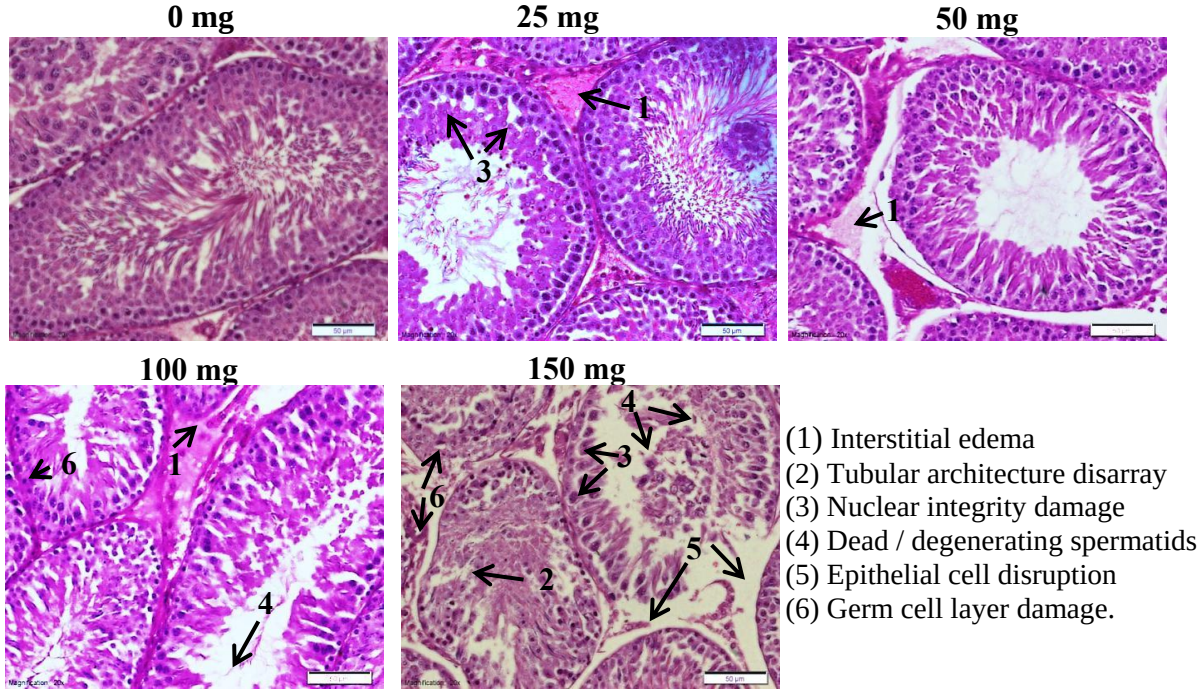

Figure 3.10. *Histopathology of testes in the allethrin treated rats. Arrows indicate site of lesion. Number indicates the type of lesion.*

Table 3.3. Histological scores of caput, cauda and testis obtained from allethrin treated rats.

Caput	Damage	25 mg	50 mg	100 mg	150 mg
1	Dead/degenerating spermatozoa	+	+ +	+ +	+ + +
2	Tubular architecture disarray	+	+ +	+ + +	+ + + +
3	Congestion	+	+ +	++	+ + +
4	Epithelial lining cell degeneration	+	+	+ +	+ +
Cauda					
1	Dead/degenerating spermatozoa	+	+ +	+ +	+ +
2	Tubular architecture disarray	+	+	+ +	+ + +
3	Congestion	+	+	+	+
4	Epithelial lining cell degeneration	++	+ + +	+ + +	+ + +
Testis					
1	Interstitial edema	+ + +	+ + + +	+ + + +	+ + + +
2	Tubule architecture disarray	+	+ +	+ + +	+ + + +
3	Nuclear integrity damage	+	+ +	+ + +	+ + + +
4	Dead/degenerating spermatids	+ +	+ +	+ + +	+ + + +
5	Epithelial cell disruption	+	+	+ +	+ +
6	Germ cell layer damage	+	+ +	+ + +	+ + + +
7	Lumen morphology anamoly	+ +	+ + +	+ + +	+ + + +

On a score of 5 points:- *+++++ : maximum damage; ++++ : high damage; +++ : Moderately damage; ++ : Low/moderate; + : Low.*

7. Gene expression analysis of spermatogenic, sperm maturation and capacitation factors

Maintenance of fertility is one of the major functions of the male reproductive tract. This is accomplished by the constant production of spermatozoa in a two stage process involving spermatogenesis and sperm maturation, which occur in the testis and epididymis respectively. Spermatogenesis is the process of formation of functional spermatozoa from germ cells, which is tightly regulated through transcriptional and post-transcriptional mechanisms. Spermatid formation from germ cells in testis requires interaction with Sertoli cells. Here, we evaluated the expression pattern of genes important for germ cell development in testis of allethrin treated rats. Further, the ability of sperm to fertilize depends on factors that facilitate capacitation and recognition of proteins on the ovum. Since we observed morphological, biochemical and molecular changes in the male reproductive tract due to allethrin exposure, it is possible that the gene expression pattern of spermatogenic factors (*Tgf-β1, Scf and C-Kit*), DNA-binding proteins (heat shock factor 2 (*Hsf2*), *Ovol1* and *Wt1*), chromatin-associated factors (bromodomain testis-specific protein (*Brdt*), RNA-binding proteins (gonadotrophin-regulated testicular RNA helicase (*Grth*), *Dazap1*, *Kdm3a*, *Ybx-2*), sperm maturation factors (*Spag11e*) and capacitation factors (*Defb22*) could be compromised. To the best of our knowledge, there are no reports on the effect of allethrin on the molecular changes that can occur in the male reproductive tract and thereby affect germ cell production and function. *Scf* and *c-Kit* mRNA expression levels were significantly down-regulated in the testis obtained from rats treated with 100 and 150 mg allethrin (Figure 3.11A and B). *Tgf-β1* mRNA levels were also reduced significantly in the testes of rats treated with all the doses tested (Figure 3.11C).

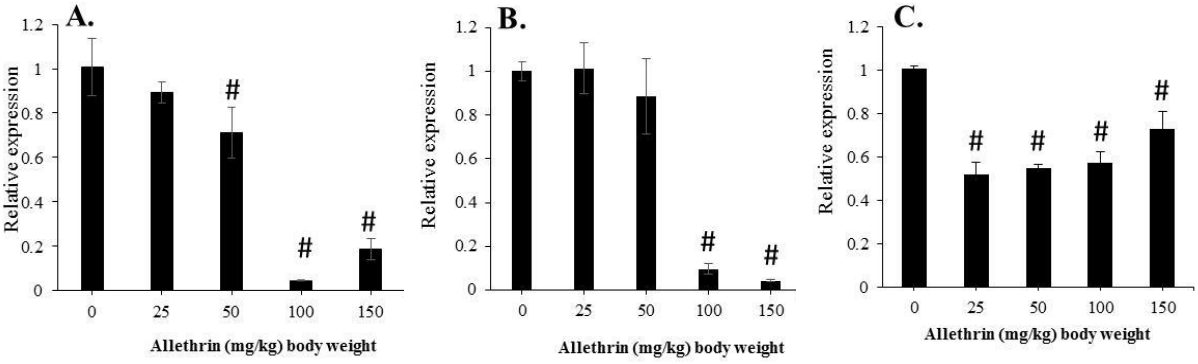

Figure 3.11. *mRNA expression of factors that influence sperm production in testes of allethrin treated rats.* ***(A)*** *Scf;* ***(B)*** *C-kit;* ***(C)*** *Tgf-β1. Values shown are Mean ± S.D. # denotes p < 0.001.*

Heat shock factor 2 (HSF2) and OVOL1 are DNA-binding proteins expressed in germ cells and directly act on genes that control spermatogenesis. Disruption of HSF-2 and OVOL1s leads to apoptosis of pachytene spermatocytes and degeneration of germ-cells resulting in reduced numbers of round and elongated spermatids in rats. In allethrin treated rats, expression of *Hsf2* and *Ovol1* are down-regulated (Figure 3.12A and B).

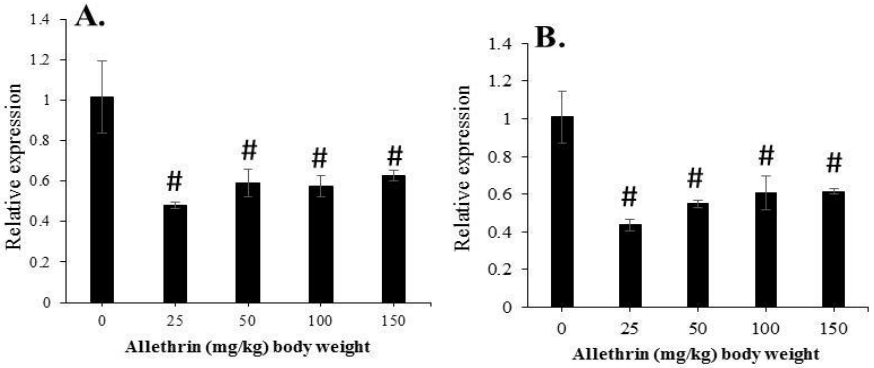

Figure 3.12. *mRNA expression of transcription factors that influence sperm production in testes of allethrin treated rats.* ***(A)*** *Hsf2;* ***(B)*** *Ovol1. Values shown are Mean ± S.D. # denotes p < 0.001.*

Bromodomain testis specific protein (BRDT), Lysine demethylase 3A (KDM3A) are chromatin associated factors regulate spermatogenesis. Down-regulation of KDM3A and BRDT led to drastically reduced sperm count and defects in male germ cells. mRNA levels of both chromatin associated factors were significantly reduced in testis of allethrin treated rats (Figure 3.13A and B). RNA-binding proteins that regulate spermatogenesis include Gonadotrophin-regulated testicular RNA helicase (GRTH) and Y box binding protein 2 (YBX2). *Grth* mRNA expression levels were significantly down-regulated in the testis of rats treated with varying doses of allethrin, whereas *Ybx2* levels were decreased in testes of 100 and 150 mg of allethrin (Figure 3.14A and B).

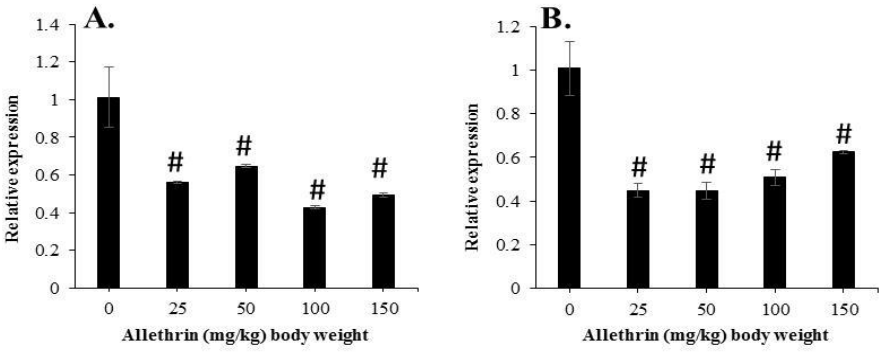

Figure 3.13. *mRNA expression of chromatin associated factors that influence sperm production in testes of allethrin treated rats.* ***(A)*** *Brdt;* ***(B)*** *Kdm3a. Values shown are Mean ± S.D. # denotes p < 0.001.*

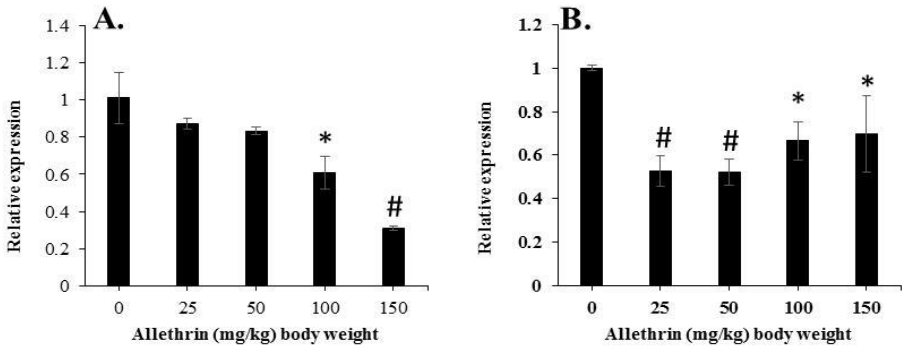

Figure 3.14. *mRNA expression of RNA binding proteins that influence sperm production in testes of allethrin treated rats.* ***(A)*** *Ybx2;* ***(B)*** *Grth. Values shown are Mean ± S.D. * and # denotes p <0.01 and p < 0.001, respectively.*

A drastic reduction was observed in the mRNA expression of *Spag11e* and *Defb22* in the epididymides of allethrin treated rats (Figure 3.15A and B). These results in combination with the histopathological analyses suggest allethrin exposure to rats may cause abnormal sperm morphology and alterations in the factors that affect sperm production and function.

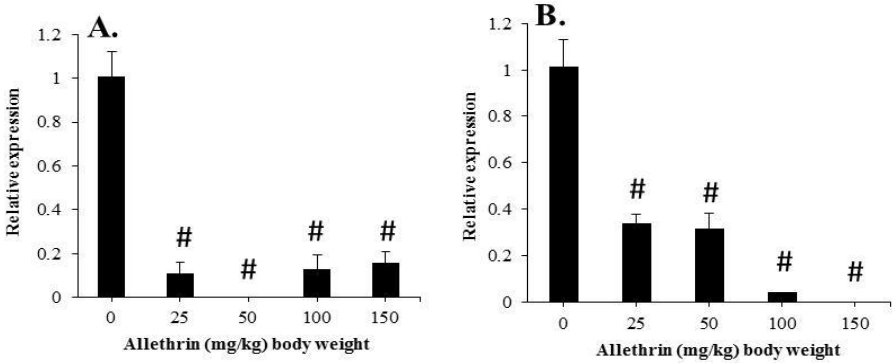

Figure 3.15. *mRNA expression of sperm maturation factors in epididymides of allethrin treated rats.* ***(A)*** *Spag11e;* ***(B)*** *Defb22. Values shown are Mean ± S.D. # denotes p < 0.001.*

8. Effect on steroidogenesis and serum testosterone levels

Significant decrease in the mRNA expression of cholesterol transporter (*StAR*) was observed in the testis obtained from 100 and 150 mg/kg allethrin treated rats. However, the mRNA expression of androgen receptor (*AR*) was decreased in the testis of rats treated with all the doses of allethrin (Figure 3.16A and B)**.** The steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β- hydroxysteroid dehydrogenase (17β-HSD) are crucial in the synthesis of testosterone. The activities of these enzymes were lowered at the highest dose of allethrin tested (Figure 3.17A and B).

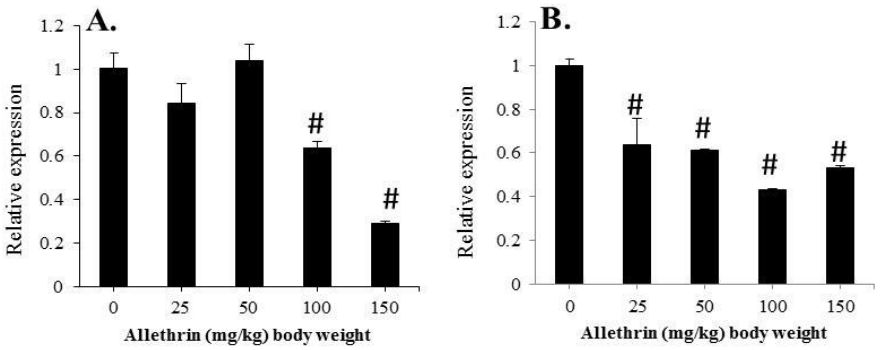

Figure 3.16. *mRNA expression of* ***(A)*** *StAR;* ***(B)*** *AR in the testis of allethrin treated rats. Values shown are Mean ± S.D. # denotes p < 0.001.*

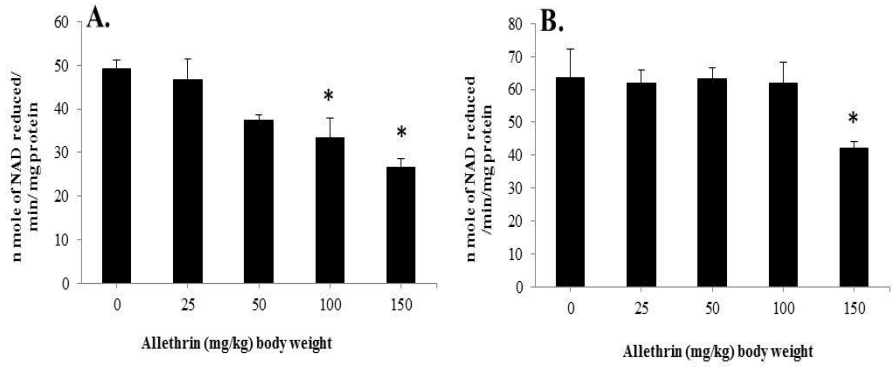

Figure 3.17. *Steroidogenic enzyme activities in testis of allethrin treated rats.* ***(A)*** *3β-HSD;* ***(B)*** *17β-HSD. Values shown are Mean ± S.D. * denotes p <0.01.*

As decrease in the activities of steroidogenic enzymes was observed, we measured the serum testosterone levels in allethrin treated rats. There was a significant decrease in the serum testosterone levels in rats treated with 100 and 150 mg allethrin (Figure 3.18).

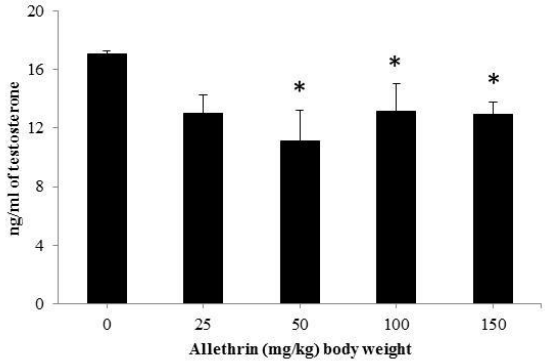

Figure 3.18. *Serum testosterone in allethrin treated rats. Values shown are Mean ± S.D. * denotes p< 0.01.*

9. Daily sperm production, sperm count and motility parameters

The mRNA expression levels of spermatogenic factor, *Tgf-β1* was significantly lowered in the testis of allethrin treated rats suggesting that the production of spermatids

in the testis may be impaired. As shown in figure 3.19, testicular daily sperm production was significantly decreased in rats treated with 100 and 150 mg allethrin.

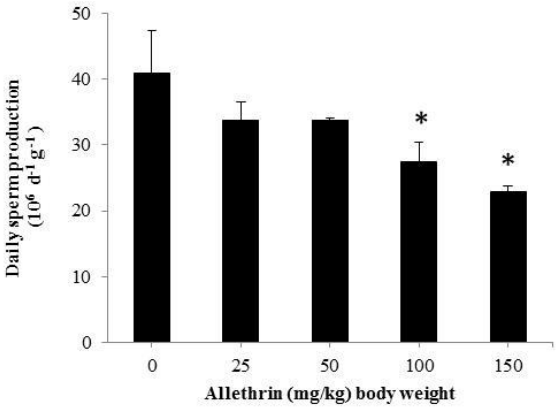

Figure 3.19. *Daily sperm production in the testes of allethrin treated rats. Values shown are Mean ± S.D. * denotes p <0.05.*

Spermatozoa collected from cauda epididymis were counted and we observed that allethrin treatment causes decreased sperm count (Table 3.4).

Table 3.4. Total sperm count in cauda epididymis of allethrin treated rats.

Allethrin dose (mg/kg body weight)	**Total sperm count (10^6)** **(**Mean ± S.D. * p <0.05).
0	166 ± 11.1
25	159.4 ± 13.4
50	155.9 ± 14.7
100	**146.2*** ± 6.2
150	**141.3*** ± 2.1

Motility parameters

Since allethrin significantly reduced the production of spermatids in the testis and decreased count, we also analysed its effect on the motility of spermatozoa. Sperm motility parameters like linear velocity (LIN), straight line velocity (VSL), curvilinear velocity (VCL), straightness (STR), beat cross frequency (BCF) and average path velocity (VAP) were analyzed. The VCL, BCF, VSL, VAP, STR and LIN were not altered in spermatozoa obtained from allethrin treated rats (Figure 3.20 A-F).

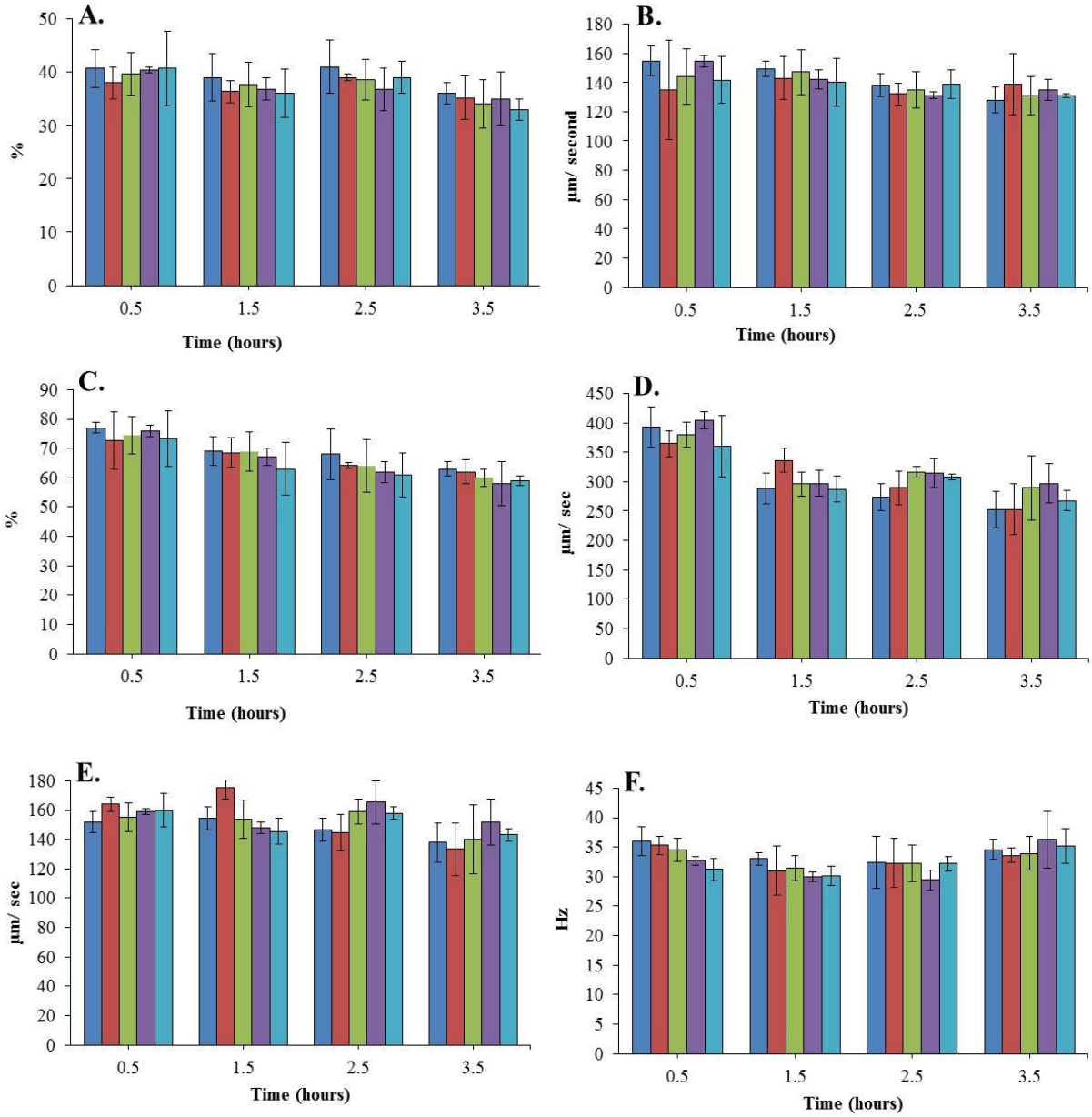

Figure 3.20: *Mean values of* ***(A)*** *Linear velocity (LIN);* ***(B)*** *Straight line velocity (VSL).* ***(C)*** *Straightness (STR);* ***(D)*** *Curvilinear velocity (VCL);* ***(E)*** *Average path velocity (VAP);* ***(F)*** *Beat cross frequency (BCF) of spermatozoa obtained from allethrin treated rats. (■) 0; (■) 25; (■) 50; (■) 100 and (■) 150 mg/kg body weight.*

10. Expression of cell cycle regulators

Increased lipid peroxidation in allethrin treated rats indicates elevated oxidative stress, which leads to altered gene expression of the stress response genes. DNA damage is a hall mark of oxidative stress during which the cell initiates multiple repair mechanisms. A key regulator that determines the fate of the cell either to undergo apoptosis or cell cycle arrest or to be hyper proliferative (cancer initiation) is the *p53* gene. Since increased lipid peroxidation was observed in all the tissues obtained from allethrin treated rats, changes in the expression pattern of *p53* were analyzed using real

time PCR. The expression of *p53* gene was significantly reduced in the epididymides and testes obtained from all the treatment groups (Figure 3.21A and B), suggesting that increased oxidative stress leads to suppression of *p53* mRNA expression, thereby allowing cancer initiation.

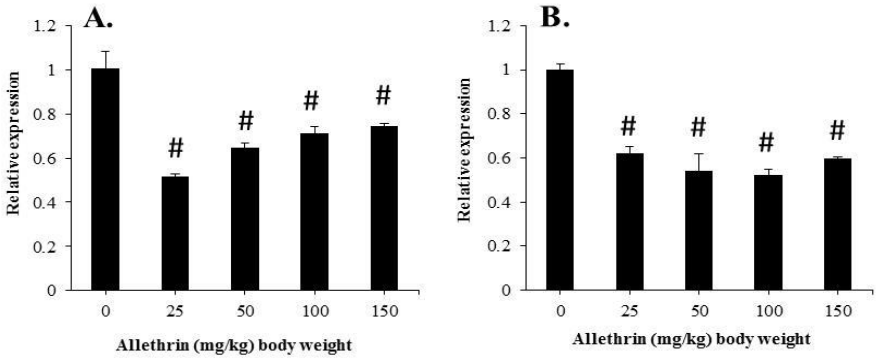

Figure 3.21*. mRNA expression of p53 in the epididymides and testes of allethrin treated rats*. ***(A)*** *Epididymis;* ***(B)*** *Testis. Values shown are Mean ± S.D. # denotes p <0.001.*

11. MAPK phosphorylation

Many cellular processes depend on the phosphorylation of a wide variety of proteins including MAPKs, whose role in cellular motility, proliferation, differentiation and death is well documented. P44/42 signaling pathway is activated by external stimuli are considered play an important role in initiation of cancer (Roberts and Der, 2007). To determine whether long term allethrin exposure could affect MAPK pathway, the levels of phospho p44/p42 was analyzed in the epididymis and testis. We observed increased phosphorylation of p44/p42 in the epididymis and testis (Figure 3.22) obtained from rats exposed to varying doses of allethrin, when compared with untreated control, suggesting that allethrin could affect cell proliferation.

12. Proteome analysis of caput epididymides

Proteome analysis using 2D gel electrophoresis was carried out in the caudal homogenates obtained from control and 100 mg/kg body weight allethrin treated rats. We observed differential expression of many proteins (Figure 3.23). Some of the differentially expressed proteins are given in the table 3.5. A two fold increase in the levels of sperm motility kinase W-like and protein disulfide isomerase associated 3, isoform CRA_a was observed. Endoplasmin precursor and glucose-regulated protein 78 (GRP78) was found to be downregulated significantly.

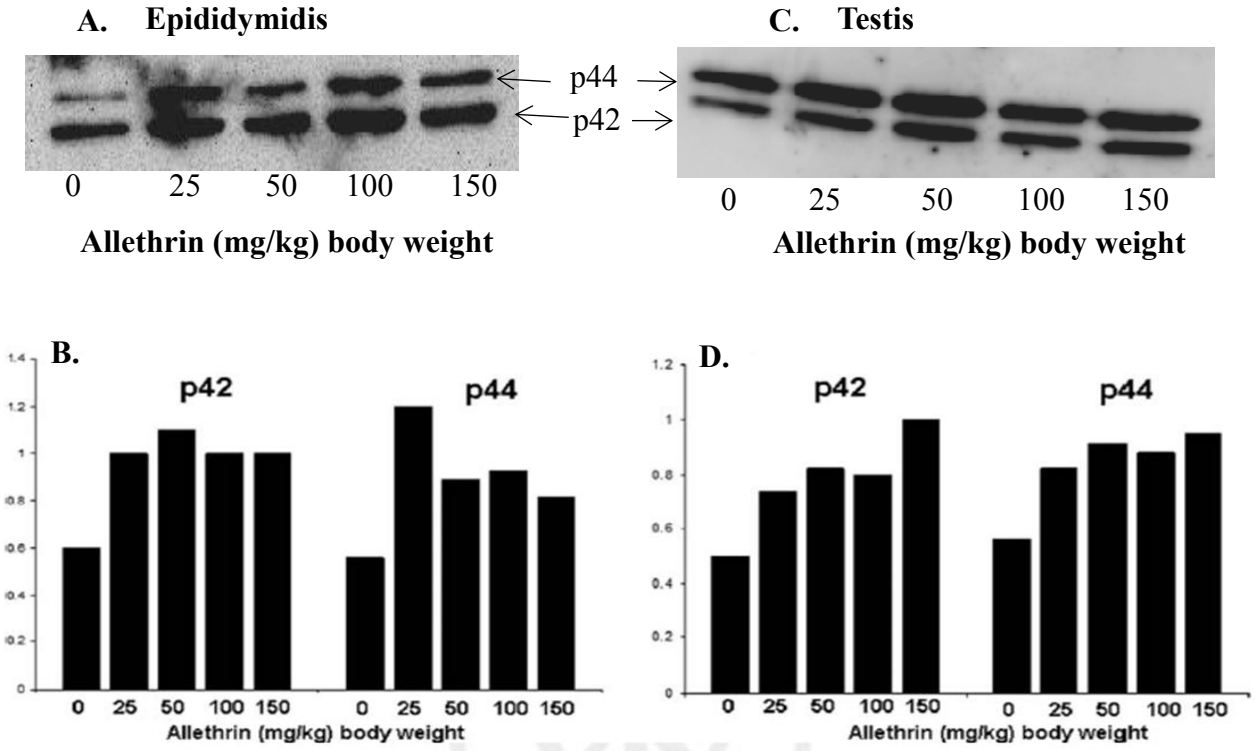

Figure 3.22. *Phosphorylation of MAPKs in the epididymis and testis obtained from allethrin treated rats. Western blot analyses for p44/42 in the epididymis* ***(A)*** *and testis* ***(C)***. *Densitometric analyses for phospho p44/p42 in the epididymis* ***(B)*** *and testis* ***(D)***. *Result shown is a representative of three independent experiments.*

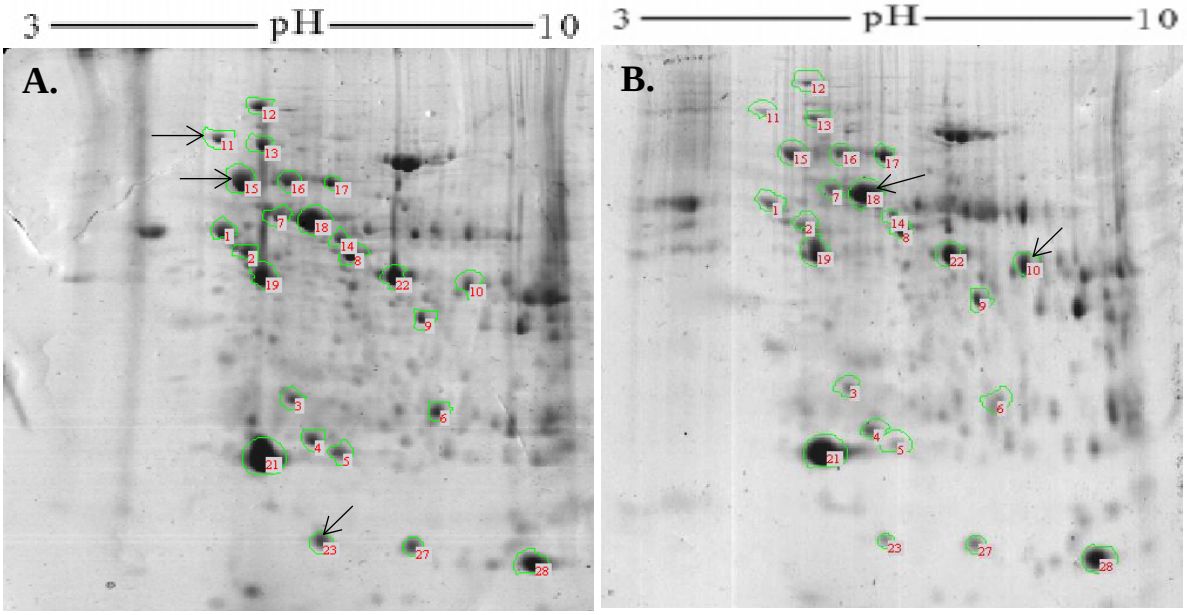

Figure 3.23: *2-D images of protein extracts of caput epididymides.* ***(A)*** *Control;* ***(B)*** *Allethrin treated. Arrows indicates match ID.*

Table 3.5. Differentially expressed proteins.

Match ID	Control	Treated	Ratio	Identity
10	6.75957	14.6306	2.164428	Sperm motility kinase W-like
11	6.01602	2.89476	0.481175	Endoplasmin precursor
15	9.57688	5.31648	0.555137	Glucose-regulated protein 78 (GRP78)
18	6.77479	11.8795	1.753486	Protein disulfide isomerase associated 3, isoform CRA_a (*Rattus norvegicus*)

Discussion

The use of pesticides for pest management has been on the rise over the decade. However, comprehensive analyses on the effect of pyrethroid based pesticides including allethrin on the male reproductive tract received little attention.

Environmental contaminants contribute to oxidative stress and initiates a variety of pathways leading to pathological conditions (Reuter *et al.,* 2010). In this study, we observed increased lipid peroxidation in the caput, corpus and cauda of allethrin treated rats. Earlier studies have demonstrated that exposure to pesticides caused an increase in lipid peroxidation in the brain, liver and kidney (Gabbianelli *et al.,* 2002; Giray *et al.,* 2001; Kale *et al.,* 1999; Vontas *et al.,* 2001). Similarly, LPO was observed in the male reproductive tract of rats exposed to pyrethroid compounds (Issam *et al.,* 2009; Wang *et al.,* 2009). The increased LPO observed in our study could be due to generation of free radicals by allethrin in the organ system. Increased LPO generates signals both at cellular and organ level to initiate defense mechanisms. NO is a free radical scavenger and is considered to play an antioxidant role during oxidative stress. We observed that NO levels were increased in the caput and unaltered in the cauda and testes of rats orally treated with allethrin, suggesting tissue specific responses. Alterations in the levels of NO were also observed in the testes of cypermethrin treated rats (Wang *et al.,* 2009). Further, it was demonstrated that pyrethrin based pesticides alter the levels of NO in the serum of mice (Kanbur *et al.,* 2008). We also observe that NO/MDA levels were unaltered in the caput, whereas it was lower in the cauda and testes of allethrin treated rats. NO seems to be an important free radical scavenger in the caput and not in the cauda and testes during allethrin induced toxicity.

Pyrethroid toxicity results in oxidative stress, thereby leading to alteration in the antioxidant status. For example, oral administration of cypermethrin increased the hepatic activities of SOD, GPx and catalase (Jin *et al.*, 2011). In contrast, a significant decline in the activities of antioxidant enzymes were observed in mice exposed to cypermethrin (Kanbur *et al.,* 2008). However, the effect of allethrin oral treatment on the antioxidant status in the male reproductive tract is not reported until. We observed that the activities of catalase, GPx and glutathione-s-transferase were increased in the caput and cauda but were decreased in the testes. Caput and cauda (epididymis) initiate free radical scavenging activity in response to allethrin induced oxidative stress, where as the antioxidant mechanisms in the testes seem to have failed. These contrasting results suggest organ specific responses and the susceptibility of different organs to the same stress.

The pathomorphological changes that occur in the reproductive tract during pyrethroid exposure are unreported. We observed that allethrin exposure to rats caused damage to tubular architecture, congestion, degeneration of epithelial cell lining and intestinal edema in the epididymis and testis. Further, dead or degenerating spermatids were observed. Pathological changes in different organ systems including the male reproductive tract due to pyrethroid toxicity are reported (Abdallah *et al.,* 2010; Dahamna *et al.,* 2010; Issam *et al.,* 2011). Free radicals because of their reactive nature interact with membrane lipids and alter membrane integrity. In this study, the pathomorphological damages observed in the testis and epididymides and the presence of dead spermatozoa could be due to harmful effects of free radicals generated and alterations in the antioxidant mechanisms.

Spermatogenesis is a complex process that involves a variety of proteins such as DNA binding factors (HSF-2, OVOL1, WT1, RHOX5 and SOX8), chromatin associated factors (JHDM2A, PYGO2, BRDT and TAF4B) and RNA binding proteins (GRTH, SAM68 and MSY2) (Bettegowda and Wilkinson, 2010). mRNA expression of *Hsf-2, Ovol-1, Brdt, Kdm3a, Ybx-2* and *Grth* were significantly down regulated in the testis obtained from allethrin treated rats. Spermatogenesis and sperm function are known to be sensitive to environmental stress including exposure to pesticides (Bal *et al.,* 2012; Piña-Guzmán *et al.,* 2009). Previous studies demonstrated that steroidogenesis, sperm motility and sperm production was decreased in rats exposed to a mixture of pesticides (Arena *et*

al., 2008; Perobelli *et al.,* 2010; Wang *et al.,* 2010; Zhang *et al.,* 2010). However, the effect of pyrethroid pesticides that affect factors that govern germ cell production are not reported yet. Besides these, other proteins that bind to the sperm surface such as TGF-β1, SPAG11E and DEFB22 also play a crucial role in sperm production (Olaso *et al.,* 1998; Yudin *et al.,* 2005; Zhao *et al.,* 2011b; Zhou *et al.,* 2004). We observed a decrease in *Tgf-b1, Spag11e and Defb22* (factors that influence germ cell production and maturation) expression. This is in agreement with the previous observation that the pyrethroid bifenthrin disrupts *Tgfb1*expression in rat ovarian granulose cells (Liu *et al.,* 2011). Decreased gene expression observed in this study could be due to the ability of allethrin to influence gene transctiption machinery, which may involve complex signaling pathways. Fenvalerate induces germ cell apoptosis through the Fas/FasL pathway (Zhao *et al.,* 2011a). It is also possible that other genes that are crucial for spermatogenesis may also be affected during allethrin toxicity. The mechanisms and the signaling pathways by which allethrin affects the expression of *Tgf-b1, Spag11e and Defb22* needs further investigation.

The consequences of free radical generation at the cellular level involves shift in the dynamics of gene expression resulting in the induction of apoptosis or transformation of the proliferative properties of the cell to become cancerous. One of the vital factors that determine the fate of the cell under stress is the *p53* gene. Decreased *p53* expression results in genomic instability and reduced ability of tumor suppression (Han *et al.,* 2008; Reuter *et al.,* 2010). Alterations in the expression of apoptosis related genes in the testes were reported earlier (Hsu *et al.,* 2007). We observed a reduced expression of p53 in the epididymides and testes at all the administered doses of allethrin, indicating a possibility of tumor development in these organs. MAP kinases regulate cell proliferation in response to external stimuli including environmental toxicants (Roberts and Der, 2007). We observed increased phosphorylation of p44/42 MAPKs in the epididymis and testis obtained from allethrin treated rats. Earlier reports suggest that the pyrethroids affect MAPK phosphorylation (Liu *et al.,* 2009; Qu *et al.,* 2012). It is possible that the increased levels of p44/p42 observed in this study could contribute to increased cell proliferation, thereby initiation of cancer in the male reproductive tract. Although previous studies demonstrated the toxic effects of allethrin inhalation due to use of mosquito coils and vaporizers, we for the first time report the effects due to oral administration (Madhubabu and Yenugu, 2012; Vences-Mejía *et al.,* 2012). Pesticides

dihydrorotenone (DHR) and bifenthrin alters the levels of stress protein, GRP78 (Skandrani *et al.,* 2006; Zhang *et al.,* 2013). In our study, we observed decreased expression of glucose-regulated protein (GRP78) in caput epididymis obtained from 100 mg allethrin treated rats.

Decreased expression of a spermatogenic factors in the testis impairs the formation of spermatids, thus a decrease in the production of sperms. Our results showed decreased daily sperm production in the testis when treated with 100 and 150 mg dose of allethrin. Sperm motility factors like linear velocity (LIN), straight line velocity (VSL), curvilinear velocity (VCL), straightness (STR), beat cross frequency (BCF) and average path velocity (VAP) were not altered in allethrin rats which indicate that produced sperms didn't show any abnormalities. These results indicate that, allethrin induces its effects at the level of spermatogenesis which causes decreased sperm production.

To summarize, we reported that oral administration of allethrin to rats for 60 days results in increased lipid peroxidation in the caput, cauda and testes accompanied by an alteration in the antioxidant status. Allethrin treatment resulted in pathomorphological changes of the male reproductive tract tissues. The expression of spermatogenic and sperm maturation factors were significantly reduced thereby possibly affecting sperm production. Oxidative stress induced by allethrin reduced p53 gene expression. Results of this study support our hypothesis that increased oxidative stress induced by oral administration of allethrin affects fertility and may contribute to disregulated cell cycle in the male reproductive tract. The molecular mechanisms of pyrethroid toxicity involve modifying the kinetics of voltage-sensitive sodium channels of neurons and there seems to be a structure-activity relationship. We demonstrate that allethrin causes reproductive toxicity by altering the oxidant status and may influence sperm production. However, further studies are required to determine whether there is a correlation between the mechanisms of toxicity of allethrin in the reproductive and nervous systems.

REFERENCES

Abdallah FB, Slima AB, Dammak I, Keskes-Ammar L, and Mallek Z. (2010). Comparative effects of dimethoate and deltamethrin on reproductive system in male mice. *Andrologia*, *42*(3), 182–6.

Aebi H. (1984). Catalase. *Methods in Enzymology*, *105*(1947), 121–126.

Aly HA, Domènech O, and Banjar ZM. (2012). Effect of nonylphenol on male reproduction: analysis of rat epididymal biochemical markers and antioxidant defense enzymes. *Toxicology and Applied Pharmacology*, *261*(2), 134–41.

Anvita Srivastava, Srivastava MK, and Raizada RB. (2006). Ninety-day toxicity and one-generation reproduction study in rats exposed to allethrin-based liquid mosquito repellent. *The Journal of Toxicological Sciences*, *31*(1), 1–7.

Arena AC, Fernandez CD, Porto EM, Bissacot DZ, Pereira OC, and Kempinas WG. (2008). Fenvalerate, A Pyrethroid Insecticide, Adversely Affects Sperm Production and Storage in Male Rats. *Journal of Toxicology and Environmental Health, Part A*, *71*(23), 1550–1558.

Bal R, Naziroglu M, Turk G, Yilmaz O, Kuloglu T, Etem E, and Baydas G. (2012a). Insecticide imidacloprid induces morphological and DNA damage through oxidative toxicity on the reproductive organs of developing male rats. *Cell Biochemistry and Function*, *30*(6), 492–9.

Bal R, Turk G, Tuzcu M, Yilmaz O, Ozercan I, Kuloglu T, Gur S, Nedzvetsky VS, Tykhomyrov AA, Andrievsky GV, Baydas G, and Naziroglu M. 2011. Protective effects of nanostructures of hydrated C(60) fullerene on reproductive function in streptozotocin-diabetic male rats. Toxicology 282:69–81.

Bal R, Turk G, Yılmaz O, Etem E, Kuloglu T, Baydaş G, and Naziroglu M. (2012b). Effects of clothianidin exposure on sperm quality, testicular apoptosis and fatty acid composition in developing male rats. *Cell Biology and Toxicology*, *28*(3), 187–200.

Bernheim F. (1964). The oxidative desulfuration of thio-acids. *Biochim. Biophys. Acta*, *90*, 426–428.

Bettegowda A, and Wilkinson MF. (2010). Transcription and post-transcriptional regulation of spermatogenesis. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *365*(1546), 1637–51.

Casida JE. (1995). Metabolism and synergism of Pyrethrins. In Pyrethrum Flowers Production, Chemistry,Toxicology, and Uses. (J. E. Q. Casida, G.B., Ed.). *Oxford University Press, New York.*, 258–276.

Dahamna S, Bencheikh F, Harzallah D, Boussahel S, Belgeit A, Merghem M, and Bouriche H. (2010). Cypermetherin toxic effects on spermatogenesis and male mouse reproductive organs. *Communications in Agricultural and Applied Biological Sciences*, *75*(2), 209–16.

Eil C, and Nisula BC. (1990). The binding properties of pyrethroids to human skin fibroblast androgen receptors and to sex hormone binding globulin. *Journal of Steroid Biochemistry*, *35*(3-4), 409–14.

Gabbianelli R, Falcioni G, Nasuti C, and Cantalamessa F. (2002). Cypermethrin-induced plasma membrane perturbation on erythrocytes from rats: reduction of fluidity in the hydrophobic core and in glutathione peroxidase activity. *Toxicology*, *175*(1-3), 91–101.

Giray B, Gürbay A, Hincal F. (2001). Cypermethrin-induced oxidative stress in rat brain and liver is prevented by Vitamin E or allopurinol. *Toxicology Letters*, *118*(3), 139–146.

Han ES, Muller FL, Pérez VI, Qi W, Liang H, Xi L, Fu C, Doyle E, Hickey M, Cornell J, Epstein CJ, Roberts JL, Remmen HV, and Richardson A. (2008). The in vivo gene expression signature of oxidative stress. *Physiological Genomics*, *34*(1), 112–26.

Hsu PC, Pan MH, Li LA, Chen CJ, Tsai SS, and Guo YL. (2007). Exposure in utero to 2,2',3,3',4,6'-hexachlorobiphenyl (PCB 132) impairs sperm function and alters testicular apoptosis-related gene expression in rat offspring. *Toxicology and Applied Pharmacology*, *221*(1), 68–75.

Institoris L, Undeger U, Siroki O, Nehez M, and Desi I. (1999). Comparison of detection sensitivity of immuno- and genotoxicological effects of subacute cypermethrin and permethrin exposure in rats. *Toxicology*, *137*(1), 47–55.

Issam C, Samir H, Zohra H, Monia Z, and Hassen BC. (2009). Toxic responses to deltamethrin (DM) low doses on gonads , sex hormones and lipoperoxidation in male rats following subcutaneous treatments. *The Journal of Toxicological Sciences*, *34*(6), 663–670.

Issam C, Zohra H, Monia Z, and Hassen BC. (2011). Effects of dermal sub-chronic exposure of pubescent male rats to permethrin (PRMT) on the histological structures of genital tract, testosterone and lipoperoxidation. *Experimental and Toxicologic Pathology*, *63*(4), 393–400.

Jakoby WB. (1978). The glutathione S-transferases: a group of multifunctional detoxification proteins. *Advances in Enzymology and Related Areas of Molecular Biology*, *46*, 383–414

Jana K, Jana S, and Samanta PK. (2006). Effects of chronic exposure to sodium arsenite on hypothalamo-pituitary-testicular activities in adultrats: possible an estrogenic mode of action. *Reproductive Biology and Endocrinology, 4*, 9–22.

Jin Y, Wang L, Ruan M, Liu J, Yang Y, Zhou C, Xu B, and Fu Z. (2011). Cypermethrin exposure during puberty induces oxidative stress and endocrine disruption in male mice. *Chemosphere*, *84*(1), 124–30.

Kakko I, Toimela T, and Tähti H. (2003). The synaptosomal membrane bound ATPase as a target for the neurotoxic effects of pyrethroids, permethrin and cypermethrin. *Chemosphere*, *51*(6), 475–80.

Kale M, Rathore N, John S, and Bhatnagar D. (1999). Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes : a possible involvement of reactive oxygen species, *105*, 197–205.

Kanbur M, Liman BC, Eraslan G, and Altinordulu S. (2008). Effects of Cypermethrin , Propetamphos , and Combination Involving Cypermethrin and Propetamphos on Lipid Peroxidation in Mice. *Environment Toxicology*, *23*(4), 473–479.

Lepoivre M, Chenais B, Yapo A, Lemaire G, Thelander L, and Tenu JP. (1990). Alterations of ribonucleotide reductase activity following induction of the nitrite-generating pathway in adenocarcinoma cells. *The Journal of Biological Chemistry*, *265*(24), 14143–9.

Li H, Shi N, Chen D, Dai Z, Lu W, Wang B, and Li, Y. (2005). [Oxidative stress of deltamethrin on rat nervous system]. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi*, *23*(2), 97–101.

Liu H, Xu L, Zhao M, Liu W, Zhang C, and Zhou S. (2009). Enantiomer-specific, bifenthrin-induced apoptosis mediated by MAPK signalling pathway in Hep G2 cells. *Toxicology*, *261*(3), 119–25.

Liu J, Yang Y, Yang Y, Zhang Y, and Liu W. (2011). Disrupting effects of bifenthrin on ovulatory gene expression and prostaglandin synthesis in rat ovarian granulosa cells. *Toxicology*, *282*(1-2), 47–55.

McCord JM and Fridovich I. (1969). Superoxide Dismutase. An enzymic function for Erythrocuprein (Hemocuprein). *The Journal of Bological Chemistry*, *244*(22), 6049–6055.

Madhubabu G, and Yenugu S. (2012). Effect of continuous inhalation of allethrin-based mosquito coil smoke in the male reproductive tract of rats. *Inhalation Toxicology*, *24*(3), 143–52.

Nasuti C, Gabbianelli R, Falcioni ML, Di Stefano A, Sozio P, and Cantalamessa F. (2007). Dopaminergic system modulation, behavioral changes, and oxidative stress after neonatal administration of pyrethroids. *Toxicology*, *229*(3), 194–205.

Naziroglu M, Butterworth PJ, and Sonmez TT. (2011). Dietary vitamin C and E modulates antioxidant levels in blood, brain, liver, muscle, and testes in diabetic aged rats. *International Journal for Vitamin and Nutrition Research*, *81*(6), 347–57.

Olaso R, Pairault C, Boulogne B, Durand P, and Habert R. (1998). Transforming growth factor beta1 and beta2 reduce the number of gonocytes by increasing apoptosis. *Endocrinology*, *139*(2), 733–40.

Paglia DE, and Valentine WN. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of Laboratory and Clinical Medicine*, *70*(1), 158–69.

Perobelli JE, Martinez MF, da Silva Franchi CA, Fernandez CDB, de Camargo J LV, and Kempinas WDG. (2010). Decreased sperm motility in rats orally exposed to single or mixed pesticides. *Journal of Toxicology and Environmental Health. Part A*, *73*(13-14), 991–1002.

Piña-Guzmán B, Sánchez-Gutiérrez M, Marchetti F, Hernández-Ochoa I, Solís-Heredia MJ, and Quintanilla-Vega B. (2009). Methyl-parathion decreases sperm function and fertilization capacity after targeting spermatocytes and maturing spermatozoa. *Toxicology and Applied Pharmacology*, *238*(2), 141–9.

Pontecorvo G, and Fantaccione S. (2006). Recombinogenic activity of 10 chemical compounds in male germ cells of Drosophila melanogaster. *Ecotoxicology and Environmental Safety*, *65*(1), 93–101.

Qu JH, Fei J, Hong X, Chen JF, Gu AH, Sun H, Xu XL, Song L, Wang SL, and Wang XR. (2012). Involvement of IGF-I signaling pathway in the regulation of steroidogenesis in mouse Leydig cells treated with fenvalerate. *Toxicology*, *292*(2-3), 151–5.

Rao J, Herr JC, Reddi PP, Wolkowicz MJ, Bush LA, Sherman NE., Black M, and Flickinger CJ. (2003). Cloning and characterization of a novel sperm-associated isoantigen (E-3) with defensin- and lectin-like motifs expressed in rat epididymis. *Biology of Reproduction*, *68*(1), 290–301.

Reuter S, Gupta SC, Chaturvedi MM, and Aggarwal BB. (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radical Biology and Medicine*, *49*(11), 1603–16.

Robb GW, Amann RP, and Killian GJ. (1978). Daily sperm production and epididymal sperm reserves of pubertal and adult rats. *Journal of Reproduction and Fertility*, *54*(1), 103–7.

Roberts PJ and Der CJ. (2007). Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene*, *26*(22), 3291–310.

Sattin RW, Roisin A, Kafrissen ME, Dugan JB, and Farer LS. (1984). Epidemic of gynecomastia among illegal Haitian entrants. *Public Health Reports*, *99*(5), 504–10.

Seed J, Chapin RE, Clegg ED, Dostal LA, Foote RH, Hurtt ME, Klinefelter GR, Makris SL, Perreault SD, Schrader S, Seyler D, Sprando R, Treinen KA, Veeramachaneni DN, and Wise LD. (1996). Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: A consensus report. *Reproductive Toxicology*, *10*(3), 237–244.

Sharpe RM, and Irvine DS. (2004). How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health? *BMJ*, *328*(February), 447–451.

Shukla Y, Yadav A, and Arora A. (2002). Carcinogenic and cocarcinogenic potential of cypermethrin on mouse skin. *Cancer Letters*, *182*(1), 33–41.

Shum W W, Da Silva N, Brown D, and Breton S. (2009). Regulation of luminal acidification in the male reproductive tract via cell-cell crosstalk. *The Journal of Experimental Biology*, *212*(Pt 11), 1753–61.

Skandrani D, Gaubin Y, Vincent C, Beau B, Claude Murat J, Soleilhavoup JP, and Croute F. (2006). Relationship between toxicity of selected insecticides and expression of stress proteins (HSP, GRP) in cultured human cells: effects of commercial formulations versus pure active molecules. *Biochimica et Biophysica acta, 1760(1)*, 95-103

Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, Stevens JT, and Weiner ML. (2002). Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology*, *171*(1), 3–59.

Tan J, and Soderlund DM. (2010). Divergent actions of the pyrethroid insecticides S-bioallethrin, tefluthrin, and deltamethrin on rat Na(v)1.6 sodium channels. *Toxicology and Applied Pharmacology*, *247*(3), 229–37.

Tollner TL, Yudin AI, Tarantal AF, Treece CA, Overstreet JW, and Cherr GN. (2008). Beta-defensin 126 on the surface of macaque sperm mediates attachment of sperm to oviductal epithelia. *Biology of Reproduction*, *78*(3), 400–12.

Tomlin C. (1994). *A world compendium: The pesticide manual. British Crop Protection Council*. Farnham Surrey; Cambridge.

Vences-Mejía A, Gómez-Garduño J, Caballero-Ortega H, Dorado-González V, Nosti-Palacios R, Labra-Ruíz N, and Espinosa-Aguirre JJ. (2012). Effect of mosquito mats (pyrethroid-based) vapor inhalation on rat brain cytochrome P450s. *Toxicology Mechanisms and Methods*, *22*(1), 41–6.

Vijverberg HP, and de Weille JR. (1985). The interaction of pyrethroids with voltage-dependent Na channels. *Neurotoxicology*, *6*(2), 23–34.

Vontas JG, Small GJ, and Hemingway J. (2001). Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens. *Biochemical Journal*, *357*, 65–72.

Wang H, Wang Q, Ping XZ, Tao XM, Ji YY, Zhang H, Zhang C, Zhang Y, and Xu DX. (2010). Cypermethrin exposure during puberty disrupts testosterone synthesis via downregulating StAR in mouse testes. *Archives of Toxicology*, *84*, 53–61.

Wang XZ, Liu SS, Sun Y, Wu JY, Zhou YL, and Zhang JH. (2009). Beta-cypermethrin impairs reproductive function in male mice by inducing oxidative stress. *Theriogenology*, *72*(5), 599–611.

Yokohira M, Arnold LL, Lautraite S, Sheets L, Wason S, Stahl B, Eigenberg D, Pennington KL, Kakiuchi-Kiyota S, and Cedex RD. (2011). The effects of oral treatment with transfluthrin on the urothelium of rats and its metabolite, tetrafluorobenzoic acid on urothelial cells in vitro. *Food and Chemical Toxicology*, *49*(6), 1215–1223.

Yudin AI, Tollner TL, Li MW, Treece CA, Overstreet JW, and Cherr GN. (2003). ESP13.2, a member of the beta-defensin family, is a macaque sperm surface-coating protein involved in the capacitation process. *Biology of Reproduction*, *69*(4), 1118–28.

Yudin AI, Generao SE, Tollner TL, Treece CA, Overstreet JW, and Cherr GN. (2005). Beta-defensin 126 on the cell surface protects sperm from immunorecognition and binding of anti-sperm antibodies. *Biology of Reproduction*, *73*(6), 1243–52.

Zhang H, Wang H, Wang Q, Zhao XF, Liu P, Ji YL, and Xu DX. (2010). Pubertal and early adult exposure to fenvalerate disrupts steroidogenesis and spermatogenesis in mice at adulthood. *Journal of Applied Toxicology : JAT*, *30*(4), 369–77.

Zhang J, Tang J, Cao B, Zhang Z, Li J, Schimmer AD, He S, and Mao X. (2013). The natural pesticide dihydrorotenone induces human plasma cell apoptosis by triggering endoplasmic reticulum stress and activating p38 signaling pathway. PLoS One, 8(7), e69911.

Zanich A, Pascall JC, and Jones R. (2003). Secreted epididymal glycoprotein 2D6 that binds to the sperm's plasma membrane is a member of the beta-defensin superfamily of pore-forming glycopeptides. *Biology of Reproduction*, *69*(6), 1831–42.

Zhao XF, Wang Q, Ji YL, Wang H, Liu P, Zhang C, and Xu DX. (2011a). Fenvalerate induces germ cell apoptosis in mouse testes through the Fas/FasL signaling pathway. *Archives of Toxicology*, *85*(9), 1101–8.

Zhao Y, Diao H, Ni Z, Hu S, Yu H, and Zhang Y. (2011b). The epididymis-specific antimicrobial peptide β-defensin 15 is required for sperm motility and male fertility in the rat (Rattus norvegicus). *Cellular and Molecular Life Sciences*, *68*(4), 697–708.

Zhou CX, Zhang YL, Xiao L, Zheng M, Leung KM, Chan MY, and Chan HC. (2004). An epididymis-specific beta-defensin is important for the initiation of sperm maturation. *Nature Cell Biology*, *6*(5), 458–64.

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Chapter 4

Effect of exposure of prenatal rats to allethrin-based mosquito coil smoke.

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Introduction

Allethrin, the first synthetic pyrethroid is used as insecticide for crop protection and in domestic settings to protect from diseases transmitted by mosquitoes, flies etc. In domestic settings, extra precautions are taken to prevent mosquito bites to pregnant women and the new borns. Such an exposure throughout pregnancy to mosquito repellents may cause health effects to the mother and the fetus. The reproductive toxicity of pesticides is best studied by multigenerational studies (Janer et *al.,* 2007). Exposure of humans during fetal and lactational stages of development to organochlorines like 2, 3, 7, 8-tetrachlorodibenzo-p-dioxon (TCDD) alters testosterone biosynthesis (Cooke *et al.,* 1998). Endocrine-disrupting chemicals significantly affect the fetal vulnerability. During development, the fetus is particularly sensitive to hormonal fluctuations and exposures to low levels of exogenous hormones or toxicants may result in permanent physiologic changes that are not seen in adults exposed at similar levels (Bigsby *et al.,* 1999). Any disorders in sex steroid hormone balance during the fetal development may interfere with male reproductive success and health later in life (Sharpe, 2001). A number of anthropogenic chemicals referred to as xenohormones, which include pesticides, industrial chemicals and pharmaceuticals, have the capacity to interfere with hormonal balance (Norgil *et al.,* 1996; Toppari *et al.,* 1996). Herbicide atrazine exposure during gestation shows delayed puberty in female rats (Davis *et al.,* 2011). Linuron, a herbicide when exposed during the development alters the expression of genes involved in differentiation of the upper Wolffian duct (Turner *et al.,* 2003). Rat pups exposed to liquid mosquito repellent during prenatal, postnatal and perinatal stages of development shows cholinergic dysfunction (Sinha *et al.,* 2006). Synthetic pyrethroid cypermethrin when exposed to mouse during puberty downregulates steroid-acute regulatory protein (StAR) protein (Wang *et al.,* 2010). Fenvalerate exposure to adult rats by inhalation obviously induced the decrease in testicular weight, epididymal sperm counts, and sperm motility (Mani *et al.,* 2002).

However, the effect of exposure to allethrin based mosquito repellents during pregnancy and neonatal stages are not evaluated. The purpose of the study is to determine whether prenatal and postnatal exposure to mosquito coil smoke could have any effects on the male reproductive development in the off spring.

Materials and Methods

1. Animals and mosquito coil smoke exposure

One day pregnant female Wistar rats were obtained from National Center for Laboratory Animals (NCLAS), National Institute of Nutrition (NIN), Hyderabad. They were maintained on a 12L: 12D lighting schedule, at 22–25°C, with food and water *ad libitum*. Rats were randomly divided into two groups (n = 6), housed in separate rooms and body weights were recorded. To simulate the human exposure settings, in a closed room, mosquito coils were lit (one coil per night, which is expected to burn for 8 hours) and the animals were allowed to inhale coil smoke up to 21 days of gestation (Figure 4.1). Control animals were maintained under normal animal house conditions in a separate room. After parturition, pups were exposed to coil smoke during lactation period and up to post natal day 21 (pre-weaning). Males were separated after pre-weaning and exposed to coil smoke up to 90 days (adult). Animals were sacrificed at 10, 30 and 90 days of age by CO_2 asphyxiation followed by cervical dislocation. Testis and epididymis were collected from control and mosquito coil smoke exposed rats. Body and organ weights were recorded. Blood was collected by cardiac puncture and serum was collected by centrifugation at 5,000 rpm for 15 min at 4°C. Tissues were frozen in liquid nitrogen, and stored at −80°C until use. Experiments were conducted using the guidelines for the care and use of laboratory animals, approved by the Institutional Animal Ethics Committee of University of Hyderabad.

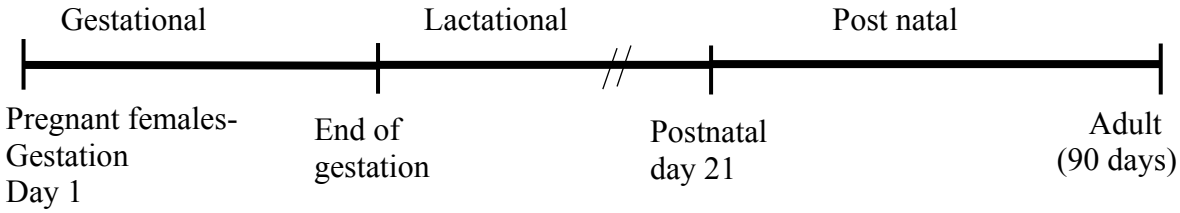

Figure 4.1. *Experimental design for exposure of mosquito coil smoke to pregnant female rats and the resulting offspring.*

2. Estimation of lipid peroxidation products

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA), the product of lipid peroxidation, under acidic conditions and high temperature (95°C) to form a pink colored complex with absorbance maxima at 532 nm. 10% (w/v) homogenates of caput, cauda and testes obtained from the control and experimental animals were prepared in ice cold phosphate buffer saline, pH 7.4 and the extent of lipid peroxidation was

estimated using the TBA method as described earlier (Bernheim, 1964). 1,1,3,3-tetraethoxy-propane (TEP) was used as the standard. Levels of lipid peroxidation was expressed as nano moles MDA/ mg protein.

3. Estimation of Nitric oxide as its stable metabolite nitrite

NO being a free radical is highly unstable and gets converted to an equimolar ratio of its stable metabolites, nitrite and nitrate. The estimation of NO is therefore done by estimating its stable metabolite. The amount of nitrite/ nitrate formed is an index of the amount of NO generated in the samples. Nitrite was measured by the method of Griess as described earlier (Lepoivre *et al.*, 1990).

4. Antioxidant enzyme assays

Antioxidant enzyme activity was measured using spectrophotometric methods. Caput, cauda, and testes collected from vehicle control and allethrin treated rats. 10% (w/v) homogenates were prepared in ice cod phosphate buffer saline, pH 7.4 and centrifuged at 3000 rpm to remove the debris. The supernatant was collected and the activities of catalase, glutathione-s-transferase (GST), glutathione peroxidase (GPx), and superoxide dismutase (SOD) were performed using standard protocols described earlier (Aebi, 1984; Jakoby, 1978; McCord and Fridovich, 1969; Paglia and Valentine, 1967). The activity of the antioxidant enzymes were calculated from the change in optical density measured on a spectrophotometer. Detailed methodologies for estimation of antioxidant enzyme activity are described in chapter 1.

5. Real time polymerase chain reaction (RT-PCR)

Tissues obtained from vehicle control and allethrin treated rats were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted. Total RNA (2 µg) was reverse transcribed using 50 U Stratascript (Invitrogen, USA) and 0.5 µg of oligodT (Invitrogen) according to the manufacturer's instructions. The resultant cDNA (2 µl) was used for real time PCR analysis using SYBR master mix kit (Applied Biosystems, Warrington, UK) in a real time thermal cycler (Applied Biosystems, Warrington, UK). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression was used as the internal control. A typical real time PCR reaction consisted of the following conditions: initial denaturation at 94°C for 10 min; 40 cycles with 94°C for 15 secs and 60°C for 1 min. Negative controls (no template control and minus RT control (only with RNA) were included in the assays to detect non-specific amplification.

The amplicons were sequenced to confirm their identity. Gene specific primers used were listed in table 4.1.

Table 4.1. Gene Specific primers

Primer	Sequence (5'-3')
17-β HSD F	CACTATCAGCGTGGTTATGAGCAAGC
17-β HSD R	GTGTCTGGATCCCCTGAAACTTGG
3-β HSD F	GGGCATCTCTGTTGTCATCCACAC
3-β HSD R	GATGGTCTTCTTGTAGGAGTTGGGC
Ar F	CACTACGGAGCTCTCACTTGTG
Ar R	CCAGAGTCATCCCTGCTTCATAAC
Brdt F	CAGTTCCTGCAGAGAGTCGT
Brdt R	CGTAGTACCTGTTTTCCAGCC
c-kit F	GGCCTCAGGAGCTCTATTTAC
c-kit R	GAGAGATTTCCCATCACACTC
Defb 22	CACTTGGTCACAGGTAACTG
Defb 22	CAGCAGCTTGGTTTCCATCTG
Gapdh F	CCAATGTATCCGTTGTGGATCTG
Gapdh R	GAGTTGCTGTTGAAGTCACAG
Grth/Ddx25 F	CCAGAACCTCATAGCACAGAG
Grth/Ddx25 F	CAGAGCCAGTTCATAGGTG
Hsf-2 F	TAAATGGCTCCTCCAACCTG
Hsf-2 R	CCAACAGCTCAACCTTTCCTA
Kdm3a F	CAAGTCAACTGTGAGGAG
Kdm3a R	GACTTGCGTTTCACAGCTC
Ovol1 F	GACCTCAAGAGACATGTCC
Ovol1 R	GGCGCTCCTTATATGCATAC
p53 F	CAAGGCAACTATGGCTTCCAC
p53 R	GACTTCTTGTAGATGGCCATG
Scf F	GATGACCTCGTGGCATGTATG
Scf R	CAGGACCTCATGTTGAAGAGAG
Spag11e F	CACATCTGCTTTCCTGCACAG
Spag11e R	GCACCCCACATCTCAGATCTTC
StAR F	CTCCTACAGACATATGCGGAAC
StAR R	CTGGTCACTGTAGAGTGTTG
Tgf-β1 F	GCAACAATTCCTGGCGTTAC
Tgf-β1 R	CCACGTGGAGTACATTATCTTTGC

6. Estimation of serum testosterone

Blood was collected by cardiac puncture and serum was separated by centrifugation at 5,000 rpm for 15 min at 4°C. Serum was stored at -20°C until further

analysis. Testosterone was estimated by using ELISA kit according to manufacturer's protocol (Omega diagnostics, UK).

7. Assessment of acrosome reaction

Pisum sativum agglutinin (PSA) binds to the glycoproteins present on the sperm membrane and acrosomal matrix. During acrosome reaction there is loss of acrosomal membrane matrix and thus leading to reduced fluorescence, which can be used to assess acrosome reaction. To determine the effect of mosquito coil smoke exposure on acrosome reaction, caudal spermatozoa were allowed to capacitate in M2 medium. Acrosome reaction was induced in spermatozoa ($5x10^6$ cells/ml) using the ionophore A23287 (20 µM). They were then washed with PBS and stained with PSA-FITC (50 µg/ml) which binds to acrosome specific glycoproteins (Jaiswal *et al.,* 1999). The intensity of fluorescence was measured using flow cytometer (BD biosciences). Decrease in fluorescence intensity indicates acrosome reaction. The acrosome reacted population showing less intensity and the acrosome intact spermatozoa with high fluorescence intensity were gated and analysed.

8. Statistical analyses

Changes in the parameters analyzed in this study were compared with the vehicle control. Statistical analyses were performed using one way ANOVA and Student's t-test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are Mean ± S.D. * and # denotes $p < 0.05$ and $p < 0.01$ respectively.

Results

1. Litter size and organ weights

The litter size of control and mosquito coil smoke exposed female rats were not altered and there was no significant mortality. Though two pups died within three days, it may be attributed to suffocation experienced (Figure 4.2). No significant changes in reproductive organ weights was observed when analysed at different time points and decreased body weight was observed only in pups exposed to smoke for 90 days (Table 4.2).

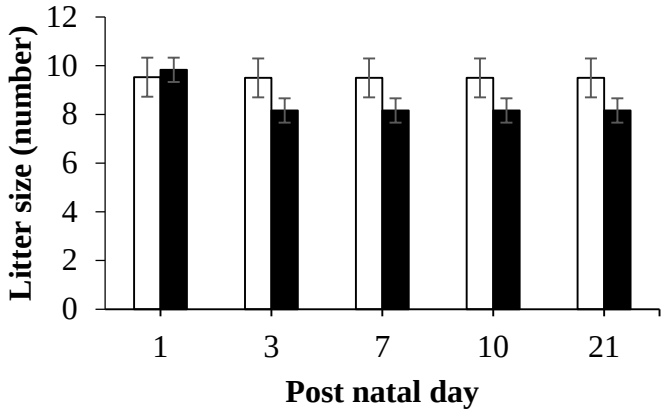

Figure 4.2. *Litter size of female rats exposed to mosquito coil smoke. Control (□); Mosquito coil smoke exposed (■).*

Table 4.2. Body and reproductive organs weight (grams) of unexposed and mosquito coil smoke exposed rats. * denotes p <0.05. (MCS: Mosquito coil smoke exposed)

	Body weight (gm)		**Epididymides (gm)**		**Testis (gm)**	
	Control	**MCS**	**Control**	**MCS**	**Control**	**MCS**
10 days	25.6 ± 2.8	23.2 ± 0.34	0.075 ± 0.01	0.07 ± 0.01	0.25 ± 0.08	0.27 ± 0.06
30 days	93.4 ± 1.5	88.3 ± 2.7	0.18 ± 0.04	0.2 ± 0.01	1.09 ± 0.2	0.89 ± 0.16
90 days	244 ± 4.8	**231.1*** ± 3.1	0.43 ± 0.02	0.39 ± 0.07	2.01 ± 0.17	1.82 ± 0.1

2. Lipid peroxidation

Lipid peroxidation was significantly increased in the testis (Figure 4.3B) of developing rats exposed to mosquito coil smoke, whereas no changes were observed in epididymides (Figure 4.3A).

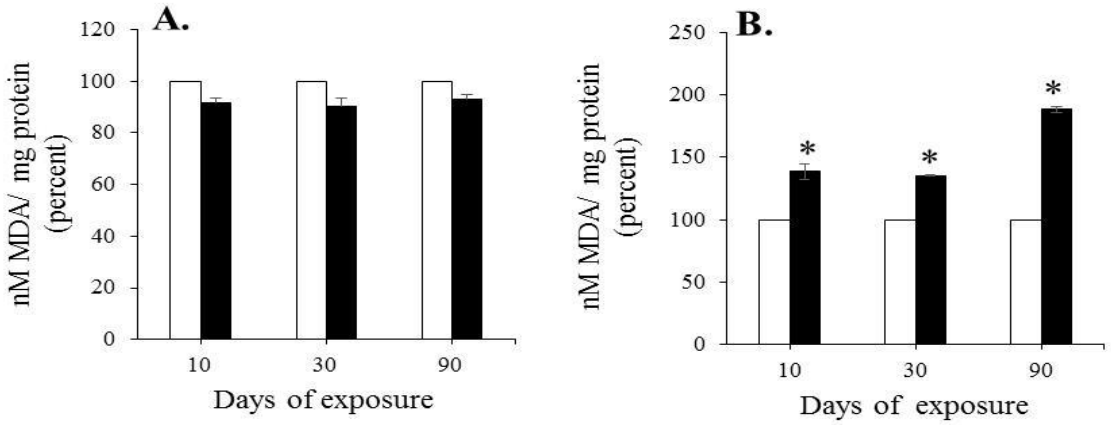

Figure 4.3. *Lipid peroxidation in the epididymides and testis of developing rats exposed to mosquito coil smoke.* ***(A)*** *Epididymides;* ***(B)*** *Testis. Control (□); Mosquito coil smoke exposed (■).* denotes p < 0.05.*

3. Nitric oxide

Nitric oxide levels were significantly increased in the epididymides obtained from rats exposed to coil smoke for 10 days (Figure 4.4A), and were unaltered in other groups. Nitric oxide levels were significantly decreased in the testis (Figure 4.4B).

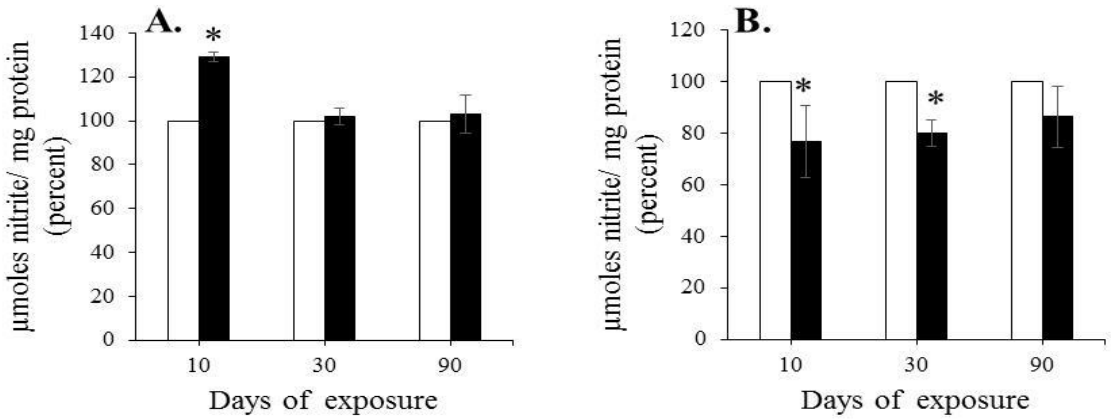

Figure 4.4. *Nitric oxide production in the epididymides and testis of developing rats exposed to mosquito coil smoke.* ***(A)*** *Epididymides;* ***(B)*** *Testis. Control (□); Mosquito coil smoke exposed (■). * denotes p < 0.05.*

NO being highly reactive, couples with free radicals to maintain the oxidant to antioxidant status in the tissues. Hence, calculating the ratio of NO and MDA (NO/MDA) provides information on the role of NO in preventing oxidative stress. In epididymides no significant changes in NO/MDA ratio was observed (Figure 4.5A) whereas in the testis of mosquito coil smoke exposed rats, the NO/MDA ratio was significantly lower when compared with the unexposed control (Figure 4.5B), indicating loss of the protective role of NO in these tissues.

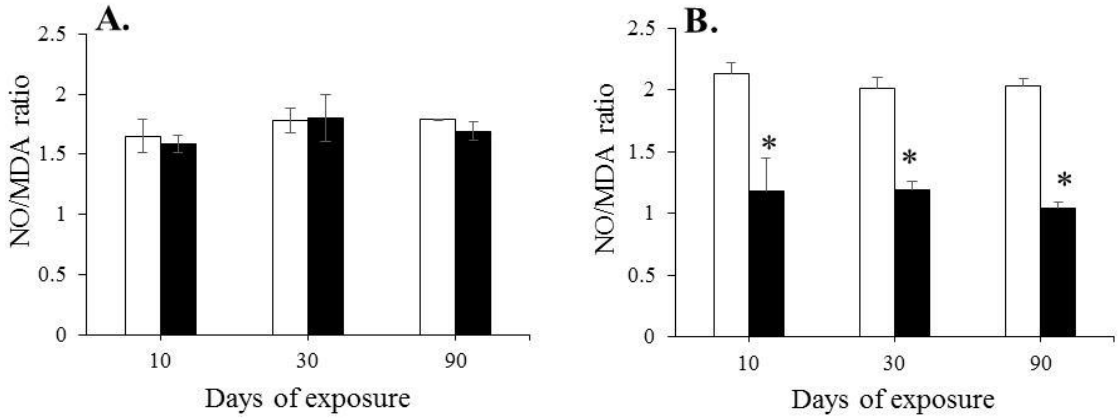

Figure 4.5. *NO/MDA ratio in the epididymides and testis of developing rats exposed to mosquito coil smoke.* ***(A)*** *Epididymides;* ***(B)*** *Testis. Control (□); Mosquito coil smoke exposed (■). * denotes p < 0.05.*

4. Antioxidant enzymes

Catalase activity was significantly decreased in the epididymides and testes of developing rats exposed to mosquito coil smoke for 10, 30 and 90 days (Figure 4.6A and B). Glutathione-S-transferase activity was increased in the epididymides of 10 day mosquito coil smoke exposed rats and decreased in other group samples (Figure 4.7A and B), whereas in testis, the activity was increased in 10, 30 and 90 days mosquito coil smoke exposed rats. In epididymides and testis of mosquito coil smoke exposed rats, glutathione peroxidase activity was increased significantly (Figure 4.8A and B). Superoxide dismutase activity in epididymis obtained from 10 and 30 days mosquito coil smoke exposed animals was increased significantly. In the testis of 10 and 90 days mosquito coil smoke exposed rats, significant increase in super oxide dismutase activity was observed (Figure 4.9A and B).

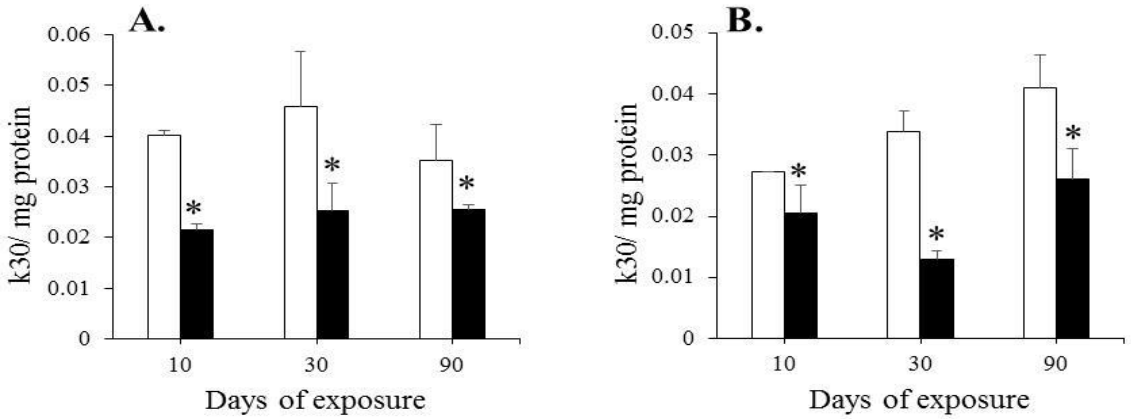

Figure 4.6*. Catalase activity in the epididymides and testis of developing rats exposed to mosquito coil smoke.* ***(A)*** *Epididymides;* ***(B)*** *Testis. Control (□); Mosquito coil smoke exposed (■). * denotes p < 0.05.*

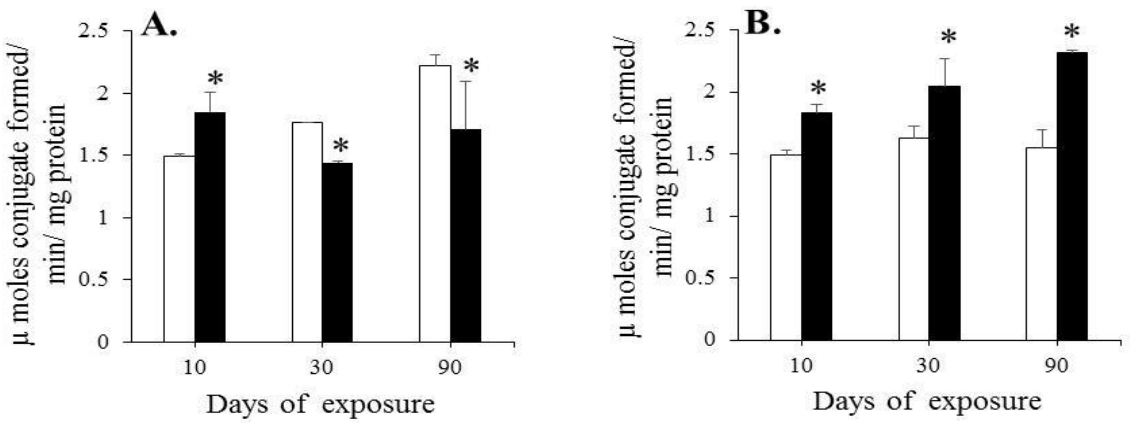

Figure 4.7. *Glutathione-S-transferase activity in the epididymides and testis of developing rats exposed to mosquito coil smoke.* ***(A)*** *Epididymides;* ***(B)*** *Testis. Control (□); Mosquito coil smoke exposed (■). * denotes p < 0.05.*

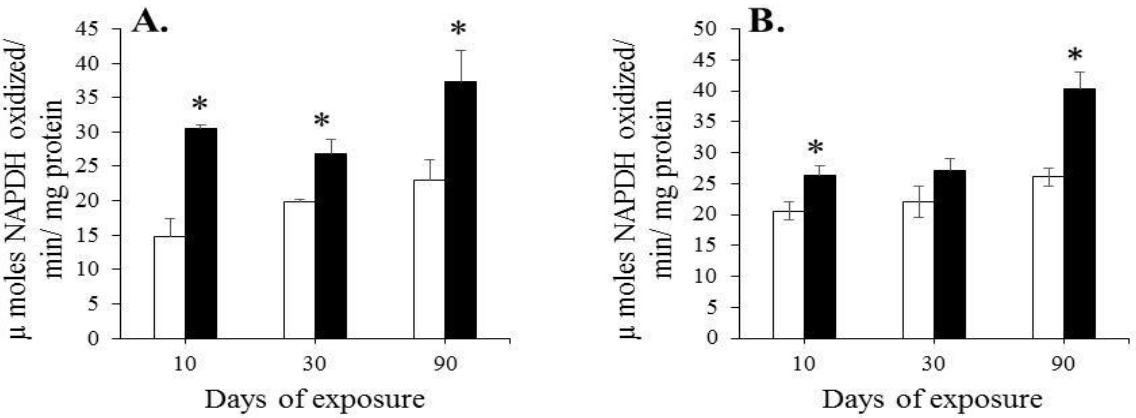

Figure 4.8. *Glutathione peroxidase activity in the epididymides and testis of developing rats exposed to mosquito coil smoke.* ***(A)*** *Epididymides;* ***(B)*** *Testis. Control (□); Mosquito coil smoke exposed (■). * denotes p < 0.05.*

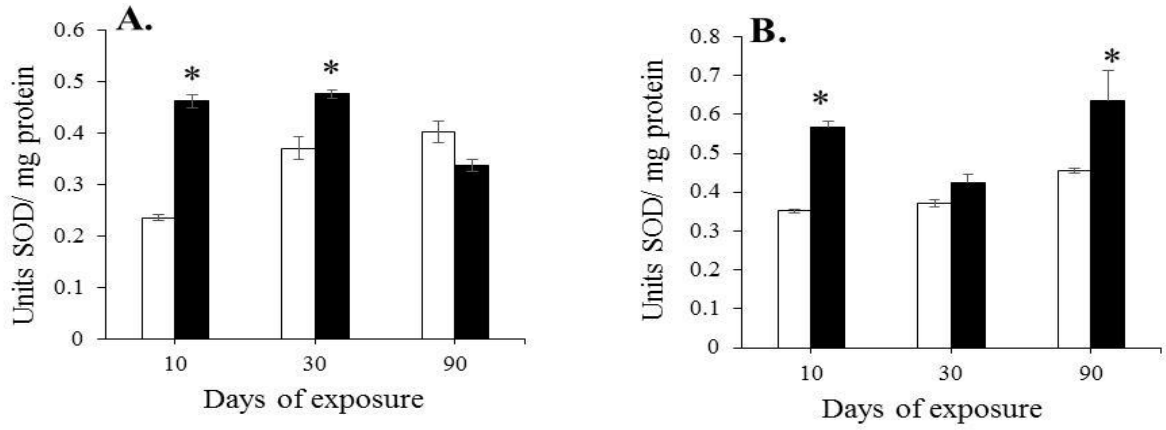

Figure 4.9. *Superoxide dismutase activity in the epididymides and testis of developing rats exposed to mosquito coil smoke.* ***(A)*** *Epididymides;* ***(B)*** *Testis. Control (□); Mosquito coil smoke exposed (■). * denotes p < 0.05.*

5. Effect of exposure to mosquito coil smoke on cell cycle regulators

Altered antioxidant status leads to oxidative stress, which in turn activates cellular repair mechanisms. p53 is a key regulator, which allows the cell to undergo repair or death by apoptosis. In the epididymides, p53 expression was significantly increased when the rats were exposed to mosquito coil smoke for 90 days (4.10A). In testis of rats exposed to mosquito coil smoke for 10 days, *p53* mRNA levels were down regulated whereas significant increase was observed in other groups (Figure 4.10B).

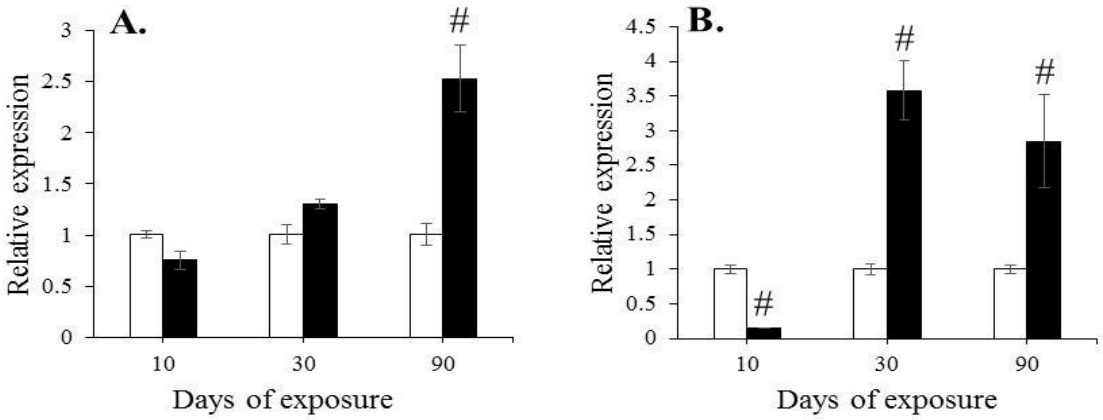

Figure 4.10. *mRNA expression of p53 in the epididymides and testis of developing rats exposed to mosquito coil smoke.* ***(A)*** *Epididymides;* ***(B)*** *Testis. Control (□); Mosquito coil smoke exposed (■). # denotes p < 0.01.*

6. Effect of exposure on genes involved in spermatogenesis

In the testis of rats exposed to mosquito coil smoke during development, mRNA expression of germ cell transcription factors were analyzed. In general, the mRNA expression of all these transcription factors were down regulated at all the time points tested (Figure 4.11A-K). Spermatozoa that emerge from the testis are immature, non-motile, and lack the ability to fertilize an egg. Sperm maturation factors (*Spag11e*) and capacitation factor (*Defb22*) are involved in maturation of sperm. A drastic reduction in the mRNA expression of *Spag11e* and *Def22* was observed in these developing rats exposed to mosquito coil smoke (Figure 4.12A and B).

7. Daily sperm production and total sperm count

Since, the factors that are involved in spermatogenesis and sperm maturation were severely affected, we analyzed the daily sperm production. Daily sperm production was significantly decreased in rats exposed to mosquito coil smoke for 90 days (Figure

4.13). Further, we also observed a decrease in total sperm count in mosquito coil smoke exposed rats (Table 4.3).

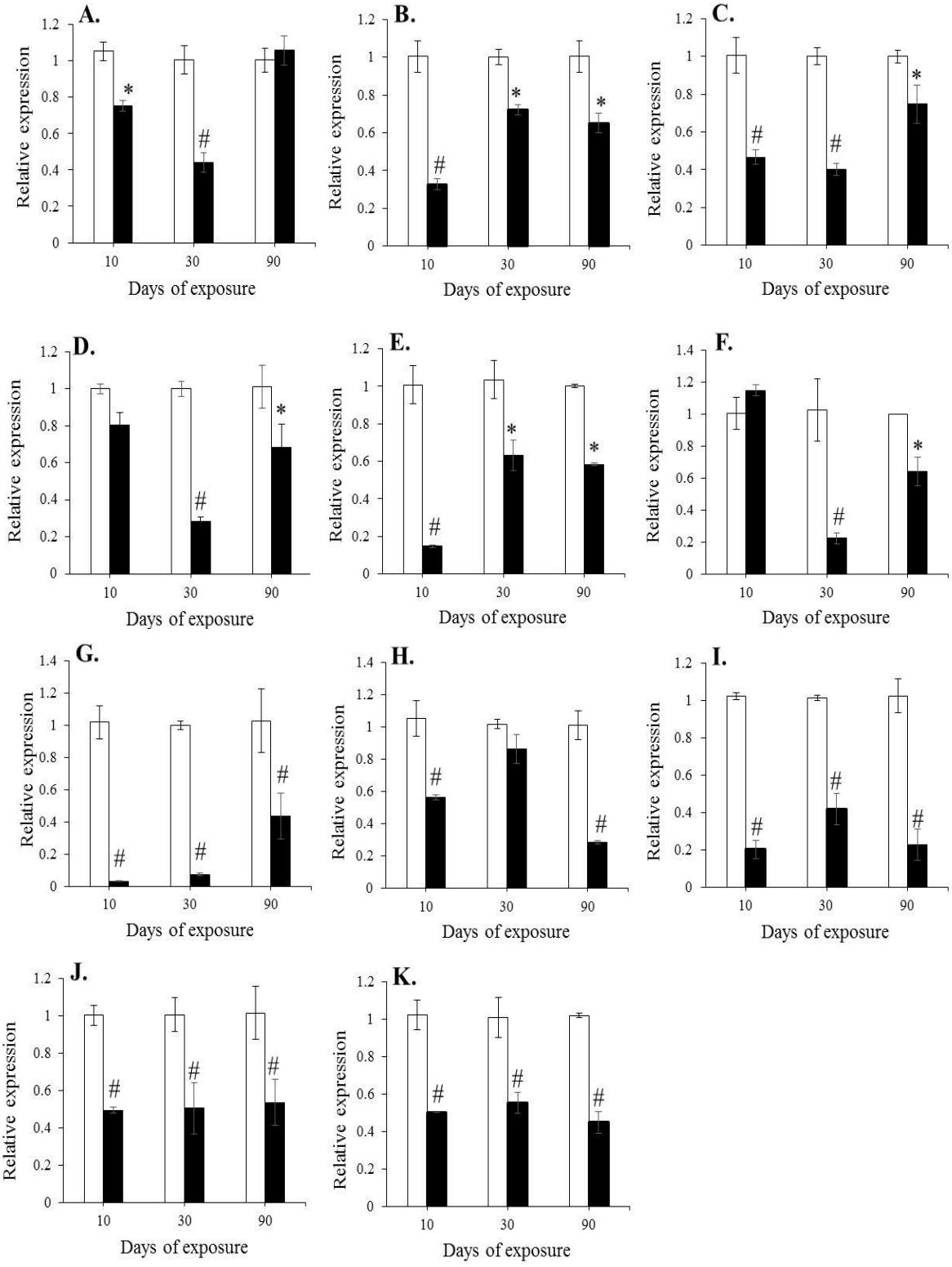

Figure 4.11. *mRNA expression of migration and spermatogenic factors in the testis of developing rats exposed to mosquito coil smoke.* ***(A)*** *Hsf2;* ***(B)*** *Ovol1;* ***(C)*** *Wt1;* ***(D)*** *Brdt;* ***(E)*** *Kdm3a;* ***(F)*** *Grth;* ***(G)*** *Ybx-2;* ***(H)*** *Scf;* ***(I)*** *c-kit;* ***(J)*** *Tgf-β1;* ***(K)*** *Ddx3y. Control (□); Mosquito coil smoke exposed (■).* and # denotes p < 0.05, p <0.01 respectively.*

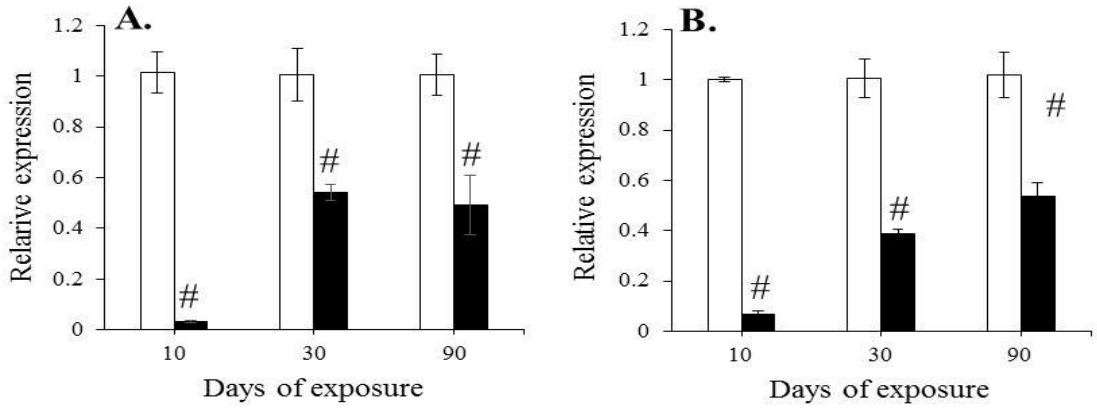

Figure 4.12. *mRNA expression sperm maturation factor in epididymis of developing rats exposed to mosquito coil smoke.* ***(A)*** *Spag11e;* ***(B)*** *Defb22. Control (□); Mosquito coil smoke exposed (■). # denotes p < 0.01.*

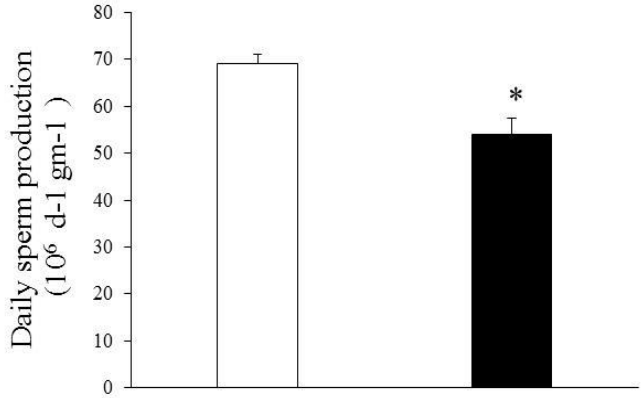

Figure 4.13. *Daily sperm production in testis of developing rats exposed to mosquito coil smoke. Control (□); Mosquito coil smoke exposed for 90 days (■).*denotes p < 0.05.*

Table 4.3. Total sperm count. *denotes p < 0.05.

Total sperm count (10^6)	
Control	**Mosquito Coil exposed**
152.5 ± 7.13	**127.13*** ± 4.23

8. Effect on steroidogenesis

To determine whether exposure to allethrin may affect steroidogenesis, the mRNA expression of enzymes involved in this process was analyzed. Compared to controls, mRNA expression of *StAR*, (which is responsible for transport of cholesterol to the inner mitochondrial membrane), *3β-HSD* (a rate limiting enzyme in cholesterol biosynthesis) and *17β-HSD* (for testosterone synthesis) were significantly reduced in the testes of rats exposed to mosquito coil smoke for 90 days (Figure 4.14).

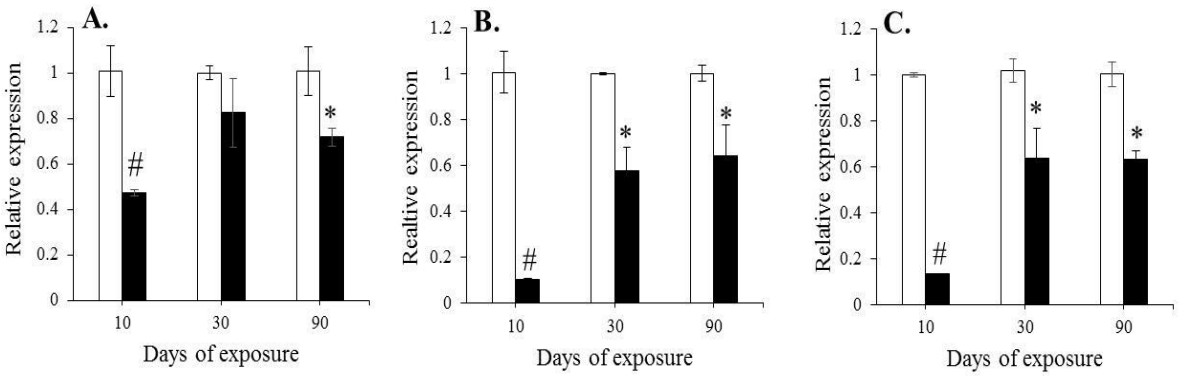

Figure 4.14. *mRNA expression of factors involved in steroidogenesis.* ***(A)*** *StAR;* ***(B)*** *3β-HSD;* ***(C)*** *17β-HSD. Control (□); Mosquito coil smoke exposed (■).* and # denotes p < 0.05, p <0.01 respectively.*

Further, we analyzed the mRNA expression of androgen receptor (AR), which plays a crucial role in steroidogenesis. Significant decrease in mRNA expression of AR was observed in the testes obtained at different intervals after mosquito coil smoke exposure (Figure 4.15A). The serum testosterone levels were also decreased in rats exposed to mosquito coil smoke exposure for 90 days (Figure 4.15B), suggesting that steroidogenesis may be affected due to disruption in the production of AR gene expression.

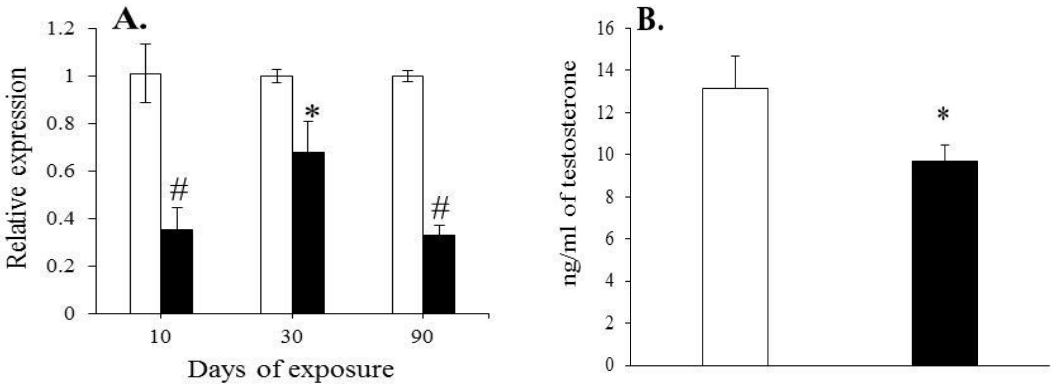

Figure 4.15. (A) *mRNA expression of androgen receptor and* ***(B)*** *Serum testosterone in developing rats exposed to mosquito coil smoke. Control (□); Mosquito coil smoke exposed for 90 days (■).* and # denotes p < 0.05, p <0.01 respectively.*

9. Effect on Acrosome reaction

The number of acrosome intact and acrosome reacted spermatozoa estimated by flow cytometry in control and mosquito coil exposed rats are shown (Figure 4.16A and B). Spermatozoa collected from epididymis of rats exposed to mosquito coil smoke for 90 days showed decreased ability to undergo acrosome reaction when compared to

control rats, indicating that decreased acrosome reaction may lead to infertility (Figure 4.16C).

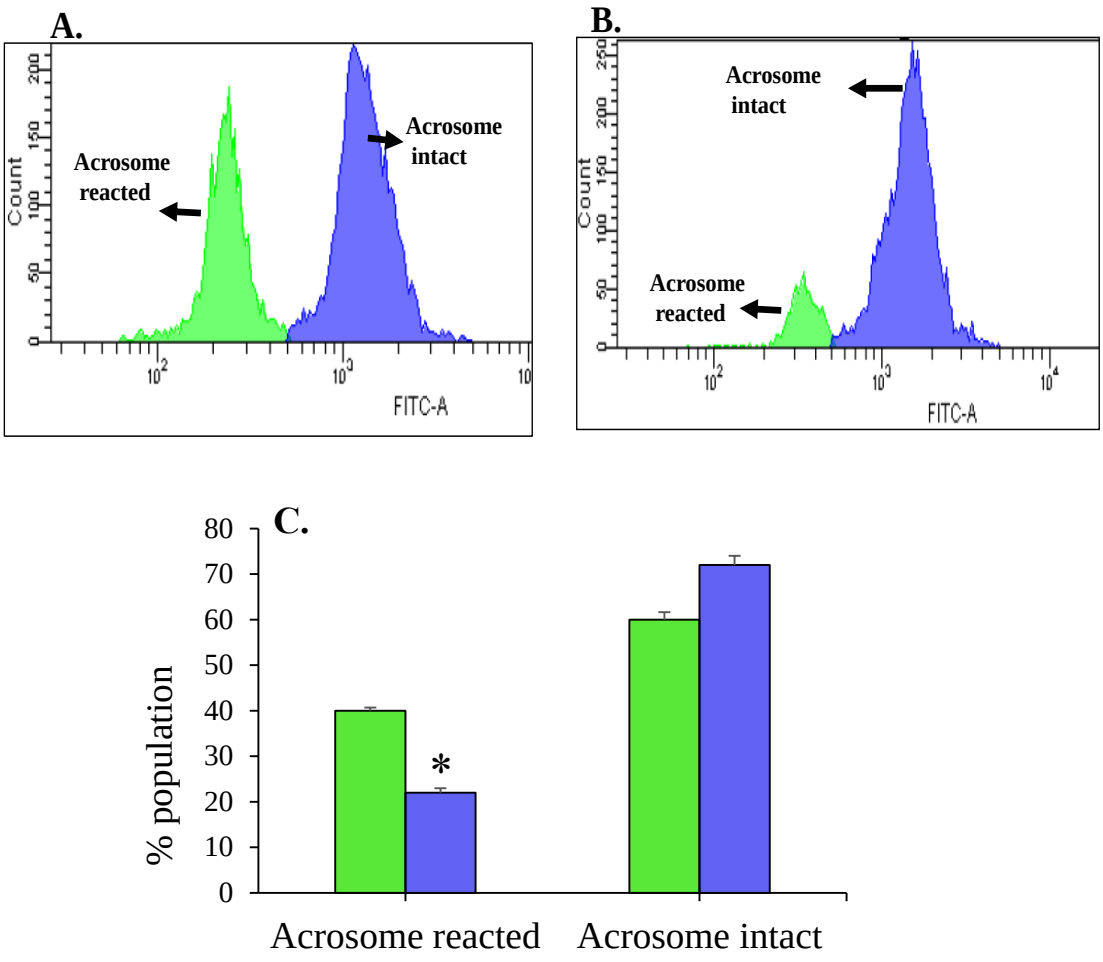

Figure 4.16. *FACS analysis to determine acrosome reaction in spermatozoa.* ***(A)*** *Control;* ***(B)*** *Mosquito coil smoke exposed;* ***(C)*** *% population of acrosome reacted and acrosome intact spermatozoa. Control (■); Mosquito coil smoke exposed (■). * denotes p < 0.05.*

Discussion

Till now we have investigated the effects of allethrin toxicity that happens due to consumption of food containing this chemical. The other common mode of exposure to allethrin by many people, especially the low income groups is by inhaling the fumes generated by burning allethrin based mosquito coils. These coils are used to prevent ourselves from mosquito bites to avoid malaria. On one hand we try to prevent one disease and at the same time, we invite problems by use of allethrin based mosquito coils. The effects of continuous usage of allethrin based mosquito repellants on the general health and reproductive functioning in particular are not studied. Another important aspect is that pregnant women and children receive extra care, in an attempt to protect them from diseases and in this process mosquito coils or other allethrin based mosquito repellents are rampantly used to prevent mosquito bites and thereby malaria. People create a situation wherein the newer generations are exposed to allethrin right from the day they are in the mother's womb and the children are exposed continuously due to over care by parents and such issues are never thought of seriously. Hence using rat models, we made an attempt to study the effects on reproductive function in the offspring of mothers who are constantly exposed to allethrin based mosquito coil smoke during their pregnancy and in the offspring who are continuously exposed until they reach adulthood.

Significant difference in litter size and mortality was not observed though a significant decrease in body weight was observed after 90 days of mosquito coil smoke exposure. Significant decrease in body weight of offspring of rats exposed to decamethylcyclopentasiloxane was observed (Siddiqui *et al.,* 2007). In another study, pesticides administered to Swisss CD-1 mice showed no abnormalities in litter size and body weight (Heindel *et al.,* 1994). In this study, lipid peroxidation was found to be decreased in epididymis and increased in testes. Nitric oxide production was increased in epididymis and decreased in testes. In the epididymis, NO/MDA ratio was significantly increased whereas in testes obtained from coil exposed rats, the ratio was decreased when compared with the unexposed groups indicating loss of the protective role of NO in these tissues. Lactational exposure to the insecticide, chlorpyrifos cause increased lipid peroxidation in lactating pups (Mansour and Mossa, 2010). The activities of catalase and GST in epididymis was decreased, whereas GPx and SOD activities were increased in epididymis and testis obtained from mosquito coil smoke exposed rats.

Embryonic and early life exposure of rats to bisphenol A (BPA), which is an endocrine disruptor, induces oxidative stress in testis of male offspring (Kabuto *et al.,* 2004).

Exposure to insecticides such as permethrin and DEET during gestation showed increase in spermatogenic cell apoptosis in rats (Manikkam *et al.,* 2012) which is correlating to our results where *p53* expression is significantly altered in testis. StAR is responsible for the transport of cholesterol into mitochondria (Miller, 2007). The synthetic pyrethroids cypermethrin and permethrin decreased serum testosterone levels in adult mice by disrupting StAR expression (Wang *et al.,* 2010; Zhang *et al.,* 2010). In the present study, we investigated the effect of allethrin exposure during prenatal and postnatal life on the mRNA expression of *StAR* in testis after mosquito coil smoke exposure for 10, 30 and 90 days. Our results showed that mRNA levels of testicular *StAR* was significantly decreased at all the time points tested. Decreased serum testosterone levels in our study correspond to the decreased StAR levels. These results suggest that decreased testosterone synthesis might be associated with downregulation of StAR. 3β-HSD and 17β-HSD are crucial enzymes in the synthesis of testosterone in Leydig cells. 3β-HSD converts pregnenolone to progesterone, whereas 17β-HSD converts androstenedione to testosterone (Stocco and Clark, 1997). We found that mRNA levels of *3β-HSD* and 17*β-HSD* was significantly decreased in all the days of coil exposure. Earlier studies showed that, fenevalerate exposure to rats significantly downregulated the expression of steroidogenic enzymes (Mani *et al.,* 2002). Taken together, it appears that down regulation of steroidogenic enzymes during mosquito coil smoke exposure results in decreased testosterone production. The molecular mechanisms that are involved in these effects needs further investigation.

Spermatogenesis is the process of formation of functional spermatozoa from germ cells, which is tightly regulated through transcriptional and post- transcriptional mechanisms. Vinclozolin, a fungicide when administered to adult Japanese quail for one-generation shows inhibition of spermatogenesis (Niemann *et al.,* 2004). Spermatid formation from germ cells in testis requires their interaction with a variety of factors produced by Sertoli cells (Bettegowda and Wilkinson, 2010). Our results demonstrated decreased mRNA expression of spermatogenic factors due to mosquito coil smoke exposure. Decreased expression of Sertoli cell factors may compromise sperm production. A variety of factors govern sperm maturation and capacitation. SPAG11E is an important sperm maturation factor and we found that its mRNA expression was

significantly decreased in testis of mosquito coil smoke exposed rats. Such an observation was also made for DEFB22, which is involved in capacitation, Down regulation of these genes may result in the production of immature and non-functional spermatozoa. To the best of our knowledge this is the first report that demonstrates such an effect. We also observed reduced daily sperm production and total sperm count in rats exposed to mosquito coil smoke. Cypermethrin causes decreased sperm count in rats (Ahmad *et al.,* 2009; Elbetieha *et al.,* 2001). Further, neonicotinoid pesticides acetamiprid (ACE) and imidacloprid (IMI) affects the fertilizing ability of sperm (Gu *et al.,* 2013). In our study, we observed decreased ability of spermatozoa to undergo acrosome reaction in rats exposed to mosquito coil smoke when compared to control rats. The effects observed in spermatozoa could be due to the direct action of allethrin present in mosquito coil smoke or could be due to consequence of disrupted steroidogenesis and sperm maturation.

In conclusion, we report that rats exposed to allethrin-based mosquito coil smoke during prenatal and post natal stages exhibits defects in steroidogenesis and sperm quality.

REFERENCES

Ahmad M, Hussain I, Khan A, and Najib-ur-Rehman. (2009). Deleterious effects of cypermethrin on semen characteristics and testes of dwarf goats (*Capra hircus*). *Experimental and Toxicologic Pathology*, *61*(4), 339–46.

Bettegowda A, and Wilkinson M F. (2010). Transcription and post-transcriptional regulation of spermatogenesis. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *365*(1546), 1637–51.

Bigsby R, Chapin RE, Daston GP, Davis BJ, Gorski J, Gray LE, Howdeshell KL, Zoeller RT, and vom Saal FS. (1999). Evaluating the effects of endocrine disruptors on endocrine function during development. *Environmental Health Perspectives*, *107 Suppl* , 613–8.

Cooke GM, Price CA, and Oko RJ. (1998). Effects Of In Utero and Lactational Exposure to 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) on Serum Androgens and Steroidogenic Enzyme Activities in the Male Rat Reproductive Tract. *The Journal of Steroid Biochemistry and Molecular Biology*, *67*(4), 347–54.

Davis LK, Murr AS, Best DS, Fraites MJ, Zorrilla LM, Narotsky MG, Stoker TE, Goldman JM, Cooper RL. (2011). The effects of prenatal exposure to atrazine on pubertal and postnatal reproductive indices in the female rat. *Reproductive Toxicology*, *32*(1), 43–51.

Elbetieha A, Da'as SI, Khamas W, and Darmani H. (2001). Evaluation of the toxic potentials of cypermethrin pesticide on some reproductive and fertility parameters in the male rats. *Archives of Environmental Contamination and Toxicology*, *41*(4), 522–8.

Gu YH, Li Y, Huang XF, Zheng JF, Yang J, Diao H, Yuan Y, Xu Yan, Liu M; Shi Hui-juan, and Xu WP. (2013). Reproductive effects of two neonicotinoid insecticides on mouse sperm function and early embryonic development in vitro. *PloS One*, *8*(7), e70112.

Heindel JJ, Chapin RE, Gulati DK, George JD, Price CJ, Marr MC, Myers CB, Barnes LH, Fail PA, and Grizzle TB *et al.* (1994). Assessment of the reproductive and developmental toxicity of pesticide/fertilizer mixtures based on confirmed pesticide contamination in California and Iowa groundwater. *Fundamental and Applied Toxicology*, *22*(4), 605–21.

Jaiswal BS, Eisenbach M, and Tur-Kaspa I. (1999). Detection of partial and complete acrosome reaction in human spermatozoa: which inducers and probes to use? *Molecular Human Reproduction*, *5*(3), 214–9.

Janer G, Hakkert BC, Slob W, Vermeire T, and Piersma AH. (2007). A retrospective analysis of the two-generation study: what is the added value of the second generation? *Reproductive Toxicology*, *24*(1), 97–102.

Kabuto H, Amakawa M, and Shishibori T. (2004). Exposure to bisphenol A during embryonic/fetal life and infancy increases oxidative injury and causes underdevelopment of the brain and testis in mice. *Life Sciences*, *74(24)*, 2931-40.

Mani U, Islam F, Prasad AK, Kumar P, Suresh Kumar V, Maji BK, and Dutta KK. (2002). Steroidogenic alterations in testes and sera of rats exposed to formulated Fenvalerate by inhalation. *Human and Experimental Toxicology*, *21*(11), 593–7.

Manikkam M, Guerrero-Bosagna C, Tracey R, Haque MM, and Skinner MK. (2012). Transgenerational actions of environmental compounds on reproductive disease and identification of epigenetic biomarkers of ancestral exposures. *PloS One*, *7*(2), e31901.

Mansour SA and Mossa AH. 2010. Adverse effects of lactational exposure to chlorpyrifos in suckling rats. *Human and Experimenatl Toxicology, 29(2),* 77-92.

Miller WL. (2007). StAR search--what we know about how the steroidogenic acute regulatory protein mediates mitochondrial cholesterol import. *Molecular Endocrinology (Baltimore, Md.)*, *21*(3), 589–601.

Niemann L, Selzsam B, Haider W, Gericke C, and Chahoud I. (2004). Effects of vinclozolin on spermatogenesis and reproductive success in the Japanese quail (*Coturnix japonica*). *Archives of Environmental Contamination and Toxicology*, *46*(4), 528–33.

Norgil DI, Main KM, Toppari J, and Skakkebaek NE. (2002). Impact of exposure to endocrine disrupters in utero and in childhood on adult reproduction. *Best Practice and Research. Clinical Endocrinology and Metabolism*, *16*(2), 289–309.

Paglia DE and Valentine WN. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of Laboratory and Clinical Medicine*, *70*(1), 158–69.

Sharpe RM. (2001). Hormones and testis development and the possible adverse effects of environmental chemicals. *Toxicology Letters*, *120*(1-3), 221–32.

Sinha C, Seth K, Islam F, Chaturvedi RK, Shukla S, Mathur N, Srivastava N, and Agrawal AK. (2006). Behavioral and neurochemical effects induced by pyrethroid-based mosquito repellent exposure in rat offsprings during prenatal and early postnatal period. *Neurotoxicology and Teratology*, *28*(4), 472–81.

Siddiqui WH, Stump DG, Reynolds VL, Plotzke KP, Holson JF, and Meeks RG. (2007). A two-generation reproductive toxicity study of decamethylcyclopentasiloxane (D5) in rats exposed by whole-body vapor inhalation. *Reproductive Toxicology*, *23*(2), 216–25.

Stocco DM and Clark BJ. (1997). The role of the steroidogenic acute regulatory protein in steroidogenesis. *Steroids*, *62*, 29–36.

Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jégou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan MA, Meyer O, Müller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J, and Skakkebaek NE. (1996). Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives*, *104 Suppl* , 741–803.

Turner KJ, Mcintyre BS, Phillips SL, Barlow NJ, Bowman CJ, and Foster PM. (2003). Altered gene expression during rat Wolffian duct development in response to in utero exposure to the antiandrogen linuron. *Toxicological Sciences*, *74*, 114–128.

Wang H, Wang Q, Ping XZ, Tao XM, Ji YY, Zhang H, Zhang C, Zhang Y, and Xu DX. (2010). Cypermethrin exposure during puberty disrupts testosterone synthesis via downregulating StAR in mouse testes. *Archives of Toxicology*, *84*, 53–61.

Zhang H, Wang H, Wang Q, Zhao XF, Liu P, Ji YL, and Xu DX. (2010). Pubertal and early adult exposure to fenvalerate disrupts steroidogenesis and spermatogenesis in mice at adulthood. *Journal of Applied Toxicology : JAT*, *30*(4), 369–77.

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Summary

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SUMMARY

Objective 1: Decipher the molecular mechanisms of allethrin toxicity *in vitro.*

In this study, we demonstrate that allethrin exhibited a dose-dependent cytotoxicity on Leydig cell carcinoma cells (LC540) and isolated primary Leydig cells with an IC_{50} of 125 µM and 59 µM respectively. Cytotoxicity was associated with generation of reactive oxygen species, increased lipid peroxidation and alterations in antioxidant enzyme status. Morphological analyses of LC540 cells treated with allethrin revealed the presence of apoptotic bodies. Using flow cytometry, we observed that the number of cells that displayed early apoptotic features and entering into G_0 phase of cell cycle increased significantly with loss of mitochondrial membrane potential. The levels of *p53* mRNA and cleaved PARP-1 protein were increased, whereas BCL-2, pro-Caspase-3 and PARP-1 were decreased. Allethrin induced apoptosis was associated with voltage gated calcium channel mediated intracellular calcium release. Results of our study demonstrate that allethrin toxicity in the male reproductive tract may involve Leydig cell apoptotic death.

Objective 2: Effect of continuous exposure to allethrin-based mosquito coil smoke on the male reproductive tract.

Continuous inhalation of allethrin-based mosquito coil smoke may affect fertility, an aspect that has not received much attention. In rats exposed to allethrin-based mosquito coil smoke for 15–180 days, compared to the unexposed controls, lipid peroxidation was increased in the cauda and testes. The activity of antioxidant enzymes remained largely unchanged in the all the tissues analyzed. Histopathological analyses revealed loss of tubule architecture, epithelial cell disruption, increase in lumen size, interstitial edema, and presence of dead spermatozoa. *p53* gene expression was differentially altered in the epididymis and testes. The expression of spermatogenic factors, namely, stem cell factor and its ligand *c-Kit* was unaltered though decreased levels of *Tgf-β1* were observed. Results of this study demonstrate that prolonged exposure to allethrin-based mosquito coil smoke could lead to oxidative stress and compromise germ cell production.

Objective 3: Study the effect of oral administration of allethrin in adult rats.

In rats treated orally with allethrin for 60 days, alteration in the levels of lipid peroxidation products and nitric oxide was observed in caput, cauda and testis accompanied by an changes in the antioxidant status. Allethrin treatment caused pathomorphological changes of the male reproductive tract tissues. The expression of spermatogenic and sperm maturation factors was significantly reduced thereby possibly affecting sperm production. Oxidative stress induced by allethrin reduced *p53* gene expression. Results of this study support our hypothesis that increased oxidative stress induced by oral administration of allethrin affects fertility and may contribute to disregulated cell cycle in the male reproductive tract of rats.

Objective 4: Effect of exposure of prenatal rats to allethrin-based mosquito coil smoke.

Prenatal and post natal exposure to allethrin based mosquito coil smoke effects oxidant and antioxidant profile in the reproductive tract tissues. Post natal exposure to mosquito coil smoke affects lipid peroxidation profile and nitric oxide levels in developing rats. Transcription factors involved in spermatogenesis were severely affected resulting in decreased sperm count. The expression of steroidogenic enzymes were decreased with a concomitant decrease in testosterone. Further, spermatozoa obtained from these rats displayed impaired acrosome reaction, thus may affect fertility.

In conclusion, we report that the molecular mechanism of allethrin toxicity involves apoptotic mechanisms. Nasal or oral exposure to this compound results in damage to the male reproductive tract and compromised sperm function.

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Publications

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Inhalation Toxicology, 2012; 24(1-4): 143–152

ISSN 0895-8378 print/ISSN 1091-7691 online
DOI: 10.3109/08958378.2011.649189

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RESEARCH ARTICLE

Effect of continuous inhalation of allethrin-based mosquito coil smoke in the male reproductive tract of rats

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Abstract

Objectives: Continuous inhalation of allethrin-based mosquito coil smoke may affect fertility, an aspect that has not received much attention. In this study, we attempt to understand the harmful effects on the male reproductive system caused by continuous exposure to allethrin-based mosquito coil smoke.

Methods: Adult Wistar rats were allowed to inhale mosquito coil smoke for 15–180 days, and male reproductive tract tissues (caput, cauda, and testes) were collected. Using standard biochemical techniques, changes in oxidative stress (lipid peroxidation) and antioxidant status was measured. Histopathological analyses were carried out to assess pathomorphological damage in the caput, cauda, and testis. Real-time polymerase chain reaction was carried out to determine the expression pattern of the stress-response gene, *p53*, and the spermatogenic factors *c-Kit*, *Scf*, and *Tgf-β1*.

Results: In rats exposed to allethrin-based mosquito coil smoke for 15–180 days, compared to the unexposed controls, lipid peroxidation was increased in the cauda and testes. The activity of antioxidant enzymes remained largely unchanged in the all the tissues analyzed. Histopathological analyses revealed loss of tubule architecture, epithelial cell disruption, increase in lumen size, interstitial edema, and presence of dead spermatozoa. *p53* gene expression was differentially altered in the epididymis and testes. The expression of spermatogenic factors, namely, stem cell factor and its ligand *c-Kit* was unaltered though decreased levels of *Tgf-β1* were observed.

Conclusion: Results of this study demonstrate that prolonged exposure to allethrin-based mosquito coil smoke could lead to oxidative stress and compromise germ cell production.

Keywords: Allethrin, epididymidis, oxidative stress, sperm maturation

Introduction

Humans are exposed to a variety of insecticides that are used for domestic and agricultural purposes. These insecticides primarily contain organochlorines, organophosphates, carbamates, and pyrethroids such as allethrin, resmethrin, tetramethrin, cypermethrin, deltamethrin, etc. Pyrethrins exhibit low levels of toxicity and are rapidly metabolized (Casida, 1995). Basing on their structure and toxicity, they are classified into type I and II (Verschoyle & Aldridge, 1980) and are known to mainly act on the nervous system (Soderlund et al., 2002). The principal mechanism of pyrethroid toxicity involves the inhibition of voltage sensitive sodium channels besides inhibition of sodium-potassium ATPase in the neuronal membranes (Vijverberg & de Weille, 1985; Kakko et al., 2003; Tan & Soderlund, 2010). The effects of pyrethroids on other organ systems including the reproductive system are also reported. Pyrethroids are shown to induce oxidative stress and alter antioxidant system in different organ systems of rats (Kale et al., 1999; Giray et al., 2001; Maiti et al., 1995; Li et al., 2005). They are known to be estrogenic and antiprogestagenic in endometrial and breast cancer cell lines (Go et al., 1999; Garey & Wolff, 1998; Kasat et al., 2002), inhibit testosterone binding to the androgen receptor and sex hormone binding globulin at high concentrations (Eil & Nisula, 1990) and cause gynecomastia (Sattin et al., 1984). Further, it is reported that pyrethroid compounds cause oxidative stress in the

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(Received 17 October 2011; revised 01 December 2011; accepted 07 December 2011)

male reproductive tract (Issam et al., 2011; Issam et al., 2009; Wang et al., 2009).

Pyrethroid pesticides because of their high potency and selectivity are commonly used in agriculture and urban settings especially as insecticides. Pyrethroids form an important component of mosquito and other insect repellants. Mosquito repellants (mats, coils, and vaporizers) contain d-allethrin, a mixture of eight stereoisomers that belong to the type I pyrethroids. In developing countries, mosquito coils are widely used in households (in closed door settings) and human exposure occurs due to inhalation of allethrin emitted along with the smoke. Analysis of air samples after mosquito coil usage in a room showed a maximum concentration of allethrin (0.0120 ppm) within 30–45 min of use followed by a decline up to 6 h (Ramesh & Vijayalakshmi, 2001). Allethrin exposure via inhalation leads to moderate toxicity in rodent models (Tomlin, 1994). Though information about allethrin toxicity on the nervous system is available and to some extent on other organ systems, its effect on the reproductive tract is not well understood. Exposure to environmental pollutants such as pesticides and heavy metals contributes to the development of cancer and reduction in reproductive functions (Sharpe & Skakkebaek, 1993; Sharpe & Irvine, 2004; Wang et al., 2009; Issam et al., 2009; Tan & Soderlund, 2010). Previous studies indicated that rats exposed to allethrin-based liquid mosquito repellants (using a vaporizer) for 90 days did not show any adverse effect on the clinical enzyme profile and reproductive indices in weaning and adult rats (Srivastava et al., 2006). Further, it is demonstrated that allethrin exhibits recombinogenic activity in the male germ cells of *Drosophila melanogaster* (Pontecorvo & Fantaccione, 2006).

Because of the wide spread use of allethrin-based mosquito repellent coils, adults and children are exposed and their direct effects on the functioning of the reproductive tract need investigation. Previous studies on the effect of mosquito repellents on reproductive tract used vaporizers, which creates a milder toxic environment when compared to mosquito coils. Studies focusing on harsher environments created by mosquito coils in different organ systems are not available. Hence, in this study, we attempt to determine the effect of mosquito coil smoke exposure (similar to that is experienced in a household setting) in the male reproductive tract of rat. Though fertility studies were undertaken to determine the effect of vaporizers, the effect of exposure to mosquito coil smoke on the pathological changes that occur in the reproductive tract is not reported till now. These morphological, cellular, and hormonal effects may involve prior disturbance in molecular mechanisms, and there are scanty reports in the literature citing the effect of exposure to mosquito coil smoke on disturbances in oxidant and antioxidant status, induction of apoptosis, inflammation and expression of male reproductive tract genes involved in spermatogenesis.

Free radical generation in the male reproductive tract affects fertility (Ochsendorf, 1999). Spermatozoa lose their shape and motility when incubated with oxygen due to the peroxidation of membrane lipids. Further, increased free radical generation was observed in oligozoospermic patients, clearly suggesting the role of free radicals in fertility (Ochsendorf, 1999). It is imperative to suggest that prolonged exposure to pesticides may increase free radical generation in the male reproductive tract and leading to decreased sperm count and motility, thus affecting fertility. Hence, the effect of exposure to allethrin-based mosquito coil smoke on the oxidative and antioxidant status in the male reproductive tract of rat was investigated in this study. Apoptosis is a consequence of free radical generation and p53 being the major effector of apoptosis, the expression pattern p53 was along with free radical generation is analyzed in the male reproductive tract.

Fertility depends on the proper production of spermatozoa. The structural integrity of the male reproductive organs determines the germ cell production quality and quantity. A variety of germ cell differentiation factors such as the tyrosine kinase receptor, c-KIT, or its ligand, stem cell factor (SCF), and transforming growth factor-β-1 (TGF-β1) have been implicated during spermatogenesis (Besmer et al., 1993; Olaso et al., 1998; Meehan et al., 2000). Hence in this study, the expression pattern of spermatogenic factors was analyzed in the male reproductive tract of rats exposed to allethrin-based mosquito coil smoke.

We demonstrate that rats exposed to allethrin-based mosquito coil smoke for 15–180 days display pathomorpholocial changes in the male reproductive tract tissues. Prolonged exposure leads to increased oxidant stress in the epididymis and testes without a marked change in the antioxidant status. Further, the stress-response gene p53 expression was altered suggesting a loss of cell-cycle regulation. Though the expression pattern of sperm maturation factors *c-Kit* and *Scf* were unaltered, a significant decrease in *Tgf-β1* was observed in the testes.

Materials and methods

Mosquito coils

Mosquito repellent coils were obtained from the local markets. The composition (in terms of w/w) of a typical mosquito coil as per the information given with the product is as follows: 0.1% d-trans allethrin, 40% coconut shell powder, 10% starch binder, 0.1% Lo88 emulsifier, 0.1% red dye, 0.5% fragrance, 0.3% sodium benzoate, 0.1% potassium nitrate, and 6% jiggat (joss). Each mosquito coil is expected to burn for 8 hours.

Animals and tissue specimens

Adult male Wistar rats (90-day-old) were obtained from National Center for Laboratory Animals, National Institute of Nutrition, Hyderabad. They were maintained on a 12L:12D lighting schedule, at 22–25°C, with food

and water *ad libitum*. After acclimatization, rats were divided into two groups ($n=5$) and were housed in separate rooms. To simulate the human exposure settings, in a closed room mosquito coils were lit (one coil per night) and the animals were allowed to inhale coil smoke. Control animals were maintained under normal animal house conditions in a separate room. Animals were sacrificed by cervical dislocation and tissues were collected after 15–180 days from control and mosquito coil smoke exposed rats. Testis, caput, and cauda epididymis were identified, stripped off the connective tissues, noted the weight, frozen in liquid nitrogen, and kept at –70°C until use. Experiments were conducted using the guidelines for the care and use of laboratory animals, approved by the Institutional Animal Ethics Committee of University of Hyderabad (LS/IAEC/YS/2010/02).

Estimation of lipid peroxidation products

Malondialdehyde, the product of lipid peroxidation, present in the biological samples reacts with thiobarbituric acid (TBA) under acidic conditions at 95°C to form a pink colored complex with absorbance maxima at 532 nm. The total amount of lipid peroxidation products present in the caput, cauda, and testes of the controls and experimental animals was estimated using the TBA method as described earlier (Bernheim et al., 1948). 1,1,3,3-tetraethoxy-propane was used as the standard.

Enzyme assays

Tissues collected from control and experimental animals were homogenized in phosphate buffer saline, pH 7.4. Assays to measure the activities of catalase, glutathione-s-transferase (GST), and glutathione peroxidase (GPx) were performed using standard protocols described earlier (Aebi, 1984; Jakoby, 1978; Paglia & Valentine, 1967).

Real time polymerase chain reaction

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from the epididymides and testis obtained from control and mosquito coil smoke exposed rats. Total RNA (2 μg) was reverse transcribed using 50 U Stratascript (Invitrogen, USA) and 0.5 μg of oligodT (Invitrogen) according to the manufacturer's instructions. Two microliter of the resultant cDNA was used for real-time polymerase chain reaction (PCR) analysis using SYBR master mix kit (Applied Biosystems, Warrington, UK). Glyceraldehyde-6-phosphate dehydrogenase (*Gapdh*) expression was used as the internal control. A typical real-time PCR reaction was carried out with the following conditions: initial denaturation at 94°C for 10 min; 40 cycles with 94°C for 15 s and 60°C for 1 min. Negative controls (no template control and minus RT control only with RNA) were included in the assays to detect nonspecific amplification. The amplicons were sequenced to confirm their identity.

Histopathology

Epididymides and testes obtained from control and experimental rats were fixed in 4% paraformaldehyde and Bouin's solution respectively and embedded in paraffin. Five-micron thick sections were taken and stained with hematoxylin–eosin for microscopic examination.

Statistical analyses

Changes in the parameters analyzed in this study were compared with the respective control at each time point. Statistical analyses were performed using one-way ANOVA and student's *t*-test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are mean ± SD.

Results

Organ weights

No significant changes in the organ weights were observed between the control and mosquito coil smoke exposed animals at all the time points tested (Table 1). Further, no mortality was observed during the course of study.

Lipid peroxidation

Since pyrethroids are known to induce free radical generation, we measured lipid peroxidation products in the caput, cauda, and testes obtained from mosquito coil exposed rats. The levels of lipid peroxidation products in the control animals at different time points were considered to be 100%. Changes in lipid peroxidation in the treated groups were compared to their respective controls for each time point. In the caput, no significant changes in the lipid peroxidation status was evident (Figure 1A) at the time points analyzed. Significant increase in lipid peroxidation was observed in the cauda obtained from rats exposed to 120 and 180 days (Figure 1B). In the testes, significant increase in lipid peroxidation was observed at the earlier time points (30 and 60 days) (Figure 1C). Surprisingly, decreased levels of lipid peroxidation were observed in the cauda obtained from rats exposed to mosquito coil smoke for 15 days (Figure 1B).

Table 1. Organ weights (in grams) in mosquito coil smoke exposed rats.

	15 days		30 days		60 days		90 days		120 days		180 days	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Caput	0.35 ± 0.10	0.36 ± 0.14	0.5 ± 0.10	0.45 ± 0.15	0.59 ± 0.30	0.56 ± 0.20	0.55 ± 0.17	0.50 ± 0.20	0.52 ± 0.30	0.56 ± 0.40	0.49 ± 0.30	0.48 ± 0.20
Cauda	0.29 ± 0.02	0.28 ± 0.02	0.46 ± 0.01	0.4 ± 0.02	0.44 ± 0.02	0.42 ± 0.03	0.44 ± 0.06	0.42 ± 0.05	0.43 ± 0.07	0.46 ± 0.09	0.37 ± 0.06	0.35 ± 0.03
Testis	2.58 ± 0.40	2.74 ± 0.70	2.90 ± 0.19	2.90 ± 0.90	3.10 ± 0.60	2.90 ± 0.40	2.92 ± 0.90	2.80 ± 0.30	2.78 ± 0.20	3.01 ± 0.20	2.53 ± 0.80	2.94 ± 0.50

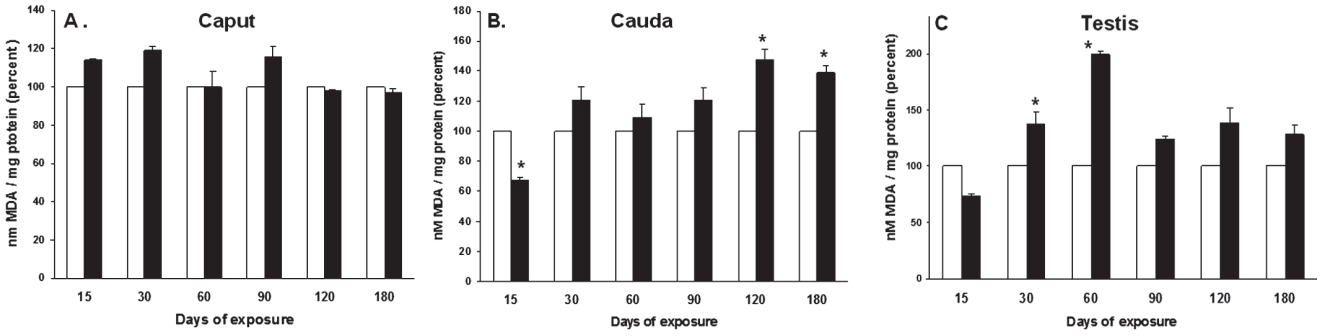

Figure 1. Lipid peroxidation in the male reproductive tract of rats exposed to mosquito coil smoke. (A) Caput; (B) Cauda; (C) Testis. Untreated control (□); Mosquito coil smoke exposed (■). * denotes $p < 0.05$.

Antioxidant enzymes

Changes in oxidant status affect antioxidant defense mechanisms in many organ systems. To determine whether mosquito coil smoke-induced lipid peroxidation may have an affect on antioxidant status, the activity of catalase, GPx, and GST was measured in the caput, cauda, and testes. When compared to their respective controls, catalase activity was not altered in the caput, cauda, and testes of mosquito coil exposed animals, except for a significant increase in catalase activity in cauda obtained from 120 and 180 time point (Figure 2A, 2B, and 2C). No significant changes in GPx activity were observed in the caput, cauda, and testes obtained from mosquito coil smoke exposed rats, except for a decrease at the 15 day time point in the caput and testis (Figure 3A, 3B, and 3C). Similarly, GST activity was largely unaltered at all the time points analyzed (Figure 4A, 4B, and 4C). A decrease was observed in the cauda obtained at the 180 day time point and in the testes obtained at 30 day time point.

Histopathological analyses

Microscopic examination of caput, cauda, and testes obtained from control and mosquito coil smoke exposed rats for 15–180 days was performed to determine pathomorphological changes. Caput and cauda appeared apparently normal except for an occasional finding of pyknotic nuclei of the lining epithelial cells. Cells undergoing vacuolar changes were found in animals that were exposed to longer durations (Figures 5 and 6). The lesions may be categorized as mild changes only. Though no significant changes in morphology of testes were observed in rats exposed to mosquito coil smoke for 15–60 days, interstitial edema, loss of tubule architecture, epithelial cell disruption, and increase in lumen size were observed in the testes of rats exposed to 90–180 days (Figure 7). Similarly, dead spermatozoa, germ cell layer damage, and nuclear integrity of the gametes were observed in rats exposed to mosquito coil smoke for 90–180 days. The histopathology scores obtained are given in Table 2.

Gene expression

Oxidative stress leads to altered gene expression of the stress response genes. Increased free radical production contributes to DNA damage thereby initiating repair mechanisms at the cellular level. During DNA damage, p53 is known to activate a number of DNA repair proteins, thereby inducing cell-cycle arrest or allowing the cells to undergo apoptosis. In order to determine whether increased lipid peroxidation in the reproductive system due to short-term and long-term mosquito coil exposure could initiate cell death, real-time PCR was carried out to analyze the expression of p53. In the epididymides obtained from mosquito coil exposed animals, p53 gene expression increased significantly until 90 days of exposure after which the levels reduced below the controls (Figure 8A). In contrast, p53 mRNA levels in the testes of mosquito coil exposed animals were decreased when compared to their respective controls throughout the experimental period (Figure 8B). These results suggest differential stress responses that can occur in the organ systems of the male reproductive tract.

Spermatogenesis, a complex process that occurs in the testes, is the major function of this organ system. Morphological, biochemical, and molecular changes due to a variety of factors including oxidative stress can affect spermatogenesis. To the best of our knowledge, there are no reports on the effect of short- and long-term mosquito coil smoke exposure on the molecular changes that can occur in the testes and thereby affecting spermatogenesis. A variety of spermatogenic factors such as TGF-β1, SCF, and c-KIT play a key role in spermatogenesis. To determine whether stress induced by mosquito coil smoke may have an effect on the expression profile of spermatogenic factors, real-time PCR was carried out for *Tgf-β1*, *Scf*, and *c-Kit*. *C-Kit* mRNA levels remained unchanged in the testes of mosquito coil smoke exposed rats at all the time points analyzed (Figure 9A). Similarly, *Scf* mRNA levels also remained unchanged during the entire experimental period (Figure 9B). However, *Tgf-β1* mRNA levels were reduced significantly at all the time points in the testes obtained from mosquito coil smoke

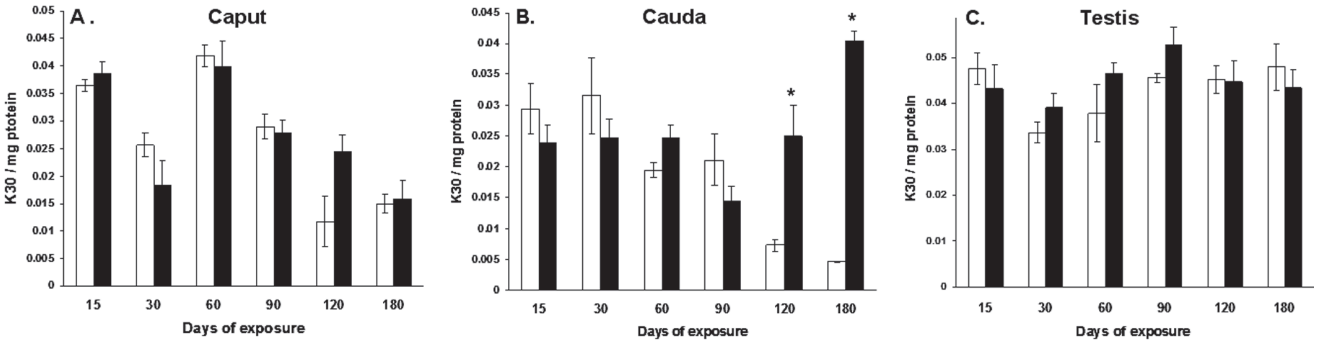

Figure 2. Catalase activity in the male reproductive tract of rats exposed to mosquito coil smoke. (A) Caput; (B) Cauda; (C) Testis. Untreated control (□); Mosquito coil smoke exposed (■). * denotes $p < 0.05$.

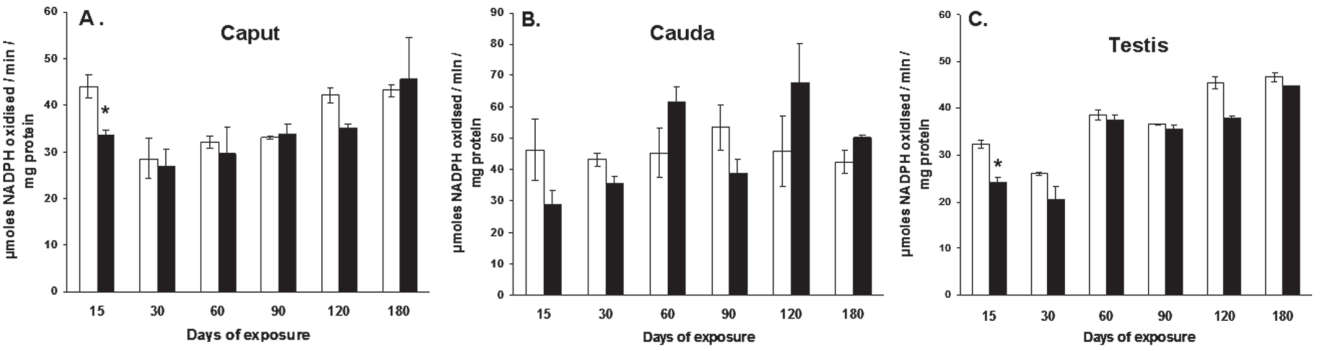

Figure 3. Glutathione peroxidase activity in the male reproductive tract of rats exposed to mosquito coil smoke. (A) Caput; (B) Cauda; (C) Testis. Untreated control (□); Mosquito coil smoke exposed (■). * denotes $p < 0.05$.

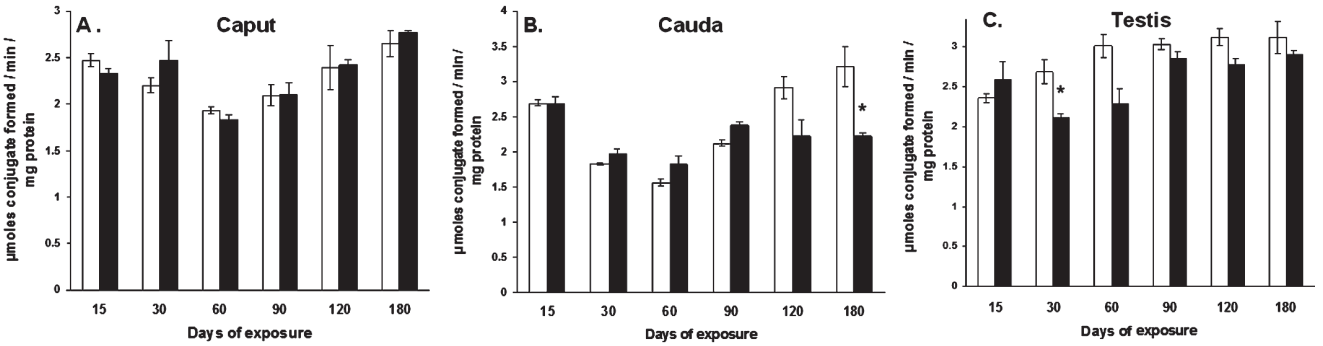

Figure 4. Glutathione-s-transferase activity in the male reproductive tract of rats exposed to mosquito coil smoke. (A) Caput; (B) Cauda; (C) Testis. Untreated control (□); Mosquito coil smoke exposed (■). * denotes $p < 0.05$.

exposed animals when compared to their respective controls (Figure 9C).

Discussion

Environmental toxicants are known to have significant effects on male reproductive function (Cheng et al., 2011, Wong & Cheng, 2011). Use of allethrin-based mosquito repellants for longer durations is a common practice because of which majority of the population are exposed to the active components (Huailu et al., 1995). Though a large number of studies described the toxic effects of mosquito repellants on various organ systems, their effects on the reproductive system have received little attention. Because of the cost factor, mosquito coils are more preferred than the liquid vaporizers especially among the lower income strata population. To the best of our knowledge, there are no reports describing the toxic effects of short- and long-term mosquito coil smoke on the functionality of the male reproductive tract.

Oxidative stress due to environmental contaminants initiates of a variety of pathways leading to pathological conditions (Reuter et al., 2010). Our results demonstrate that increased lipid peroxidation was observed in the testis and the cauda. This is in agreement with previous reports wherein increased lipid peroxidation was observed in various organs of animals exposed to pesticides (Gupta et al., 1999; Kale et al., 1999; Giray et al., 2001;

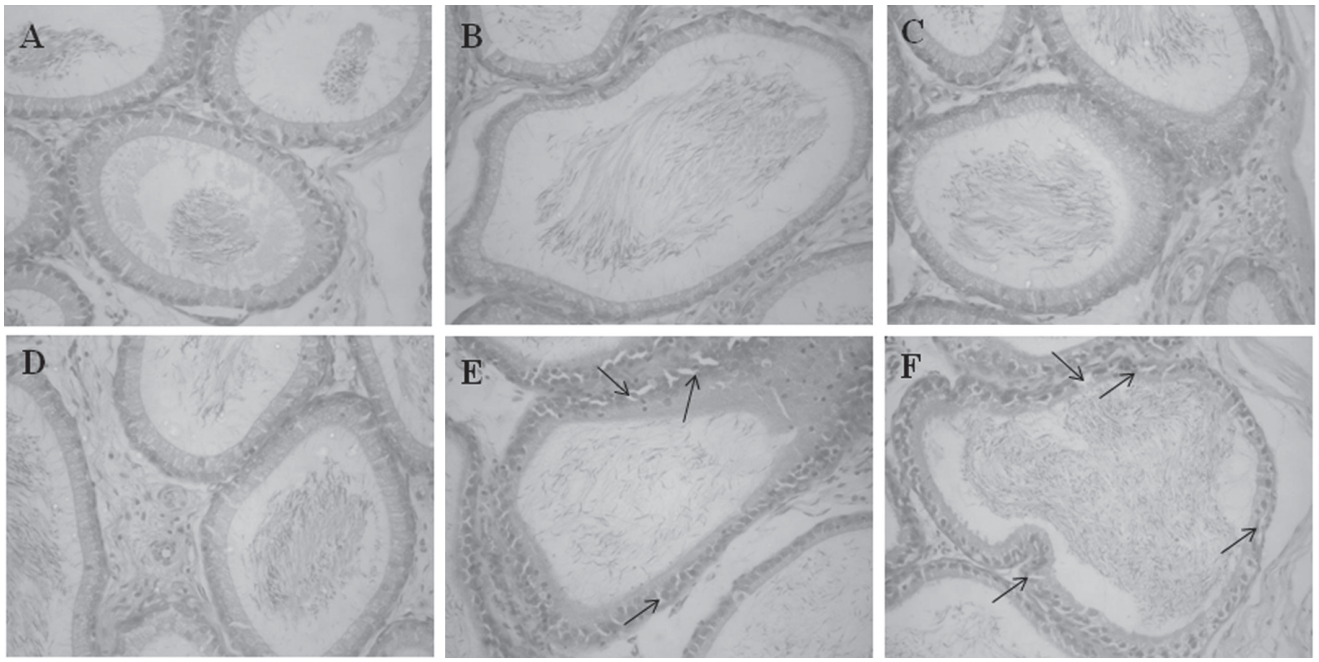

Figure 5. Histopathology of caput in the rats subjected to inhalation of mosquito coil smoke. (A) 15 days; (B) 30 days; (C) 60 days; (D) 90 days; (E) 120 days; (F) 180 days. Arrows indicate epithelial cell damage. Magnification: 40×. (See colour version of this figure online at www.informahealthcare.com/iht)

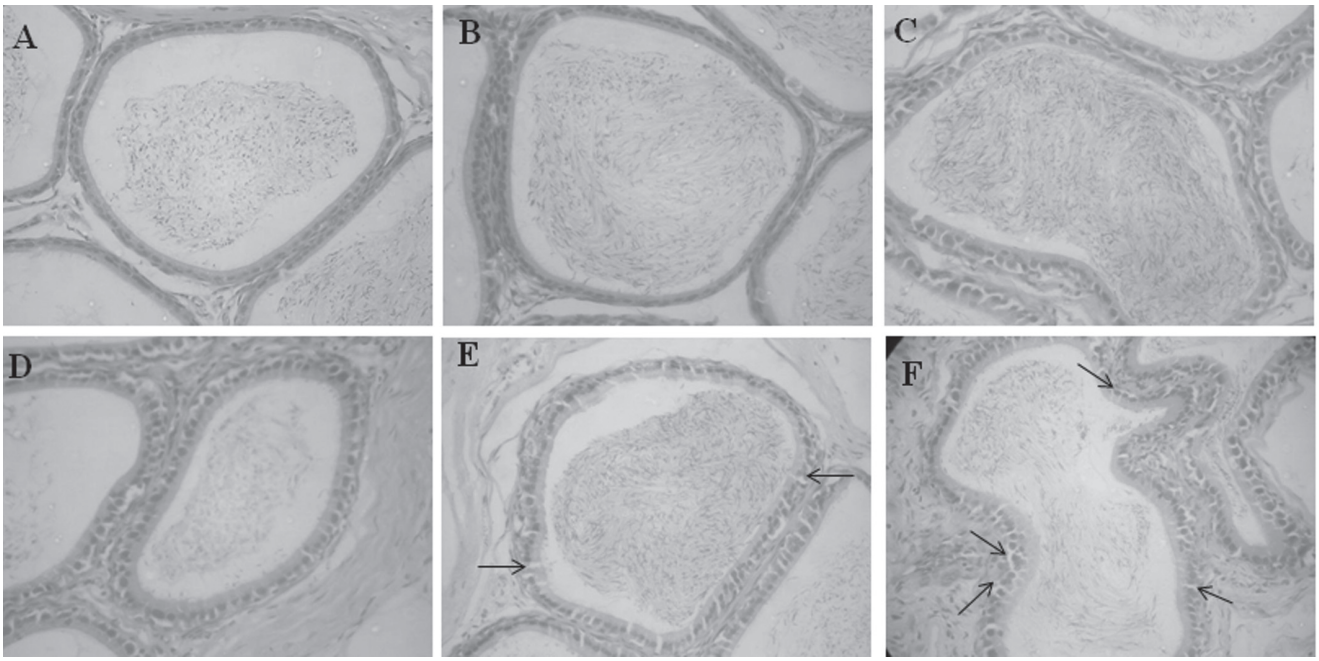

Figure 6. Histopathology of cauda in the rats subjected to inhalation of mosquito coil smoke. (A) 15 days; (B) 30 days; (C) 60 days; (D) 90 days; (E) 120 days; (F) 180 days. Arrows indicate epithelial cell damage. Magnification: 40×. (See colour version of this figure online at www.informahealthcare.com/iht)

Vontas et al., 2001; Gabbianelli et al., 2002). For example, increased oxidative stress was observed in the brain and liver of rats exposed to cypermethrin (Giray et al., 2001; El-Demerdash, 2011b; El-Demerdash, 2011a; Tuzmen et al., 2008). Increased lipid peroxidation was observed in the brain, liver, and kidney of rats subjected to mosquito repellant inhalation (Gupta A, 1999). In the male reproductive tract, pyrethroid compounds were shown to induce lipid peroxidation (Issam et al., 2009; Wang et al., 2009). However, there are no reports on oxidant stress due to mosquito coil exposure in the male reproductive tract. In this study, we demonstrate for the first time that increased lipid peroxidation is observed in the testes and cauda of rats subjected to prolonged inhalation of allethrin-based mosquito coil smoke, which may lead to high levels of free radicals. It is well-known that free

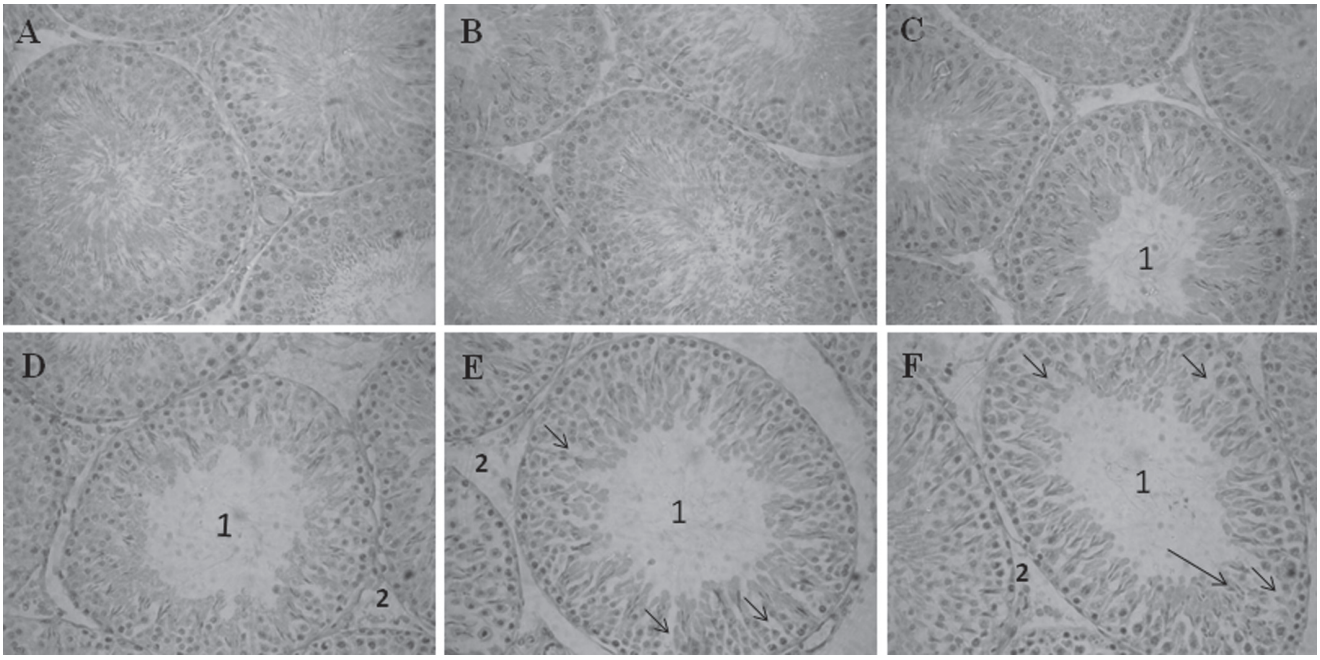

Figure 7. Histopathology of testes in the rats subjected to inhalation of mosquito coil smoke. (A) 15 days; (B) 30 days; (C) 60 days; (D) 90 days; (E) 120 days; (F) 180 days. Arrows indicate tubular damage/dead spermatozoa. Magnification: 40×. "1" indicates lumen size and "2" indicates interstitial edema. (See colour version of this figure online at www.informahealthcare.com/iht)

Table 2. Histopathological score[a] of caput, cauda, and testes.

S.No	Damage	15 days	30 days	60 days	90 days	120 days	180 days
A. Caput							
1	Dead/dry spermatozoa	–	–	–	–	–	+
2	Epithelial cell disruption	–	–	–	+	+	++
B. Cauda							
1	Dead/dry spermatozoa	–	–	–	–	+	++
2	Epithelial cell disruption	–	–	–	+	++	+++
C. Testes							
1	Interstitial edema	–	–	+	+	++	++
2	Tubule architecture disarray	–	±	+±	+±	++±	+++
3	Nuclear integrity damage	–	–	±	±	+±	++
4	Dead/dry spermatozoa	–	±	±	+	+	++
5	Epithelial cell disruption	–	–	–	–	–	+
6	Germ cell layer damage	–	±	+	++	++	+++
7	Lumen	–	±	++	++	+++	+++

[a]On a score of five points.
+++++: maximum damage; ++++: high damage; +++: moderately damage; ++: low/moderate; +: low; ±: more than zero, less than one; –: no lesions/damage.

radicals interact with membrane lipids and alter membrane integrity. In this study, we observed damage of sperm nuclear and membrane integrity, testicular tubule and epithelial cell damage besides interstitial edema, which could be due to harmful effects of free radicals generated as a result of increased lipid peroxidation.

Alteration in antioxidant status is a consequence of increased oxidative stress and the same is also demonstrated during pyrethroid toxicity in different organ systems. Decreased levels of reduced glutathione (Gupta A, 1999), catalase, superoxide dismutase, and GPx were observed during pyrethroid toxicity. The changes in antioxidant status in the male reproductive tract in rats exposed to allethrin-based mosquito coil smoke were not reported till now. Increased lipid peroxidation observed in our study may lead to oxidative stress thereby affecting antioxidant status. Surprisingly, in general, antioxidant status was not altered in the caput, cauda, and testes except for a significant increase of catalase activity in the cauda obtained from 120 and 180 day exposed rats. It was reported earlier that a 90-day exposure to liquid mosquito repellants did not affect the gonadal enzymes such as alkaline phosphatase, sorbitol dehydrogenase, lactate dehydrogenase, and glucose-6-phosphate dehydrogenase (Srivastava et al., 2006). In this study, a general lack of changes in antioxidant status in the caput, cauda, and

testes and the increased catalase activity in the cauda obtained from 120 and 180 day exposed rats could be due to the protective mechanisms adopted by the male reproductive tract to prevent loss of fertility under stress conditions.

The effects of inhalation of allethrin-based mosquito repellants on cellular architecture of reproductive organs and sperm parameters are reported earlier. Pathomorphological changes in the testes, sperm number, and motility were not observed in rats that were subjected to inhalation of liquid mosquito repellants using vaporizers for 90 days (Srivastava et al., 2006). Similarly, no decrease in spermatogenesis was observed in rats exposed to a single pesticide; however, sperm motility was decreased in rats exposed to a mixture of pesticides (Perobelli et al., 2010). Fenvalerate, a pyrethroid insecticide, adversely affected sperm production and storage in male rats (Arena et al., 2008). Our histopathological analyses revealed increased germ cell damage, loss of germ cell nuclear integrity, and increased number of dead spermatozoa in the testes of rats exposed to allethrin-based mosquito coil smoke. It is possible that prolonged exposure to mosquito coil smoke (>90 days) is required to cause a loss of germ cell integrity; since no changes in pathomorphological features of the caput, cauda, and testes and sperm parameters are reported for 15–90 day samples in this study as well as in a previous 90-day toxicity report (Srivastava et al., 2006). These pathomorphological changes observed could be due to increased free radical generation.

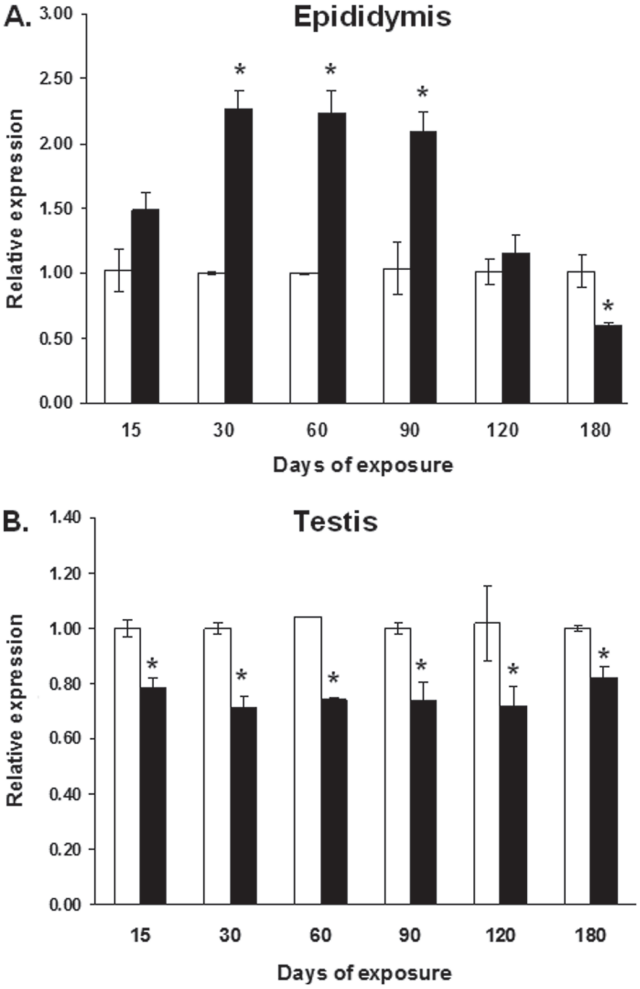

Figure 8. mRNA expression of p53 in the epididymides and testes. (A) Epididymides; (B) Testis. Untreated control (□); Mosquito coil smoke exposed (■). * denotes $p < 0.05$.

Of the many responses that occur during stress, the role of p53 is regarded as a key factor, since its dynamics dictates the fate of the cell to either undergo apoptosis or initiate repair mechanisms by temporarily halting the cell cycle. In our study, we observed increased p53 mRNA expression in the epididymides obtained from rats exposed to mosquito coil smoke for 15–90 days and the levels gradually decreased until 180 days. In the testes, lower levels of p53 were observed when compared to the controls at all the time points. The initial increase of p53 expression in the epididymis may be a stress response to allow cells to initiate repair mechanisms. Loss of p53 expression results in genomic instability and severely reduces tumor suppression (Han et al., 2008; Reuter et al., 2010). Reduced expression of p53 in the testes and at later time points in the epididymides could lead to development of tumors, though no such features were observed in our histopathological analyses. However, further studies are required to determine whether tumor development due to loss of p53 can occur in the male reproductive tract of rats exposed to mosquito coil smoke for more than 180 days.

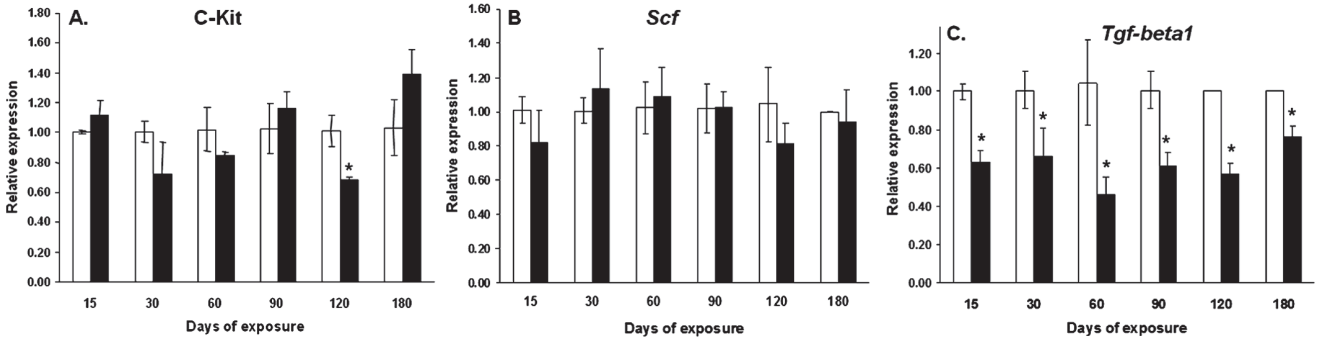

Figure 9. mRNA expression of spermatogenic factors in the testes of mosquito coil smoke exposed rats. (A) *c-Kit*; (B) Stem cell factor (*Scf*); (C) Transforming growth factor-β-1 (*Tgf-β1*). Untreated control (□); Mosquito coil smoke exposed (■). * denotes $p < 0.05$.

Spermatozoa are produced in the male reproductive tract by a series of complex mechanisms that are broadly classified in to spermatogenesis and sperm maturation, the former occurring in the testes and latter in the epididymis respectively. A wide variety of molecules such as TGF-β1, stem cell factor, and its ligand, *c-kit* play a key role in spermatogenesis. Expression of spermatogenic factors depends on the microenvironment and can be influenced by various factors such as hormonal status, stress, etc. In our study, we demonstrate that the expression of *Scf* and its ligand *c-Kit* are not altered, whereas decreased levels of *Tgf-β1* are observed. Though *Tgf-β-1* levels were decreased, there was no marked change in the density of spermatids in the testes as revealed by our histopathological analyses. It is possible that the decreased levels of *Tgf-β1* alone may not be functionally significant.

Manufacturers of leading brands of mosquito coils available in the Indian market do not disclose the complete composition of these coils. As per the information provided in the leaflet along with the product, the composition of the coils used in our study contained 0.1% w/w d-tansallethrin, 40% w/w wood floor, 40% w/w coconut shell powder, 10% w/w starch and 9.9% other ingredients. The composition of mosquito coils available in retail outlets of Asian countries and those available in the Asian markets of United States is reported (Liu & Sun, 1988; Liu et al., 1987; Krieger et al., 2003). They are known to contain octadichlorodipropyl ester (S-2) as a synergist. Further, use of mosquito coils increases the risk of exposure to bis(chloromethyl) ether and submicron particles (<1 μm) coated with heavy metals, allethrin, and phenol O-cresol. Though we observe biochemical and toxicogenomic effects in the male reproductive tract of rats exposed to allethin-based mosquito coil smoke, caution is exercised to attribute all of the observed effects to be result of inhalation of allethrin released during burning of mosquito coils. It is possible that the effects observed could also be due to the other toxic materials released during mosquito coil combustion. Further, burning one mosquito coil smoke can emit particulate matter equivalent to that of 75–137 cigarettes (Liu et al., 2003). It is well-known that cigarette (tobacco) smoking causes severe impairment to the reproductive function (Dechanet et al., 2011). Hence, it can be expected that the impairment induced by mosquito coil smoke is far greater than cigarette smoke.

In conclusion, we report that in the male reproductive tract of rats exposed to mosquito coil smoke for 15–180 days, there are no significant changes in the organ weight. Further, increased lipid peroxidation was observed in the cauda and testes without any major changes in the antioxidant status, except for an increase in the catalase activity in the cauda obtained from 120 and 180 day mosquito coil smoke exposed rats. Pathomorphological changes were predominantly observed in the cauda and testes at the later time points. The expression pattern of stress response gene, p53, was altered in the epididymides and testes, whereas the mRNA expression of sperm maturation factors *C-Kit* and *Scf* remained largely unaltered, though decreased levels of *Tgf-β1* was observed. Results of this study indicate that exposure to mosquito coil smoke for prolonged periods of time increases oxidative stress with concomitant changes in tumor suppressor response gene, thereby increasing the susceptibility to develop cancers of the male reproductive tract. Further, severe damage to male reproductive tract function may occur due to loss of organ and spermatozoa architectural integrity.

Acknowledgments

Financial support from University Grants Commission, Government of India, is acknowledged. Golla Madhubabu thanks University of Hyderabad and Council of Scientific and Industrial Research for providing the fellowship. We thank the facilities extended by UGC-SAP, UGC-CAS, DBT-CREBB, and FIST programmes at University of Hyderabad.

Declaration of interest

The authors declare no conflict of interest with the study or preparation of the manuscript.

References

Aebi H. 1984. Catalase in vitro. Meth Enzymol 105:121–126.

Arena AC, Fernandez CD, Porto EM, Bissacot DZ, Pereira OC, Kempinas WG. 2008. Fenvalerate, a pyrethroid insecticide, adversely affects sperm production and storage in male rats. J Toxicol Environ Health Part A 71:1550–1558.

Bernheim FM, Bernheim MLC, Wilbur KM. 1948. The reaction between thiobarbutiric acid and the oxidation products of certain lipids. J Biol Chem 174:257–264.

Besmer P, Manova K, Duttlinger R, Huang EJ, Packer A, Gyssler C, Bachvarova RF. 1993. The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. Dev Suppl :125–137.

Casida JE. 1995. Metabolism and synergism of Pyrethrins. In: Casida JEQ, GB, ed. Pyrethrum Flowers Production, Chemistry, Toxicology, and Uses. New York: Oxford University Press.

Cheng CY, Wong EW, Lie PP, Li MW, Su L, Siu ER, Yan HH, Mannu J, Mathur PP, Bonanomi M, Silvestrini B, Mruk DD. 2011. Environmental toxicants and male reproductive function. Spermatogenesis 1:2–13.

Dechanet C, Anahory T, Mathieu Daude JC, Quantin X, Reyftmann L, Hamamah S, Hedon B, Dechaud H. 2011. Effects of cigarette smoking on reproduction. Hum Reprod Update 17:76–95.

Eil C, Nisula BC. 1990. The binding properties of pyrethroids to human skin fibroblast androgen receptors and to sex hormone binding globulin. J Steroid Biochem 35:409–414.

El-Demerdash FM. 2011a. Lipid peroxidation, oxidative stress and acetylcholinesterase in rat brain exposed to organophosphate and pyrethroid insecticides. Food Chem Toxicol 49:1346–1352.

El-Demerdash FM. 2011b. Oxidative stress and hepatotoxicity induced by synthetic pyrethroids-organophosphate insecticides mixture in rat. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 29:145–158.

Gabbianelli R, Falcioni G, Nasuti C, Cantalamessa F. 2002. Cypermethrin-induced plasma membrane perturbation on erythrocytes from rats: reduction of fluidity in the hydrophobic core and in glutathione peroxidase activity. Toxicology 175:91–101.

Garey J, Wolff MS. 1998. Estrogenic and antiprogestagenic activities of pyrethroid insecticides. Biochem Biophys Res Commun 251:855–859.

Giray B, Gürbay A, Hincal F. 2001. Cypermethrin-induced oxidative stress in rat brain and liver is prevented by vitamin E or allopurinol. Toxicol Lett 118:139–146.

Go V, Garey J, Wolff MS, Pogo BG. 1999. Estrogenic potential of certain pyrethroid compounds in the MCF-7 human breast carcinoma cell line. Environ Health Perspect 107:173–177.

Gupta A, Nigam D, Gupta A, Shukla GS, Agarwal AK. 1999. Effect of pyrethroid-based liquid mosquito repellent inhalation on the blood-brain barrier function and oxidative damage in selected organs of developing rats. J Appl Toxicol 19:67–72.

Han ES, Muller FL, Pérez VI, Qi W, Liang H, Xi L, Fu C, Doyle E, Hickey M, Cornell J, Epstein CJ, Roberts LJ, Van Remmen H, Richardson A. 2008. The *in vivo* gene expression signature of oxidative stress. Physiol Genomics 34:112–126.

Huailu C, Wen Y, Waumin K, Chougyi L. 1995. Large scale spraying of bed nets to control mosquito vector and malaria in Sichuan, China. Bulletin WHO 73:321–328.

Issam C, Samir H, Zohra H, Monia Z, Hassen BC. 2009. Toxic responses to deltamethrin (DM) low doses on gonads, sex hormones and lipoperoxidation in male rats following subcutaneous treatments. J Toxicol Sci 34:663–670.

Issam C, Zohra H, Monia Z, Hassen BC. 2011. Effects of dermal sub-chronic exposure of pubescent male rats to permethrin (PRMT) on the histological structures of genital tract, testosterone and lipoperoxidation. Exp Toxicol Pathol 63:393–400.

Jakoby WB. 1978. The glutathione-S-transferases: a group of detoxification proteins. New York: John Wiley.

Kakko I, Toimela T, Tähti H. 2003. The synaptosomal membrane bound ATPase as a target for the neurotoxic effects of pyrethroids, permethrin and cypermethrin. Chemosphere 51:475–480.

Kale M, Rathore N, John S, Bhatnagar D. 1999. Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: a possible involvement of reactive oxygen species. Toxicol Lett 105:197–205.

Kasat K, Go V, Pogo BG. 2002. Effects of pyrethroid insecticides and estrogen on WNT10B proto-oncogene expression. Environ Int 28:429–432.

Krieger RI, Dinoff TM, Zhang X. 2003. Octachlorodipropyl ether (s-2) mosquito coils are inadequately studied for residential use in Asia and illegal in the United States. Environ Health Perspect 111:1439–1442.

Li HY, Shi N, Chen D, Dai ZH, Lu WH, Wang B, Li YR. 2005. Oxidative stress of deltamethrin on rat nervous system. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi 23:97–101.

Liu W, Zhang J, Hashim JH, Jalaludin J, Hashim Z, Goldstein BD. 2003. Mosquito coil emissions and health implications. Environ Health Perspect 111:1454–1460.

Liu WK, Sun SE. 1988. Ultrastructural changes of tracheal epithelium and alveolar macrophages of rats exposed to mosquito coil smoke. Toxicol Lett 41:145–157.

Liu WK, Wong MH, Mui YL. 1987. Toxic effects of mosquito coil (a mosquito repellent) smoke on rats. I. Properties of the mosquito coil and its smoke. Toxicol Lett 39:223–230.

Maiti PK, Kar A, Gupta P, Chaurasia SS. 1995. Loss of membrane integrity and inhibition of type-I iodothyronine 5'-monodeiodinase activity by fenvalerate in female mouse. Biochem Biophys Res Commun 214:905–909.

Meehan T, Schlatt S, O'Bryan MK, de Kretser DM, Loveland KL. 2000. Regulation of germ cell and Sertoli cell development by activin, follistatin, and FSH. Dev Biol 220:225–237.

Ochsendorf FR. 1999. Infections in the male genital tract and reactive oxygen species. Hum Reprod Update 5:399–420.

Olaso R, Pairault C, Boulogne B, Durand P, Habert R. 1998. Transforming growth factor beta1 and beta2 reduce the number of gonocytes by increasing apoptosis. Endocrinology 139:733–740.

Paglia DE, Valentine WN. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70:158–169.

Perobelli JE, Martinez MF, da Silva Franchi CA, Fernandez CD, de Camargo JL, Kempinas Wde G. 2010. Decreased sperm motility in rats orally exposed to single or mixed pesticides. J Toxicol Environ Health Part A 73:991–1002.

Pontecorvo G, Fantaccione S. 2006. Recombinogenic activity of 10 chemical compounds in male germ cells of Drosophila melanogaster. Ecotoxicol Environ Saf 65:93–101.

Ramesh A, Vijayalakshmi A. 2001. Monitoring of allethrin, deltamethrin, esbiothrin, prallethrin and transfluthrin in air during the use of household mosquito repellents. J Environ Monit 3:191–193.

Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. 2010. Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med 49:1603–1616.

Sattin RW, Roisin A, Kafrissen ME, Dugan JB, Farer LS. 1984. Epidemic of gynecomastia among illegal Haitian entrants. Public Health Rep 99:504–510.

Sharpe RM, Irvine DS. 2004. How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health? BMJ 328:447–451.

Sharpe RM, Skakkebaek NE. 1993. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 341:1392–1395.

Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, Stevens JT, Weiner ML. 2002. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. Toxicology 171:3–59.

Srivastava A, Srivastava MK, Raizada RB. 2006. Ninety-day toxicity and one-generation reproduction study in rats exposed to allethrin-based liquid mosquito repellent. J Toxicol Sci 31:1–7.

Tan J, Soderlund DM. 2010. Divergent actions of the pyrethroid insecticides S-bioallethrin, tefluthrin, and deltamethrin on rat Na(v)1.6 sodium channels. Toxicol Appl Pharmacol 247:229–237.

Tomlin C. 1994. A world compendium: the pesticide manual. British Crop Protection Council.

Tuzmen N, Candan N, Kaya E, Demiryas N. 2008. Biochemical effects of chlorpyrifos and deltamethrin on altered antioxidative defense mechanisms and lipid peroxidation in rat liver. Cell Biochem Funct 26:119–124.

Verschoyle RD, Aldridge WN. 1980. Structure-activity relationships of some pyrethroids in rats. Arch Toxicol 45:325–329.

Vijverberg HP, de Weille JR. 1985. The interaction of pyrethroids with voltage-dependent Na channels. Neurotoxicology 6:23–34.

Vontas JG, Small GJ, Hemingway J. 2001. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens. Biochem J 357:65–72.

Wang XZ, Liu SS, Sun Y, Wu JY, Zhou YL, Zhang JH. 2009. Beta-cypermethrin impairs reproductive function in male mice by inducing oxidative stress. Theriogenology 72:599–611.

Wong EW, Cheng CY. 2011. Impacts of environmental toxicants on male reproductive dysfunction. Trends Pharmacol Sci 32:290–299.

Allethrin Induced Toxicity in the Male Reproductive Tract of Rats Contributes to Disruption in the Transcription of Genes Involved in Germ Cell Production

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Received 1 November 2012; revised 12 March 2013; accepted 16 March 2013

ABSTRACT: Pyrethroids are known to be neurotoxic. However, their toxic effects including that of allethrin on the male reproductive tract are not elucidated. Adult male rats were treated orally with 25, 50, 100, and 150 mg/kg body weight allethrin every day for 60 days. Lipid peroxidation was increased ($p < 0.001$) in the caput, cauda, and testes. Nitric oxide production was increased ($p < 0.001$) in the caput, but unaltered in the cauda and testes. The activities of catalase, glutathione peroxidase, glutathione-*S*-transferase, and superoxide dismutase were decreased in the caput and cauda where as a decrease was observed in the testis obtained from allethrin treated rats. In the epididymides and testes, damage to tubular architecture, congestion, degeneration of epithelial cell lining, intestinal edema, and presence of dead or degenerating spermatids were observed in a dose dependent manner. The expression profile of genes involved in spermatogenesis (*Tgf-beta1*), sperm maturation (*Spag11e*), and sperm function (*Defb22*) were reduced ($p < 0.001$) in allethrin rats. The expression of p53 gene was decreased and increased phosphorylation of MAPK (p42/p44) expression was observed the male reproductive tract tissues of allethrin treated rats. Although earlier studies have reported the effects of allethrin inhalation because of the use of mosquito coils and vaporizers, our results for the first time prove that oral exposure to allethrin could affect fertility and may contribute to deregulation of cell cycle in the male reproductive tract. Environ Toxicol 29: 1330–1345, 2013.

Key words: allethrin; epididymis; testis; oxidative stress; sperm maturation

INTRODUCTION

Synthetic pyrethroids are used for pest control in crop production and households and exposure to pyrethrin based pesticides by humans occurs during such usage. Although, pyrethroids are known to exhibit low levels of toxicity (Casida, 1995), they primarily act on nervous system (Soderlund et al., 2002) to inhibit voltage sensitive sodium channels and sodium-potassium ATPase (Kakko et al., 2003; Tan and Soderlund, 2010). Further, pyrethroids are known to be hepatotoxic, carcinogenic, immunosuppressive, estrogenic, and antiprogestagenic in mammals (Institoris et al., 1999; Shukla et al., 2002; Nasuti et al., 2007; Yokohira et al., 2011). Pyrethroids induce oxidative stress and alter antioxidant system in different organ systems of rats (Giray et al., 2001; Li et al., 2005). Their effects on the reproductive

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Published online 18 April 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/tox.21864

system include gynecomastia, inhibition of testosterone binding to the androgen receptor and sex hormone binding globulin at high concentrations (Sattin et al., 1984; Eil, 1990). Further, it is reported that pyrethroid compounds cause oxidative stress in the male reproductive tract (Issam et al., 2009, 2011; Wang et al., 2009). Endocrine disruption and oxidative stress caused by pyrethroid based pesticides (Jin et al., 2011) is an important area of investigation in toxicology.

D-allethrin, a mixture of eight stereoisomers belongs to the type I pyrethroids and is used widely in developing countries for household pest control. It was found to be moderately toxic in rodent models (Tomlin, 1994). Although the effects of allethrin based pesticides are well studied in the nervous system, information about allethrin toxicity in the reproductive tract is limited. Rats exposed nasally to allethrin based mosquito repellents for 90 days did not show any change in their clinical enzyme profile and reproductive indices (Srivastava et al., 2006). Further, it is demonstrated that allethrin exhibits recombinogenic activity in the male germ cells of *Drosophila melanogaster* (Pontecorvo and Fantaccione, 2006).

Sperm production is seriously compromised due to oxidative stress (Naziroglu et al., 2011; Bal et al., 2012a). Nitric oxide (NO) produced in the reproductive tract (Shum et al., 2009) is considered a free radical scavenger during oxidative stress. Increased oxidative stress initiates apoptosis, a process tightly under the control of p53 gene product. Production of male germ cells in the testis is controlled by spermatogenic factors such as *Tgf-β1* (Olaso et al., 1998). Immature spermatids undergo maturation in the epididymis and it is demonstrated that SPAG11E plays an important role in spermatid maturation (Zhou et al., 2004; Zhao et al., 2011b) Further, the macaque *Defensin 126* (*DEFB126*), the rat homologue of *Defensin 22* (*Defb22)*, is a key factor involved in sperm capacitation (Yudin et al., 2003), mediates attachment of sperm to the epithelium of oviduct (Tollner et al., 2008) and protects the sperm from immuno recognition (Yudin et al., 2005). The rat *Defb22* is expressed in the epididymis and its protein product is localized on the sperm implying roles similar to its macaque counterpart (Rao et al., 2003; Zanich et al., 2003). The effects of allethrin based pesticides on the possible changes at the architectural and molecular levels besides alterations in the expression of genes that regulate spermatogenesis, sperm maturation, capacitation, and fertilization is not yet investigated.

Environmental pollutants such as pesticides and heavy metals contribute to the development of neoplasia and reduction in reproductive functions (Issam et al., 2009; Wang et al., 2009; Tan and Soderlund, 2010; Aly et al., 2012). It can be hypothesized that prolonged exposure to pyrethroid based pesticides such as allethrin could cause oxidative stress and alteration in the antioxidant status, thereby contribute to the initiation of cancer in the male reproductive tract. Further, allethrin exposure could lead to disturbances in the morphological and cellular architecture thereby affecting fertility, that is, spermatogenesis, sperm maturation, and capacitation. Hence, in this study we attempt to study whether oral exposure of allethrin to rats could cause initiation of cancer via the induction of oxidative stress in the male reproductive tract and whether spermatogenesis and sperm maturation are compromised.

We observed increased oxidative stress and altered antioxidant enzyme status in the testes and epididymides of rats orally exposed to allethrin for 60 days. Significant pathomorphological changes in the male reproductive tract tissues were observed. Altered *p53* gene expression and increased MAPK phosphorylation suggests loss of cell cycle regulation. The expression of *Tgf-β1, Spag11e*, and *Defb22* were reduced indicating a loss of fertility.

ABBREVATIONS

Defb	Beta Defensin
Gpx	glutathione peroxidase
GST	glutathione-S-transferase
LPO	lipid peroxidation
MAPK	MAP Kinase
MDA	malondialdehyde
NO	nitric oxide
SOD	superoxide dismutase
Spag11	Sperm associated antigen 11
TBA	thiobarbutiric acid
TGF-β	transforming growth factor beta.

MATERIALS AND METHODS

Animals and Tissue Specimens

Male Wistar rats aged 90 days were obtained from National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad and were maintained on a 12L:12D lighting schedule, at 22–25°C, with food and water *ad libitum*. After acclimatization, rats were divided into different groups ($n = 5$) and were orally administered every day with 25, 50, 100, and 150 mg/kg body weight allethrin (mixture of stereo isomers; Sigma Aldrich) dissolved in corn oil for 60 days. Treatment doses chosen were based on previous reports (Soderlund et al., 2002). Control animals were administered corn oil orally for 60 days. Animals were sacrificed by cervical dislocation and tissues were collected after 60 days from vehicle treated control and allethrin treated rats. Testis, caput and cauda epididymides were identified, stripped off the connective tissues, noted the weight, frozen in liquid nitrogen and kept at −70°C until use. Experiments were conducted using the guidelines for the care and use of laboratory animals, approved by the Institutional Animal Ethics Committee of University of Hyderabad.

TABLE I. Organ weights in allethrin treated rats

	Allethrin Dose (mg/kg body weight)				
Organ	0	25	50	100	150
Caput Epididymides	0.48 ± 0.4	0.48 ± 0.2	0.52± 0.3	0.56± 0.4	0.49± 0.3
Cauda Epididymides	0.46 ± 0.4	0.42 ± 0.5	0.43± 0.7	0.46± 0.9	0.37± 0.6
Testis	2.9 ± 0.7	2.8 ± 0.3	2.78± 0.18	3.01± 0.2	2.53±0.8

Estimation of Lipid Peroxidation (LPO) Products

LPO was estimated using the thiobarbituric acid (TBA) test as described earlier (Bernheim, 1964). TBA reacts with malondialdehyde (MDA), the product of LPO, under acidic conditions and high temperature (95°C) to form a pink colored complex with absorbance maxima at 532 nm. Caput, cauda, and testes obtained from the control and experimental animals were homogenized in phosphate buffer saline, pH 7.4. To 0.5 mL of the homogenate, 1 mL of 10% TCA was added to precipitate the protein. The samples were then centrifuged at 3000 rpm for 10 min and to the supernatant, 0.25 mL of 0.33% TBA was added and incubated at 95°C for 1 h. Absorbance of the pink color formed was measured at 532 nm using a spectrophotometer. 1,1,3,3-Tetraethoxy-propane (TEP) was used as the standard. Levels of LPO were expressed as nano moles MDA/mg protein.

Estimation of NO as Its Stable Metabolite Nitrite

NO being a free radical is highly unstable and get converted to an equimolar ratio of its stable metabolites, nitrite and nitrate. The estimation of NO is therefore done by estimating its stable metabolite. The amount of nitrite/nitrate formed is an index of the amount of NO generated in the samples. Nitrite was measured by the method of Griess as described earlier (Lepoivre et al., 1990). Briefly, 0.5 mL of caput, cauda, and testicular homogenates obtained from allethrin exposed rats, were mixed with 70% Sulfosalicylic acid to precipitate the protein. After centrifugation at 15,000 rpm for 30 min, the supernatant was mixed with Griess reagent and incubated in dark for 30 min at room temperature. The intensity of the purple color formed was measured at 540 nm using a spectrophotometer. Sodium nitrite was used as the standard.

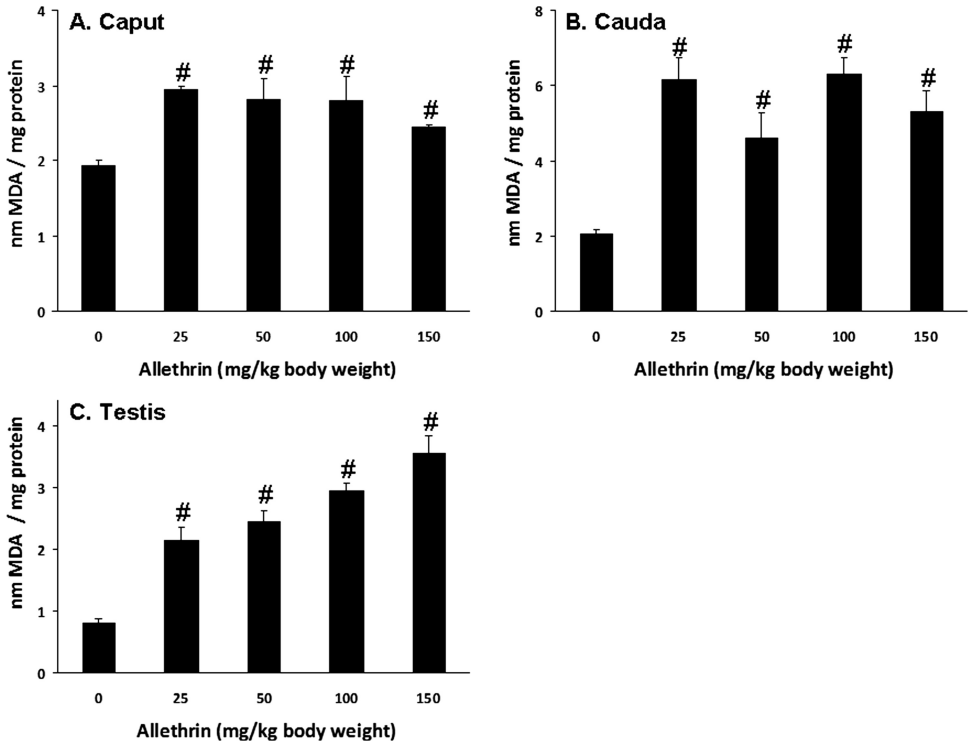

Fig. 1. LPO in the male reproductive tract of allethrin treated rats. Male Wistar rats received 25–150 mg/kg body weight allethrin orally for 60 days. Reproductive tract tissues were collected and homogenates prepared and LPO estimated. A: Caput; B: Cauda; C: Testis. Values shown are Mean ± S.D. * and # denotes $p < 0.01$ and 0.001, respectively.

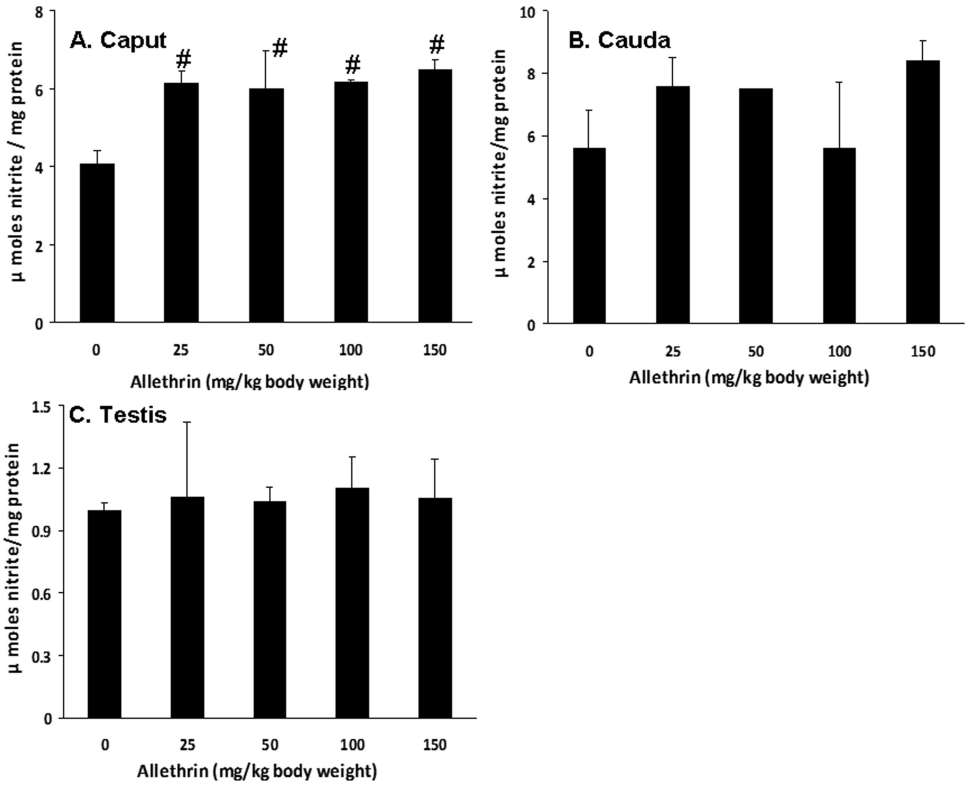

Fig. 2. NO production in the male reproductive tract of allethrin treated rats. Male Wistar rats received 25–150 mg/kg body weight allethrin orally for 60 days. Reproductive tract tissues were collected and homogenates prepared and nitrite estimated. A: Caput; B: Cauda; C: Testis. Values shown are Mean ± S.D. * and # denotes $p < 0.01$ and 0.001, respectively.

Antioxidant Enzyme Assays

Antioxidant enzyme activity was measured using spectrophotometric methods. Caput, cauda, and testes collected from control and allethrin treated rats were homogenized in phosphate buffer saline, pH 7.4 and centrifuged at 3000 rpm to remove the debris. The supernatant was collected and the activities of catalase, glutathione-*s*-transferase (GST), glutathione peroxidase (GPx), and superoxide dismutase (SOD) were performed using standard protocols described earlier (Paglia and Valentine, 1967; McCord and Fridovich, 1969; Jakoby, 1978; Aebi, 1984). The activity of the antioxidant enzymes were calculated from the change in optical density measured on a spectrophotometer.

Real Time Polymerase Chain Reaction (RT-PCR)

Tissues obtained from control and allethrin treated rats were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA was extracted. Total RNA (2 µg) was reverse transcribed using 50 U Stratascript (Invitrogen) and 0.5 µg of oligodT (Invitrogen) according to the manufacturer's instructions. The resultant cDNA (2 µL) was used for real time PCR analysis using SYBR master mix kit (Applied Biosystems, Warrington, UK). Glyceraldehyde-6-phosphate dehydrogenase expression was used as the internal control. A typical real time PCR reaction consisted of the following conditions: initial denaturation at 94°C for 10 min; 40 cycles with 94°C for 15 s and 60°C for 1 min. Negative controls (no template control and minus RT control (only with RNA) were included in the assays to detect nonspecific amplification. The amplicons were sequenced to confirm their identity.

Histopathology

Epididymides and testes fixed in Bouin's fluid for 24 h and then into 70% ethanol, which was changed 3–4 times every day until the yellow color of Bouin's fluid completely disappeared. Tissues were hydrated serially in 80, 90, and 100% ethanol and stored in isopropanol overnight at 60°C before embedding in paraffin. Five micron sections were made for further analysis. Sections were deparaffinized by xylene and were re-hydrated serially in 100, 90, 80, 70, and 50% ethanol, and then rinsed briefly in distilled water. Slides were immersed into Harris hematoxylin solution for 10 min, subsequently washed in distilled water for a while and differentiated in 1% hydrochloric acid for 30 s and then immersed in 0.2% ammonia water for 30 s to 1 min. After washing in running tap water for 10 min, sections were counterstained with 0.2% eosin Y solution for 1 min. Sections were washed in distilled water and

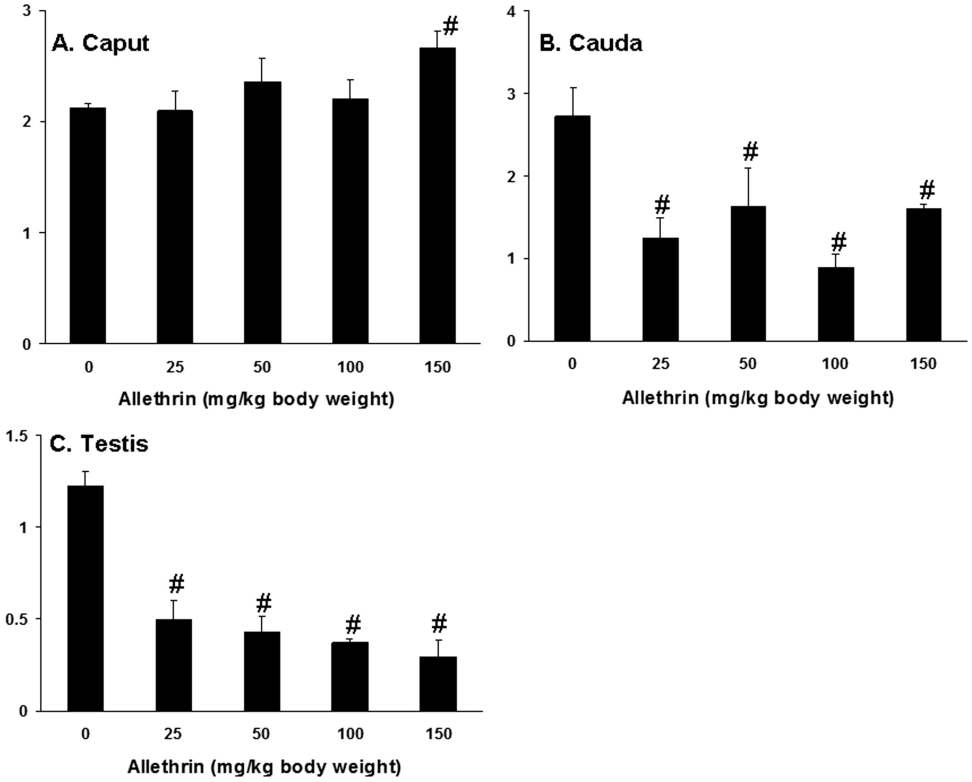

Fig. 3. NO/MDA ratio in the male reproductive tract tissues of allethrin treated rats. A: Caput; B: Cauda; C: Testis. Values shown are Mean ± S.D. * and # denotes $p < 0.01$ and 0.001, respectively.

followed by serial dehydration using 50, 70, 80, 90, and 100% alcohol. Slides were cleaned with three changes of xylene and then mounted with xylene based mounting medium. Photographs were taken on a Leica microscope.

Immunoblotting

Total protein from tissues obtained from allethrin treated and control rats was harvested in radioimmuno precipitation assay buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, and 0.1% SDS) supplemented with proteinase cocktail inhibitor Tissues were then homogenized and centrifuged at 10,000 rpm. The supernatant was collected and the concentration of the total protein was determined by Lowry's method using bovine serum albumin as a standard. Hundred microgram of each protein sample was separated on a 10% SDS-PAGE and transferred on to a 0.2 μm pore size nitrocellulose membrane (HybondTM ECLTM, GE Healthcare, UK). The nonspecific binding sites were blocked by incubating the membranes in 5% nonfat milk in Tris-buffered saline (TBS) with 0.1% Tween (TBS-T) for 1 h at room temperature. The membranes were then incubated with specific antibodies (Cell Signaling Technology, Danvers, MA) directed against rat phospho p44/42 MAPK for 3 h at room temperature. The membrane was then washed and incubated with secondary antibody against rabbit IgG conjugated with horse radish peroxidase (Cell Signaling Technology). Immunoreactive proteins were detected using an ECL prime Western blot detection Kit (GE Healthcare life sciences, MA), according to the manufacturer's instructions.

Statistical Analyses

Changes in the parameters analyzed in this study were compared with the vehicle control. Statistical analyses were performed using one way ANOVA Holm-Sidak test available in Sigma Plot software (SPSS, Chicago, IL). Values shown are Mean ± S.D. *a* and *b* indicates $p < 0.01$ and 0.001, respectively.

RESULTS

Organ Weights

When compared with vehicle treated control, no significant changes in the weights of caput, corpus, and testes were observed in the animals treated with varying doses of allethrin (Table I).

Lipid Peroxidation

Pyrethroids being potent inducers of free radical generation, LPO products were measured in the caput, cauda and testes

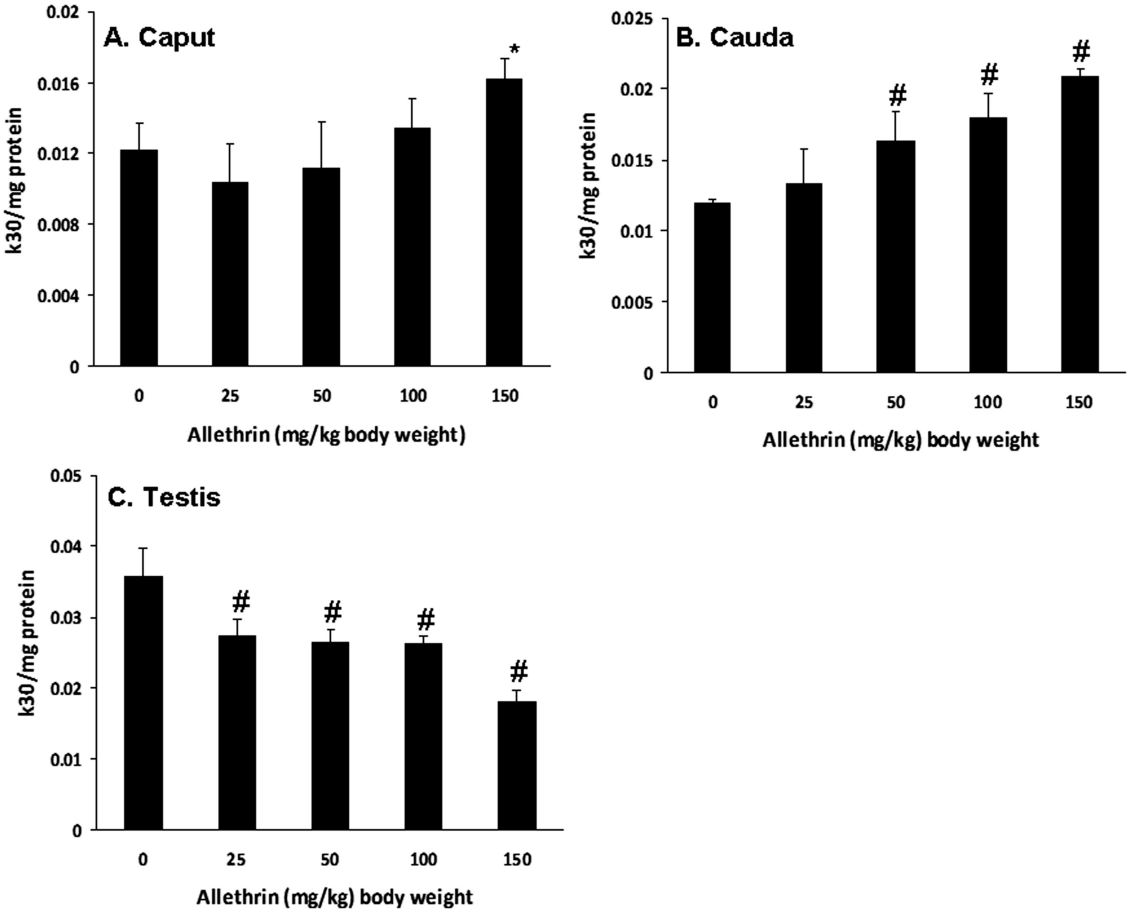

Fig. 4. Catalase activity in the male reproductive tract of allethrin treated rats. Male Wistar rats received 25–150 mg/kg body weight allethrin was administered orally for 60 days. Homogenates of reproductive tissues were prepared and catalase activity estimated. A: Caput; B: Cauda; C: Testis. Values shown are Mean ± S.D. * and # denotes $p < 0.01$ and 0.001, respectively.

obtained from control and allethrin treated rats. Increased levels of LPO products were observed in the caput, cauda, and testes of allethrin treated rats [Fig. 1(A–C); $p < 0.001$]. In the testes, the increase in LPO seems to dose dependent [Fig. 1(C)].

Nitric Oxide

NO plays an important role in the maintaining the oxidant and pro-oxidant mileu in the tissues. In the caput, increased levels of NO was observed at all the doses tested [Fig. 2(A)], when compared with the vehicle treated control ($p < 0.001$). However, no changes in the levels of NO were observed in the cauda [Fig. 2(B)] and testes [Fig. 2(C)] of allethrin treated rats.

NO being highly reactive, couples with free radicals to maintain the oxidant to antioxidant status in the tissues. Hence, calculating the ratio of NO and MDA (NO/MDA) provides information on the role of NO in preventing oxidative stress. In the caput, no significant changes were observed in the NO/MDA ratio [Fig. 3(A)], suggesting that the increased LPO (oxidative stress) is being quenched by NO. In the cauda and testes obtained from allethrin treated rats, the NO/MDA ratio was significantly lower ($p < 0.001$) when compared with the vehicle treated control [Fig. 3(B,C)], indicating loss of the protective role of NO in these tissues.

Antioxidant Enzymes

The defense against oxidative stress is provided by the antioxidant systems that primarily consist of the antioxidant enzymes. The activity of such enzymes namely, catalase, GPx, GST, and SOD were measured in the caput, cauda and testes. When compared with the vehicle treated control, catalase activity in the caput of allethrin treated rats remained largely unchanged except for a significant increase ($p < 0.01$) in the 150 mg/kg body weight treated animals [Fig. 4(A)]. Significant increase ($p < 0.001$) in catalase activity was observed in the cauda obtained from 50, 100, and 150 mg/kg body weight treated rats [Fig. 4(B)]. In contrast, there was a dose dependent decrease ($p < 0.001$) in catalase activity in the testes of allethrin treated rats [Fig. 4(C)].

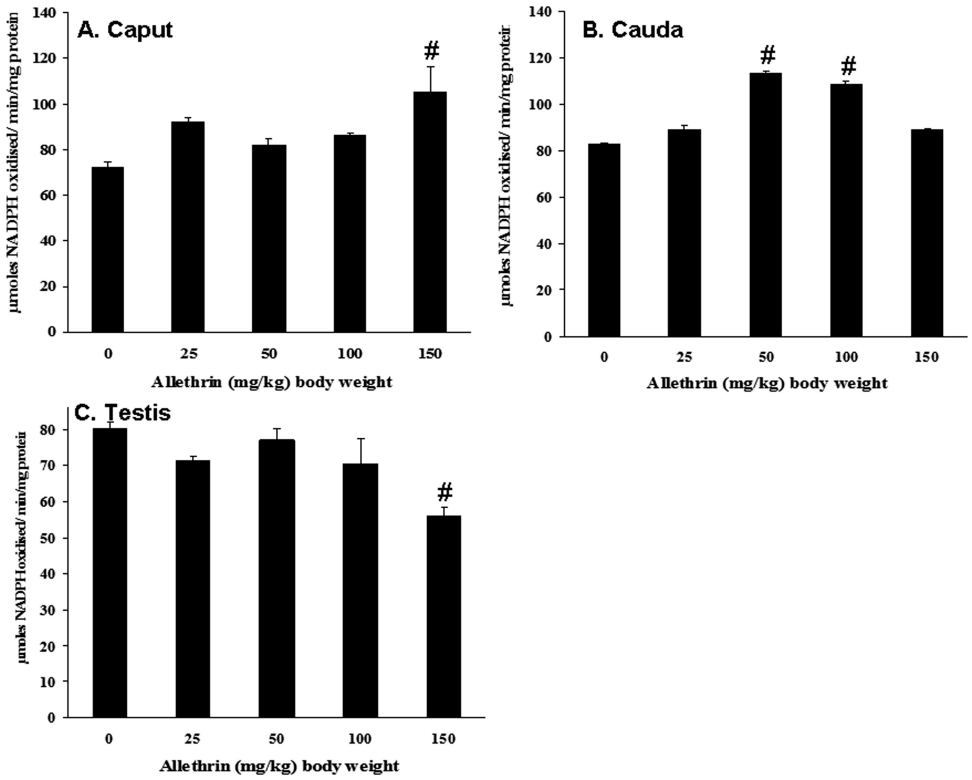

Fig. 5. GPx activity in the male reproductive tract of allethrin treated rats. Male Wistar rats received 25–150 mg/kg body weight allethrin orally for 60 days. Reproductive tract tissues were collected and homogenates prepared and GPx activity estimated. A: Caput; B: Cauda; C: Testis. Values shown are Mean ± S.D. * and # denotes $p < 0.01$ and 0.001, respectively.

GPx activity was significantly increased ($p < 0.001$) in the caput obtained from 150 mg/kg body weight allethrin treated rats [Fig. 5(A)]. In the cauda, it was found to be increased significantly ($p < 0.001$) in the 50 and 100 mg/kg allethrin treated groups [Fig. 5(B)]. In contrast, the activity of GPx activity was significantly decreased ($p < 0.001$) in the testes of rats treated with 150 mg/kg body weight allethrin [Fig. 5(C)].

GST activity was found to be increased significantly ($p < 0.001$) in a dose dependent manner in the caput and cauda of allethrin treated rats [Fig. 6(A,B)]. However, in the testes, a dose dependent decrease was observed [Fig. 6(C)], but this decrease was significant ($p < 0.001$) only in the testes of rats treated with the highest dose (150 mg/kg body weight).

In the caput, significant increase ($p < 0.001$) in the activity of SOD was observed in the 150 mg/kg body weight allethrin treated rats [Fig. 7(A)]. In the cauda and testes, allethrin treatment resulted in increase ($p < 0.001$) of SOD activity at all the doses tested [Fig. 7(B,C)].

Histopathological Analyses

The possible damages to the caput, cauda, and testes obtained in the allethrin treated rats was analyzed by studying the pathomorphological changes. The effects observed were assessed as histological scores (Table II). In the caput, presence of dead spermatozoa, damage to tubular architecture disarray, congestion (accumulation of blood or other fluid in the body part), and degeneration of epithelial cell lining were observed (Fig. 8). The damages observed seemed to be dose dependent with the maximum effects seen in the highest dose (150 mg/kg body weight) group. In the cauda, similar pathomorphological changes were observed and seem to be dose dependent (Fig. 9). In the testis, intestinal edema, tubule architecture disarray, changes in nuclear integrity, presence of dead or degenerating spermatids, epithelial cell disruption, germ cell layer damage, and anomalies in lumen morphology were evident (Fig. 10); and the effects observed were dose dependent.

Gene Expression

Maintenance of fertility is one of the major functions of the male reproductive tract. This is accomplished by the constant production of spermatozoa in a two-stage process involving spermatogenesis and sperm maturation, which occur in the testis and epididymis respectively. Further, the ability of sperm to fertilize depends on factors that facilitate capacitation and recognition of proteins on the ovum. Since we observed morphological, biochemical, and molecular changes in the male reproductive tract due to allethrin exposure, it is possible that the expression pattern of spermatogenic factors (*Tgf-β1*), sperm maturation factors (*Spag11e*),

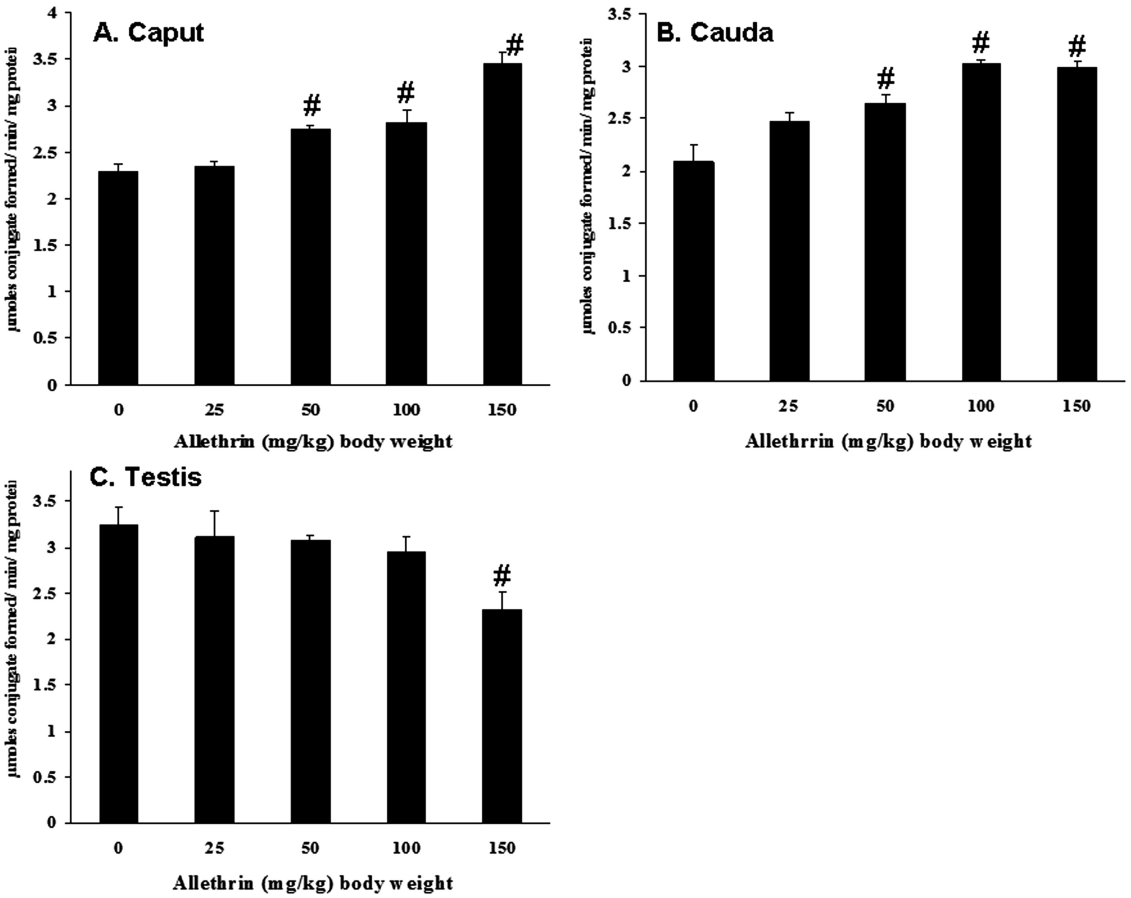

Fig. 6. GST activity in the male reproductive tract of allethrin treated rats. Male Wistar rats received 25–150 mg/kg body weight allethrin was administered orally for 60 days. Homogenates of reproductive tissues were prepared and GST activity estimated. A: Caput; B: Cauda; C: Testis. Values shown are Mean ± S.D. * and # denotes $p < 0.01$ and 0.001, respectively.

and capacitation factors (*Defb22*) could be compromised. To the best of our knowledge, there are no reports on the effect of allethrin on the molecular changes that can occur in the male reproductive tract and thereby affect germ cell production and function. *Tgf-β1* mRNA levels were reduced significantly ($p < 0.001$) in the testes of allethrin treated rats at all the doses tested [Fig. 11(A)]. Similarly a drastic reduction ($p < 0.001$) was observed in the mRNA expression of *Spag11e* and *Defb22* [Fig. 11(B,C)]. These results in combination with the histopathological analyses suggest allethrin exposure in rats causes abnormal sperm morphology and alterations in the factors that affect sperm production and function.

Increased LPO in allethrin treated rats indicates elevated oxidative stress, which leads to altered gene expression of the stress response genes. DNA damage is a hall mark of oxidative stress during which the cell initiates multiple repair mechanisms. A key regulator that determines the fate of the cell either to undergo apoptosis or cell cycle arrest or to be hyper proliferative (cancer initiation) is the *p53* gene. Since increased LPO was observed in all the tissues obtained from allethrin treated rats, changes in the expression pattern of *p53* were analyzed using real time PCR. The expression of *p53* gene was significantly reduced ($p < 0.001$) in the epididymidis and testes obtained from all the treatment groups (Fig. 12), suggesting that increased oxidative stress leads to suppression of *p53* mRNA expression, thereby allowing disrupting cell cycle regulation.

MAPK Phosphorylation

Many cellular processes depend on the phosphorylation of a wide variety of proteins including MAPKs, whose role in cellular motility, proliferation, differentiation, and death is well documented. P44/42 signaling pathway is activated by external stimuli are considered play an important role in initiation of cancer (Roberts and Der, 2007). To determine whether long term allethrin exposure could affect MAPK pathway, the levels of phospho p44/p42 was analyzed in the epididymis and testis. We observed increased phosphorylation of p44/p42 in the epididymis and testis (Fig. 13) obtained from rats exposed to varying doses of allethrin, when compared with untreated control, suggesting that allethrin could affect cell proliferation.

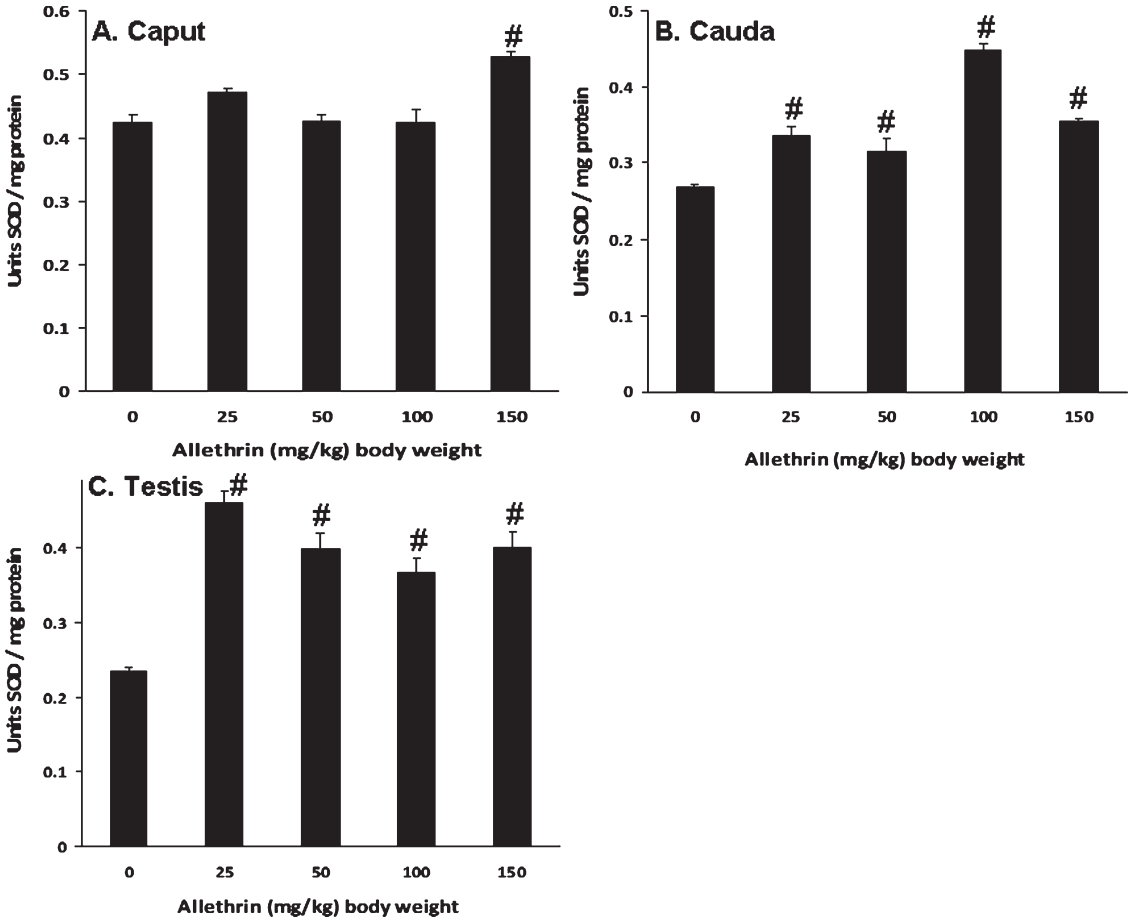

Fig. 7. SOD activity in the male reproductive tract of allethrin treated rats. Male Wistar rats received 25–150 mg/kg body weight allethrin orally for 60 days. Reproductive tract tissues were collected and homogenates prepared and SOD activity estimated. A: Caput; B: Cauda; C: Testis. Values shown are Mean ± S.D. * and # denotes $p < 0.01$ and 0.001, respectively.

DISCUSSION

The use of pesticides for pest management has been on the rise over the decade. However, comprehensive analyses on the effect of pyrethroid based pesticides including allethrin on the male reproductive tract have received little attention.

Environmental contaminants contribute to oxidative stress and initiates of a variety of pathways leading to pathological conditions (Reuter et al., 2010). In this study, we observed increased LPO in the caput, corpus, and cauda of allethrin treated rats (Fig. 1). Earlier studies have demonstrated that exposure to pesticides caused an increase in LPO in the brain, liver, and kidney (Kale et al., 1999; Giray et al., 2001; Vontas et al., 2001; Gabbianelli et al., 2002). Similarly, LPO was observed in the male reproductive tract of rats exposed to pyrethroid compounds (Issam et al., 2009; Wang et al., 2009). The increased LPO observed in our study could be due to generation of free radicals by allethrin in the organ system. Increased LPO generates signals both at cellular and organ level to initiate defense mechanisms. NO is a free radical scavenger and is considered to play an antioxidant role during oxidative stress. We observed that NO levels were increased in the caput and unaltered in the cauda and testes of rats orally treated with allethrin (Fig. 2), suggesting tissue specific responses. Alterations in the levels of NO were also observed in the testes of cypermethrin treated rats (Wang et al., 2009). Further, it was demonstrated that pyrethrin based pesticides alter the levels of NO in the serum of mice (Kanbur et al., 2008). We also observe that NO/MDA levels were unaltered in the caput, whereas it was lower in the cauda and testes of allethrin treated rats. NO seems to be an important free radical scavenger in the caput and not in the cauda and testes during allethrin induced toxicity.

Pyrethroid toxicity results in oxidative stress, thereby leading to alteration in the antioxidant status. For example, oral administration of cypermethrin increased the hepatic activities of SOD, GPx, and catalase (Jin et al., 2011). In contrast, a significant decline in the activities of antioxidant enzymes were observed in mice exposed to cypermethrin

TABLE II. Histological scores of caput, cauda and testis obtained from allethrin treated rats

Sl. No	Lesion	25 mg	50 mg	100 mg	150 mg
Caput epididymides					
1	Dead/degenerating spermatozoa	+	++	++	+++
2	Tubular architecture disarray	+	++	+++	++++
3	Congestion[a]	+	++	++	+++
4	Epithelial lining cell degeneration	+	+	++	++
Cauda epididymides					
1	Dead/degenerating spermatozoa	+	++	++	++
2	Tubular architecture disarray	+	+	++	+++
3	Congestion[a]	+	+	+	+
4	Epithelial lining cell degeneration	++	++	+++	+++
Testes					
1	Interstitial edema	+++	++++	++++	++++
2	Tubule architecture disarray	+	++	+++	++++
3	Nuclear integrity damage	+	++	+++	++++
4	Dead/degenerating spermatids	++	++	+++	++++
5	Epithelial cell disruption	+	+	++	++
6	Germ cell layer damage	+	++	+++	++++
7	Lumen morphology anomaly	++	+++	+++	++++

On a score of 5 points: +++++: maximum damage; ++++: high damage; +++: Moderately damage; ++: Low/moderate; +: Low; ±: more than zero, less than one; −: No lesions.

[a]Congestion: Excessive accumulation of blood or other fluid in a body part.

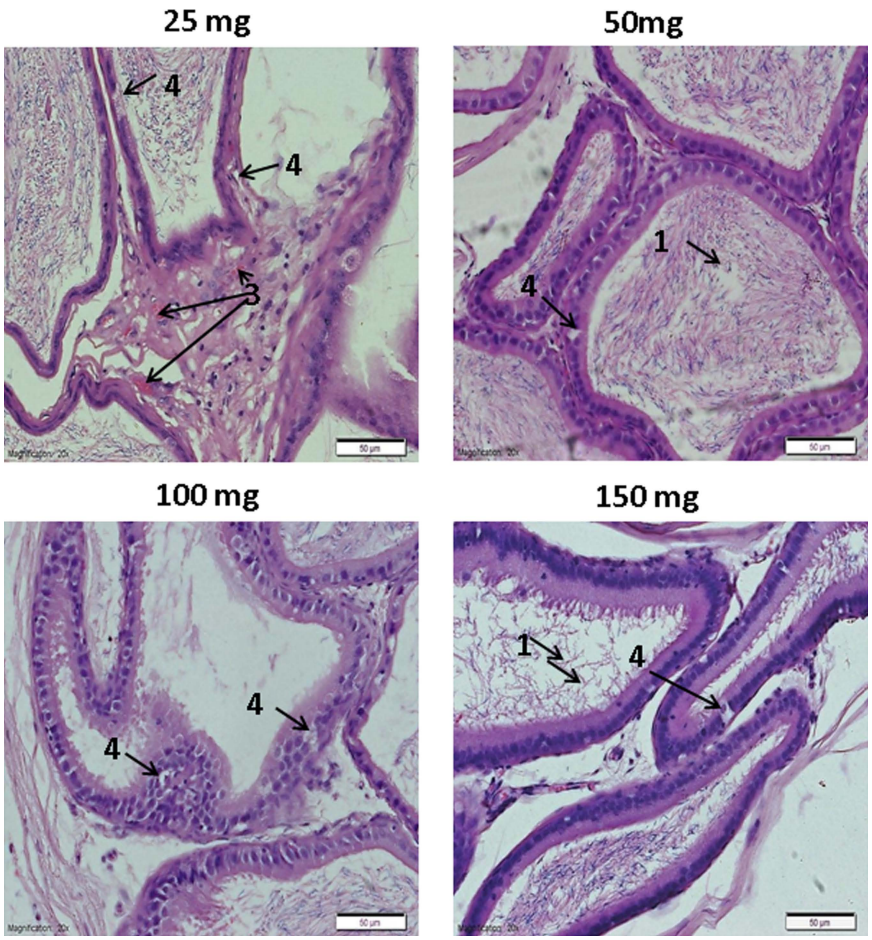

Fig. 8. Histopathology of caput in the allethrin treated rats. A: 25 mg; B: 50 mg; C: 100 mg; D: 150 mg. Arrows indicate site of lesion. Number indicates the type of lesion: (1) dead/degenerating spermatozoa; (2) tubular architecture disarray; (3) congestion; (4) epithelial cell degeneration. Magnification: 40×. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

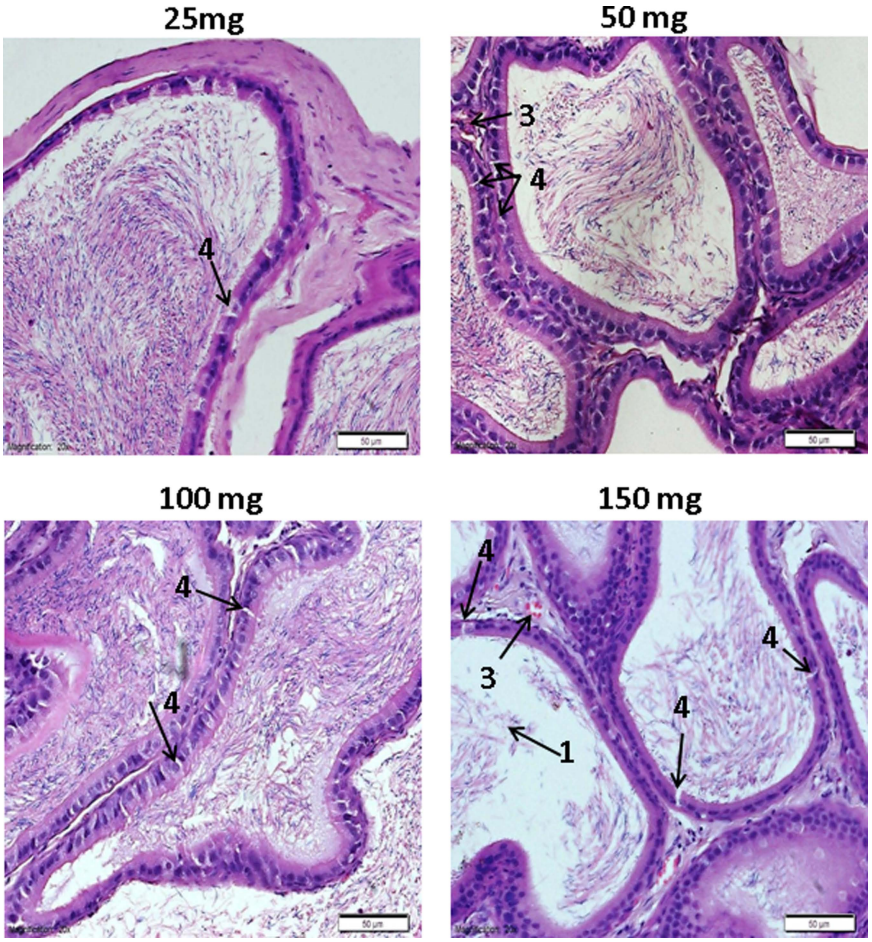

Fig. 9. Histopathology of cauda in the allethrin treated rats. A: 25 mg; B: 50 mg; C: 100 mg; D: 150 mg. Arrows indicate site of lesion. Number indicates the type of lesion: (1) dead/degenerating spermatozoa; (2) tubular architecture disarray; (3) congestion; (4) epithelial cell degeneration. Magnification: 40×. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Kanbur et al., 2008). However, the effect of allethrin oral treatment on the antioxidant status in the male reproductive tract is not reported until. We observed that the activities of catalase, GPx and glutathione-*s*-transferase were increased in the caput and cauda but were decreased in the testes. Caput and cauda (epididymis) initiate free radical scavenging activity in response to allethrin induced oxidative stress, where as the antioxidant mechanisms in the testes seem to have failed. These contrasting results suggest organ specific responses and the susceptibility of different organs to the same stress.

The pathomorphological changes that occur in the reproductive tract during pyrethroid exposure are underreported. We observed that allethrin exposure to rats caused damage to tubular architecture, congestion, degeneration of epithelial cell lining, and intestinal edema in the epididymis and testis. Further, dead or degenerating spermatids were observed. Pathological changes in different organ systems including the male reproductive tract due to pyrethroid toxicity are reported (Abdallah et al., 2010; Dahamna et al., 2010; Issam et al., 2011). Free radicals because of their reactive nature interact with membrane lipids and alter membrane integrity. In this study, the pathomorphological damages observed in the testis and epididymides and the presence of dead spermatozoa could be due to harmful effects of free radicals generated and alterations in the antioxidant mechanisms.

Spermatogenesis is a complex process that involves a number of genes that encode a variety of proteins, which include DNA binding factors (HSF-2, OVOL1, WT1, RHOX5, and SOX8), chromatin associated factors (JHDM2A, PYGO2, BRDT, and TAF4B) and RNA binding proteins (GRTH, SAM68, and MSY2; Bettegowda and Wilkinson, 2010). Besides these, other proteins that bind to the sperm surface such as TGF- β1, SPAG11E, and DEFB22 also play a crucial role in sperm production (Olaso et al., 1998; Zhou et al., 2004; Yudin et al., 2005; Zhao et al., 2011b).

Spermatogenesis and sperm function are known to be sensitive to environmental stress including exposure to pesticides (Pina-Guzman et al., 2009; Bal et al., 2011, b). Previous studies demonstrated that steroidogenesis, sperm

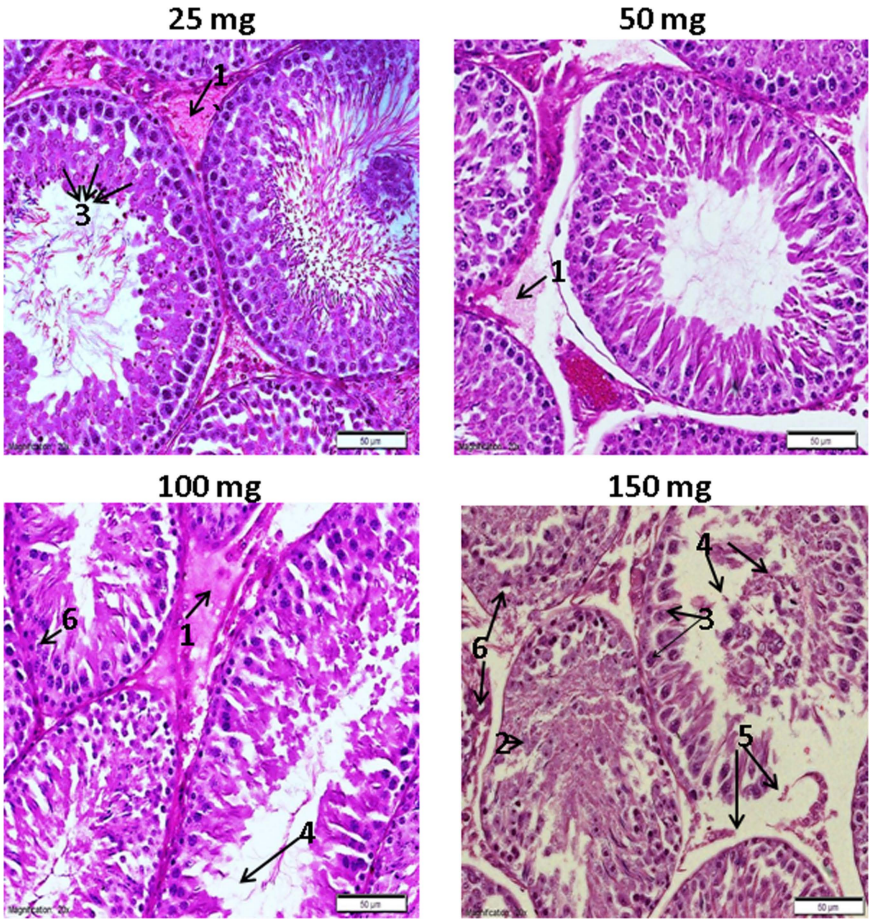

Fig. 10. Histopathology of testis in the allethrin treated rats. A: 25 mg; B: 50 mg; C: 100 mg; D: 150 mg. Arrows indicate site of lesion. Number indicates the type of lesion: (1) interstitial edema; (2) tubular architecture disarray; (3) nuclear integrity damage; (4) dead/degenerating spermatids; (5) epithelial cell disruption; (6) germ cell layer damage. Magnification: 40×. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

motility, and sperm production was decreased in rats exposed to a mixture of pesticides (Arena et al., 2008; Perobelli et al., 2010; Wang et al., 2010; Zhang et al., 2010). However, the effect of pyrethroid pesticides that affect factors that govern germ cell production are not reported yet. We observed a decrease in *Tgf-β1*, *Spag11e*, and *Defb22* (factors that influence germ cell production and maturation) expression was observed. This is in agreement with the previous observation that the pyrethroid bifenthrin disrupts *Tgf-β1* expression in rat ovarian granulose cells (Liu et al., 2011). Decreased gene expression observed in this study could be due to the ability of allethrin to influence gene transctiption machinery, which may involve complex signaling pathways. Fenvalerate induces germ cell apoptosis through the Fas/FasL pathway (Zhao et al., 2011a). It is also possible that other genes that are crucial for spermatogenesis may also be affected during allethrin toxicity. The mechanisms and the signaling pathways by which allethrin affects the expression of *Tgf-β1*, *Spag11e*, and *Defb22* needs further investigation.

The consequences of free radical generation at the cellular level involves shift in the dynamics of gene expression resulting in the induction of apoptosis or transformation of the proliferative properties of the cell to become cancerous. One of the vital factors that determine the fate of the cell under stress is the *p53* gene. Decreased *p53* expression results in genomic instability and reduced ability of tumor suppression (Han et al., 2008; Reuter et al., 2010). Alterations in the expression of apoptosis related genes in the testes were reported earlier (Hsu et al., 2007). We observed a reduced expression of *p53* in the epididymides and testes at all the administered doses of allethrin, indicating a possibility of tumor development in these organs. MAP kinases regulate cell proliferation in response to external stimuli including environmental toxicants (Roberts and Der, 2007). We observed increased phosphorylation of p44/42 MAPKs in the epididymis and testis obtained from allethrin treated rats. Earlier reports suggest that the pyrethroids affect MAPK phosphorylation (Liu et al., 2009; Qu et al., 2012). It is possible that the

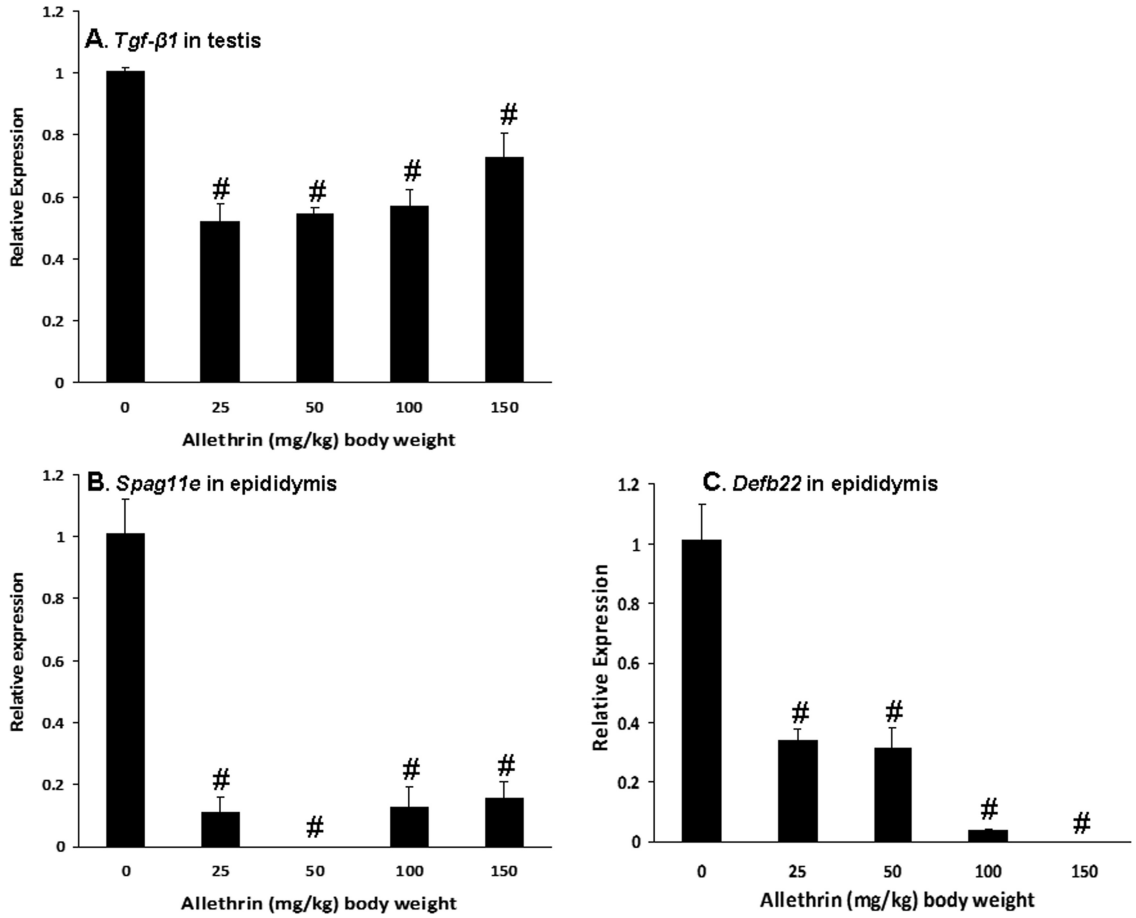

Fig. 11. mRNA expression of factors that influence sperm production and function in testes and epididymides of allethrin treated rats. A: *Tgf-β1*; B: *Spag11e*; C: *Defb22*. Values shown are Mean ± S.D. * and # denotes $p < 0.01$ and 0.001, respectively.

increased levels of p44/p42 observed in this study could contribute to increased cell proliferation, thereby initiation of cancer in the male reproductive tract.

Although previous studies demonstrated the toxic effects of allethrin inhalation due to use of mosquito coils and vaporizers, we for the first time report the effects due to oral

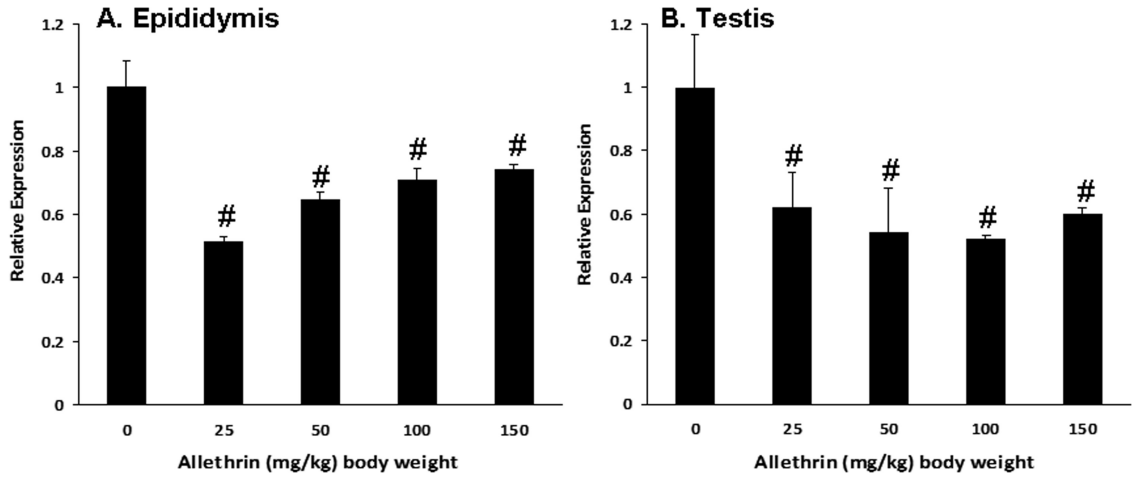

Fig. 12. mRNA expression of p53 in the epididymides and testes of allethrin treated rats. A: Epididymis; B: Testis. Values shown are Mean ± S.D. * and # denotes $p < 0.01$ and 0.001, respectively.

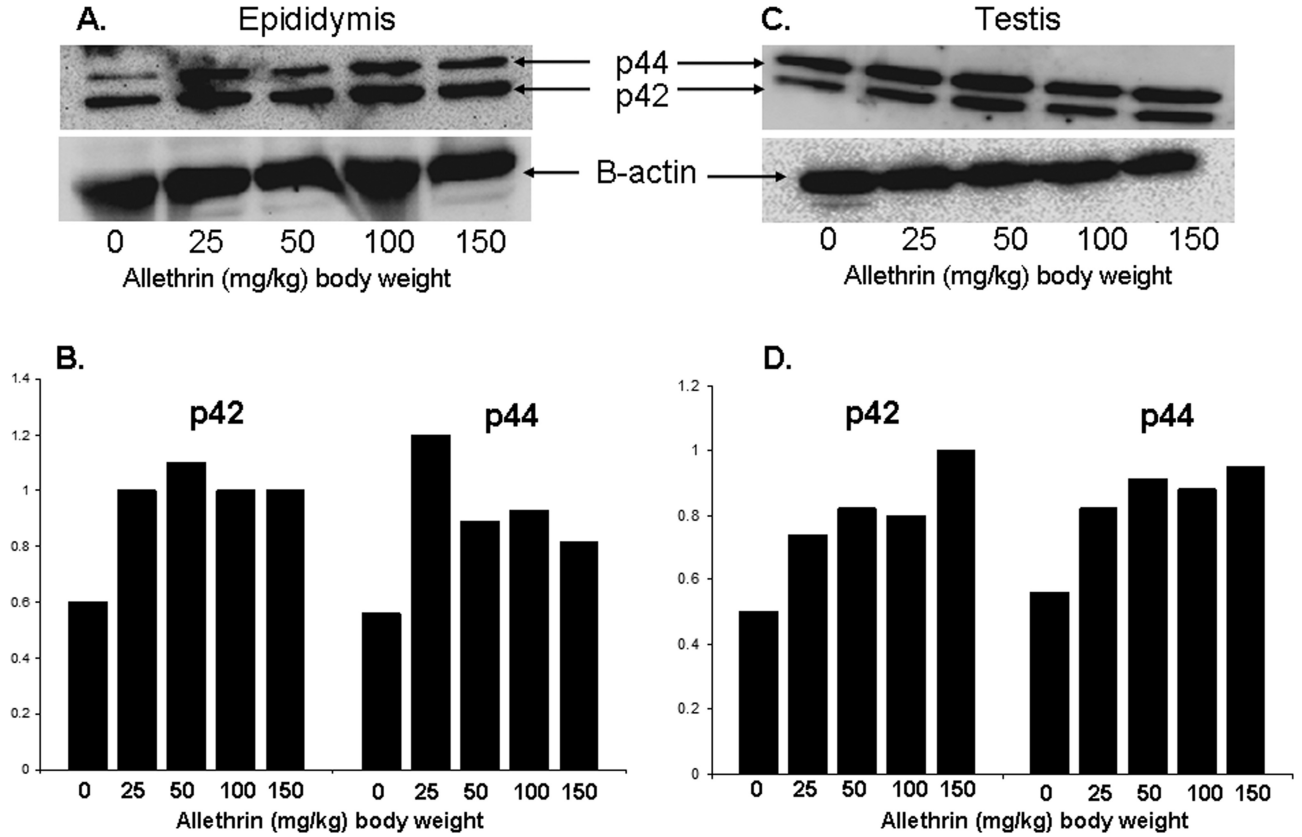

Fig. 13. Phosphorylation of MAPKs in the epididymis and testis obtained from allethrin treated rats. Western blot analyses for p44/42 in the epididymis (A) and testis (C). Densitometric analyses for phospho p44/p42 in the epididymis (B) and testis (D). Result shown is a representative of three independent experiments.

administration (Madhubabu and Yenugu, 2012; Vences-Mejia et al., 2012). We report that oral administration of allethrin to rats for 60 days results in increased LPO in the caput, cauda, and testes accompanied by an alteration in the antioxidant status. Allethrin treatment resulted in pathomorphological changes of the male reproductive tract tissues. The expression of *Tgf-β1*, *Spag11e*, and *Defb22* was significantly reduced thereby possibly affecting sperm production. Oxidative stress induced by allethrin reduced p53 gene expression. Results of this study support our hypothesis that increased oxidative stress induced by oral administration of allethrin affects fertility and may contribute to disregulated cell cycle in the male reproductive tract. The molecular mechanisms of pyrethroid toxicity involve modifying the kinetics of voltage-sensitive sodium channels of neurons and there seems to be a structure-activity relationship. We demonstrate that allethrin causes reproductive toxicity by altering the oxidant status and may influence sperm production. However, further studies are required to determine whether there is a correlation between the mechanisms of toxicity of allethrin in the reproductive and nervous systems.

The authors thank the facilities extended by UGC-SAP, UGC-CAS, DBT-CREBB, and FIST programs at School of Life Sciences, University of Hyderabad.

REFERENCES

Abdallah FB, Slima AB, Dammak I, Keskes-Ammar L, Mallek Z. 2010. Comparative effects of dimethoate and deltamethrin on reproductive system in male mice. Andrologia 42:182–186.

Aebi H. 1984. Catalase. Meth Enzymol 105:121–126.

Aly HA, Domenech O, Banjar ZM. 2012. Effect of nonylphenol on male reproduction: Analysis of rat epididymal biochemical markers and antioxidant defense enzymes. Toxicol Appl Pharmacol 261:134–141.

Arena AC, Fernandez CD, Porto EM, Bissacot DZ, Pereira OC, Kempinas WG. 2008. Fenvalerate, a pyrethroid insecticide, adversely affects sperm production and storage in male rats. J Toxicol Environ Health A 71:1550–1558.

Bal R, Naziroglu M, Turk G, Yilmaz O, Kuloglu T, Etem E, Baydas G. 2012a. Insecticide imidacloprid induces morphological and DNA damage through oxidative toxicity on the reproductive organs of developing male rats. Cell Biochem Funct 30:492–499.

Bal R, Turk G, Tuzcu M, Yilmaz O, Ozercan I, Kuloglu T, Gur S, Nedzvetsky VS, Tykhomyrov AA, Andrievsky GV, Baydas G, Naziroglu M. 2011. Protective effects of nanostructures of hydrated C(60) fullerene on reproductive function in streptozotocin-diabetic male rats. Toxicology 282:69–81.

Bal R, Turk G, Yilmaz O, Etem E, Kuloglu T, Baydas G, Naziroglu M. 2012b. Effects of clothianidin exposure on sperm quality, testicular apoptosis and fatty acid composition in developing male rats. Cell Biol Toxicol 28:187–200.

Bernheim F. 1964. The oxidative desulfuration of thio-acids. Biochimica et Biophysica Acta 90:426–428.

Bettegowda A, Wilkinson MF. 2010. Transcription and post-transcriptional regulation of spermatogenesis. Philos Trans R Soc Lond B Biol Sci 365:1637–1651.

Casida JE. 1995. Metabolism and synergism of Pyrethrins. In: Casida JE, Quistad GB, editors. Pyrethrum Flowers Production, Chemistry,Toxicology, and Uses. New York: Oxford University Press. p 258–276.

Dahamna S, Bencheikh F, Harzallah D, Boussahel S, Belgeit A, Merghem M, Bouriche H. 2010. Cypermetherin toxic effects on spermatogenesis and male mouse reproductive organs. Commun Agric Appl Biol Sci 75:209–216.

Eil C NB. 1990. The binding properties of pyrethroids to human skin fibroblast androgen receptors and to sex hormone binding globulin. J Steroid Biochem 35:409–414.

Gabbianelli R, Falcioni G, Nasuti C, Cantalamessa F. 2002. Cypermethrin-induced plasma membrane perturbation on erythrocytes from rats: reduction of fluidity in the hydrophobic core and in glutathione peroxidase activity. Toxicology 175:91–101.

Giray B, Gurbay A, Hincal F. 2001. Cypermethrin-induced oxidative stress in rat brain and liver is prevented by vitamin E or allopurinol. Toxicol Lett 118:139–146.

Han ES, Muller FL, Perez VI, Qi W, Liang H, Xi L, Fu C, Doyle E, Hickey M, Cornell J, Epstein CJ, Roberts JL, Remmen HV, Richardson A. 2008. The in vivo gene expression signature of oxidative stress. Physiol Genomics 34:112–126.

Hsu PC, Pan MH, Li LA, Chen CJ, Tsai SS, Guo YL. 2007. Exposure in utero to 2,2',3,3',4,6'-hexachlorobiphenyl (PCB 132) impairs sperm function and alters testicular apoptosis-related gene expression in rat offspring. Toxicol Appl Pharmacol 221:68–75.

Institoris L, Undeger U, Siroki O, Nehez M, Desi I. 1999. Comparison of detection sensitivity of immuno- and genotoxicological effects of subacute cypermethrin and permethrin exposure in rats. Toxicology 137:47–55.

Issam C, Samir H, Zohra H, Monia Z, Hassen BC. 2009. Toxic responses to deltamethrin (DM) low doses on gonads, sex hormones and lipoperoxidation in male rats following subcutaneous treatments. J Toxicol Sci 34:663–670.

Issam C, Zohra H, Monia Z, Hassen BC. 2011. Effects of dermal sub-chronic exposure of pubescent male rats to permethrin (PRMT) on the histological structures of genital tract, testosterone and lipoperoxidation. Exp Toxicol Pathol 63:393–400.

Jakoby WB. 1978. The glutathione-S-transferases: a group of detoxification proteins. In Meister A, editor. New York: Wiley. p 383–414.

Jin Y, Wang L, Ruan M, Liu J, Yang Y, Zhou C, Xu B, Fu Z. 2011. Cypermethrin exposure during puberty induces oxidative stress and endocrine disruption in male mice. Chemosphere 84:124–130.

Kakko I, Toimela T, Tahti H. 2003. The synaptosomal membrane bound ATPase as a target for the neurotoxic effects of pyrethroids, permethrin and cypermethrin. Chemosphere 51:475–480.

Kale M, Rathore N, John S, Bhatnagar D. 1999. Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: a possible involvement of reactive oxygen species. Toxicol Lett 105:197–205.

Kanbur M, Liman BC, Eraslan G, Altinordulu S. 2008. Effects of cypermethrin, propetamphos, and combination involving cypermethrin and propetamphos on lipid peroxidation in mice. Environ Toxicol 23:473–479.

Lepoivre M, Chenais B, Yapo A, Lemaire G, Thelander L, Tenu JP. 1990. Alterations of ribonucleotide reductase activity following induction of the nitrite-generating pathway in adenocarcinoma cells. J Biol Chem 265:14143–14149.

Li HY, Shi N, Chen D, Dai ZH, Lu WH, Wang B, Li YR. 2005. [Oxidative stress of deltamethrin on rat nervous system]. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi 23:97–101.

Liu H, Xu L, Zhao M, Liu W, Zhang C, Zhou S. 2009. Enantiomer-specific, bifenthrin-induced apoptosis mediated by MAPK signalling pathway in Hep G2 cells. Toxicology 261:119–125.

Liu J, Yang Y, Zhang Y, Liu W. 2011. Disrupting effects of bifenthrin on ovulatory gene expression and prostaglandin synthesis in rat ovarian granulosa cells. Toxicology 282:47–55.

Madhubabu G, Yenugu S. 2012. Effect of continuous inhalation of allethrin-based mosquito coil smoke in the male reproductive tract of rats. Inhal Toxicol 24:143–152.

McCord JM, Fridovich I. 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244:6049–6055.

Nasuti C, Gabbianelli R, Falcioni ML, Di Stefano A, Sozio P, Cantalamessa F. 2007. Dopaminergic system modulation, behavioral changes, and oxidative stress after neonatal administration of pyrethroids. Toxicology 229:194–205.

Naziroglu M, Butterworth PJ, Sonmez TT. 2011. Dietary vitamin C and E modulates antioxidant levels in blood, brain, liver, muscle, and testes in diabetic aged rats. Int J Vitam Nutr Res 81:347–357.

Olaso R, Pairault C, Boulogne B, Durand P, Habert R. 1998. Transforming growth factor beta1 and beta2 reduce the number of gonocytes by increasing apoptosis. Endocrinology 139:733–740.

Paglia DE, Valentine WN. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70:158–169.

Perobelli JE, Martinez MF, da Silva Franchi CA, Fernandez CDB, Viana de Camargo JJ, Kempinas DG. 2010. Decreased sperm motility in rats orally exposed to single or mixed pesticides. J Toxicol Environ Health 793:991–1002.

Pina-Guzman B, Sanchez-Gutierrez M, Marchetti F, Hernandez-Ochoa I, Solis-Heredia MJ, Quintanilla-Vega B. 2009. Methylparathion decreases sperm function and fertilization capacity after targeting spermatocytes and maturing spermatozoa. Toxicol Appl Pharmacol 238:141–149.

Pontecorvo G, Fantaccione S. 2006. Recombinogenic activity of 10 chemical compounds in male germ cells of Drosophila melanogaster. Ecotoxicol Environ Saf 65:93–101.

Qu JH, Fei J, Hong X, Chen JF, Gu AH, Sun H, Xu XL, Song L, Wang SL, Wang XR. 2012. Involvement of IGF-I signaling pathway in the regulation of steroidogenesis in mouse Leydig cells treated with fenvalerate. Toxicology 292:151–155.

Rao J, Herr JC, Reddi PP, Wolkowicz MJ, Bush LA, Sherman NE, Black M, Flickinger CJ. 2003. Cloning and characterization of a novel sperm-associated isoantigen (E-3) with defensin- and lectin-like motifs expressed in rat epididymis. Biol Reprod 68:290–301.

Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. 2010. Oxidative stress, inflammation, and cancer: How are they linked? Free Radic Biol Med 49:1603–1616.

Roberts PJ, Der CJ. 2007. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 26:3291–310.

Sattin RW RA, Kafrissen ME, Dugan JB, Farer LS. 1984. An epidemic of gynecomastia among illegal Haitian entrants. Public Health Rep 99:504–510.

Shukla Y, Yadav A, Arora A. 2002. Carcinogenic and cocarcinogenic potential of cypermethrin on mouse skin. Cancer Lett 182:33–41.

Shum WW, Da Silva N, Brown D, Breton S. 2009. Regulation of luminal acidification in the male reproductive tract via cell-cell crosstalk. J Exp Biol 212(Pt 11):1753–1761.

Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, Stevens JT, Weiner ML. 2002. Mechanisms of pyrethroid neurotoxicity: Implications for cumulative risk assessment. Toxicology 171:3–59.

Srivastava A, Srivastava MK, Raizada RB. 2006. Ninety-day toxicity and one-generation reproduction study in rats exposed to allethrin-based liquid mosquito repellent. J Toxicol Sci 31:1–7.

Tan J, Soderlund DM. 2010. Divergent actions of the pyrethroid insecticides S-bioallethrin, tefluthrin, and deltamethrin on rat Na(v)1.6 sodium channels. Toxicol Appl Pharmacol 247:229–237.

Tollner TL, Yudin AI, Tarantal AF, Treece CA, Overstreet JW, Cherr GN. 2008. Beta-defensin 126 on the surface of macaque sperm mediates attachment of sperm to oviductal epithelia. Biol Reprod 78:400–412.

Tomlin C. 1994. A world compendium: The pesticide manual. British Crop Protection Council, United Kingdom.

Vences-Mejia A, Gomez-Garduno J, Caballero-Ortega H, Dorado-Gonzalez V, Nosti-Palacios R, Labra-Ruiz N, Espinosa-Aguirre JJ. 2012. Effect of mosquito mats (pyrethroid-based) vapor inhalation on rat brain cytochrome P450s. Toxicol Mech Methods 22:41–46.

Vontas JG, Small GJ, Hemingway J. 2001. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens. Biochem J 357(Pt 1):65–72.

Wang H, Wang Q, Zhao XF, Liu P, Meng XH, Yu T, Ji YL, Zhang H, Zhang C, Zhang Y, Xu DX. 2010. Cypermethrin exposure during puberty disrupts testosterone synthesis via down-regulating StAR in mouse testes. Arch Toxicol 84:53–61.

Wang XZ, Liu SS, Sun Y, Wu JY, Zhou YL, Zhang JH. 2009. Beta-cypermethrin impairs reproductive function in male mice by inducing oxidative stress. Theriogenology 72:599–611.

Yokohira M, Arnold LL, Lautraite S, Sheets L, Wason S, Stahl B, Eigenberg D, Pennington KL, Kakiuchi-Kiyota S, Cohen SM. 2011. The effects of oral treatment with transfluthrin on the urothelium of rats and its metabolite, tetrafluorobenzoic acid on urothelial cells in vitro. Food Chem Toxicol 49:1215–1223.

Yudin AI, Tollner TL, Li MW, Treece CA, Overstreet JW, Cherr GN. 2003. ESP13.2, a member of the beta-defensin family, is a macaque sperm surface-coating protein involved in the capacitation process. Biol Reprod 69:1118–1128.

Yudin AI, Generao SE, Tollner TL, Treece CA, Overstreet JW, Cherr GN. 2005. Beta-defensin 126 on the cell surface protects sperm from immunorecognition and binding of anti-sperm antibodies. Biol Reprod 73:1243–1252.

Zanich A, Pascall JC, Jones R. 2003. Secreted epididymal glycoprotein 2D6 that binds to the sperm's plasma membrane is a member of the beta-defensin superfamily of pore-forming glycopeptides. Biol Reprod 69:1831–1842.

Zhang H, Wang H, Wang Q, Zhao XF, Liu P, Ji YL, Ning H, Yu T, Zhang C, Zhang Y, Meng XH, Xu DX. 2010. Pubertal and early adult exposure to fenvalerate disrupts steroidogenesis and spermatogenesis in mice at adulthood. J Appl Toxicol 30:369–377.

Zhao XF, Wang Q, Ji YL, Wang H, Liu P, Zhang C, Zhang Y, Xu DX. 2011a. Fenvalerate induces germ cell apoptosis in mouse testes through the Fas/FasL signaling pathway. Arch Toxicol 85:1101–1108.

Zhao Y, Diao H, Ni Z, Hu S, Yu H, Zhang Y. 2011b. The epididymis-specific antimicrobial peptide beta-defensin 15 is required for sperm motility and male fertility in the rat (Rattus norvegicus). Cell Mol Life Sci 68:697–708.

Zhou CX, Zhang YL, Xiao L, Zheng M, Leung KM, Chan MY, Lo PS, Tsang LL, Wong HY, Ho LS, Chung YW, Chan HC. 2004. An epididymis-specific beta-defensin is important for the initiation of sperm maturation. Nat Cell Biol 6:458–464.

Toxicology in Vitro 28 (2014) 1386–1395

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Allethrin induces oxidative stress, apoptosis and calcium release in rat testicular carcinoma cells (LC540)

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ARTICLE INFO

Article history:
Received 16 March 2014
Accepted 19 July 2014
Available online 27 July 2014

Keywords:
Allethrin
Testis
Oxidative stress
Apoptosis
Calcium
Spermatogenesis

ABSTRACT

Over the years, pyrethroids, including D-allethrin, are widely used for domestic and agricultural purposes and are found to be toxic to many organ systems including the male reproductive system. However, the molecular mechanisms of allethrin toxicity are not well understood. In this study, we demonstrate that allethrin exhibited a dose-dependent cytotoxicity on Leydig cell carcinoma cells (LC540) and isolated primary Leydig cells with an IC_{50} of 125 μM and 59 μM respectively. Cytotoxicity was associated with generation of reactive oxygen species, increased lipid peroxidation and alterations in antioxidant enzyme status. Morphological analyses of LC540 cells treated with allethrin revealed the presence of apoptotic bodies. Using flow cytometry, we observed that the number of cells that displayed early apoptotic features and entering into G_0 phase of cell cycle increased significantly with loss of mitochondrial membrane potential. The levels of *p53* mRNA and cleaved PARP-1 protein were increased, whereas BCL-2, pro-Caspase-3 and PARP-1 were decreased. Allethrin induced apoptosis was associated with voltage gated calcium channel mediated intracellular calcium release. Results of our study demonstrate that allethrin toxicity in the male reproductive tract may involve Leydig cell apoptotic death.

1. Introduction

Among a variety of insecticides used for agricultural and domestic purposes, pyrethroid based are the most common. Pyrethroids are mainly classified as type I and type II depending on the presence of a cyano group. They are known to cause insecticidal effects and the mechanism involves prolonged activation of voltage-gated sodium channels (VGSC) and delayed channel activation in neuronal cells (Narahashi, 1986; Ray and Fry, 2006; Neal et al., 2010). Symptoms of pyrethroid poisoning in insects range from body tremors to convulsions resulting in death. Besides their neurotoxic effects, they are known to be carcinogenic, hepatotoxic, immunosuppressive, estrogenic and antiprogestagenic in a variety of species (Yokohira et al., 2011; Institoris et al., 1999; Nasuti et al., 2007; Shukla et al., 2002).

The type I pyrethroid, D-allethrin (mixture of eight stereoisomers) is one of the preferred household pest control insecticide throughout the world. It is used in mosquito repellants (mats, coils and vaporizers) and human exposure occurs due to inhalation of allethrin emitted along with the smoke. In many animals and humans, it is moderately toxic and primarily acts on the nervous system (Tomlin, 1994). Though pyrethroids are used at low concentrations in domestic set ups, prolonged or recurrent use at these levels is known to cause locomotory defects in rats (Abou-Donia et al., 2001). We previously demonstrated alterations in antioxidant enzyme profile and reproductive parameters in male rats exposed to allethrin nasally or orally (Madhubabu and Yenugu, 2012, 2013). On the contrary, the clinical enzyme profile and reproductive indices in rats nasally exposed to allethrin were found to be unaltered (Srivastava et al., 2006). Further, pyrethroids are known to contribute to recombinogenic activity in the male germ cells of *Drosophila melanogaster* (Pontecorvo and Fantaccione, 2006), estrogenic and antiprogestagenic activity in endometrial and breast cancer cells respectively (Go et al., 1999; Garey and Wolff, 1998; Kasat et al., 2002), gynecomastia (Eil, 1990; Sattin et al., 1984) and oxidative stress in the male reproductive system (Madhubabu and Yenugu, 2012, 2013; Issam et al., 2011, 2009; Wang et al., 2009).

Pyrethroids are actively metabolized *in vivo* to generate free radicals that can damage the integrity of cellular membranes and initiate a variety of events leading to cell death. Free radical generation during pyrethroid metabolism causes oxidative stress (Romero et al., 2012; Banerjee et al., 2001; Giray et al., 2001; El-Demerdash, 2007; Sadowska-Woda et al., 2010) and alter antioxidant status in different organ systems of rats (Giray et al., 2001;

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http://dx.doi.org/10.1016/j.tiv.2014.07.008
0887-2333/

Kale et al., 1999; Maiti et al., 1995; Li et al., 2005). Further, studies including ours demonstrated that pyrethroid compounds cause oxidative stress in the male reproductive system and disturbs the antioxidant status (Madhubabu and Yenugu, 2012, 2013; Issam et al., 2011, 2009; Wang et al., 2009). It is well established that sperm production is seriously compromised due to oxidative stress (Naziroglu et al., 2011; Bal et al., 2012). Free radical generation during oxidative stress promotes apoptosis; affected by a variety of signaling events that involve alteration in the mitochondrial membrane potential, release of cytochrome C from mitochondria and changes in the levels of apoptotic effectors such as p53, BCL-2, PARP-1 and Caspase-3 (Booth et al., 2013). Though *in vivo* studies have demonstrated the ability of pyrethroids to induce oxidative stress, alter antioxidant enzyme activities and loss of germ cell production, limited information is available on the molecular mechanisms of pyrethroid toxicity (Romero et al., 2012; Chi et al., 2014; Yokohira et al., 2011). Further, the molecular mechanism of allethrin induced cell death has not been reported till date. Hence, in this study, we attempt to decipher the toxic actions of allethrin at the cellular and molecular level with emphasis on the role of apoptotic factors.

Intracellular calcium plays a key role in factor in many cellular functions including apoptosis (Bootman et al., 2002). Alterations in intracellular calcium concentrations are due to release of calcium by store operated calcium entry from endoplasmic reticulum stores or by influx through the plasma membrane. Loss of calcium from endoplasmic reticulum leads to apoptotic cell death (Yoshida et al., 2006). Similarly, high calcium influx also favors release of apoptotic factors resulting in cell death (Paschen, 2000). It is reported that oxidative stress caused by toxic chemicals including pyrethroids induces apoptosis that is associated with calcium dynamics. Deltamethrin causes apoptosis in human oral cancer cells by altering intracellular calcium levels (Chi et al., 2014). However, the role of calcium in allethrin induced cytotoxicity is not reported. Voltage gated calcium channels (VGCCs) are multimeric proteins with a common pore forming motif and are involved in a variety of physiological events. The ten members of the VGCC family are divided into L-, N-, P/Q-, R- and T-types depending on the calcium current. Pyrethroids interact with VGCCs to facilitate neurotransmitter release and increased miniature excitatory post-synaptic current (Clark and Symington, 2008; Meyer and Shafer, 2006) It is demonstrated that allethrin modulates VGCCs in rat PC12 cells (Neal et al., 2010). The involvement of VGCCs in allethrin induced apoptosis remains to be investigated.

Though the effects of allethrin at the organ level are documented, the mechanism of its toxicity at the cellular levels remains largely unknown. The primary goal of this study is to understand the mechanism of allethrin toxicity in cells related to the male reproductive system. Analyzing the cellular toxicity of allethrin at the testicular level requires treatment of animals with the compound and the isolation of the specific cell type (Leydig or Sertoli). The yields of these cells isolated from testis are low and non-homogenous and makes it difficult to analyze a variety of parameters. To circumvent this, as an initial step, the mechanisms of allethrin induced cell death were analyzed using rat Leydig cell tumor cell line (LC540 cells). In this study, we evaluated the cytotoxicity of allethrin in rat Leydig carcinoma cells with emphasis on oxidant-antioxidant dynamics, molecular events of apoptotic cell death and the expression of factors that govern germ cell production. We observed that allethrin is cytotoxic to Leydig cells and initiates a cascade of events that involves generation of reactive oxygen species (ROS), lipid peroxidation and altered antioxidant enzyme profile. Mitochondrial membrane potential dependent apoptotic cell death mediated by BCL-2, PARP-1, Caspase-3 and *p53* was evident. Allethrin induced apoptotic cell death seems to involve VGCC mediated release of intracellular calcium.

2. Materials and methods

2.1. Cell culture and treatments

LC540 cells (derived from rat Leydig cell tumor) obtained from National Centre for Cell Science, Pune, India, were used in this study. They were cultured in Eagle's Minimal Essential Medium (MEM) containing 5% newborn calf serum, 1.2 g/L $NaHCO_3$ and antibiotic-antimycotic solution (10,000 units of Penicillin, 10 mg Streptomycin and 25 μg amphotericin/mL) in a 5% humidified incubator at 37 °C. Cells were treated with varying concentrations of allethrin (Sigma Aldrich, USA) dissolved in DMSO for different time periods. To test the effect of the solvent in which allethrin is dissolved, cells were also treated with DMSO equivalent to the amount present in the allethrin added to cells.

2.2. Isolation of primary Leydig cells

Leydig cells were isolated as described earlier (Abarikwu et al., 2011). We used 27–30 day old rats since the yield of Leydig cells were higher in these animals when compared to adult rats. Briefly, testes dissected out were decapsulated and subjected to enzymatic digestion in HBSS containing 0.25 mg/mL collagenase type IV at 37 °C in a shaking water bath (80 cycles/min) for 45 min. Following digestion, the debris in the sample was allowed to settle and the supernatant was aspirated carefully and filtered through a nylon mesh. The suspension was centrifuged at 800 g for 5 min and the pellet resuspended in DMEM-F12 and plated in culture flasks or plates. Cell count was determined by a hemocytometer and the viability by Trypan blue staning. The purity of the cells obtained was assessed by 3β-hydroxysteroid dehydrogenase (3β-HSD) staining (Aldred and Cooke, 1983) and was found to be more than 95%.

2.3. Measurement of cytotoxicity

MTT assay was used to assess the cytotoxicity of allethrin (Mosmann, 1983). In brief, LC540 cells or primary Leydig cells (0.5×10^6 cells/well) were seeded into 24 well microtiter plates and kept in CO_2 incubator for 18–24 h to adhere. The medium was replaced with fresh medium containing varying different concentrations (0.001–250 μM) of allethrin. The plates were then incubated for 24 h in a humidified CO_2 incubator. At the end of treatment, fresh medium containing MTT (40 μl/mL of 5 mg/mL) was added and incubated for 3 h. The medium was removed and 500 μl of 40 mM HCl/Isopropanol was added to each well to stop the MTT reduction and to dissolve the formazan crystals. The contents were collected and absorbance was measured at 570 nm in a spectrophotometer. The IC_{50} value for LC540 cells was found to be 125 μM and further experiments were carried out with the same dose.

2.4. Estimation of reactive oxygen species (ROS)

The ability of allethrin to generate ROS in testicular cells was estimated as described earlier (Eruslanov and Kusmartsev, 2010). Briefly, 2×10^6 cells after treatment with 125 μM allethrin for 0–5 h were incubated for 15 min with 10 μM 2,7-dichloro dihydro fluorescein diacetate (DCFH-DA), a non-fluorescent cell-permeable dye. DCFH-DA upon oxidation by ROS generated in the cell is converted to 2,7-dichloro-fluorescein, which exhibits fluorescence at 525 nm when excited with light of 488 nm. Cells were then collected, washed with PBS and subjected to FACS analysis using a FACS Calibur flow cytometer (BD Biosciences) with detection settings of FITC range.

2.5. Estimation of lipid peroxidation (LPO) products

Thiobarbutitic acid (TBA) test was used to estimate lipid peroxidation as described earlier (Bernheim, 1964). 6×10^6 cells were treated with 125 μM allethrin for 0–48 h and to the collected cells, 1 mL of 10% TCA was added to precipitate the protein. After centrifugation at 3000 rpm for 10 min, to the supernatant, 0.25 mL of 0.33% TBA was added and incubated at 95 °C for 1 h. Absorbance of the pink color formed was measured at 532 nm using a spectrophotometer. 1,1,3,3-tetraethoxy-propane (TEP) was used as the standard. Levels of LPO were expressed as nano moles TBARS formed/mg protein.

2.6. Enzyme assays

6×10^6 cells plated in 100 mm cell culture plate were treated with 125 μM allethrin for 0–48 h were collected and sonicated in phosphate buffer saline, pH 7.4. Catalase, superoxide dismutase, glutathione-S-transferase (GST) and glutathione peroxidase (GPx) activities were determined by using standard protocols described earlier (Aebi, 1984; Jakoby, 1978; Paglia and Valentine, 1967; McCord and Fridovich, 1969). Solvent controls were maintained for each time point tested. Enzyme activity of the control at each time point is taken as 100% and the activity in allethrin treated cells is expressed as percent of the respective control (without allethrin) at each time point.

2.7. Cell cycle analyses

Cells were seeded at a density of 2×10^6 per well in 6-well plates and treated with 125 μM allethrin for 24 h. Following treatment, cells were fixed in 70% ice cold ethanol, washed with PBS, incubated with 0.1 mg/mL RNase A and stained with propidium iodide (final concentration 50 mg/mL) for DNA content analysis using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) (Hadnagy et al., 1999).

2.8. Measurement of mitochondrial membrane potential

LC540 cells (2×10^6) were plated in a 60 mm cell culture plate and treated with 125 μM allethrin for 24 h. To measure mitochondrial membrane potential, the cells were incubated with rhodamine (10 μg/mL) for 20 min followed by measuring fluorescence intensity in a FACS Calibur flow cytometer (Scaduto and Grotyohann, 1999). As a positive control, 10 μM doxorubicin was also used. Data were collected using the Cell Quest software (Becton Dickinson, San Jose, CA, USA) using a FL-1 detector.

2.9. Detection of early apoptosis

The early apoptotic events initiated by allethrin treatment was detected using Annexin-V staining kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Following treatment with 125 μM allethrin for 24 h, 2×10^6 cells were collected and washed with PBS. The cells were then resuspended in 300 μl of 1X binding buffer and 5 μl of annexin V and incubated in dark for 15 min. The suspension was centrifuged at 4000 rpm for 4 min and washed with 200 μl of 1X binding buffer. The samples resuspended in 195 μl of binding buffer and 10 μl of propidium iodide were analyzed on FACS Calibur flow cytometer (BD Biosciences) to detect cells displaying features of early apoptosis.

2.10. Measurement of intracellular calcium release

The effect of allethrin on the dynamics of intracellular calcium was measured as described earlier (Chi et al., 2014). 3×10^6 cells suspended in PBS were incubated with 2 μg/mL FURA 2-AM (Molecular Probes, Eugene, USA) for 45 min in dark. After incubation, the cells were washed and resuspended in 2.5 mL of PBS and the fluorescence intensity measured using a multidimensional fluorescence spectrophotometer (ISS VINCI, Champaign, IL, USA). Fluorescence measurements were made in slow kinetics mode and data acquired as ratio metric measurement since FURA has dual excitation wavelengths at 340 and 380 nm with a single emission at 505 nm. Fluorescence intensity at 340 and 380 nm indicate the amount of calcium bound FURA and unbound FURA respectively. Baseline readings were obtained for 130 sec, after which the cells were treated with varying concentrations of allethrin. As a positive control, 10 μM H_2O_2 was used in the assays. Data were recorded until there was no appreciable change in fluorescence intensity. To test the validity of the assay, readings were obtained by adding 20 μl of Triton X-100 (to release the intracellular calcium) were followed by 50 μl 0.5 M EGTA (to chelate calcium). To determine whether the changes in calcium concentration during allethrin toxicity involves VGCCs, LC540 cells were pre treated with 5 and 10 μM Nimodipine (NME; L-type VGCC blocker) or Ruthenium Red (RTR; P/Q type VGCC blocker), prior to allethrin treatment.

2.11. Real time polymerase chain reaction (RT-PCR)

LC540 cells (2×10^6 in a 60 mm culture plate) after treatment with allethrin were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA extracted. 2 μg of total RNA was reverse transcribed using 50 U Stratascript (Invitrogen, USA) and 0.5 μg of oligodT (Invitrogen, Eugene, USA) to generate cDNA. 2 μl of cDNA was used for real time PCR analysis using gene specific primers and SYBR master mix kit (Applied Biosystems, Warrington, UK). As an internal control, the expression of Glyceraldehyde-6-phosphate dehydrogenase (*Gapdh*) expression was determined. Real-time PCR was performed under the following conditions: initial denaturation at 94 °C for 10 min; 40 cycles with 94 °C for 15 s and 60 °C for 1 min. To detect non-specific amplification, negative controls (no template control and minus RT control (only with RNA) were included in the assays. The amplicons were sequenced to confirm their identity.

2.12. Immunoblotting

Total protein was obtained from control and allethrin treated LC540 cells (6×10^6) by harvesting in radioimmuno precipitation assay buffer (25 mM Tris-HCl pH 7.6, containing 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) supplemented with proteinase cocktail inhibitor. The lysates were then centrifuged at 10,000 rpm and the concentration of the total protein in the supernatant was determined by Lowry's method using bovine serum albumin as a standard. Each protein sample (100 μg) was separated on a 10% SDS–PAGE and transferred on to a 0.2 μm pore size nitrocellulose membrane (HybondTM ECLTM, GE Healthcare, UK). The non-specific binding sites were blocked by incubating the membranes in 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% Tween (TBS-T) for 1 h at room temperature. The membranes were then incubated with specific antibodies (Cell Signalling Technology, Danvers, USA) directed against rat BCL-2, pro-Caspase 3 and PARP-1 for 3 h at room temperature. The membrane was then washed and incubated with secondary antibody against rabbit IgG conjugated with horse radish peroxidase (Cell Signaling Technology). Immunoreactive proteins were detected using an ECL prime Western blot detection Kit (GE Healthcare life sciences, MA, USA), according to the manufacturer's instructions.

2.13. Statistical analyses

Changes in the parameters analyzed in this study were compared with the DMSO control. Statistical analyses were performed using one way ANOVA and Holm-Sidak test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are Mean ± S.D. a, b and c indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

3. Results

The cytotoxicity of allethrin on LC540 cells was evaluated by MTT assay. Allethrin at low concentrations did not display appreciable cell killing activity up to 50 μM when incubated for 24 h (Fig. 1A). At concentrations above 100 μM, cell killing was observed. Based on the results obtained, the IC_{50} of allethrin was found to be 125 μM (Fig. 1A). All further experiments in this study were carried with this dose. In order to find out whether allethrin can also display similar cytotoxic effects on primary testicular cells, MTT assay was carried out with Leydig cells isolated from adult rats. Allethrin was found to be cytotoxic to primary Leydig cells in a dose-dependent manner (Fig. 1B) with an IC_{50} value of 59 μM. In all the assays conducted, the cells were incubated with DMSO equivalent to the amount present in 125 μM allethrin. DMSO alone did not contribute to any cell killing when incubated up to 48 h (data not shown). To study the mechanism of allethrin toxicity, LC540 cells were preferred over primary Leydig cells because of the difficulty in obtaining high number of primary cells that required sacrificing many animals.

To determine whether allethrin induced cytotoxicity involves ROS generation, the oxidation of DCFH-DA to 2,7-dichloro-fluorescein was measured. The mean fluorescence intensity increased significantly when compared to control within 1 h after allethrin treatment (Fig. 1C) indicating generation of ROS. However, a time dependent increase in the generation of ROS was not observed. A time dependent increase in the concentration of lipid peroxidation products was observed (Fig. 1D).

Catalase, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase activities were measured in LC540 cells treated with or without allethrin for 0–48 h. Cells treated with the solvent control for each time point were maintained and the enzyme activity observed in the allethrin treated cells was expressed as percent activity relative to the solvent control at each time point. When compared to the respective controls, no significant change in catalase activity was found up to 12 h after allethrin treatment. A significant increase at 24 h followed by sharp decline at the 48 h time point (Fig. 2A) was evident. Superoxide dismutase activity increased significantly within 3 h after allethrin treatment and remained elevated until 9 h (Fig. 2B). At the later time points, a time dependent decline up to 24 h followed by a sharp increase at the 48 h time point was observed (Fig. 2B). Glutathione-S-transferase was decreased significantly in a time dependent manner (Fig. 2C). Glutathione peroxidase activity increased significantly at 9 h time point followed by a sharp decline at the later time points (Fig. 2D).

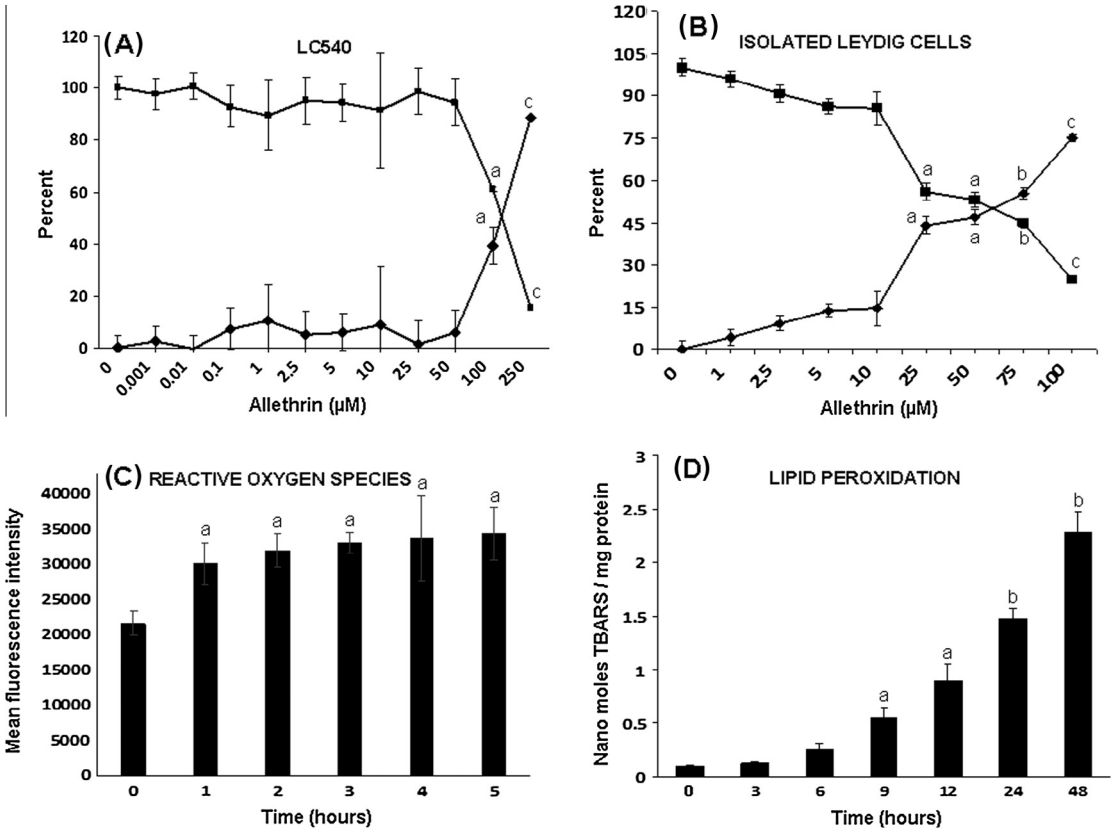

Fig. 1. Allethrin induced cytotoxicity and oxidative stress. LC540 cells (A) and isolated primary Leydig cells (B) were treated with 0–250 μM allethrin for 24 h. The viability of the cells was measured using MTT assay and represented as percent survival (■) or death (♦). (C) LC540 cells treated with allethrin (125 μM) for 0–5 h were incubated with 10 μM 2,7-dichloro dihydro fluorescein diacetate (DCFH-DA). The oxidation of DCFH-DA by the ROS generated in the cells was analyzed in a flow cytometer by measuring the fluorescence intensity of oxidized DCFH-DA. (D) The TCA precipitates of LC540 cells treated with 125 μM allethrin for 0–48 h were mixed with thiobarbutirc acid and incubated for 1 h at 95 °C. The pink color formed was estimated at 532 nm in a spectrophotometer. The concentration of lipid peroxidation products formed was calculated using 1,1,3,3-tetraethoxy-propane (TEP) as the standard. Values shown are Mean ± S.D. a, b and c indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

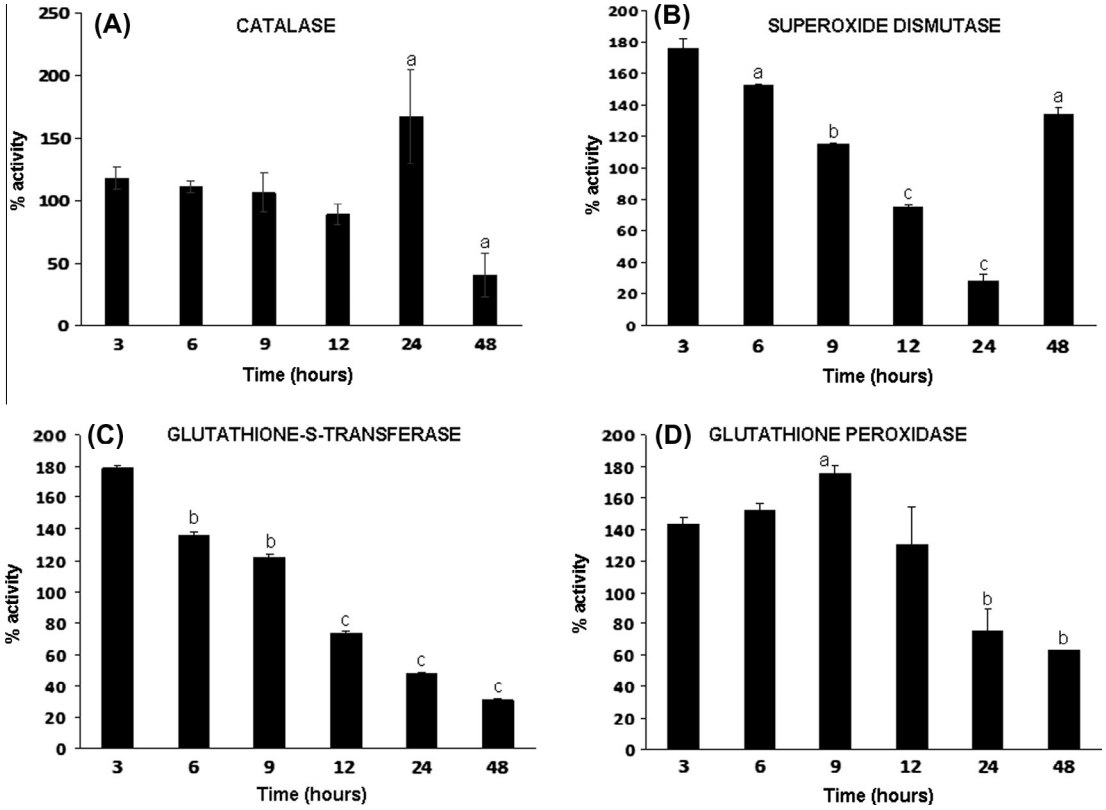

Fig. 2. Antioxidant enzyme activity. 6×10^6 cells were treated with 125 μM allethrin for 0–48 h. Catalase (A), superoxide dismutase (B), glutathione-s-transferase (C) and glutathione peroxidase (D) enzyme activity was measured in the cell lysates. Values shown are Mean ± S.D. a, b and c indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Morphological examination of LC540 cells treated with allethrin revealed the presence of apoptotic bodies (Fig. 3A–F); and their frequency increased with the duration of treatment. Annexin-V staining and FACS analyses indicated the distribution of cells at different stages namely the necrotic, late apoptotic, live and early apoptotic cells in the Q1, Q2, Q3 and Q4 quadrants of the cytogram (Fig. 4B). The percentage of cells displaying early apoptotic features increased significantly after allethrin treatment (Fig. 4A and B). On the same lines, cells that are in late apoptosis are also increased, though not significant. Cell cycle arrest is one of the key indicators that drive the fate of the cell. Propidium iodide staining followed by FACS analyses indicated the distribution of cells in different phases of cell cycle such as G_0, G_1, S and G_2/M in the M1, M2, M3 and M4 regions of the cytogram (Fig. 4C). We observed that cells entering into G_0 phase increased significantly in a time dependent manner after allethrin treatment (Fig. 4D). The changes in MMP in cells treated with allethrin were assessed by Rhodamine staining followed by FACS analyses. The fluorescence intensity decreased significantly in cells treated with 125 μM allethrin indicating loss of MMP (Fig. 5A); and the decrease was more than that induced by the MMP lowering drug doxorubicin. From the above results, it is clear that allethrin induces apoptosis in LC540 cells. A number of molecular factors such as p53, BCL-2, Caspase-3 and PARP-1 play a key role in the execution of apoptosis. We analyzed

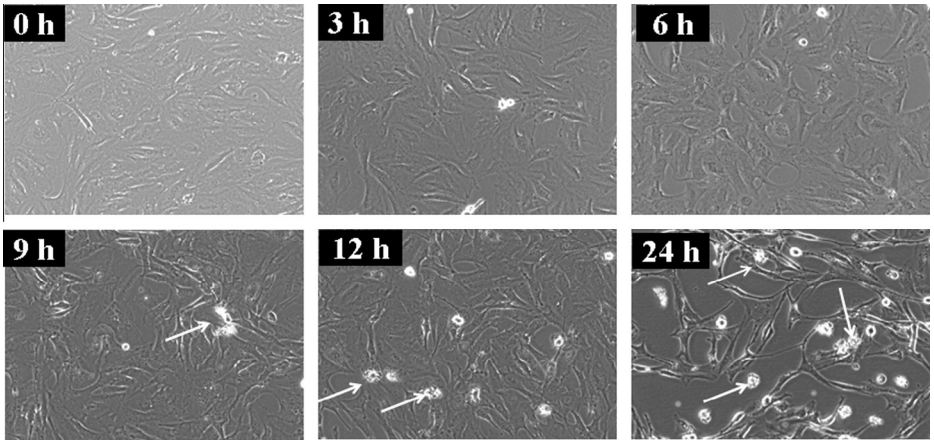

Fig. 3. Morphological features of allethrin treated cells. LC540 cells treated for 0–24 h with 125 μM allethrin were examined under a phase contrast microscope. Arrows indicate location of apoptotic bodies.

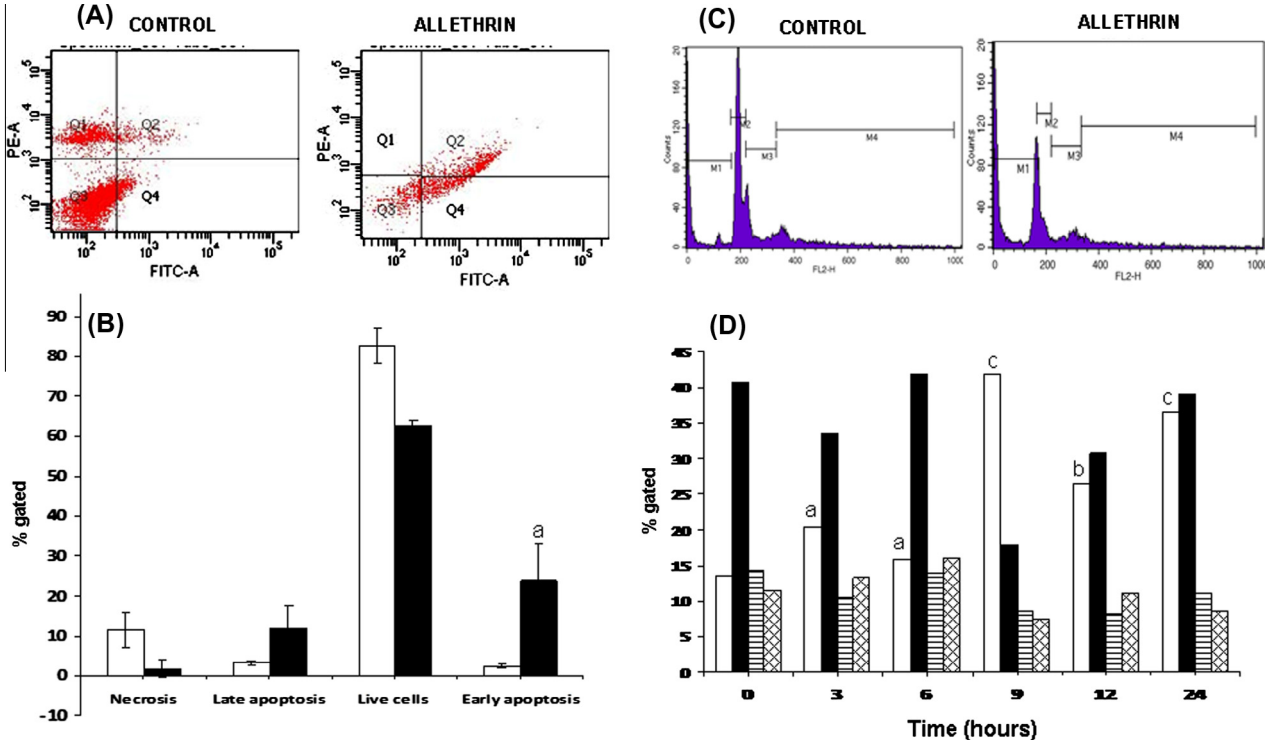

Fig. 4. Apoptotic features in allethrin treated cells. LC540 cells after treatment with 125 μM allethrin for 24 h were collected and used for the assessment of apoptotic parameters. (A) and (C) are representative FACS cytograms of Annexin-V and propidium iodide stained cells respectively. Events in the Q1, Q2, Q3 and Q4 quadrants of the cytogram indicate necrotic, late apoptotic, live and early apoptotic cells respectively. M1, M2, M3 and M4 in the cytogram indicate cells that are in G_0, G_1, S and G_2/M phases of cell cycle respectively. (B) Annexin-V assay to detect early apoptotic cells in control (□) and allethrin treated cells (■). (D) Cell cycle analyses in control and allethrin treated cells using propidium iodide staining. Cells in the G_0 phase are indicated in (□). Values shown are Mean ± S.D. a, b and c indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

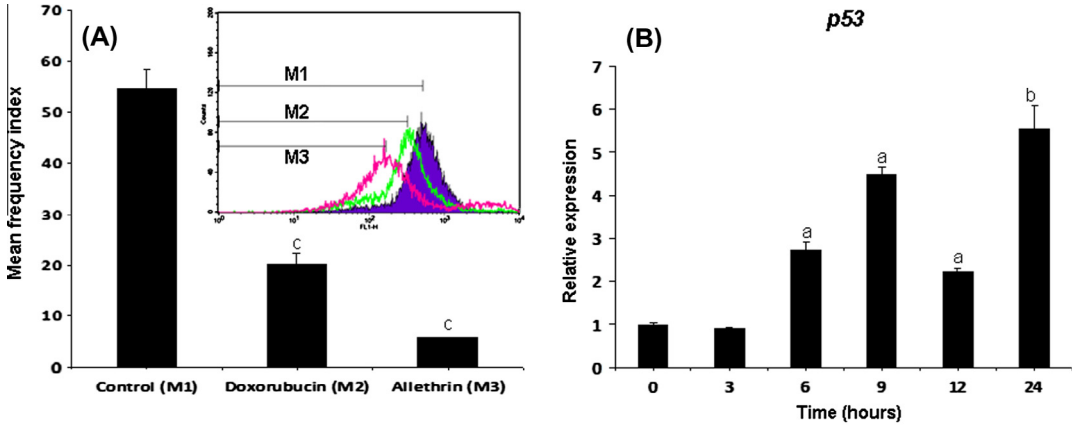

Fig. 5. Apoptotic indicators in allethrin treated cells. (A) Mitochondrial membrane potential measurement in cells treated with allethrin or doxorubicin (10 μM). M1, M2 and M3 in the cytogram indicate mitochondrial membrane dependent fluorescence in control, doxorubicin and allethrin treated cells. (B) *p53* gene expression. RNA isolated from allethrin treated cells was reverse transcribed and the expression of *p53* analyzed using real time PCR. Values shown are Mean ± S.D. a, b and c indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

their expression pattern n LC540 cells treated with allethrin. The mRNA levels of *p53* increased significantly at all the time points 6 h after allethrin treatment (Fig. 5B). BCL-2, pro-Caspase-3 and PARP-1 protein expression decreased significantly with an increase in cleaved PARP-1 levels (Fig. 6A and B).

The ability of allethrin to induce calcium release from intracellular sources was evaluated. Increased fluorescence (emission at 340 nm) exhibited by calcium bound FURA was observed immediately after allethrin addition in a dose-dependent fashion (Fig. 7A). Surprisingly, a dose-dependent increase in the fluorescence intensity (emission at 380 nm) of free FURA 2-AM also increased in allethrin treated cells (Fig. 7B). The increase in the intensity of fluorescence exhibited by free FURA was such that when the ratio of fluorescence exhibited at 340 and 380 nm was plotted, the net effect seems to appear that there is an overall decrease in fluorescence (data not shown). Hence, the changes in the fluorescence emitted at 340 and 380 nm were plotted separately (Fig. 7A and B). Pretreatment of LC540 cells with L- and P-type VGCC blockers inhibited allethrin induced release of intracellular calcium (Fig. 8).

4. Discussion

Understanding the molecular mechanisms of allethrin cytotoxicity in a variety of *in vitro* models has been an active area of

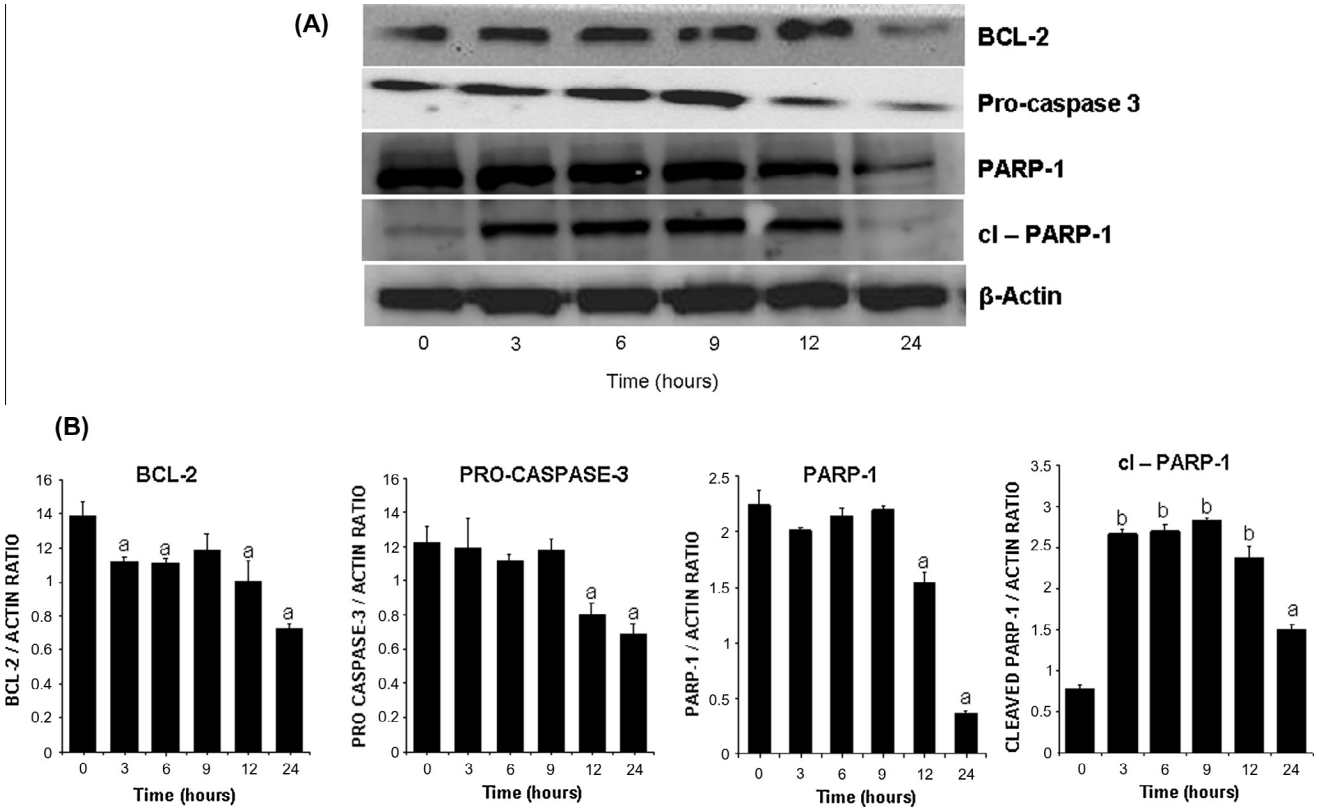

Fig. 6. Expression pattern of apoptotic effectors. (A) Western blotting analyses for BCL-2, pro-Caspase-3, PARP-1 and cleaved (cl) PARP-1. Result shown is a representative of three independent experiments. (B) Densitometric analyses to demonstrate the differential expression levels. Values shown are Mean ± S.D. a and b indicates $p < 0.05$ and $p < 0.01$ respectively.

investigation because of its widespread domestic use. However, there is little information on the molecular mechanisms of toxicity in testicular cells, despite the fact that allethrin is demonstrated to be toxic to the male reproductive system (Madhubabu and Yenugu, 2012, 2013). The central focus of this study was to determine the mechanism by which allethrin causes cell death in testicular carcinoma cells. A dose-dependent cell death was observed when LC540 cells were treated with allethrin with an IC_{50} value of 125 μM. The structural, functional and molecular features of normal and cancer cells differ to a great extent and could result in variation in their susceptibility to toxic compounds. We observed that isolated primary Leydig cells were susceptible to the cytotoxic effects of allethrin with an IC_{50} of 59 μM. The variation in the IC_{50} values could be due to the differences in membrane composition, proliferation ability, endogenous expression of cellular proteins and overall survival mechanisms of LC540 cells and primary Leydig cells. It would be interesting to study the physiological, biochemical and molecular factors that contribute to the resistance of LC540 cells towards allethrin induced toxicity. However, the cytotoxicity observed in this study reaffirms our earlier observation that allethrin is toxic to the male reproductive system (Madhubabu and Yenugu, 2013) and the Leydig cells could be one of the target cell types.

The primary objective of this study was to evaluate the molecular mechanisms of allethrin toxicity at the cellular level. Hence, we used rat Leydig tumor cell lines as our model system, since they are easy to propagate to conduct a series of experiments. It is possible that allethrin may have deleterious effects on germ cells or Sertoli cells or other cells of the male reproductive tract. Further, allethrin dose used in this study is not precisely related to our previous *in vivo* studies (Madhubabu and Yenugu, 2012, 2013). Despite many reports on the toxic effects of pyrethroids on male reproductive function, their exact concentrations at the organ (testicular/epididymal) level during the experimental period are not documented. In this study, we focused on the molecular mechanisms of allethrin toxicity using IC_{50} concentration, though the best possible approach would be to treat the cells with the doses that exist at the organ level after allethrin exposure. Hence, our results are only an indicative of the possible toxic effects of allethrin *in vivo*.

It is well established that environmental factors including pyrethroid toxicity induces oxidative stress in many organ systems. Oxidative stress results in the generation of ROS, which in turn attack cellular membranes and DNA to induce repair mechanisms or initiate programmed cell death. ROS interaction with cellular membranes and DNA leads to the formation of lipid peroxidation products and strand breaks respectively. Oxidative stress during pyrethroid metabolism has been demonstrated both *in vitro* and *in vivo* (Giray et al., 2001; El-Demerdash, 2007). In this study, ROS generated in LC540 cells could be due to the intermediate products of allethrin metabolism. Lipid peroxidation products were also found to be increased suggesting that the free radicals generated during allethrin treatment contribute to the peroxidation of membrane lipids and subsequently damage to cellular membranes. It is interesting to note that ROS generation increased within 1 h after allethrin treatment without any further increase in a time dependent manner. However, formation of lipid peroxidation products increased in a time dependent manner. It is possible that allethrin could have been metabolized immediately after addition and the free radicals generated could have long term actions on membrane lipids, thus generating lipid peroxidation products for longer durations. Increased oxidative stress leads to antioxidant responses that involve modulation of the activity of antioxidant enzymes.

Antioxidant enzymes such as catalase, glutathione peroxidase and superoxide disumutase, in general, aid in scavenging free

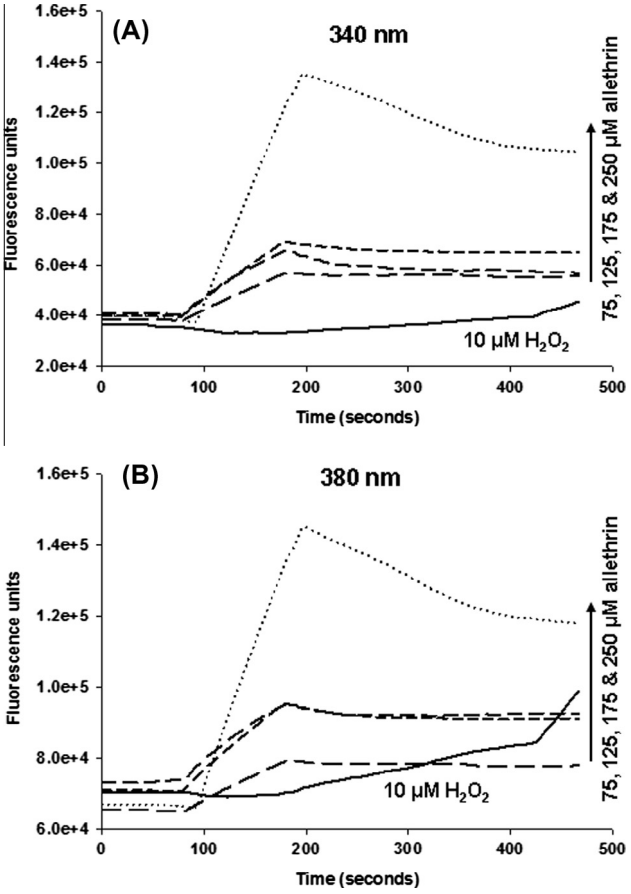

Fig. 7. Dynamics of calcium concentration. LC540 cells were incubated with FURA-2AM and treated with 75–250 µM allethrin and fluorescence levels measured at 340 (A) and 380 nm (B) to detect calcium bound FURA and free FURA. As a positive control hydrogen peroxide was used. Result shown is a representative of three independent experiments.

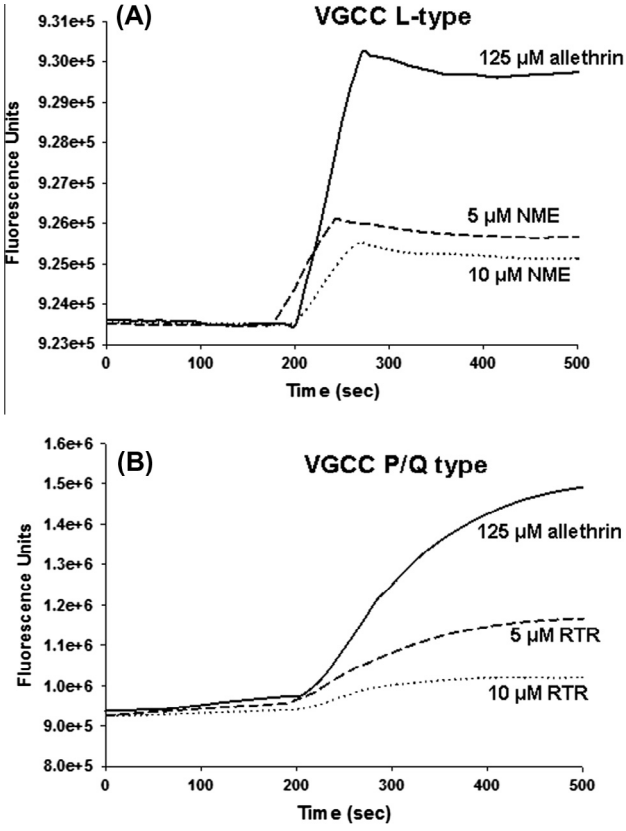

Fig. 8. Voltage gated calcium channel mediated calcium release. Prior to treatment with 125 µM allethrin, LC540 cells were preincubated with 5 and 10 µM (A) Nimodipine (NME) or (B) Ruthenium Red (RTR) for 15 min. Fluorescence was measured at 340 nm to detect calcium bound FURA.

radical generation, whereas glutathione-S-transferase plays a crucial role in xenobiotic metabolism to conjugate toxic compounds and facilitating clearance from the cell. Pyrethroid induced oxidative stress and the consequent lipid peroxidation is reported earlier (Sadowska-Woda et al., 2010). Disruption in the antioxidant enzyme profile by pyrethroids was reported both *in vitro* and *in vivo* (Madhubabu and Yenugu, 2012, 2013). In general, we observed increased antioxidant enzyme activity at the initial time points after allethrin treatment followed by a decline. It appears that there is a protective effect conferred by the antioxidant enzymes immediately after allethrin exposure to scavenge free radicals. However, prolonged free radical presence could have led to failure of antioxidant defense mechanisms which is evident as a decrease of antioxidant enzyme activity. The marked induction of glutathione peroxidase and catalase at 9 and 24 h respectively could be one of the many protective mechanisms initiated by cells to combat allethrin induced toxicity. Decreased glutathione-S-transferase activity after allethrin treatment suggests failure of xenobiotic metabolism machinery.

Oxidative stress initiates of a number of biochemical and molecular events that direct the cells to either mount repair mechanisms or undergo self destruction. It is well known that apoptosis is the ubiquitous form of cell death under stress conditions induced by a variety of environmental toxicants including pyrethroids. The shift in dynamics of pro and anti apoptotic gene expression during cellular stress determines the fate of the cell to either undergo apoptotic cell death or survive by initiating defense mechanisms. Transfluthrin and its metabolite tetrafluorobenzoic acid cytotoxicity on urothelial cells of the rat involved oxidative stress mediated apoptosis (Yokohira et al., 2011). Deltamethrin induces apoptosis in the hippocampal neurons (Grosse et al., 2002) and brain cells of the rat (Wu et al., 2003). Similarly, pyrethroids induce apoptotic cell death in various organ systems including the male reproductive system (Hossain and Richardson, 2011; El-Gohary et al., 1999). However, the molecular changes that occur during allethrin induced apoptosis are not reported. We observed the formation of apoptotic bodies, increased number of cells with cycle arrest and signs of early apoptosis, loss of membrane potential, decreased levels of BCL-2, pro-Caspase-3 and PARP-1 and increased levels of *p53* mRNA and cleaved PARP-1, all of which are the classical events that occur during apoptotic cell death. During apoptotic cell death, pro-caspase-3 is cleaved to form active caspase-3, which in turn acts on PARP-1. The decrease in the levels of pro-Caspase-3 after allethrin treatment could be due to its cleavage and subsequent activation. PARP-1 is a substrate for activated caspase-3. Increased levels of cleaved PARP-1 observed in this study may be due to cleavage of full length PARP-1 by the activated caspase-3. The dynamics of gene expression during oxidative stress determines the fate of the cell to either undergo apoptosis or mount defense mechanisms. The expression of *p53* gene during stress is vital for the cell to undergo apoptosis. Alterations in the expression of apoptosis related genes during oxidative stress in the testes were reported (Hsu et al., 2007). We observed decreased *p53* expression after allethrin treatment and this could be the signal for the cells to undergo apoptosis. To the best of our knowledge, these results for the first time suggest that allethrin mediated cell death involves apoptotic mechanisms. It would be interesting to conduct further studies to determine whether allethrin induced apoptosis could be due to the

direct modulation of pro apoptotic genes at the promoter level, besides the classical pathways.

Intracellular calcium levels are crucial in regulating a variety of cellular functions. Increase in intracellular calcium level is a key signal for apoptosis and necrosis, whereas lower calcium levels compromise cellular processes (Clapham, 1995). We observed oxidative stress induced apoptosis in LC540 cells treated with allethrin. The correlation between oxidative stress induced apoptosis with the calcium dynamics during allethrin toxicity, especially in the male reproductive cells are not reported. Increased calcium levels as indicated by enhanced fluorescence emitted by calcium bound FURA, indicated that during allethrin toxicity, release of calcium could be due to the direct of allethrin so as to facilitate the opening of calcium channels located on the membranes of calcium storage organelles; or it could be due to the secondary effects of oxidative stress induced by allethrin. These changes in calcium dynamics are strengthened by the fact that the assays were performed in cells suspended in calcium free medium. It is demonstrated in oral cancer cells that deltamethrin affects calcium release in a PLC independent mechanism and its uptake is mediated by nifedipine sensitive calcium channels (Chi et al., 2014). Further studies that involves pretreatment of LC540 cells with antioxidant molecules such as reduced glutathione will help to pin point whether induction of apoptosis observed in this study is due to allethrin itself (by modulating the voltage gated calcium channels) or due to the resultant oxidative stress. Surprisingly, we also found that the fluorescence emitted by calcium free FURA is also increased following allethrin treatment, indicating that allethrin may affect calcium efflux from the cells or compete with FURA for its binding and subsequently chelating it. Sudden decrease in intracellular calcium is also detrimental to the cells and in the event of allethrin chelating the calcium, the cellular death could also be due to this physiological change. Decreased calcium release in LC540 cells pretreated with L- and P/Q-type VGCC blockers indicate that allethrin causes release of calcium from intracellular stores. It will be worthwhile to determine the role of specific VGCC subtypes involved in calcium ion dynamics (release from intracellular stores and/or uptake from the extra cellular medium) during allethrin toxicity. More importantly, the possible calcium chelating or calcium effluxing ability of allethrin needs attention.

5. Conclusions

In conclusion, we report that the apoptotic cell death in rat Leydig cells induced by allethrin involves generation of free radicals, lipid peroxidation and alterations in the oxidant and antioxidant status. The up regulation of p53 and cleaved PARP-1; decreased levels of BCL-2, pro-Caspase-3 and PARP-1, with a concomitant release of calcium from intracellular stores may contribute to the cytotoxic effects of allethrin. Besides cytotoxicity, the reduced expression of spermatogenic factors namely *Tgf-β1* and *Scf* suggests the potential of allethrin to affect spermatogenesis. Results of this study provide further understanding into the molecular mechanisms of pyrethroid toxicity in the male reproductive system.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

MG thanks Council of Scientific and Industrial Research Government of India, for providing the fellowship. We thank the facilities extended by UGC-SAP, UGC-CAS, DBT-CREBB and FIST programs at School of Life Sciences, University of Hyderabad.

References

Abarikwu, S.O., Farombi, E.O., Kashyap, M.P., Pant, A.B., 2011. Atrazine induces transcriptional changes in marker genes associated with steroidogenesis in primary cultures of rat Leydig cells. Toxicol. In Vitro 25, 1588–1595.
Abou-Donia, M.B., Goldstein, L.B., Jones, K.H., Abdel-Rahman, A.A., Damodaran, T.V., et al., 2001. Locomotor and sensorimotor performance deficit in rats following exposure to pyridostigmine bromide, DEET, and permethrin, alone and in combination. Toxicol. Sci. 60, 305–314.
Aebi, H., 1984. Catalase. Meth. Enzymol. 105, 121–126.
Aldred, L.F., Cooke, B.A., 1983. The effect of cell damage on the density and steroidogenic capacity of rat testis Leydig cells, using an NADH exclusion test for determination of viability. J. Steroid Biochem. 18, 411–414.
Bal, R., Naziroglu, M., Turk, G., Yilmaz, O., Kuloglu, T., et al., 2012. Insecticide imidacloprid induces morphological and DNA damage through oxidative toxicity on the reproductive organs of developing male rats. Cell Biochem. Funct. 30, 492–499.
Banerjee, B.D., Seth, V., Ahmed, R.S., 2001. Pesticide-induced oxidative stress: perspectives and trends. Rev. Environ. Health 16, 1–40.
Bernheim, F., 1964. The oxidative desulfuration of thio-acids. Biochim. Biophys. Acta 90, 426–428.
Booth, L.A., Tavallai, S., Hamed, H.A., Cruickshanks, N., Dent, P., 2013. The role of cell signalling in the crosstalk between autophagy and apoptosis. Cell. Signal. 26, 549–555.
Bootman, M.D., Berridge, M.J., Roderick, H.L., 2002. Calcium signalling: more messengers, more channels, more complexity. Curr. Biol. 12, R563–565.
Chi, C.C., Chou, C.T., Liang, W.Z., Jan, C.R., 2014. Effect of the pesticide, deltamethrin, on Ca2+ signaling and apoptosis in OC2 human oral cancer cells. Drug Chem. Toxicol. 37, 25–31.
Clapham, D.E., 1995. Intracellular calcium. Replenishing the stores. Nature 375, 634–635.
Clark, J.M., Symington, S.B., 2008. Neurotoxic implications of the agonistic action of CS-syndrome pyrethroids on the N-type Ca(v)2.2 calcium channel. Pest Manage. Sci. 64, 628–638.
Eil, C.N.B., 1990. The binding properties of pyrethroids to human skin fibroblast androgen receptors and to sex hormone binding globulin. J. Steroid Biochem. 35, 409–414.
El-Demerdash, F.M., 2007. Lambda-cyhalothrin-induced changes in oxidative stress biomarkers in rabbit erythrocytes and alleviation effect of some antioxidants. Toxicol. In Vitro 21, 392–397.
El-Gohary, M., Awara, W.M., Nassar, S., Hawas, S., 1999. Deltamethrin-induced testicular apoptosis in rats: the protective effect of nitric oxide synthase inhibitor. Toxicology 132, 1–8.
Eruslanov, E., Kusmartsev, S., 2010. Identification of ROS using oxidized DCFDA and flow-cytometry. Methods Mol. Biol. 594, 57–72.
Garey, J., Wolff, M.S., 1998. Estrogenic and antiprogestagenic activities of pyrethroid insecticides. Biochem. Biophys. Res. Commun. 251, 855–859.
Giray, B., Gurbay, A., Hincal, F., 2001. Cypermethrin-induced oxidative stress in rat brain and liver is prevented by vitamin E or allopurinol. Toxicol. Lett. 118, 139–146.
Go, V., Garey, J., Wolff, M.S., Pogo, B.G., 1999. Estrogenic potential of certain pyrethroid compounds in the MCF-7 human breast carcinoma cell line. Environ. Health Perspect. 107, 173–177.
Grosse, G., Thiele, T., Heuckendorf, E., Schopp, E., Merder, S., et al., 2002. Deltamethrin differentially affects neuronal subtypes in hippocampal primary culture. Neuroscience 112, 233–241.
Hadnagy, W., Seemayer, N.H., Kuhn, K.H., Leng, G., Idel, H., 1999. Induction of mitotic cell division distrubances and mitotic arrest by pyrethroids in V79 cell cultures. Toxicol. Lett. 107, 81–87.
Hossain, M.M., Richardson, J.R., 2011. Mechanism of pyrethroid pesticide-induced apoptosis: role of calpain and the ER stress pathway. Toxicol. Sci. 122, 512–525.
Hsu, P.C., Pan, M.H., Li, L.A., Chen, C.J., Tsai, S.S., et al., 2007. Exposure in utero to 2,2′,3,3′,4,6′-hexachlorobiphenyl (PCB 132) impairs sperm function and alters testicular apoptosis-related gene expression in rat offspring. Toxicol. Appl. Pharm. 221, 68–75.
Institoris, L., Undeger, U., Siroki, O., Nehez, M., Desi, I., 1999. Comparison of detection sensitivity of immuno- and genotoxicological effects of subacute cypermethrin and permethrin exposure in rats. Toxicology 137, 47–55.
Issam, C., Samir, H., Zohra, H., Monia, Z., Hassen, B.C., 2009. Toxic responses to deltamethrin (DM) low doses on gonads, sex hormones and lipoperoxidation in male rats following subcutaneous treatments. J. Toxicol. Sci. 34, 663–670.
Issam, C., Zohra, H., Monia, Z., Hassen, B.C., 2011. Effects of dermal sub-chronic exposure of pubescent male rats to permethrin (PRMT) on the histological structures of genital tract, testosterone and lipoperoxidation. Exp. Toxicol. Pathol. 63, 393–400.
Jakoby, W.B., 1978. In: Meister, A. (Ed.), The Glutathione-S-Transferases: a Group of Detoxification Proteins. John Wiley, New York, pp. 383–414.

Kale, M., Rathore, N., John, S., Bhatnagar, D., 1999. Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: a possible involvement of reactive oxygen species. Toxicol. Lett. 105, 197–205.
Kasat, K., Go, V., Pogo, B.G., 2002. Effects of pyrethroid insecticides and estrogen on WNT10B proto-oncogene expression. Environ. Int. 28, 429–432.
Li, H.Y., Shi, N., Chen, D., Dai, Z.H., Lu, W.H., et al., 2005. Oxidative stress of deltamethrin on rat nervous system. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi 23, 97–101.
Madhubabu, G., Yenugu, S., 2012. Effect of continuous inhalation of allethrin-based mosquito coil smoke in the male reproductive tract of rats. Inhal. Toxicol. 24, 143–152.
Madhubabu, G., Yenugu, S., 2013. Allethrin induced toxicity in the male reproductive tract of rats contributes to disruption in the transcription of genes involved in germ cell production. Environ. Toxicol..
Maiti, P.K., Kar, A., Gupta, P., Chaurasia, S.S., 1995. Loss of membrane integrity and inhibition of type-I iodothyronine 5′-monodeiodinase activity by fenvalerate in female mouse. Biochem. Biophys. Res. Commun. 214, 905–909.
McCord, J.M., Fridovich, I., 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.
Meyer, D.A., Shafer, T.J., 2006. Permethrin, but not deltamethrin, increases spontaneous glutamate release from hippocampal neurons in culture. Neurotoxicology 27, 594–603.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
Narahashi, T., 1986. Toxins that modulate the sodium channel gating mechanism. Ann. NY Acad. Sci. 479, 133–151.
Nasuti, C., Gabbianelli, R., Falcioni, M.L., Di Stefano, A., Sozio, P., et al., 2007. Dopaminergic system modulation, behavioral changes, and oxidative stress after neonatal administration of pyrethroids. Toxicology 229, 194–205.
Naziroglu, M., Butterworth, P.J., Sonmez, T.T., 2011. Dietary vitamin C and E modulates antioxidant levels in blood, brain, liver, muscle, and testes in diabetic aged rats. Int. J. Vitam. Nutr. Res. 81, 347–357.
Neal, A.P., Yuan, Y., Atchison, W.D., 2010. Allethrin differentially modulates voltage-gated calcium channel subtypes in rat PC12 cells. Toxicol. Sci. 116, 604–613.
Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70, 158–169.
Paschen, W., 2000. Role of calcium in neuronal cell injury: which subcellular compartment is involved? Brain Res. Bull. 53, 409–413.
Pontecorvo, G., Fantaccione, S., 2006. Recombinogenic activity of 10 chemical compounds in male germ cells of *Drosophila melanogaster*. Ecotoxicol. Environ. Saf. 65, 93–101.
Ray, D.E., Fry, J.R., 2006. A reassessment of the neurotoxicity of pyrethroid insecticides. Pharmacol. Ther. 111, 174–193.
Romero, A., Ramos, E., Castellano, V., Martinez, M.A., Ares, I., et al., 2012. Cytotoxicity induced by deltamethrin and its metabolites in SH-SY5Y cells can be differentially prevented by selected antioxidants. Toxicol. In Vitro 26, 823–830.
Sadowska-Woda, I., Wojcik, N., Karowicz-Bilinska, A., Bieszczad-Bedrejczuk, E., 2010. Effect of selected antioxidants in beta-cyfluthrin-induced oxidative stress in human erythrocytes in vitro. Toxicol. In Vitro 24, 879–884.
Sattin, R.W.R.A., Kafrissen, M.E., Dugan, J.B., Farer, L.S., 1984. An epidemic of gynecomastia among illegal Haitian entrants. Public Health Rep. 99, 504–510.
Scaduto Jr., R.C., Grotyohann, L.W., 1999. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys. J. 76, 469–477.
Shukla, Y., Yadav, A., Arora, A., 2002. Carcinogenic and cocarcinogenic potential of cypermethrin on mouse skin. Cancer Lett. 182, 33–41.
Srivastava, A., Srivastava, M.K., Raizada, R.B., 2006. Ninety-day toxicity and one-generation reproduction study in rats exposed to allethrin-based liquid mosquito repellent. J. Toxicol. Sci. 31, 1–7.
Tomlin, C., 1994. A World Compendium: The Pesticide Manual. British Crop Protection Council.
Wang, X.Z., Liu, S.S., Sun, Y., Wu, J.Y., Zhou, Y.L., et al., 2009. Beta-cypermethrin impairs reproductive function in male mice by inducing oxidative stress. Theriogenology 72, 599–611.
Wu, A., Li, L., Liu, Y., 2003. Deltamethrin induces apoptotic cell death in cultured cerebral cortical neurons. Toxicol. Appl. Pharmacol. 187, 50–57.
Yokohira, M., Arnold, L.L., Lautraite, S., Sheets, L., Wason, S., et al., 2011. The effects of oral treatment with transfluthrin on the urothelium of rats and its metabolite, tetrafluorobenzoic acid on urothelial cells in vitro. Food Chem. Toxicol. 49, 1215–1223.
Yoshida, I., Monji, A., Tashiro, K., Nakamura, K., Inoue, R., et al., 2006. Depletion of intracellular Ca2+ store itself may be a major factor in thapsigargin-induced ER stress and apoptosis in PC12 cells. Neurochem. Int. 48, 696–702.

Toxicology in Vitro 27 (2013) 2242–2248

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Allethrin toxicity on human corneal epithelial cells involves mitochondrial pathway mediated apoptosis

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ARTICLE INFO

Article history:
Received 25 March 2013
Accepted 11 September 2013
Available online 19 September 2013

Keywords:
Allethrin
Corneal epithelial cells
Apoptosis

ABSTRACT

Pyrethroids including allethrin are the most common commercial household insecticides. The detrimental effects caused by pyrethroids on humans are gaining considerable attention. The present study was aimed to elucidate the effects of allethrin on the human corneal epithelial (HCE) cells. Allethrin inhibited the proliferation of HCE cells in a dose-dependent manner. In the presence of allethrin, cells showed membrane blebbing and nuclear fragmentation along with significant decrease in mitochondrial membrane potential resulting in increased cytochrome c (Cyt c) release into the cytosol. Further, flow cytometry analysis demonstrated a marked increase in sub G_0–G_1 cells, characteristic of apoptosis. Increased expression of pro-apoptotic protein, Bax, a simultaneous decrease of anti-apoptotic protein, Bcl-2, and activation of Caspase 3 was evident in the treated cells. In addition, extracellular matrix digesting metalloproteinase 9 (MMP-9) was also stimulated. Furthermore, significant increase in the gene expression of inflammatory cytokines, TNF-α and IL-1β was observed. Taken together, these findings suggest that allethrin ($IC_{50} \approx 85$ μM) is toxic to HCE cells causing death through mitochondrial pathway.

1. Introduction

Pyrethroids including allethrin are used to control a broad range of pests in agriculture, public health, and households, accounting for about 25% of the worldwide insecticide market (Casida and Quistad, 1998). In the recent past, pyrethroid usage greatly increased on account of restrictions posed on organophosphorus insecticides; however, there have been relatively few reports of systemic toxicity. Pyrethroids primarily act on the nervous system by inhibiting voltage sensitive sodium channels and sodium–potassium ATPase (Soderlund and Bloomquist, 1989; Soderlund et al., 2002; Bradberry et al., 2005; Wolansky and Harrill, 2008; Tan and Soderlund, 2010). These compounds are also known to be hepatotoxic (Giray et al., 2001), carcinogenic (Go et al., 1999), immunosuppressive (Institóris et al., 1999), estrogenic, and anti-progestagenic (Garey and Wolff, 1998). Pyrethroids also induce oxidative stress (Srivastava et al., 2012) and alter the plasma biochemical profile (Narendra et al., 2008). Further, deleterious effects of these compounds on the human reproductive system are emerging (Cheng et al., 2011; Issam et al., 2011; Liu et al., 2011; Madhubabu and Yenugu, 2012). Evidently, toxicity of pyrethroid-based compounds is of great concern and warrants for a much detailed investigation of their effect(s) on various human organ systems and/or cell lines.

D-allethrin is a widely used synthetic pyrethroid that accumulates on different household surfaces and persists in the environment for an extended period of time (Ramesh and Vijayalakshmi, 2002). Allethrin degradation via oxidative pathway (Miyamoto, 1976) leads to the generation of free radicals causing DNA damage and various adverse health effects in exposed populations (Kale et al., 1999; Srivastava et al., 2012). Earlier studies assessed allethrin's neurotoxicity in rodents (Bloomquist, 1996; Mubarak Hossain et al., 2006) and genotoxicity in bacterial systems (Hour et al., 1998). The usage of mosquito coil repellants release high concentrations of allethrin into the surrounding air within short period of time (0.0120 ppm/45 min), which is several folds greater than the level required to control mosquitoes (Ramesh and Vijayalakshmi, 2001). In this context, it would be logical to examine the effect(s) of air-borne allethrin on the sensitive corneal epithelial surface that is directly exposed.

Integrity of the ocular surface contributes significantly to proper vision. Ocular surface epithelium (limbus, cornea, and conjunctiva) is covered by a tear film that acts as a physical barrier from external agents. These cells are continually at risk from infection (Kernacki et al., 1998), pollutants and environmental toxins (Bhattacharya et al., 2007), drugs and drug preservatives (Ammar et al., 2010; Baudouin et al., 2010) as well as chemical and physical injuries (Wagoner, 1997; Ambrósio et al., 2009). These might lead to alterations in tear film composition, increased inflammatory reaction, corneal cell death or a delayed wound healing process (Noecker et al., 2004; Kahook and Noecker, 2008). The ocular surface is easily prone to pyrethroid toxicity (Xiong et al., 2010). However, information

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http://dx.doi.org/10.1016/j.tiv.2013.09.011

RESEARCH ARTICLE

Molecular Reproduction & Development 78:633–641 (2011)

Identification and Characterization of *Wfdc* Gene Expression in the Male Reproductive Tract of the Rat

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SUMMARY

WFDC (Whey Acidic Protein Four Disulfide Core)-containing proteins have been reported in many species, yet they remain uncharacterized in the rat. In this study, we report the identification and characterization of four rat *Wfdc* genes, *Wfdc6a*, *Wfdc8*, *Wfdc11* and *Wfdc16.* Their expression profile in a variety of tissues including the male reproductive tract is analyzed. *Wfdc8*, *Wfdc11* and *Wfdc16* expression is confined to the epididymis, while *Wfdc6a* is expressed widely. Since gene expression in the male reproductive tract is largely androgen-dependent, *Wfdc* expression was analyzed in the developing (20–60-day-old) and castrated rats. Their expression pattern in developing rats does not correlate with changes in testosterone. *Wfdc* genes are, however, down-regulated in castrated adult rats, indicating that their dependence on androgens for expression is more pronounced in the adult than in the developing rat. To test the anti-microbial potential of WFDC8, a recombinant WFDC8 C-terminal protein was produced, which exhibited potent anti-bacterial activity against *Eschericia coli*. Induction of anti-microbial genes is one of the responses during infections in many organ systems. To determine if WFDCs form the components of male reproductive tract innate immunity, *Wfdc8* expression pattern was observed in rats challenged with lipopolysaccharide (LPS). For the first time we report the induction of *Wfdc8* gene expression in LPS-treated rats, indicating their contributions to the innate immune functions of the male reproductive tract.

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Mol. Reprod. Dev. 78: 633–641, 2011. © *2011 Wiley-Liss, Inc.*

Received 24 March 2011; Accepted 24 June 2011

Published online 8 July 2011 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/mrd.21361

INTRODUCTION

The male reproductive tract is a dynamic organ system involved in both endocrine and reproductive functions. Although the major male endocrine functions occur in the testis, the production and maturation of spermatozoa occur both in the testis and epididymis. Spermatozoa that emerge from the testis are immature, non-motile, and lack the ability to fertilize an egg. Their passage through the epididymis provides interactions with a wide variety of epididymal-secreted proteins, resulting in the acquisition of motility and the capacity to fertilize. Proteins secreted into the epididymal lumen (Hall et al., 2002) include defensins (Yenugu et al., 2004a, 2006), lipocalins (Hamil et al., 2003), cathelicidins (Travis et al., 2000), members of the sperm associated antigen 11 family (Hamil et al., 2000) and protease inhibitors (Hiemstra et al., 1996; Blankenvoorde et al., 1998; Hamil et al., 2002). Some of the epididymal protease inhibitors secreted into the lumen, such as eppin (Richardson et al., 2001), secretory leukocyte protease inhibitor (SLPI) (McNeely et al., 1995) and elafin

Additional Supporting Information may be found in the online version of this article.

Abbreviations: DHT, dihydrotestosterone; LPS, lipopolysaccharide; WAP, whey acidic protein; WFDC, whey acidic protein four-disulfide core.