

**Effect of a mixture of pyrethroids at doses relevant to human consumption on the
general and male reproductive physiology in rats**

*Thesis submitted to the University of Hyderabad for the award of
Doctor of Philosophy in the Department of Animal Biology*

By

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July 2021



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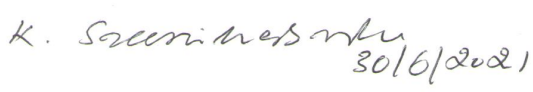
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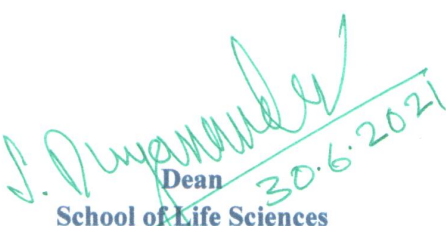

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DEDICATED TO MY PARENTS

Table of Contents

	Page No.
Abbreviations.....	i-iii
Abstract.....	ix-xi
Chapter 1.....	
General Introduction.....	1
1.1 Historical aspects of pesticides References.....	2
1.2 Pesticide usage.....	2-4
1.3. Classification of pesticides.....	4
1.3.1. Natural pesticides	5
1.3.2. Synthetic pesticides	5
1.3.3. Pyrethroids	6
1.3.4. Chemistry, classification, and properties of pyrethroids	6-8
1.3.5. Routes of exposure, absorption, metabolism and excretion of pyrethroids	8
1.3.6. Mechanism and toxicity of pyrethroids.....	9
1.3.7. Health hazardous of pyrethroids	10
1.4. The male reproductive system	11
1.4.1. Anatomical aspects	11
1.4.2. Hormonal regulation of the male reproductive system	12-13
1.4.3. Organisation and functions of testis.....	13
1.4.4. Steroidogenesis and its regulation	13-15
1.4.5. Spermatogenesis	15-17
1.4.6. Spermiogenesis	17
1.4.7. Factors involved in spermatogenesis	18
1.4.8. Hormonal regulation of spermatogenesis.....	18-19
1.5. References	20-21
Chapter 2.....	
Methodology.....	22
2.1. Reagents and chemicals.....	23
2.2 Animals and their maintenance	23
2.3. Rationale for dosage and duration of treatment.....	23-24
2.4. Animal treatments	
2.4.1. Experimental protocol – I (EP-I)	24
2.4.2. Experimental protocol –II (EP II)	25
2.5. Assessment of fecundity.....	25
2.6. Sperm count	25
2.7. Estimation of dehydrogenase enzyme activity	25-26
2.8. Estimation of hormones.....	26
2.9. Capacitation and acrosome reaction	26-27
2.10. Real-time polymerase chain reaction	27-28
2.11. Liquid Chromatography with tandem mass spectrometry	

(LC-MS-MS)	28
2.12. Histopathology	28-29
2.13. Estimation of lipid peroxidation	29
2.14. Estimation of Nitric oxide	29-30
2.15. Estimation of antioxidant enzyme activity	30-31
2.16. Estimation of liver and kidney function, lipid profile, and complete blood picture	31
2.17. Statistical analyses	31
2.18. References	35

Chapter 3.....

Evaluating the effect of a mixture of pyrethroids administered at doses relevant to human consumption on the male reproductive system.	36
3.1. Introduction	37-38
3.2. Results... ..	39
PART I: Effect of a mixture of pyrethroids on the male reproductive function when exposed to doses relevant to human consumption (Experimental protocol I)	39
3.2.1. Fecundity	39
3.2.2. Sperm count	39
3.2.3. 3 β - and 17 β -HSD activity	39
3.2.4. Acrosome reaction	41
3.2.5. Relative organ weights	41
3.2.6. Lipid peroxidation and nitric oxide levels	42
3.2.7. Antioxidant enzymes	42
3.2.8. Histopathological analyses	45-48
PART II: Effect of a mixture of pyrethroids on the male reproductive function when exposed to doses equivalent to human consumption (Experimental protocol –II).	49
3.2.9. Fecundity	49
3.2.10. Sperm count	49
3.2.11. 3 β - and 17 β -HSD activity	49
3.2.12. Capacitation and acrosome reaction	51-52
3.2.13. Hormonal profile	52
3.2.14. Relative organ weights	53
3.2.15. Lipid peroxidation and nitric oxide	53
3.2.16. Histopathological analyses	59
3.3. Discussion	61-62
3.4. References.....	63-64

Chapter 4.....

Evaluating the effect of a mixture of pyrethroids administered at doses relevant to human consumption on the factors that affect male reproduction at the transcriptomic and translational level.	65
4.1. Introduction	66-67
4.2. Results	68

PART – I: Effect of a mixture of pyrethroids on the male reproductive function when exposed to doses relevant to human consumption (Experimental protocol -I)	68
4.2.1. Expression of genes related to spermatogenesis.	68 -71
4.2.2. Expression of genes related to steroidogenesis	71-73
4.2.3. Expression of genes related to epigenetic modifications.....	73-75
PART – II: Effect of a mixture of pyrethroids on the male reproductive function when exposed to doses equivalent to human consumption (Experimental protocol –II).	76
4.3.1 Expression of genes related to spermatogenesis	76
4.3.2 Expression of genes related to steroidogenesis	77-78
4.3.3. Expression of genes related to germ cell epigenetics.....	78-79
4.3.4. Expression of genes related to germ cell apoptosis	79-80
4.4. Transgenerational sperm proteome analyses	80-81
4.5. Discussion	87-89
4.6. References	109-112

Chapter 5.....

Evaluating the effect of a mixture of pyrethroids administered at doses relevant to human consumption on the general physiological processes.....	113
5.1. Introduction	114-115
5.2. Results	116
PART – I: Effect of a mixture of pyrethroids on the liver, kidney, and lung of rats the male reproductive function when exposed to doses relevant to human consumption (Experimental protocol - I)	116
5.2.1. Body and relative organ weights	116
5.2.2. Lipid peroxidation and antioxidant status	116
5.2.3. Antioxidant enzymes	116-117
5.2.4. Serum lipid profile, liver and kidney function	120
5.2.5. Histopathology	123-125
PART- II: Effect of a mixture of pyrethroids on the liver, kidney, and lung or rats exposed to doses equivalent to human consumption	125
(Experimental protocol –II).	
5.2.6. Body and relative organ weights	125
5.2.7. Lipid peroxidation and nitric oxide	125
5.2.8. Antioxidant enzyme activities	125
5.2.9. Liver and Kidney function	130
5.2.10. Lipid profile and complete blood picture	130
5.2.11. Histopathological analyses	135
5.3. Discussion	137-139
5.4. References	140-142
Summary of the study	143-147
Publications	

List of figures

	Page No.
Figure 1. Projected usage of pesticides	3
Figure 2. Classification of pesticides	4
Figure 3. Chemical structures of pyrethroids	7
Figure 4. Molecular mechanism of action of pyrethroids on nervous system	9
Figure 5. Pathology of pyrethroid toxicity	10
Figure 6. Male reproductive system	11
Figure 7. Regulation of male reproductive function through hypothalamic-pituitary-testicular (HPT) axis	12
Figure 8. Signaling pathway of steroidogenesis in Leydig cell	15
Figure 9. Phases and factors that govern spermatogenesis.....	16
Figure 10. Acrosome reaction in the spermatozoa of pyrethroid treated rats	41
Figure 11. Histopathology of liver, kidney, and lung in rats treated with pyrethroid mixture.	46
Figure 12. Histopathology of male reproductive tract tissues in rats treated with pyrethroid mixture.....	47
Figure 13. Histopathological evaluation of reproductive tissues in rats treated with a mixture of pyrethroids.	48
Figure 14. Capacitation in the spermatozoa of pyrethroid treated rats.	51
Figure 15. Acrosome reaction in the spermatozoa of pyrethroid treated rats.	52
Figure 16. Histopathological evaluation of reproductive tissues in rats treated with a mixture of pyrethroids.	59
Figure 17. Histopathological evaluation of reproductive tissues in rats treated with a mixture of pyrethroids.	60
Figure 18. Expression pattern of spermatogenesis-related genes.	68
Figure 19. Expression pattern of spermatogenesis-related genes	69
Figure 20. Expression pattern of spermatogenesis-related genes.	70
Figure 21. Expression pattern of steroidogenesis related genes.	72
Figure 22. Expression pattern of steroidogenesis-related genes	73
Figure 23. Expression pattern of epigenetic modification-related genes.	74

Figure 24. Expression pattern of epigenetic modification-related genes.	75
Figure 25. Histopathology of liver, kidney, and lung in rats treated with pyrethroid mixture.	123
Figure 26. Histopathology of liver, kidney, and lung in rats treated with pyrethroid mixture.	124
Figure 27. Histopathological evaluation of organs of non-reproductive tissues in rats treated with a mixture of pyrethroids.	124
Figure 28. Histopathological evaluation of organs of non-reproductive tissues in rats treated with a mixture of pyrethroids.	135

List of tables

	Page No.
Table 1. Primer sequences to amplify genes related to spermatogenesis	32-33
Table 2. Primer sequences to amplify genes related to steroidogenesis	33
Table 3. Primer sequences to amplify genes related to epigenetic modifications	33-34
Table 4. Primer sequences to amplify genes related to apoptosis and oxidative stress	34
Table 5. Fecundity in rats treated with pyrethroids.	40
Table 6. Sperm count and hydroxysteroid dehydrogenase activities.	40
Table 7. Relative organ weights (grams per 100 g body weight) in the pyrethroid treated rats.	43
Table 8. Lipid peroxidation# in various tissues of rats treated with a mixture of pyrethroids.	43
Table 9. Nitric oxide levels# in various tissues of rats treated with a mixture of pyrethroids.	43
Table 10. Catalase activity# in various tissues of rats treated with a mixture of pyrethroids.	44
Table 11. Glutathione peroxidase activity# in various tissues of rats with a mixture of pyrethroids. ...	44
Table 12. Glutathione S- transferase activity# in various tissues of rats with a mixture of pyrethroids	44
Table 13. Transgenerational fecundity in pyrethroid treated rats.	50
Table 14. Sperm count and hydroxysteroid dehydrogenase activities in pyrethroid treated rats.	50
Table 15. Hormonal profile in pyrethroid treated rats.	54
Table 16. Relative organ weights of various tissues in rats treated with a mixture of pyrethroids for 1 to 15 months.	55
Table 17. Lipid peroxidation levels# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.	55
Table 18. Nitric oxide levels# in various tissues of rats treated with a	

mixture of pyrethroids for 1 to 15 months.	56
Table 19. Catalase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.	56
Table 20. Glutathione peroxidase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.	57
Table 21. Glutathione-S-transferase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.	57
Table 22. Superoxide dismutase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.	58
Table 23. Expression pattern of spermatogenesis-related genes in the testes of pyrethroid treated rats.	77
Table 24. Expression pattern of steroidogenesis-related genes in the testes of pyrethroid treated rats.	78
Table 25. Expression pattern of epigenetic modulation related genes involved in the testes of pyrethroid treated rats.	79
Table 26. Expression pattern of apoptosis and oxidative stress-related genes involved in the testes of pyrethroid treated rats.	80
Table 27. Differential expression of sperm proteome in rats treated with a mixture of pyrethroids.	82-83
Table 28. Transgenerational analyses for differential expression of proteins in the sperm lysates of control and pyrethroid treated rats.	84-86
Table 29. Body and relative organ weights in the pyrethroid treated rats.	118
Table 30. Lipid peroxidation# in various tissues of rats treated with a mixture of pyrethroids.	118
Table 31. Nitric oxide levels# in various tissues of rats treated with a mixture of pyrethroids.	118
Table 32. Catalase activity (K30#) in various tissues of rats with a mixture of pyrethroids.	118
Table 33. Glutathione peroxidase activity# in various tissues of rats with a mixture of pyrethroids	118
Table 34. Glutathione S- transferase activity# in various tissues of rats with	

a mixture of pyrethroids.	118
Table 35. Liver function in rats treated with a mixture of pyrethroids.	121
Table 36. Kidney function in rats treated with a mixture of pyrethroids.	121
Table 37. Lipid profile in rats treated with a mixture of pyrethroids.	122
Table 38. Body and relative organ weights in rats treated with a mixture of pyrethroids for 1 to 15 months.	126
Table 39. Lipid peroxidation# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.	126
Table 40. Nitric oxide levels# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.	127
Table 41. Catalase activity (K30#) in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.	127
Table 42. Glutathione peroxidase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.	128
Table 43. Glutathione S- transferase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.	128
Table 44. Superoxide dismutase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months	129
Table 45. Liver function in rats treated with a mixture of pyrethroids for 1 to 15 months.	131
Table 46. Kidney function in rats treated with a mixture of pyrethroids for 1 to 15 months.	132
Table 47. Lipid profile in rats treated with a mixture of pyrethroids for 1 to 15 months.	133
Table 48. Complete blood picture profile in rats treated with a mixture of pyrethroids for 1 to 15 months.	134
Table 49. Histopathological changes in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.	136
Appendix -1. List of proteins that were commonly present in the sperm lysates of biological triplicates of control rats.....	90-10

Abbreviations

µg	:	microgram
µM	:	micromolar
3β-HSD	:	3 beta-hydroxysteroid dehydrogenase
17β-HSD	:	17 beta-hydroxysteroid dehydrogenase
Ad4b/SF1	:	Adrenal 4 binding protein/steroidogenic factor-1
ALP	:	Alkaline phosphatase
AR	:	Androgen receptor
cAMP	:	Cyclic adenosine monophosphate
CREB	:	cAMP-responsive element binding protein
CDNB	:	1-Chloro-2,4-dinitrobenzene
Ct	:	Cycle threshold
DHT	:	Dihydrotestosterone
E2	:	Estradiol
EDTA	:	Ethylenediaminetetraacetic acid
FACS	:	Fluorescence-activated cell sorting
FITC	:	Fluorescein isothiocyanate
FSH	:	Follicle stimulating hormone
FSHR	:	Follicle stimulating hormone receptor
gm	:	gram

GnRH	:	Gonadotrophin releasing hormone
GST	:	Glutathione-S-transferase
Gpx	:	Glutathionperoxidase
HD	:	High dose
H₂O₂	:	Hydrogen peroxide
KISS1	:	Kisspeptin
LD	:	Low dose
LDH	:	Lactate dehydrogenase
LH	:	Luteinizing hormone
LHR	:	Luteinizing hormone receptor
MDA	:	Malondialdehyde
Mg	:	milligram
Min	:	minute
ml	:	millilitre
NADP	:	Nicotinamide adenine dinucleotide phosphate
PBO	:	Piperonyl butoxide
PBS	:	Phosphate-buffered saline
PCNA	:	Proliferating cell nuclear antigen
PFA	:	Paraformaldehyde
PPM	:	Parts per million
qPCR	:	Quantitative polymerase chain reaction
RT	:	Room temperature

SGPT	:	Serum glutamic pyruvic transaminase
SGOT	:	Serum glutamic oxaloacetic transaminase
SOD	:	Superoxide dismutase
STAR	:	Steroidogenic acute regulatory protein
T	:	Testosterone
TBA	:	Thiobarbituric acid
TCA	:	Trichloroacetic acid
TEP	:	1, 1, 3, 3-tetraethoxy-propane
<i>TGF-β</i>	:	Transforming growth factor- β
WNT	:	Wingless-type MMTV integration site family
WT1	:	Wilms' tumor suppressor 1
VGCC	:	Voltage gated calcium channel
WHO	:	World health organization

ABSTRACT OF THE STUDY

Background

Humans are exposed to pyrethroid-based pesticides through agricultural produce. Consumption of agricultural products that contain residual amounts of pesticides has become inevitable in the current day world. Studies on the effects of unintentional intake of pyrethroid based pesticides that are akin to actual human exposure settings are not reported.

Methods

Dosages and choice of pyrethroids to be administered for animal experimentation were fixed based on: (1) the type and residual amount of pyrethroids present in rice and vegetables as per the report of the Project Coordinating Cell, All India Network Project on Pesticide Residues, Indian Agricultural Research Institute, New Delhi – 110 012; and (2) on the amount of rice and vegetables consumed by an average Indian as per the report published by National Institute of Nutrition, Hyderabad, India. Basing on these reports, it is estimated that the daily per kg body weight exposure to a mixture of pyrethroids (allethrin, cypermethrin, deltamethrin, cyhalothrin L, and fenvalerate) through rice and vegetables in an average Indian is 15.695 µg. The mixture of pyrethroids to be administered was prepared in corn oil.

The duration of treatment was fixed on the basis of the fact that one month's lifetime in rat is equal to 2.5 years in humans. We used rats that are 45 days old (1.5 months), whose age resembles approximately to that of 4-year-old humans. A 15-month treatment in rats is equivalent to 37.5 years of exposure equivalent in humans. Thus, the duration of treatment is a direct reflection of the long-term exposure of humans to pyrethroids through food on a daily basis starting from childhood stage to middle-aged life. At different points after treatment (1, 3, 6, 9, 12, and 15 months), animals were sacrificed, tissues collected, and further analyses conducted.

Animal experimentation was conducted by two protocols. In the experimental protocol - I (**EP-I**), rats were treated daily for 9 to 12 months with a mixture of pyrethroids (allethrin, cypermethrin, deltamethrin, cyhalothrin L, and fenvalerate) with doses that are one-fifth ($15.695/5 = 3.319$ µg per kg body weight; termed as high dose; **HD**) or one-twenty fifth ($15.695/25 = 0.6278$ µg per kg body weight; termed as low dose; **LD**) of the amount being exposed by humans through rice and vegetables. In the experimental protocol – II (**EP-II**), rats were treated daily for 1 to 15 months with a mixture of pyrethroids (as mentioned above) at the equivalent amounts (15.695 µg per kg body weight) present in the rice and vegetables consumed by an average Indian.

Fecundity was determined by natural mating of the animals. Sperm function was assessed by using fluorescence-based flow cytometry. Loss of cholesterol and sperm glycoproteins during capacitation and acrosome reaction was monitored by the fluorescent dyes filipin and PNA-FITC, respectively. Dynamics of expression of genes involved in spermatogenesis, steroidogenesis, germ cell epigenetic regulation and apoptosis were assessed by real-time PCR. Differential expression of sperm proteins in the control and pyrethroid treated rats was analysed by LC-MS. Oxidative stress was measured as a function of the levels of lipid peroxidation and nitric oxide and activities of antioxidant enzymes (catalase, GST, GPx, and SOD) in different tissues were assayed by spectrometry. Anatomical changes were evaluated by histopathological analyses.

Results

A. Effects on male reproductive physiology: biochemical aspects

In both the experimental protocols (EP-I and EP-II), fecundity, sperm count, and sperm function (capacitation and acrosome reaction) were significantly compromised in the pyrethroid treated rats compared to control. Decreased levels of 3β - and 17β -HSD activity was evident in all the pyrethroid treatment groups. Disturbances in the levels of FSH, GnRH, INHB, KISS1, LH, TSH, PTL and testosterone was evident in the pyrethroid treated rats. Increased lipid peroxidation and disrupted activity of antioxidant enzymes was evident in all the tissues obtained from pyrethroid treated rats. Severe anatomical damage was noticed in the testis, caput, cauda, and prostate of pyrethroid treated rats, which correlated with the dose and duration of exposure.

B. Effects on male reproductive physiology: transcriptome and proteome level

The relative expression of genes that govern spermatogenesis, steroidogenesis, germ cell epigenetic changes and apoptosis were perturbed in the testes of pyrethroid treated rats. Differential expression of 506 proteins was evident in the sperm lysates of rats treated with a pyrethroid mixture for 6 months. To determine whether the differential expression of proteins observed in the spermatozoa of pyrethroid treated could also be transgenerational, the differentially expressed proteins identified in the sperm lysates of F0 rats were analysed in the F1, F2 and F3 generations. We observed that the differential expression observed for a majority of proteins identified in the sperm lysates of F0 rats remained the same in the next generations. 22 proteins that were down regulated in the F0 generation continued to remain under expressed in the F1, F2 and F3 generations. Similarly, 45 proteins exhibited under expression up to F2 generation, while only 1 protein exhibited continued to be under expressed up to F1

generation. 4 of the up regulated proteins in the sperm lysates of F0 generation continued to be over expressed up to F3 generation. 1 protein each was found to be over expressed up to F2 and F1 generations. Among the proteins that continued to be differentially expressed in the later generations, some of them are reported to have critical roles in male reproduction.

C. Effects on the general physiology

Liver function was compromised as evident by the elevated levels of serum SGPT, SGOT and ALP activity in the pyrethroid treated rats. No effect on kidney function was evident. Disturbances in the lipid profile (cholesterol, LDL, HDL and VLDL) in the pyrethroid treated rats was observed. Except for platelet count, no significant changes in the complete blood profile were evident. Severe anatomical damage in the liver, lung and kidney was noted at all the time points tested and the severity of damage appears to increase with the time of exposure.

Conclusions

Exposure to low doses of pyrethroids that are relevant / equivalent to the amounts routinely consumed by Indian through rice and vegetables resulted in loss of reproductive function both at physiological and molecular level. Further, the functioning of organs in general physiology was compromised. Results of this study clearly indicate that long term exposure to pyrethroids through food affects multiple organs and to the best of our knowledge, this is the first study to report such a conclusion. Interventions to reduce the usage of pyrethroid based pesticides for agriculture and enhanced technology to eliminate residual levels of pesticides during food processing are necessary to prevent the silent killing effects of these chemicals.

Chapter 1

General Introduction

1.1 Historical aspects of pesticides

Botanical derivatives as pesticides have been used for many centuries and are known for their eco-friendly nature (1,2). The oldest herbal knowledge Ebers Papyrus described more than 800 medical recipes which included pesticides as ingredients. Sulfur and arsenic were used as pesticides for pest management from the times of Romans. Nicotine sulfate, calcium arsenate, and sulfur were in use in the late 1800s for crop protection. A major breakthrough in the development of pesticides started during World War II with the production of environmentally persistent first-generation synthetic pesticides namely, the organochlorines; and prominent among them were aldrin and dichlorodiphenyltrichloroethane (DDT). Second-generation organophosphate pesticides such as malathion were discovered between 1950 and 1955 followed by triazine weedicides during 1955–1960 and their usage reached its peak by 1961. Owing to the public attention toward excessive usage of pesticides and the health hazards elegantly described in the famous book “Silent Spring”, the use of pesticides decreased drastically by 1962 (3). Following this, a new method of integrated pest management (IPM) came into existence in which biological predators or parasites were used to control pests. Albeit the population of pests reduced significantly, IPM methods did not display substantial effects akin to chemical pesticides. In the years between 1970–1980s, pyrethroids, sulfonylureas, synthetic fungicides and other chemical pesticides were introduced. DDT was completely banned in the USA followed by restrictions on usage of endosulfan, dieldrin, and lindane in the early 1970s. An international treaty derived at Stockholm Convention held in 2001 to which 179 nations were signatories, resolved to completely ban the use of twelve persistent organic pollutants including DDT. On the same lines, European Union (EU) supported to ban on the use of nicotinoids in 2013.

1.2 Pesticide usage

Pesticides, including synthetic pyrethroids are among the extremely useful as well as harmful agents to human welfare because of their widespread applications (4,5). Pesticides have multiple benefits. They are used to avert crop loss to alleviate the global problem of hunger (6). Approximately, 1/3rd of agriculture production depends on pesticide use and thus has become indispensable. Despite pesticide usage, crop loss due to pests and diseases is around 40% and it is anticipated that it could be double in absence of pesticides. Only a minute quantity (0.3%) of the pesticide sprayed on the crop reaches the target site, while the rest of it leads to contamination of the surrounding environment. Most of the pesticides are heat stable,

polar, and water-soluble, which makes it difficult to reduce their lethality. The global usage of different chemical pesticides, which include insecticides, weedicides, fungicides and bactericides were projected for the year 2020. It is estimated that about 34,71,780 metric tons will be used globally in 2020 (7). Based on these projections, the percent use of different pesticides is presented in figure 1. Worldwide, weedicidal pesticides are consumed at a higher percentage (40%) than other types of pesticides with China ranking as the top consumer (8).

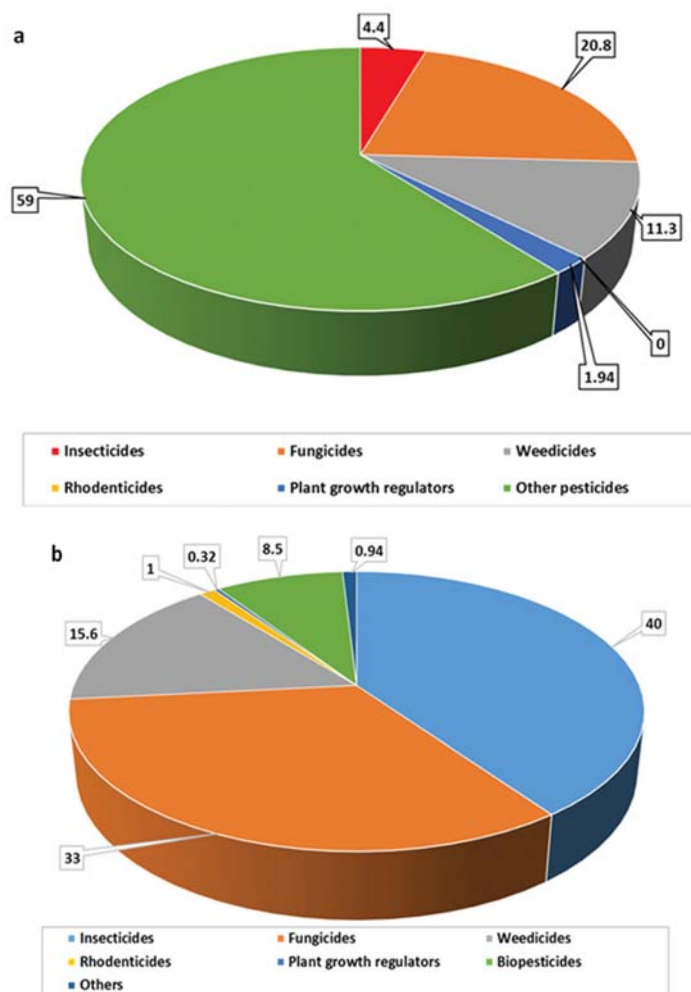


FIGURE 1. Projected usage of pesticides. a. Projected use of pesticides globally in 2020. The global consumption of different pesticides was predicted. The percent of each pesticide was calculated using its fraction out of the total pesticide amount b. Pesticide usage in India during 2018–2019. The percent of each pesticide was calculated using the data provided by the Directorate of Plant Protection, Quarantine and Storage (QPPQS). The consumption of both indigenous and imported pesticides was combined to calculate the percentages (figure from Ravula AR and Yenugu S (2021) Critical Reviews in Toxicology).

In India, The Directorate of Plant Protection, Quarantine and Storage (DPPQS), an apex agriculture institute funded by the Government of India, provided the statistics on the projected

consumption pattern of different pesticides for the year 2019–2020 (figure 1) (9). Albeit the fact that pesticides improved the living standards of human health by controlling vector-borne diseases, according to World Health Organization (WHO), indiscriminate usage of pesticides mainly in developing countries causes approximately 3,000,000 poisonings and 220,000 deaths every year. Infants, children, agriculture workers and pesticide applicators are more susceptible to pesticide toxicity than the general population. Pesticides enter into the ecosystem when the water-soluble compounds are released into the aquatic systems, while the fat-soluble compounds enter into animal tissues by bioamplification. Excessive usage of pesticides led to their entry into the food chain and thus causing respiratory, carcinogenic, neurological, reproductive and endocrine abnormalities (10). Consumption of pesticide-contaminated food is the major gateway for their entry into the human body, apart from accidental ingestion, inhalation and dermal exposure (10). The effects of pesticides on human health are highly variable and are classified as acute or short term (symptoms appear immediately or within hours) and chronic or long term (symptoms appear after months or years) (11).

1.3. Classification of pesticides

Pesticides are broadly classified based on their origin i.e. natural (biological) or chemically synthesized. Based on the chemical structure and target of action, they are divided into five major categories namely, insecticides, weedicides, fungicides, rodenticides and fumigants and other minor categories like molluscicides and nematocides (figure 2).

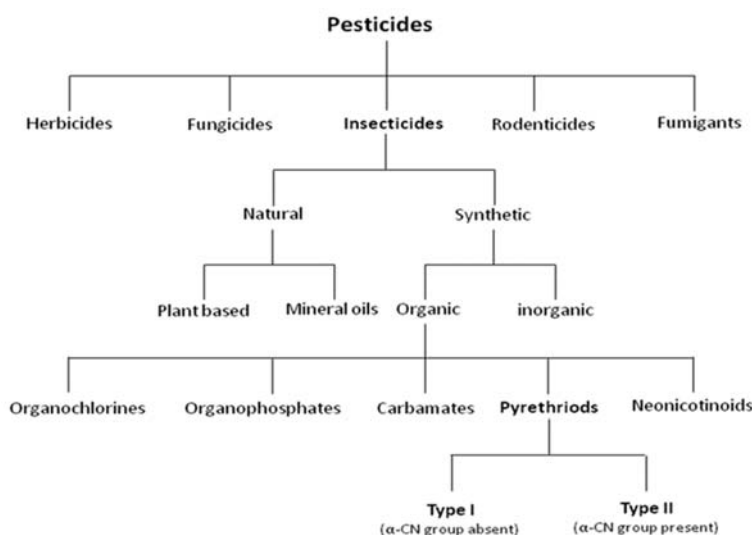


FIGURE 2. Classification of pesticides (figure from Ravula AR and Yenugu S (2021) *Critical Reviews in Toxicology*).

1.3.1. Natural pesticides

These are also referred to as biopesticides and a variety of them are botanically derived compounds, antibiotics from microbes, pheromones of insects, microbial organisms, entomophagous nematodes, etc. The most popular among them are of botanical origin that includes crude extracts or purified compounds derived from different plants. Botanical pesticides have been used for centuries to protect stored food items, repelling household pests and also as fragrances (2). Pyrethrum, azadirachtin nicotine, sabadilla, ryania, fluoroacetate, carboxin and Cry proteins of *B. thuringiensis* are used as insecticides, whereas laminarine, fennel oil, lecithin are known to exhibit fungicidal properties. Citronella and pine oil derived from products are used as repellents and herbicides, respectively (2). The target organs of the botanically based natural pesticides are diverse. The insecticides nicotine, sabadilla and pyrethrum act on the nerve cells, rotenone targets mitochondrial electron transport, ryania is a stomach poison. Biopesticides are known to have very low toxicity on mammalian physiology and are also cost-effective and hence are increasingly being recognized throughout the world for plant protection in an eco-friendly manner.

1.3.2. Synthetic pesticides

Among the synthetic pesticides, insecticides rank the highest in terms of toxicity followed by fungicides and weedicides. Insecticides include organochlorines (OCs), organophosphates (OPs), carbamates, pyrethroids (PYs) and neonicotinoids (NEs). OCs are synthetic chlorinated hydrocarbons classified as highly persistent organic pollutants (POPs) and are widely used in agriculture and mosquito control. The overall statistics indicate that 40% of all pesticides used belong to OCs (12,13). The representative compounds that are highly used in this class are DDT, hexachlorocyclohexane (HCH), aldrin and dieldrin (12,13). DDT-type insecticides cause acute toxic effects in animals by preventing the deactivation or closing of sodium gates of axons, thereby resulting in hyperexcitation of the nervous system (14). OPs or esters of phosphoric acid are ecologically good alternatives to OCs as they are not persistent in the environment. The most common of this class is glyphosate, a weedicide. The other OPs include malathion, parathion, dimethoate and chlorpyrifos. Global usage of organophosphate insecticides is more than 30% according to the reports of the Environmental Protection Agency (EPA), USA. In insects and mammals, OPs affect neuromuscular transmission by interfering with acetylcholinesterase (AChE) in cholinergic synapsis via phosphorylation of this enzyme resulting in excess accumulation of acetylcholine (ACh). CAs are derived from carbamic acid

and the first one among this class, carbaryl, was introduced in 1956 as a lawn and garden insecticide. Its broad-spectrum insect control activity and low toxicity to mammals allowed it to be a preferred choice. CAs are less persistent in the environment unlike OPs and OCs and are rapidly detoxified in animal tissues. The mode of action of CAs is similar to that of OPs as both are inhibitors of acetylcholinesterase (AChE). Pyrethrum is a natural resin extracted from dried flowers of pyrethrum daisy, *Chrysanthemum* (or *Tanacetum*) *cinerariaefolium*. This resin contains six esters of chrysanthemic and pyrethric acids. Those incorporated with pyrethrolone, form pyrethrins I and II and display insecticidal activity. Pyrethrins form 80% of the global insecticide market (2). The modern synthetic pyrethroids that are stable in the sunlight are derived from natural pyrethrins and account for about one-sixth of global insecticidal sales with a turnover of US\$1.4 billion.

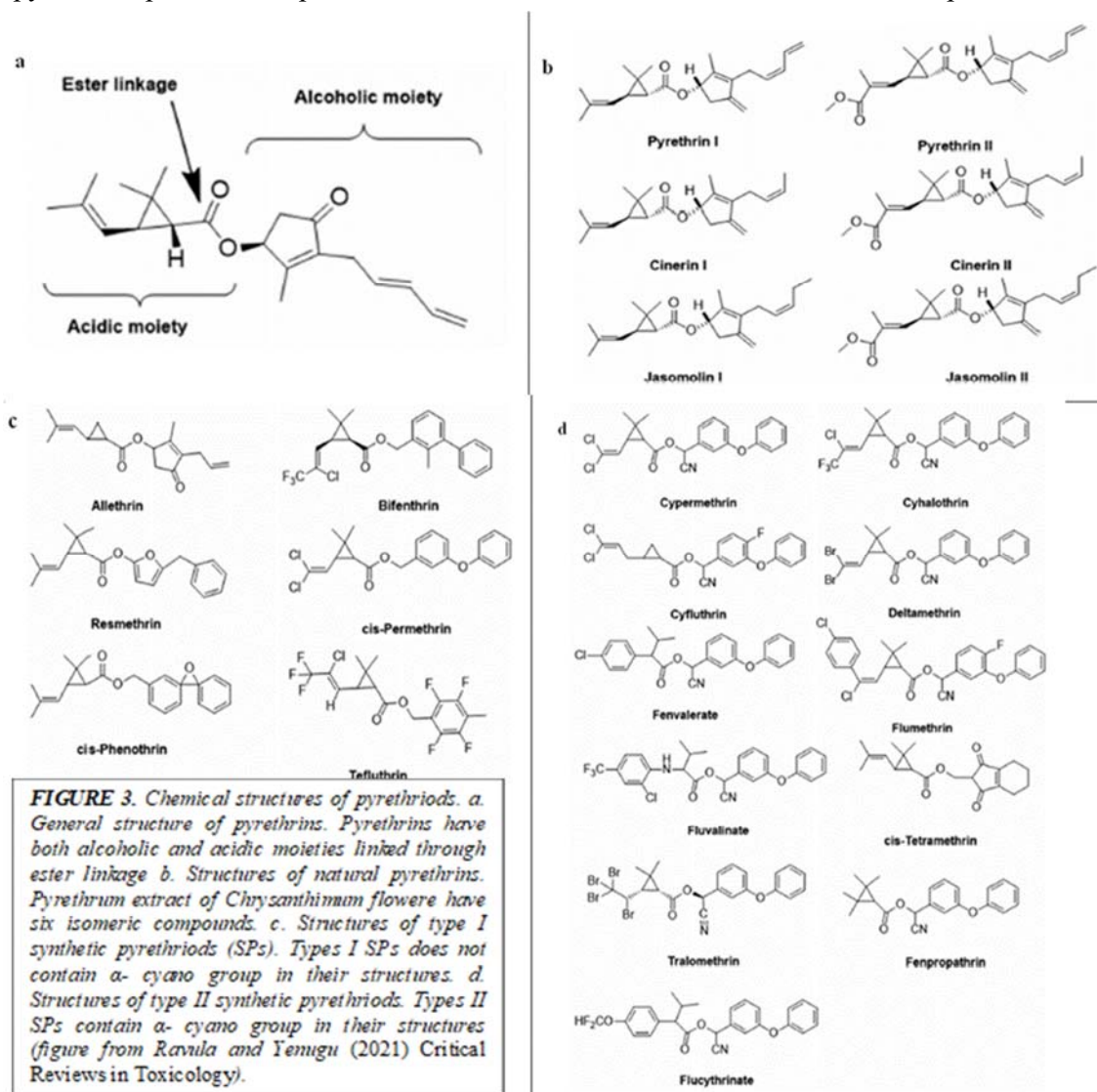
1.3.3. Pyrethroids

The first synthetic pyrethroids, allethrin and bioallethrin were developed in 1949. Resmethrin, the first generation synthetic pyrethroid was developed from naturally occurring pyrethrins by altering their structure to increase stability in sunlight and insecticidal activity in 1962. By 1983, pyrethroids were applied to crops in over 33 million hectares annually and constituted 25.1% of the global insecticide market (15). In late the 1980s, World Health Organization (WHO), recommended use of pyrethroids including deltamethrin and permethrin owing to the less environmental persistency and toxicity to humans and other mammals. Pyrethroids such as cypermethrin and deltamethrin were used in long-lasting insecticidal nets (LLINs) for the malaria control programme of WHO.

1.3.4. Chemistry, classification, and properties of pyrethroids

A naturally occurring pyrethrum extract of *chrysanthemum* flower contains six types of compounds, namely, pyrethrins I, cinerins I, jasmolins I, pyrethrins II, cinerins II and jasmolins II. The chemical structures of these compounds are shown in figure 3. The first three are esters of chrysanthemic acid whereas the latter three are esters of pyrethric acid. Pyrethrin composition in pyrethrum extract is 45–55% with the proportion of pyrethrin I and II in the ratio of 0.2:2.8, whereas the ratio of pyrethrins: cinerins: jasmolins is 71:21:7. Pyrethrin I contributes to insecticidal activity, while pyrethrin II provides rapid knock-down. Chemically, natural pyrethrins contain an acid moiety i.e cyclopropane carboxylic acid and an alcohol moiety i.e., cyclopentenolone. The diversified pyrethroids are produced by adding specific

functional groups to the moieties of natural pyrethrins. The structures of type I and type II pyrethroids differ with respect to the position of a cyano and other functional groups. Owing to the presence of two chiral or asymmetric centers at carbon- 1 and 3 of an acid moiety, pyrethrin I produce two pairs of diastereomers indicated as cis and trans that depends on the



orientation of functional group on these positions with respect to cyclopropane ring or similar structure applied to replace this ring. In the synthetic compounds, a total of eight stereoisomers are possible due to the presence of three asymmetric centers: one and two on alcohol and acid moiety, respectively. In the natural pyrethrin's, acidic moieties are specifically in the 1 R, trans (absolute) configuration. Esters with R configuration at cyclopropane C-1 of these chrysanthemic acid isomers are more insecticidal whereas those isomers with enantiomeric 1S configuration are not insecticidal albeit displaying identical physical

properties. Although both *cis* and *trans* show insecticidal activity, the *cis* isomer is relatively more potent.

Pyrethroid pesticides are categorized into two groups, type I and type II based on behavioural toxicity and on the presence or absence of α -cyano group in their structures. Most of them are placed in category class I and II based on their acute toxicity in rodents. Allethrin was the first candidate of type I synthetic pyrethroid identified. Other known type I pyrethroids are permethrin, resmethrin, bifenthrin, d-phenothrin and tetramethrin. So far, identified Type II pyrethroids are cypermethrin, deltamethrin, cyhalothrin (λ), cyfluthrin and fenvalerate. Type I pyrethroids do not have the α -cyano group and hence are less toxic, whereas Type II pyrethroids that contain α -cyano group are highly toxic. Based on the photostability, pyrethroids are divided into two groups, viz first generation and second generation. The first-generation photolabile synthetic pyrethroids (resmethrin, tetramethrin, phenothrin) are the derivatives of chrysanthemic acid esters, produced by substitutions to alcohol moiety. Second generation synthetic pyrethroids (permethrin, cypermethrin and deltamethrin) with high potency and photostability were produced by systemic changes in both acid and alcohol moieties. The physical properties of pyrethroids are low vapour pressure, low Henry's law constants, larger octanol-water ratio coefficients (K_{ow}), sparingly soluble in water, enantiomers with identical physical properties and diastereomers with different physical properties (16).

1.3.5. Routes of exposure, absorption, metabolism, and excretion of pyrethroids

The main routes for human exposure to pesticides are physical contact, ingestion and inhalation, whereas spills at storage houses also contribute to a certain extent (17). Since pyrethroids are being used in various domestic applications including household products, impregnated cloths, repellents, and shampoos, they can also be absorbed through skin and can also be exposed through floor wipes and floor dust. Children are more prone to exposure of pyrethroid-based products during treatment of scabies and lice. The rate of oral absorption of pyrethroids may also depend on the type of vehicle in which the target compound is mixed. Most pyrethroids or their metabolites are not accumulated in any tissues or organs as they are metabolized within 16–24 h after absorption. However, some of them are detected in fat tissues due to high lipophilicity. Based on several studies carried out in rodents, it is evident that the metabolism of more than 30 pyrethroids involves oxidation, hydrolysis of ester bond (phase I) and conjugation (phase II) to generate hydrophilic and lipophilic forms. Pyrethroids cause lesser acute toxicity in mammals due to their rapid metabolism. The plasma half-life of

pyrethroids is less than 8 h and some high lipophilic compounds like permethrin remain in tissues up to 24 h. 3-phenoxybenzoic acid (3-PBA), one of the common and non-specific metabolites of all pyrethroids. The metabolism of pyrethroids, primarily in humans and rat, involve oxidation and ester hydrolysis mediated by cytochrome p450 isoforms and carboxylesterases, respectively. Six rat-specific cytochrome P450 isoforms (CYP1A1, CYP1A2, CYP2C6, CYP2C11, CYP3A1 and CYP3A2) and four human-specific isoforms (CYP2C8, CYP2C9, CYP2C19 and CYP3A4) are implicated in the metabolism of most pyrethroids. The cleavage of ester bond by hydrolysis is catalyzed by carboxylesterases and the liver has the highest hydrolase activity. The biotransformation and enzymatic reactions of synthetic pyrethroids in mammals are extensively reviewed (18).

1.3.6. Mechanism and toxicity of pyrethroids

Most of synthetic pesticides including insecticides primarily target the components of nervous system. The primary insecticidal activity of pyrethroids is to disturb the nerve membrane of insects by delaying the closure or inactivation or long-term opening of voltage-sensitive sodium channels (VSSC) that leads to more sodium ions crossing the membrane and depolarizing the neural membrane beyond the normal extent (**figure 4**). Some of the pyrethroids also act on voltage-gated chloride and calcium channels as secondary target sites. The immediate signs of pyrethroidal intoxication in insects are tremors and hyperexcitation followed by paralysis. Pyrethroids are less toxic for mammals and birds than fishes and insects due to rapid detoxification and less intestinal absorption in homoeothermic organisms.

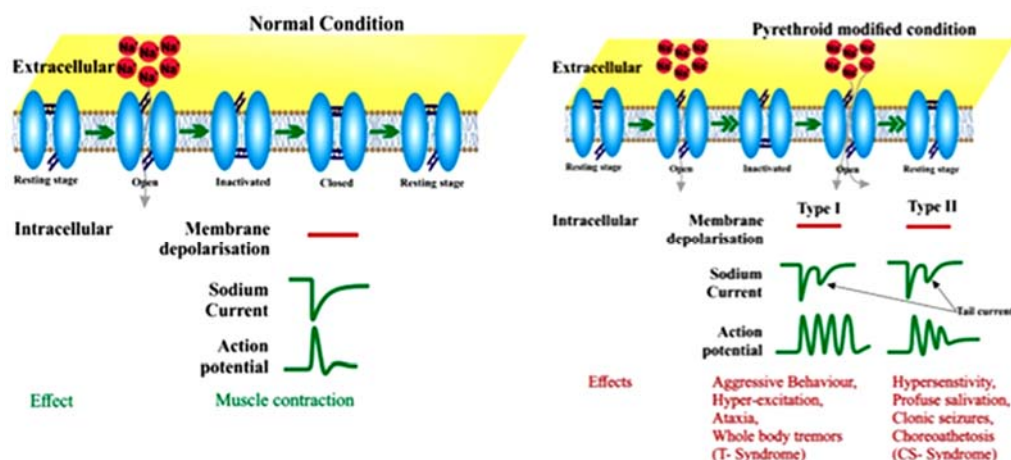


FIGURE 4. Molecular mechanism of action of pyrethroids on nervous system. Under normal conditions, VSSCs are in resting state (closed). An extracellular stimulus results in opening of VSSCs to allow influx of Na^+ ions. Entry of Na^+ creates an action potential, which diminishes when the VSSCs revert to resting state. The downstream effect of action potential generates many physiological responses such as muscle contraction. The toxic action of type-I or type-II pyrethroids results in continued opening of VSSCs to allow prolonged influx of Na^+ influx and persistent action potential. The consequences of the action of type-I or type-II pyrethroids leads to development of T- and CS-syndrome respectively (figure from Ravula and Yenugu (2021) Critical Reviews in Toxicology).

Pyrethroids are 2250 times more toxic to insects than higher organisms because of the smaller size, low body temperature and excess expression of sensitive sodium channels with small structures. Based on the acute toxicity of hazardous chemicals and their LD50 values, they are classified into five classes as per WHO and GHS guidelines. Similarly, based on the acute toxicity (LD50 values) in the rat, synthetic pyrethroids are classified as moderately hazardous (class II) for which the oral LD50 values are between 50 and 500 mg/kg bw (solids) or 200–2000 mg/kg body weight (liquids). To sustain the degree of toxicity and stability, commercial formulation of pyrethroids is mixed with synergistic compounds such as piperonylbutoxide (PBO), MGK-264, N-octyl bicycloheptene dicarboximide, sulfoxide, petroleum distillates and sesame oil (18,19)

1.3.7. Health hazardous of pyrethroids

Several studies have reported the health effects associated with synthetic pyrethroids in laboratory animals as well as in humans (general population and agriculture workers). They are summarized in figure 5.

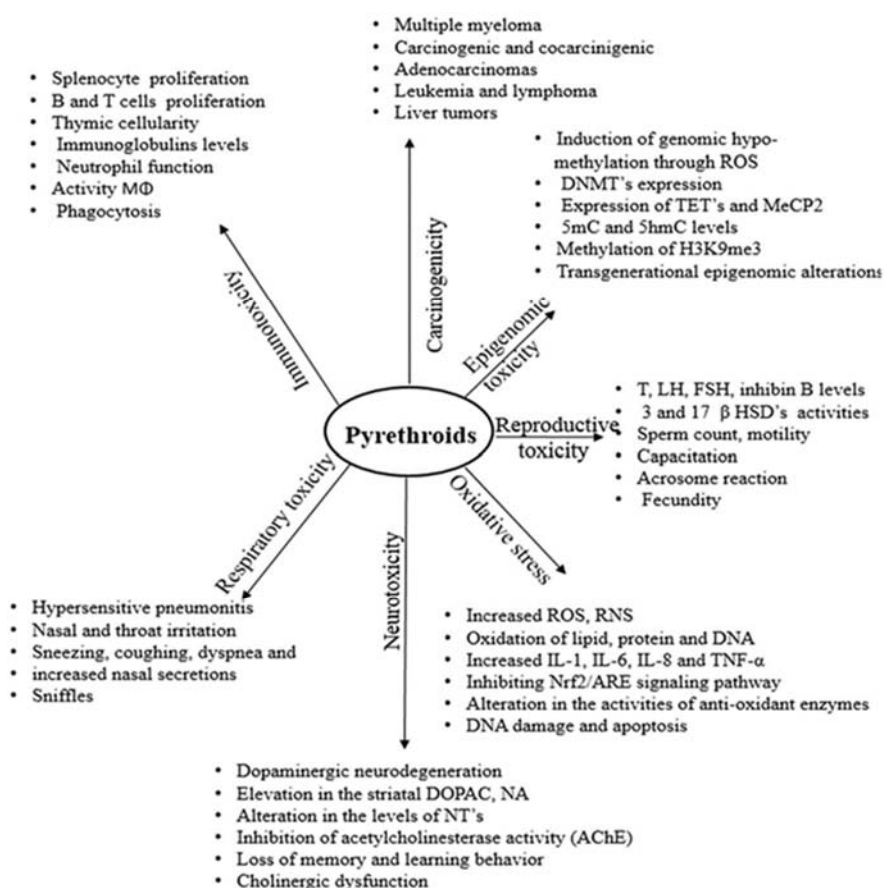


FIGURE 5. Pathology of pyrethroid toxicity. The potential health hazards of pyrethroids are depicted. The functional, biochemical, and molecular events that may occur in each of the pathologies are indicated (figure from Ravula AR and Yenugu S (2021) *Critical Reviews in Toxicology*).

1.4. The male reproductive system

1.4.1. Anatomical aspects

In mammals, the male reproductive system contains well vascularised internal structures, including a pair of testes, epididymis, vas deference, prostate, seminal vesicles and external structures like penis and scrotum, with many glands and ducts (figure 6). The testis is well organised and compartmentalised into two district regions i.e. interstitial space and seminiferous tubules. Specialised somatic cell types namely, Leydig cells (LCs; localised in the interstitial space) and Sertoli cells (SCs; present in the seminiferous tubules), are crucial for steroidogenesis and spermatogenesis. In the mammalian body, the testis is the site for immune privilege, a specialised status of sites in the body where foreign (allo) and self (auto) antigens are tolerated as it sequesters the antigens and antibodies from the immune system through blood-testis barrier (BTB). The spermatozoa in the testis are immature and acquire maturation for their motility and fertilization capabilities while transiting via epididymis, a long and coiled tube with duct-like organ composed of the initial segment, head (caput), body (corpus) and tail (cauda) regions (20). The cauda is the site for the storage of matured sperm. The prostate is a walnut-sized structure situated below the bladder and its secretions nourish the sperm. Seminal vesicles are sac-like pouches that are attached to the vas deferens, and they produce sugar (fructose) rich fluid which acts as an energy source for developing sperms to gain motility. Penis, an external male organ meant for sexual intercourse and to transport both semen and urine out of the body. The Scrotum, a pouch-like sac of the skin, hangs behind the penis, and it holds and protects the testes by controlling testicular temperature slightly cooler ($\approx 2\text{-}3\text{ }^{\circ}\text{C}$) than that of body temperature suitable for the normal development of sperm.

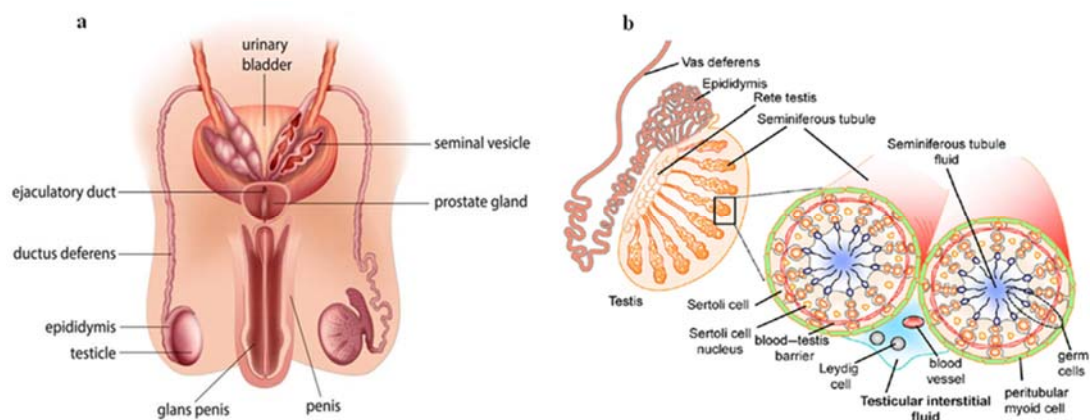


FIGURE 6. Male reproductive system. *a. The male reproductive system contains testes, epididymides, ductus deferens, ejaculatory ducts, seminal vesicles, prostate gland, and penis. b. Structure of testis. Cross-section and cellular components of testis (figure from Joseph O'Neill et al., 2017 and Cheng et al., 2017).*

1.4.2. Hormonal regulation of the male reproductive system

The male reproductive system is under the control of the master gland of the endocrine system i.e., the hypothalamus (figure 7). Most of the molecules derived from the hypothalamus of the brain are endocrine in nature since they exert their activity in extra brain tissues. Such a control on the male gonads i.e., the testes by the hypothalamus through the pituitary is called as Hypothalamo pituitary testicular (HPT) axis. Peptides of the hypothalamus (kisspeptins) are the master and principal regulators of reproduction. They are encoded by the gene *KiSS1* and act as key neuromodulators to control the onset of puberty by acting on the upstream of gonadotrophin-releasing hormone (GnRH). Kp-54 (formerly known as metastin) the major form of kisspeptins, binds to its receptor, *KiSS1R* or *GPR54* (G-protein coupled receptor 54) present on the GnRH neurons within the hypothalamus and stimulates them to release GnRH. The GnRH1, the major isoform in mammals, binds to gonadotrophs of the anterior pituitary through its receptor, *GnRH1R* to secrete luteinising hormone (LH) and follicle-stimulating hormone (FSH). The testicular functions, namely, steroidogenesis and spermatogenesis are mediated by LH and FSH through the corresponding receptors, luteinising hormone receptor (LHR) and follicle stimulating hormone receptor (FSHR) expressed on the LCs and SCs respectively.

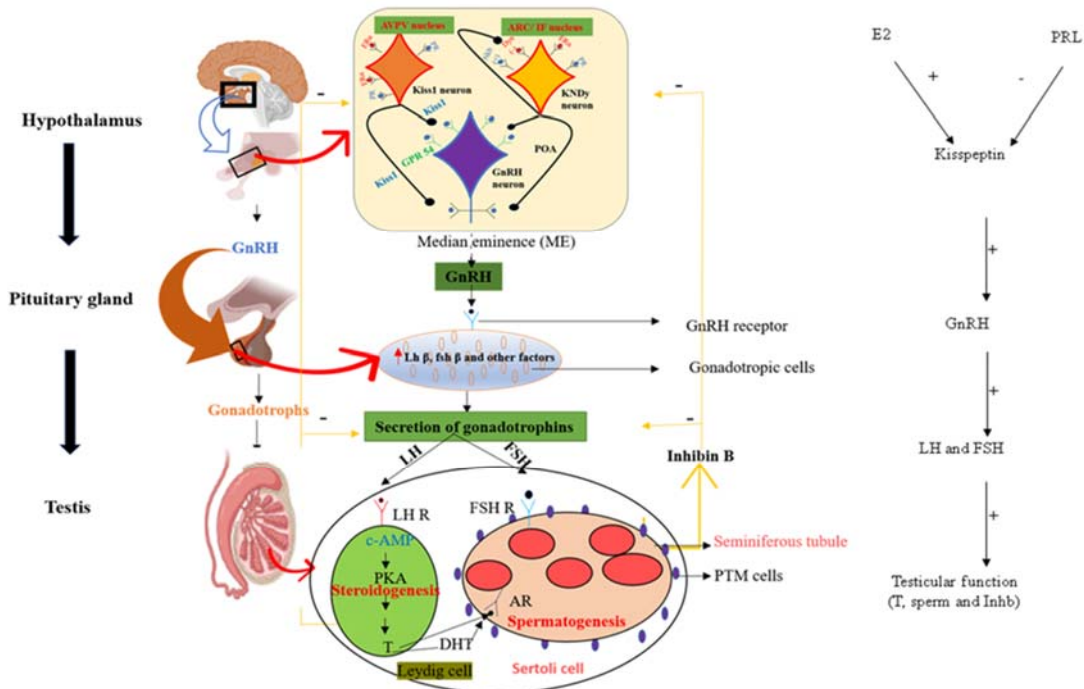


FIGURE 7. Regulation of male reproductive function through hypothalamic-pituitary-testicular (HPT) axis. Kisspeptin neurons of AVPV and ARC nucleus of hypothalamus control the secretion of GnRH which regulates the pulsatile release of pituitary gonadotrophins which in turn regulates the testicular function. The HPT axis controls the male reproduction through hormonal feedback regulation.

1.4.3. Organisation and functions of testis

Externally, the testes are covered by tunica albuginea and have two internal compartments; seminiferous tubules contain SCs and developing germ cells within the seminiferous epithelium (SE), and interstitial space having LCs and blood vessels. In adult rats, SCs occupy close to 20 % volume in SE and exhibit cytoplasmic projections to support developing germ cells. The testis has a specialised, unique, and important ultrastructure called the blood-testis barrier (BTB) (21). BTB is created by actin-based tight junctions between two adjacent SCs to support spermatogenesis by providing an adequate specialized microenvironment for the development of post-meiotic germ cells. The BTB divides SE of tubules into two compartments across the adjacent SCs i.e., the basal compartment present below or outside the barrier where self-renewal and proliferation of spermatogonial germ cells takes place; and the apical or adluminal compartment present above or behind the barrier where meiosis, spermiogenesis and spermiation occurs (22). BTB acts as an immunological barrier that restricts or limits the accessibility of immune system. LCs and SCs present in the seminiferous tubules are primarily involved in steroidogenesis (production of testosterone) and spermatogenesis respectively.

1.4.4. Steroidogenesis and its regulation

During development, steroid hormones are essential for key physiological functions and maintenance of homeostasis. In males, the testis is the major reproductive organ responsible for producing testosterone and deficiency in adults is associated with many complications such as fat accumulation, fatigue, mood disorders, cognitive dysfunction, loss of muscle weight and bone density (23), immune suppression (24) and mortality in aged men (25). Production of testosterone is a multi-step enzymatic process that takes place in the LCs. Cholesterol, the initial precursor, and backbone molecule for all steroid hormones is processed by multiple enzymes to produce testosterone. During steroidogenesis, the first and common enzymatic step in all the steroidogenic tissues, is the cleavage of side chain of cholesterol, to result in conversion to pregnenolone by cytochrome P450 side chain cleavage enzyme (P450_{scc} or CYP11A1); and this takes place in the inner mitochondrial membrane. Pregnenolone diffuses into the smooth endoplasmic reticulum, and it is converted into progesterone by 3 β -hydroxysteroid dehydrogenase (3 β -HSD), which is further converted into androstenedione by 17 α -hydroxylase or 17-20 lyase or CYP17A1. Eventually, androstenedione gets converted into C19 testosterone by 17 β -hydroxysteroid dehydrogenase 3 (17 β -HSD3). Testosterone is peripherally metabolised into either oestradiol (E2) by aromatase or

dihydrotestosterone (DHT) by 5- α reductase (S5AR1). DHT is further metabolized into 5 α -androstane-3 α , 17 β -diol (DIOL) by 3 α -hydroxysteroid dehydrogenase (HSD3A). The DIOL is a weak and abundant androgen in rat immature LCs and it accounts for close to 90% of total androgen, while testosterone accounts only for 10%. Testosterone exists in two forms, namely, unbound (free) and bound. In normal males, only 2% exists as free form and 44% is bound to high-affinity sex hormone binding globulin (SHBG) and the remaining is bound to albumin and other proteins. Only free and albumin-bound forms constitute the bioavailable testosterone. Testosterone may directly diffuse into the SE to exert its spermatogenic function or metabolized into more potent androgen, DHT, or aromatized into more potent oestrogen, E2, in peripheral tissues (26).

Many factors affect the steroidogenic output of the male reproductive system (figure 8). LH is required for trophic and acute regulation of steroidogenesis as it balances the threshold levels of steroidogenic enzymes (trophic regulation) and cholesterol transportation into inner mitochondrial membranes (acute regulation). These trophic and acute responses of LH regulate through a signaling cascade that begins with cyclic adenosine monophosphate (c-AMP), a secondary messenger. The steroidogenic signal transduction begins with binding of pituitary LH to its receptor, (LHR) that belongs to G-protein coupled receptor (GPCR) family. Interaction between LH-LHR facilitates the activation of AC and increased in the intracellular c-AMP levels, activation of protein kinase A (PKA) which phosphorylates, particularly c-AMP response element binding protein 1 (CREB1) which stimulates steroidogenic factor 1 (SF1), which finally activates the cholesterol translocator proteins namely, steroid acute regulatory protein (StAR) and translocator proteins (TSPO). The process of steroidogenesis begins with stimulation of TPSO and StAR, a critical step during steroidogenesis, as the activated StAR regulates the activities of steroidogenic enzymes. The c-AMP-PKA dependent phosphorylation of CREB/CREM (pCREB/CREM), GATAA4 and c-Jun/ c-Fos (AP1) stimulates the binding of SF1 upstream side of the transcription start site (TSS) of the promotor of StAR gene and activates StAR that modulates the cholesterol transport and steroidogenic enzyme activities. The source of cholesterol for steroidogenic cells is either derived from lipoproteins present in circulation through membrane-bound scavenger receptor class B type I (SR-B1 or also called SCARBI) or synthesized within the cells from acetyl Co-A through de novo pathway. The major enzymes such as 3-hydroxy -3- methylglutaryl coenzyme A reductase (HMG-CoAR) and 3-hydroxy -3- methylglutaryl coenzyme A synthase (HMG-CoAS) is involved in the cholesterol biosynthesis via de novo pathway and HMG-CoAR1 and HMG-CoAS1 are the

major isoforms in rat and majority of steroidogenic factors, including StAR, CYP11A1, CYP17A1, HMG-CoAS, HMG-CoAR.

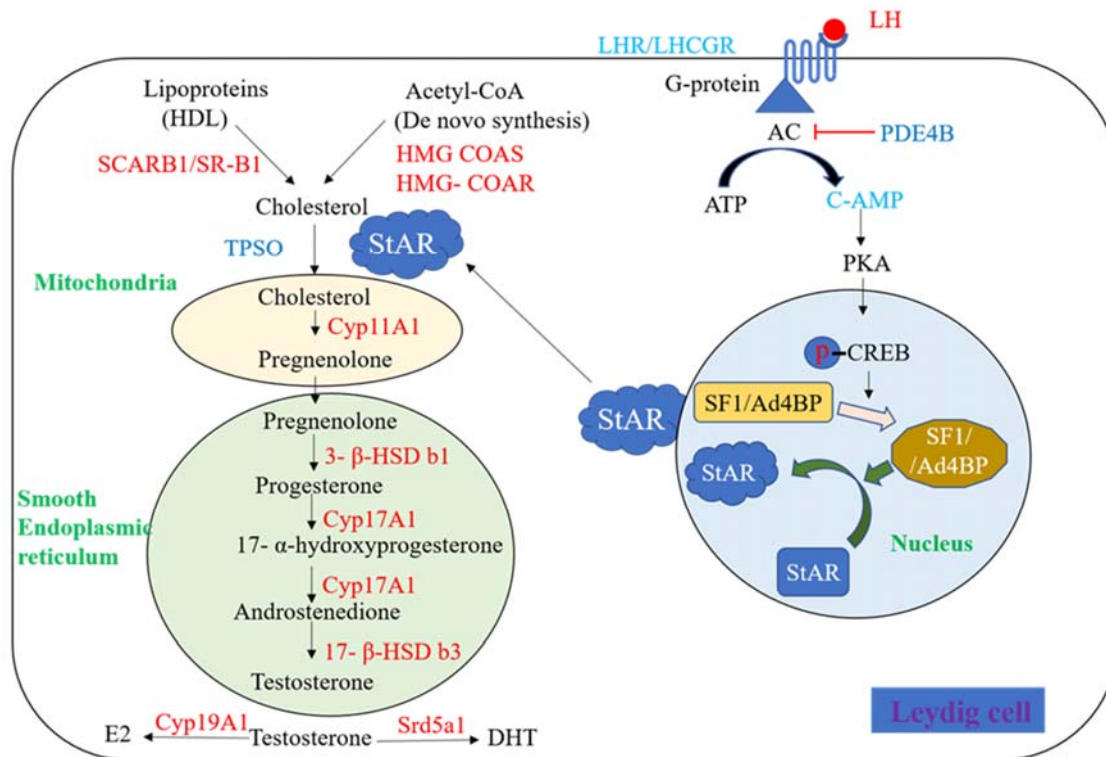


FIGURE 8. Signaling pathway of steroidogenesis in Leydig cell. The signaling cascade to produce testosterone begins with the binding of luteinising hormone (LH) to its receptor, luteinising hormone receptor (LHR) present on Leydig cell. The interaction between LH- LHR stimulates the c-AMP-PKA signaling that activates steroidogenic factor 1 (SF1), which in turn activates cholesterol transporters like steroidogenic acute regulatory protein (StAR) to transport cholesterol from OMM to IMM where it is converted into pregnenolone by a rate-limiting enzyme -CYP11A1 and then finally converted into testosterone through a series of enzymatic reactions. Testosterone is further metabolised into either E2 through aromatization or active form of testosterone, dihydrotestosterone (DHT).

1.4.5. Spermatogenesis

Spermatogenesis is a physiological, dynamic, developmental, complex, well organized and multistep sequential process to produce matured haploid (n) spermatozoa from diploid (2n) undifferentiated primitive spermatogonia (As) through the series of mitotic, meiotic and post-meiotic phases (figure 9). The size of adult testes and the outcome of sperm number is determined by the number of SCs (27). SCs secrete glycoproteins to form a basement membrane between them and peritubular cells. As the maturation or puberty reaches, the morphology and function of SCs change to facilitate the formation of BTB, decline in their proliferative activity, transform to become elongated in nature with larger nucleus and have adequate smooth and rough endoplasmic reticulum. After the formation of BTB, SCs start to produce seminiferous tubular fluid which is essential for spermatogenesis. During different stages of development, 30- 50 germ cells are supported by each Sertoli cell (28).

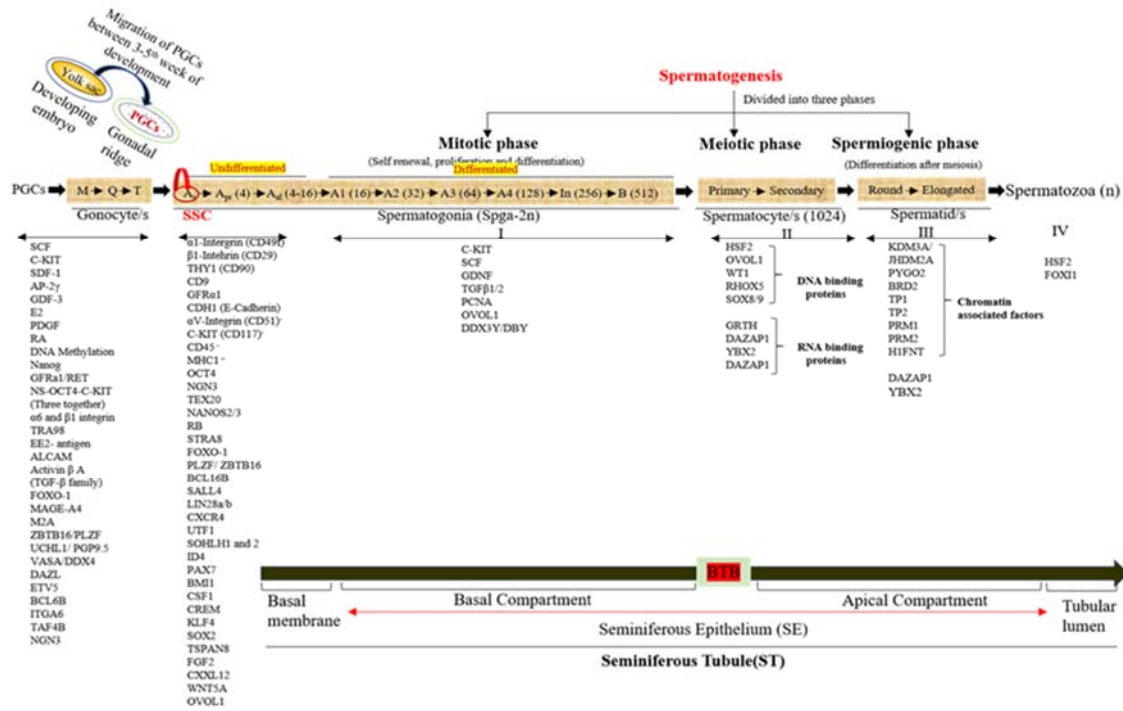


FIGURE 9. Phases and factors that govern spermatogenesis. For easy understanding, the complex spermatogenesis process is conveniently divided into three phases namely, mitotic, meiotic and spermatogenic. Through the process of spermatogenesis, haploid spermatozoa are produced from diploid spermatogonia, which are in turn derived from primordial germ cells (PGCs). The successful spermatogenesis can be achieved by involvement of several stage-specific factors which helps in the transition or differentiation of one cell type into another successive cell type. During spermatogenesis, there are four types of germ cells, i.e., spermatogonia, spermatocytes, spermatids, and spermatozoa. Blood- Testis-Barrier (BTB) divides the seminiferous epithelium into basal and apical compartments. Both the spermatogonia and primary spermatocytes are present towards basal compartment and secondary spermatocytes and spermatids are present towards apical compartment. The elongated spermatids present in the apical compartment convert into spermatozoa which are then released into the tubular lumen by the process of spermiation. Molecular factors that are involved at different stages are indicated.

A unique microenvironment with enrichment of stem cells is termed as 'niche' which supports self-renewal and keeps the stem cells in an undifferentiated state. During the 3rd to 5th week of embryonic development, the primordial germ cells start migrating from extra embryonic tissues surrounding the yolk sac to gonadal ridges and are differentiated into gonocytes, which enter the G0 phase and remain mitotically inactive till birth. Between PND1 to PND180, the gonocytes differentiate into spermatogonia which remains in quiescent state until 7 years. During embryonic development, based on the expression of BMP4 and BMP8b, primordial germ cells specify the formation of gonocytes. Specific gene and protein markers such as Nanog, GFRa1/RET, nucleostemin (NS), OCT4 (Pou5f1), c-KIT, α6 and β1 integrins, FOXO1 have been implicated to have a role during their differentiation into spermatogonial stem cells (SSCs). Based on the cellular properties and morphology, spermatogonia are of three types; type A, intermediate type (In) and type B. Based upon the cell types, type A is further divided into several types; A single (As), A paired (Apr), A aligned (Aal), A1, A2, A3, and A4

(29). The early spermatogonia As, Apr, Aal are undifferentiated whereas A1, A2, A3, A4, In and B are differentiated spermatogonia. Among the three types of undifferentiated spermatogonia, As is considered as spermatogonial stem cell (SSC). These SSCs are fundamental for spermatogenesis and male fertility and unlike other stem cells, SSCs are rare with approximately 0.03% of all germ cells in rodents. It has been reported that the approximate estimation of SSCs per testis is around 2000- 3000 only and it is close to 10% of As pool. The generated Apr initiates the spermatogenesis by forming Aal (4-16) which again divides to form A1(16), A2 (32), A3 (64) and A4 (128). The A4 spermatogonia transform into In spermatogonia which divides by mitosis to form B-type spermatogonia which further differentiated into preleptotene spermatocytes after completion of final mitotic division. In the cross-section of ST, the germ cells are organised in a chronological manner in the form of centric layers starts from basement membrane towards the lumen with sequence of immature with germ cell/s, spermatogonia (As, Apr, Pal, In and B), primary spermatocytes, primary spermatocytes, round spermatids, elongated spermatids, mature spermatozoa.

1.4.6. Spermiogenesis

It is the last phase of spermatogenesis in which transformation of round spermatids into mature sperm series of morphological changes, such as removal of cytoplasm, formation of acrosome and flagella, nuclear elongation and extensive chromatin condensation with transition of basic histones to protamines occur (30). Remodelling of chromatin starting from early post-meiotic phase (round spermatids), gradual replacement of canonical core histones with testis-specific variants of core histone H2A, H2B, H3 (non-canonical; several types), which again are replaced with transition proteins (TP1 and TP2 in elongated spermatid) and finally the replacement of TPs with protamines (PRM1/P1 and PRM2/P2) are some to the molecular events during spermiogenesis. Spermatid-specific linker histone 1 like protein (HILS1), TP1, TP2, PRM2 and PRM2 have significant role in the packaging of spermatid chromatin during spermiogenesis. Protamines (PRMs/Ps) are different from basic histones by having arginine and cystine instead of lysine. In mice and humans, the two types of protamines, protamine 1 (PRM1/P1) and protamine 2 (PRM2/P2) were first detected at step 12 and step 14 of spermiogenesis, respectively and retained in mature sperm. Soon after entry of matured sperm into the oocyte cytoplasm during fertilization, protamines are replaced with maternally supplied histones and histone variants specific to testis will disappear from the paternal genome after completion of fertilization (31).

1.4.7. Factors involved in spermatogenesis.

As shown in figure 9, a variety of factors such as SCF, SDF-1, AP-2 γ , SDF-1, GDF3, RA, E2 mediate the differentiation of PGCs into gonocytes in humans. Abundant expression of GDNF receptor complex, GRF1 α /RET, Nanog was observed in the mitotic gonocytes of mice (M), but its expression was decreased towards quiescent gonocytes (Q). Some specific factors like TRA98, EE2- antigen, ALCAM, Activin β A (TGF- β family), MAGE-A4, M2A, UCHL1/ PGP9.5, VASA/DDX4, DAZL, ETV5, BCL6B and ITGA6 are important during the differentiation of PGCs and gonocytes (32). Forkhead transcription factor 1 (FOXO1) is critical for the neonatal gonocytes and abundant expression of FOXO1 is observed in nuclei of SSCs and undifferentiated spermatogonia. Based on this, it concluded that FOXO1 is a marker for transition between gonocyte to SSCs (33). Several protein markers for SSCs, including glycosylphosphatidylinositol-anchored glycoprotein (surface) Thy1 (CD90), glial cell line derived growth factor (GDNF), GDNF receptor (GFRA-1), α 6 integrin (CD49f), β 1- integrin (CD29), CD9, EPCAM, GPR125, E-cadherin (CDH1), MCAM, B-cell specific moloney murine leukemia virus integration site 1 (BMI1), paired box7 (PAX7), inhibitor of differentiation 4 (ID4) are important during spermatogenesis. ID4 specifically expressed in As indicates that it is essential for SSC self-renewal in mice (34). Similarly, PAX7 and BMI are expressed in subpopulations of As (35). The cells which express PAX7, BMI and ID4 are considered to be ultimate SSCs. Expression of PLZF (promyelocytic leukaemia zinc finger) is restricted to undifferentiated spermatogonia, including SSCs and it may support the maintenance of undifferentiated state of spermatogonia (36). The role (TGF- β 1) in cultured Sertoli cells for differentiation of some pachytene spermatocytes into round spermatids by completion of meiosis is documented. There are other factors, such as DNA binding proteins (HSF2, OVOL1, FOXI1, WT1, SOX8 and RHOX5), chromatin-associated proteins (JHDM2A/KDM3A, PYGO2, BRDT) and a few RNA binding proteins (GRTH, YBX2, DAZAP1 and SMA68) that are involved at different levels to regulate spermatogenesis.

1.4.8. Hormonal regulation of spermatogenesis

In males, fertility and spermatogenesis are dependent on testosterone, LH, FSH and their receptors. Androgens, particularly testosterone and DHT are crucial and essential regulators of spermatogenesis, and they act by binding to the androgen receptor (AR). The LH-LHR signaling induces the LCs of the testis to produce testosterone which diffuses into the basal membrane of SE to act on the developing germ cells and thus, testosterone acts as a

paracrine factor (37). Both T and DHT act as ligands for the AR depending on the concentrations of these hormones, i.e., testosterone acts as ligand for AR at higher concentrations whereas DHT would be the preferred ligand for AR under conditions of suppressed levels of intracellular testosterone. Both testosterone and DHT show same affinity for AR and this hormone- receptor complex attaches to acceptor site in the nuclei of SCs to initiate the biological response (26). The concentrations of locally produced testicular testosterone are 25-125 folds higher than that of serum concentration and the sperm production or spermatogenesis may affect if its concentration decreases below 70 mM. Due to the presence of ERs in testis, oestrogen also is associated with spermatogenesis and the role of exogenous oestrogen has been documented in adult male rats. Both androgen and oestrogen seem to be associated with the regulation of spermiation (38).

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Chapter 2

Methodology

2.1. Reagents and chemicals

Analytical grade allethrin (Cat No. 33396), deltamethrin (Cat No. 45423), λ -cyhalothrin (Cat. 72765), cypermethrin (Cat No. 36128), fenvalerate (Cat No. 45495), corn oil (Cat No. C8267), calcium ionophore A23187 (Cat No. C7522-1), filipin (Cat No. F9765-25), protease inhibitor cocktail (Cat No. P8340-5), lectin FITC-PNA (L7381-2), M2 medium (M7167-100), nicotinamide adenine dinucleotide (NAD) (N3014), nicotinamide adenine dinucleotide phosphate (NADPH) (Cat No. N705), glutathione reductase (G3664) and SOD assay kit (Cat No. 19160) were purchased from Sigma Aldrich, USA. FLUO-3-AM (F1242) was purchased from Invitrogen, USA. All ELISA kits were purchased from Elabscience, USA. Protein reagents A (Cat No. 5000113) and B (Cat No. 5000114) were purchased from Bio-Rad.

2.2 Animals and their maintenance

30 days old male Wistar rats procured from National Centre for Laboratory animals (NCLAS), National Institute of Nutrition (NIN), Hyderabad, were kept for acclimatisation for 15 days before starting the experiment. They were maintained in sterile conditions with ample ventilation, ambient room temperature (23 ± 3 °C), relative humidity ($50 \pm 20\%$) and with a 12 h photoperiod, in animal house at the University of Hyderabad. Animals were provided with rodent diet (National Institute of Nutrition, Hyderabad, India) with free access to water ad libitum. Three to four animals were housed in each polypropylene cage (450mm $\times$ 270mm $\times$ 150 mm), with dry rice husk as bedding material. During the treatment period, rats were monitored twice a day for any signs of intolerance. Body weights were recorded once in a week.

2.3. Rationale for dosage and duration of treatment

Based on the reports provided by the Project Coordinating Cell, All India Network Project on Pesticide Residues, Indian Agricultural Research Institute, New Delhi (1), and those compounds that were detected in the dietary items, the mixture of pyrethroids was formulated by combining five individual compounds to treat the animals. As per these reports, residual levels of cypermethrin, deltamethrin, fenvalerate, cyhalothrin L, and allethrin are present in the rice and vegetables available in the Indian market. The dose of individual pyrethroids in the mixture administered was based on the amount of each of these presents in the average quantity of rice and vegetables consumed by an individual in the Indian scenario. According to the National Institute of Nutrition (NIN), a government of India funded Institute, the per-caput consumption of rice and vegetables per day is 345 and 43 g respectively (total 388 g) (2). The

amount (parts per million; PPM) of cypermethrin, deltamethrin, fenvalerate, cyhalothrin L, and allethrin present in rice and vegetables is 1.542, 0.233, 0.0833, 0.5188, 0.05 and 2.4271, respectively. Thus, the quantity of each of the above five pyrethroids present in the average amount of rice and vegetables (388 g) consumed daily by an individual would be 598.3 µg, 90.40 µg, 32.32 µg, 201.29 µg, 19.4 µg respectively and the total quantity of all of them is 941.71 µg. Accordingly, the per kg body weight exposure (for an individual of 60 kg body weight) will be 9.97 µg, 1.506 µg, 0.538 µg, 3.35 µg, and 0.323 µg of cypermethrin, deltamethrin, fenvalerate, cyhalothrin L, and allethrin respectively with a cumulative amount of 15.687 µg. Based on this calculation, the experimental protocols were designed for treatments.

Correlation of human age and life span of a rat was considered as the basis for the duration of administration. It is an accepted concept that the lifetime of one month in a rat is equal to 2.5 years in humans (3). Rats that were used in this study were 45 days old (1.5 months), which are equivalent to approximately 4-year-old humans. Treatment for 1 to 15 months turns out to be an exposure period of 2.5 to 37.5 years in a human setting, which truly reflects long-term exposure to pyrethroids via food consumption during almost half a decade of a lifetime starting from childhood.

2.4. Animal treatments

2.4.1. Experimental protocol – I (EP-I)

As stated above, the per kg body weight exposure of an individual to cypermethrin, deltamethrin, fenvalerate, cyhalothrin L and allethrin 9.97 µg is 1.506 µg, 0.538 µg, 3.35 µg and 0.323 µg of respectively with a cumulative amount of 15.687 µg. In this protocol, rats (45 days old; n=10 in each group) were treated with one-fifth of the cumulative amount, and this dose is referred to as the high dose (HD; containing 1.994 µg, 0.301 µg, 0.107 µg, 0.671 µg, 0.064 µg of cypermethrin, deltamethrin, fenvalerate, cyhalothrin L and allethrin respectively with a cumulative amount of 3.137 µg). Since it is a usual practice to extensively wash vegetables and rice before cooking, we also treated rats (45 days old; n=10 in each group) with a dose that is equal to one twenty-fifth of the cumulative amount, and this dose is referred to as the low dose (LD; containing 0.398 µg, 0.0602 µg, 0.0215 µg, 0.134 µg and 0.0129 µg of cypermethrin, deltamethrin, fenvalerate, cyhalothrin L and allethrin respectively with a cumulative amount of (0.6266 µg). The mixture of pyrethroids was prepared in corn oil. Rats in the control group (n=10) received corn oil only. The duration of treatment was 9, 12, and 15 months.

2.4.2. Experimental protocol –II (EP II)

This protocol is designed to treat rats at doses that are equivalent to human consumption. It is estimated that an average Indian consumes 345 and 43 grams of rice and vegetables per day (total 388 grams) (4). In this quantity of rice and vegetables, the concentration of cypermethrin, deltamethrin, fenvalerate, cyhalothrin L and allethrin in parts per million (PPM) is 1.542, 0.233, 0.0833, 0.5188, 0.05, and 2.4271 respectively (5,6), which is equivalent to 598.3 µg, 90.40 µg, 32.32 µg, 201.29 µg and 19.4 µg respectively (totaling to 941.71 µg). Assuming that the average body weight of an Indian individual is 60 kg, the per kg body weight exposure will be 9.97 µg, 1.506 µg, 0.538 µg, 3.35 µg and 0.323 µg of cypermethrin, deltamethrin, fenvalerate, cyhalothrin L and allethrin respectively with a cumulative amount of 15.687 µg. Based on this, rats (45 days old; n=10 for each time point) were treated with a mixture of five pyrethroids at a dose of 15.687 µg / kg body weight. Rats in the control group (n=10 for each time point) received corn oil only. The duration of treatment was 1, 3, 6, 9, 12, and 15 months.

2.5. Assessment of fecundity

Fecundity was assessed by allowing the rats to natural mating. Each control and pyrethroid-treated rat were allowed to mate with two females for one week. The females were then separated and monitored for the litter size immediately after parturition. Periodical monitoring i.e., at least once in 12 h was carried out for any signs of cannibalization by the mother and presence of dead pups.

2.6. Sperm count

The cauda epididymis was minced and placed in M2 medium (Sigma Aldrich). The sperm suspension was diluted with PBS in 1: 10 dilutions. 10 µl of diluted sperm suspension was placed on a hemacytometer and the number of sperms in all the four chambers were counted under a microscope.

2.7. Estimation of dehydrogenase enzyme activity

The activities of 3β- and 17β-hydroxysteroid dehydrogenase (HSD) were measured as per established protocols (7) with slight modification. Briefly, testes tissue homogenates were prepared in 20% glycerol containing 50 mM potassium phosphate and 1 mM EDTA, followed by centrifugation at 16,000g for 10 min at 4° C. To determine 3β-HSD activity, the reaction

cocktail was prepared by mixing 600 μ l of Na-pyrophosphate buffer (0.166 mM), 200 μ l of NAD (1mM), 2 ml of double-distilled water, 100 μ l of tissue lysate. The reaction mixture with sample lysate was kept in a spectrophotometer till the absorbance is stabilized (4- 5 minutes). Thereafter, the reaction was initiated by addition of 100 μ l of 0.1 mM dehydroepiandrosterone (DHEA), and the change in OD at 340 nm was noted for 10 minutes. To determine 17 β -HSD activity, the reaction cocktail was prepared by mixing 600 μ l of Na-pyrophosphate buffer (0.166 mM), 200 μ l of NADPH (1mM), 2 ml of double-distilled water, 100 μ l of tissue lysate. The reaction mixture with sample lysate was kept in a spectrophotometer till the absorbance is stabilized (4- 5 minutes). Thereafter, the reaction was initiated by the addition of 100 μ l of 0.1 mM 1,4-androstadiene-3,17-dione (ASD) change in the OD at 340 nm was noted for 10 minutes.

2.8. Estimation of hormones

The levels of different hormones were estimated in the serum of control and pyrethroid treated rats by ELISA using commercially available kits: luteinising hormone (Cat No. LH- E-EL-R0026), follicle-stimulating hormones (Cat No. FSH- E-EL-R0391), prolactin (Cat No. PRL- E-EL-R3006), inhibin B (Cat No. INHB-E-EL-R1027), gonadotrophin-releasing hormone (Cat No. GnRH-E-EL-0071), kisspeptin1 (Cat No. Kisspeptin 1- E-EL- R2530), testosterone (Testosterone -E-EL-0155), and thyroid-stimulating hormone (Cat No. TSH-E-EL-R0976). All the kits were purchased from Elabscience, USA. Triiodothyronine (T3), thyroxine (T4), oestradiol (E2), and progesterone (P4) were estimated in an automated analyser equipped with cobas c501 module (Roche Diagnostics, Risch-Rotkreuz, Switzerland) using commercially available electrochemiluminescence immune assay (ECLIA) kits.

2.9. Capacitation and acrosome reaction

In the female reproductive tract, the dynamic changes in the membrane composition of sperm, namely, that is the efflux of cholesterol and exocytosis of glycoproteins (along with acrosome membrane) takes place and are considered as the hallmarks for capacitation and acrosome reaction, respectively. An increase in intracellular calcium levels is also an indicator for the acrosome reaction of sperm in vitro. The fluorescent probes filipin and peanut agglutinin-FITC (PNA-FITC), which are specific to cholesterol and glycoproteins respectively were used to assess sperm function in vitro. In spermatozoa pre-labelled with filipin or PMA-FITC, it is anticipated the fluorescence intensity of these dyes will decrease as capacitation or acrosome reaction occurs, because of the loss of cholesterol or membrane glycoproteins. The

mean fluorescence intensity was measured by flow cytometry as described previously (8). The cauda epididymis was minced and placed in M2 medium (Sigma Aldrich, USA) and incubated in CO₂ incubator for 5 minutes to collect sperms. Collected sperms were counted and seeded (2×10^6 / ml) in 24 well plate with M2 medium containing BSA and incubated in CO₂ incubator (5% CO₂ 95% air) at 37 °C for three hours to allow capacitation. After incubation for three hours, the medium was discarded and stained with 200 µM of filipin for one hour. Samples were then washed thoroughly thrice with PBS and resuspended in µl of 500 PBS. The fluorescence intensity of filipin was measured in a flow cytometer using 340 nm (excitation) and 425 nm (emission) wavelengths. To assess the acrosome reaction, another batch of capacitated spermatozoa was treated with the calcium ionophore A23187 (10 µM; (Sigma Aldrich, USA) and incubated at 37 °C, 5% CO₂, and 95% air for 30 min to induce an acrosome reaction. After incubation with the calcium ionophore, the medium was discarded, and acrosome reacted spermatozoa were labelled with PNA-FITC (50 µg/ml) for 30 minutes in dark at room temperature. Samples were washed thoroughly thrice with PBS and resuspended in µl of 500 PBS. The extent of fluorescence on the spermatozoa was measured in a flow cytometer using excitation and emission wavelengths at 488 nm and 515–540 nm respectively. The acrosome reaction was also assessed as a function of calcium release using the fluorescent dye Fluo 3-AM. Acrosome reacted spermatozoa were stained with Fluo 3-AM (5 µg) (Cat. No. F1242, Invitrogen, Carlsbad, USA), and incubated for 30 minutes in dark at room temperature. The fluorescence intensity was measured by flow cytometry with excitation and emission wavelengths at 488 nm and 515–540 nm, respectively. Forward scatter (FSC), and side scatter (SSC) were collected in logarithmic and linear modes, respectively. For each fluorescence analysis, 10,000 individual sperm cells were assessed in a flow cytometer (BD Biosciences, USA) and expressed as mean fluorescence intensity.

2.10. Real-time polymerase chain reaction

Testis tissues (100 mg) were placed in liquid nitrogen and powdered and homogenized in TRIzol reagent (Invitrogen). Using standard isolation kits, total RNA was isolated and 2 µg of it was reverse transcribed to generate cDNA using 50 U Stratascript (Invitrogen) and 0.5 µg of oligo dT (Invitrogen). The resulting cDNA was subjected to real-time PCR analysis using SYBR master mix kit (Applied Biosystems, UK) using gene-specific primers (Tables 1 to 4). The expression of Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) expression was taken

as an internal control. The PCR conditions, in general, were initial denaturation at 94 °C for 10 min; 40 cycles with 94 °C for 15 s and 60 °C for 1 min.

Appropriate control reactions (negative controls - no template and without RT control (only with RNA)) were carried out to rule out non-specific amplification. By standard sequencing, the identity of the amplicons was confirmed. The expression levels of different genes presented are a representation of triplicate of triplicates. Relative quantification ($\Delta\Delta C_t$) method was adopted and relative gene expression in samples from different groups is presented.

2.11. Liquid Chromatography with tandem mass spectrometry (LC-MS-MS)

Sperm protein was extracted by sperm lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) dithiothreitol (DTT), 2mM Na₃VO₄, 50 mM NaF containing a protease inhibitor (Sigma- Aldrich, USA) (8). The quality of protein was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) or one-dimensional (1D) gel electrophoresis. 100 µg protein sample was subjected to in-solution digestion with trypsin and incubated overnight at 37°C. The resulting sample was vacuum dried and dissolved in 50µl of 0.1% formic acid in water and spun at 10000g and the supernatant was collected into a separate tube. 10µl of supernatant sample was injected onto BEH C18 UPLC column (UPLC systems, Waters) for separation of peptides and three runs were performed per sample carried out for label-free quantitation (LFQ). The peptides separated on the column were directed to Waters Synapt G2 Q-TOF instrument for MS and MSMS analysis. The raw data was processed by MassLynx 4.1 WATERS. The individual peptides of MSMS spectra were matched to the rat protein database sequence for protein identification through Protein Lynx Global Server (PLGS, WATERS) software.

2.12. Histopathology

Histopathological analyses were carried out in testis, caput, cauda, prostate, liver, kidney, lung, and pancreas obtained from control and pyrethroid mixture treated rats. They were processed as per standard protocols. In brief, after an initial soaking in Bouin's fluid for 24 h, they were extensively washed with 70% ethanol to remove the yellow colour. Stepwise dehydration of the samples was carried out by placing them serially in graded ethanol (80%, 90%, and 100%), followed by processing with isopropanol overnight at 60 °C. The processed tissues were then embedded in paraffin wax and five-micron sections were made for histological staining. The sections were then placed in xylene to allow deparaffinization

followed by rehydration in graded ethanol (100%, 90%, 80%, 70%, and 50%) and rinsing in distilled water. Staining was carried out by placing the slides in Harris haematoxylin solution for 10 min. Brief washing in distilled water, differentiation in 1% hydrochloric acid for 30 s, and immersion in 0.2% ammonia water for 30 s to 1 min were carried out. Excess reagents were washed off with distilled water and the sections were counterstained with 0.2% eosin Y solution for 1 min. This was followed by washing with distilled water and dehydration (by placing serially in 50, 70, 80%, 90%, and 100% alcohol). Finally, they were washed with 3 changes of xylene and mounted using xylene based mounting medium. The slides were photographed and evaluated by a board-certified histopathologist who had no information on the experimental setup, and treatment protocol. Multiple fields (at least 25) in the stained sections were analyzed. Histopathological changes were evaluated as per the established principles described earlier (9-11).

2.13. Estimation of lipid peroxidation

Malondialdehyde (MDA) is the end product of lipid peroxidation and thus it is marker for the degree of lipid peroxidation in the sample. The extent of lipid peroxidation in the tissues was estimated using the thiobarbituric acid reactive substances (TBARS) method (12). To 1 ml of 10% (w/v) tissue homogenate prepared in ice cold phosphate buffer saline (pH 7.4), an equal volume of trichloroacetic acid (TCA) was added and the proteins precipitated by centrifugation at 15,000 rpm for 20 min. To 1 ml of the supernatant, 0.5 ml of TBA was added and incubated at 95 °C for 1 h. The reaction mixture was cooled in ice water for 5 minutes and the optical density of the coloured TBA-MDA complex was measured in a spectrophotometer set at 532 nm. The concentration of lipid peroxides was estimated using a standard curve generated by using 1,1,3,3-tetraethoxypropane (TEP) at different concentrations. Levels of lipid peroxides were expressed as nanomoles of MDA/ mg protein.

2.14. Estimation of Nitric oxide

Griess method (13) was employed to determine the levels of nitric oxide (NO). Tissue homogenates (2 ml; 10% w/v) were mixed with 10% TCA and centrifuged at 15,000 rpm for 20 min to precipitate the protein. The resulting clear supernatant was collected and an equal amount of Griess reagent was added and incubated in dark for 30 min. The intensity of the purple colour formed was determined in a spectrophotometer set at 520 nm. Nitrite levels were

estimated using a standard curve generated with varying concentrations of sodium nitrite and the concentration of NO was expressed as $\mu\text{moles nitrite/mg protein}$.

2.15. Estimation of antioxidant enzyme activity

Tissue homogenates (10% w/v) were prepared in ice-cold phosphate buffer saline (pH 7.4), debris removed by centrifugation at 3000 rpm, and the resultant tissue lysates were used to determine the activities of catalase, glutathione-s-transferase (GST), and glutathione peroxidase (GPx) through the standard methods (14-16). The activity of superoxide dismutase (SOD) activity was determined through a commercially available assay kit (Cat No. 19160). To measure catalase activity, 10 μl of tissue homogenate was incubated with 1 ml of 30 mM H_2O_2 prepared in 50 mM phosphate buffer, pH 7.0, and the change in O.D. at 240 nm was monitored for 5 min at 1 min intervals. The activity 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) and expressed as $K_{30} / \text{mg protein}$). GST activity was assayed by incubating 10 μl of the sample with 924 μl of 0.1 M phosphate buffer (pH 6.5), 33.3 μl of 10 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 33.3 μl of reduced glutathione (GSH) and the change in O.D. was measured at 340 nm for 5 min with 1 min intervals. The activity was calculated using the formula: (Absorbance difference ($\Delta\text{OD}/\text{min}$) $\times V \times 100$ (dilution factor) / $9.6 \times \text{Conc. of protein in mg}$), where V is volume of the reaction mixture and 9.6 is the molar extinction coefficient of CDBN. The activity of GST was expressed as $\mu\text{moles of CDBN-GSH conjugate formed}/\text{min}/\text{mg protein}$. The assay for GPx involved incubation of 10 μl of the tissue homogenate with 660 μl of phosphate buffer, 1 U of glutathione reductase, 100 μl of GSH solution, and 100 μl of NADPH solution. Change in O.D. at 340 nm was monitored for 5 min with 1 min intervals. The activity was calculated using the equation: (Absorbance difference ($\Delta\text{OD}/\text{min}$) $\times 1$) / ($6.2 \times \text{volume of sample}$), where 6.2 is the molar extinction coefficient of NADPH at 340 nm; and expressed as $\mu\text{moles NADPH oxidized}/\text{min}/\text{mg protein}$. The assay for SOD activity was determined in the 96 well plate through a commercially available assay kit. Briefly, incubation of 20 μl of tissue homogenate with 20 μl of working enzyme solution (xanthine oxidase- XO) and 200 μl of working substrate solution (water-soluble tetrazolium salt-WST 1) for 20 minutes at 37 °C. They are three blanks used: 20 μl of working enzyme solution and 20 μl of ultrapure water in blank 1, 20 μl of tissue homogenate and 20 μl of dilution buffer in blank 2, and 20 μl of ultrapure water with 20 μl dilution buffer in blank 3. 200 μl of working substrate solution (water-soluble tetrazolium salt-WST 1) were added in all three blanks. OD was taken at 450

nm using a microplate reader. The activity was calculated using the equation: $(A \text{ blank 1} - A \text{ blank 3}) - (A \text{ sample} - A \text{ blank 2}) / (A \text{ blank 1} - A \text{ blank 3}) * 100 \%$ and represented in terms of inhibition rate percentage. The protein concentration was estimated by the Lowry method (17).

2.16. Estimation of liver and kidney function, lipid profile, and complete blood picture

Cholesterol, high-density lipoprotein (HDL), very low-density lipoprotein (LDL) and triglycerides were estimated in the serum to assess lipid profile. The activities of serum glutamate-pyruvate transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT) and alkaline phosphatase (ALP), total bilirubin, and total protein in the serum were determined for liver function, whereas the serum levels of creatinine, urea, uric acid, blood urea nitrogen (BUN), sodium, potassium, chloride, calcium, and phosphorus levels were measured to assess kidney function. All the above tests were carried out in an automated analyser equipped with cobas c501 module (Roche Diagnostics, Risch-Rotkreuz, Switzerland) using commercially available kits. Whole blood was used to assess complete blood picture (CBP). CBP was carried out in an automated analyser equipped with DXH-900 module (Beckman Coulter India Private Limited-Mumbai) using commercially available kits. Glycosylated haemoglobin (HbA1c) estimation was carried out by high-performance liquid chromatography (HPLC) equipped with D10 module (Bio-Rad Laboratories India Private Limited).

2.17. Statistical analyses

For each measure determined in this study, statistical analyses were carried using Student's t-test available in Sigma Plot software 12.5, Build 12.5.0.38 (SPSS Inc., Chicago, IL, USA). Comparisons were done with the respective control. The data between the two-time points for each of the treatment conditions were not analyzed to avoid complexity in the presentation. Values shown are Mean $\pm$ S.D. * denotes $p < 0.05$.

Table 1. Primer sequences to amplify genes related to spermatogenesis.

Gene	Primer Sequence (5'→3')		Amplicon size (bp)
<i>Abp</i>	FP	TGAAGGGTCCCAACTGTCTC	169
	RP	TCTCGGATGAGGTTCCAATC	
<i>Amh</i>	FP	CTAGCCACCTTCGGAGTCTG	162
	RP	AGGCTCTTGGAACCTCAGCA	
<i>Ar</i>	FP	CACTACGAGCTCTCACTTGTG	172
	RP	CCAGAGTCATCCCTGCTTCATAAC	
<i>Brdt</i>	FP	CAGTTCCTGCAGAGAGTCGT	169
	RP	CGTAGTACCTGTTTTCCAGCC	
<i>CD9</i>	FP	GGCTATACCCACAAGGACGA	176
	RP	GGGCAGATGTCCGAGATAAA	
<i>CD90/Thy1</i>	FP	GAGGGCGACTACATGTGTGA	161
	RP	AAGGAGAGGGAAAGCAGGAG	
<i>Dax1</i>	FP	ATGGCGTTCCTGTACCGTAG	185
	RP	ACCACCTGTGGATCCTTGAG	
<i>Dazap1</i>	FP	GACTGGAGTACCACTCAAGAGA	178
	RP	CATCTAGGGTGTGTGGTCTG	
<i>Ddx3y</i>	FP	GGATGCTGGATATGGGATTG	129
	RP	CACGAGGCATCTGTATCTC	
<i>Fshr</i>	FP	CTCATCAAGCGACACCAAGA	185
	RP	GGAAAGGATTGGCACAAGAA	
<i>Gdnf</i>	FP	CGAAACCAAGGAGGAAGTGA	193
	RP	ATGGTAAACCAGGCTGTCTG	
<i>Gfra1</i>	FP	TCGGGTAGCACACACCTCTGT	65
	RP	GAGGCACCAGCGAGACCAT	
<i>Grth/Ddx 25</i>	FP	CCAGAACCTCATAGCACAGAG	136
	RP	CAGAGCCAGTTCATAGGTG	
<i>Hsf-2</i>	FP	TAAATGGCTCCTCCAACCTG	99
	RP	CCAACAGCTCAACCTTTCCTA	
<i>Inhbb</i>	FP	TAACGAAGGCAACCAGAACC	193
	RP	GGTATGCCAGCCACTACGTT	
<i>Ovol1</i>	FP	GACCTCAAGAGACATGTCC	148
	RP	GGCGCTCCTTATATGCATAC	
<i>Prm1/P1</i>	FP	ATGGCCAGATACCGATGCT	80
	RP	CTCCTCCGTCTGCGACAT	
<i>Prm2/P2</i>	FP	GTTTCGCTACCGAATGAGGAG	177
	RP	GATCCTGTGAAGCCTCTTGC	
<i>PCNA</i>	FP	AGGACGGGGTGAAGTTTCT	173
	RP	CAGTGGAGTGGCTTTTGTGA	
<i>Plzf or Zbtb16</i>	FP	GCTGCACTATGGGAGAGAGG	195
	RP	TGCACATGGAGACCAGATGT	
<i>Pygo2</i>	FP	GCGAAGAAAGTCCAATACTCAGG	165
	RP	GTACAGGACTGCCAAGGAAC	
<i>Rhox5</i>	FP	CTCTAGCCATGATATCAGCC	123
	RP	TGTGCACCTTTCTCGTCTC	
<i>Scf</i>	FP	GATGACCTCGTGGCATGTATG	190
	RP	CAGGACCTAATGTTGAAGAGAG	
<i>Tgfb1</i>	FP	GCAACAATTCTGGCGTTAC	180
	RP	CCACGTGGAGTACATTATCTTTGC	
<i>Tp1/Tnp1</i>	FP	AAGAACCGAGCTCCTCACAA	109
	RP	GGTAATTGCGACTTGCATCA	
<i>Tp2/Tnp2</i>	FP	GGCCTCAAAGTCACACCAAT	161
	RP	GCTGGGTCTTGAAGGTGAGT	
<i>Vim</i>	FP	AATGCTTCTCTGGCACGTCT	100
	RP	GCTCCTGGATCTCTTCATCG	

<i>Wt1</i>	FP	CGTACCCAGGCTGCAATAA	148
	RP	CTTTGGTGCCTTTTGAGCTGGTC	
<i>Wnt4</i>	FP	TCTGGAGAAAGTGTGGCTGTG	183
	RP	GCCTCGTTGTTGTGAAGGTT	
<i>Ybx2</i>	FP	GTGACCAACAGCAGGGAGAT	189
	RP	GGAAGTAGGGACGGTTTCGT	

Table 2. Primer sequences to amplify genes related to steroidogenesis.

Gene	Primer Sequence (5'→3')		Amplicon size (bp)
<i>Hsd3b1</i>	FP	GGTGCAGGAGAAAGAACTGC	179
	RP	CGGTGTGGATGACAAACAGAG	
<i>Hsd17b3</i>	FP	CAACCTGCTCCCAAGTCATT	160
	RP	AACCCCTACTCCCGAAGAAA	
<i>Cerb1</i>	FP	CATGGACTCTGGAGCAGACA	154
	RP	TACAGTGGGAGCAGATGACG	
<i>Crem</i>	FP	TACTGCTTTGCCACAAGGTG	194
	RP	GATTTTCAAGCACAGCCACA	
<i>Cyp11A1</i>	FP	AGCTGCCTGGGATGTGATTT	155
	RP	TGTTGGCCTGGATGTTCTTG	
<i>Cyp17a1</i>	FP	ACTGAGGGTATCGTGGATGC	161
	RP	TCGAACCTTCTCCCTGCACTT	
<i>Cyp19A1</i>	FP	CAGAGTATCCGGAGGTGGAA	200
	RP	CATGACCAAGTCCACGACAG	
<i>Gata4</i>	FP	CTGTGCCAACTGCCAGACTA	165
	RP	AGATTCTTGGGCTTCCGTTT	
<i>Hmgcr</i>	FP	TGCTGCTTTGGCTGTATGTC	187
	RP	CCCTTTGGGTTACTGGGTTT	
<i>Hmgcs1</i>	FP	AAAGATGTGGGAATCGTTGC	179
	RP	TGAACCACAGTCAGGCAGAG	
<i>Lhr/ Lhcgr</i>	FP	CTGCGCTGTCTTGCC	103
	RP	CGACCTCATTAAGTCCCCTGAA	
<i>PDE4B</i>	FP	TGCATCATGTACGCCATTTT	179
	RP	GGCGTAGAGAGGAGAACGTG	
<i>Sox9</i>	FP	AGGAAGCTGGCAGACCAGTA	157
	RP	TGTAATCGGGGTGGTCTTTC	
<i>Srb1</i>	FP	GGTGCCCATCATTTACCAAC	155
	RP	GCTTGGCTTCTTGCACTACC	
<i>StAR</i>	FP	CTCCTACAGACATATGCGGAAC	175
	RP	CTGGTCACTGTAGAGTGTTG	
<i>TRPM2</i>	FP	TTCATTCCCTCCAGTCCAAG	168
	RP	CACGAGAGGGGACTTCTGAG	
<i>TSPO</i>	FP	GCTATGGTTCCCTTGGGTCT	198
		CCAGGCCAGGTAAGGATACA	

Table 3. Primer sequences to amplify genes related to epigenetic modifications.

Gene	Primer Sequence (5'→3')		Amplicon size (bp)
<i>Dnmt1</i>	FP	CGGCTCAAAGACTTGGAAG	163
	RP	TAGCCAGGTAGCCTTCCTCA	
<i>Dnmt2</i>	FP	GCAGCAGACATTGACAGGAA	176
	RP	GTGAAGCACATGGACCTCCT	
<i>Dnm3a</i>	FP	GGTGTGTGTCGAGAAGCTCA	181
	RP	AGCCTTGCCAGTGTCACTTT	
<i>Dnmt3b</i>	FP	ACTCGAGGAGGGAGCTTAGG	163

	RP	TTTGTCATTTCCCCAACCAT	
<i>Ezh2</i>	FP	ATCTGAGAAGGGACCGGTTT	178
	RP	CTGTATCCTTCGCTGCTTCC	
<i>Hdac1</i>	FP	ATCGTCCTCACAAAGCCAAC	156
	RP	AGCCATCAAATACCGGACAG	
<i>Hdac4</i>	FP	ACGGTCAAGGCTTAAGCAGA	174
	RP	ACGTTGCCAGAGCTGCTATT	
<i>Hdac10</i>	FP	CCTGGATGGGCAGATAAGAA	198
	RP	AACCCTCCAGTTGTCTGTGG	
<i>Hp1bp3</i>	FP	TTGCTGAAGGGGAAGAAGAA	173
	RP	GGTTTCCTCAGCAGACTTGC	
<i>Cbp / Crebbp / Kat3a</i>	FP	CCGGAAGATGCATCAGATTT	166
	RP	CACTCGGTCTTCCAAATGGT	
<i>P300/ Ep300/ Kat3b</i>	FP	CGGTCCATGAACAACATGAG	184
	RP	TACTAGATGGCTGGGCTGCT	
<i>Ciita or C2ta or Mhc2ta</i>	FP	AGTTTCCATCCGTGGAAGTG	159
	RP	GTGGCAGCTCCTTGTCTTTC	
<i>Ncoa7</i>	FP	GCAGCGCTTTCTTTCTATGG	176
	RP	AACACTCCGATTCTCATGG	
<i>Sirt1</i>	FP	TGGAAGGAAAGCAATTTTGG	154
	RP	GATCCTTTGGATTCTGCAA	
<i>Tet1</i>	FP	TTGTTACACATCCCCTTGA	190
	RP	AAGGGTTGGTTGTGCTTG	

Table 4. Primer sequences to amplify genes related to apoptosis and oxidative stress

Gene	Primer Sequence (5'→3')		Amplicon size (bp)
<i>Bax</i>	FP	CTGCAGAGGATGATTGCTGA	174
	RP	GATCAGCTCGGGCACTTAG	
<i>Bcl2</i>	FP	CTGGCATCTTCTCCTTCCAG	183
	RP	GACGGTAGCGACGAGAGAAG	
<i>Caspase 3</i>	FP	GGACCTGTGGACCTGAAAAA	159
	RP	GCATGCCATATCATCGTCAG	
<i>Caspase 9</i>	FP	CTCAGGCCAGAGGTTCTCAC	173
	RP	GGGCAGAAGTTCACGTTGTT	
<i>Ho-1</i>	FP	GAAGAAGATTGCGCAGAAGG	181
	RP	GAAGGCGGTCTTAGCCTCTT	
<i>Hif1 α</i>	FP	TCAAGTCAGCAACGTGGAAG	198
	RP	TATCGAGGCTGTGTGCGACTG	
<i>Nox2</i>	FP	CCAGTGTGTGCGAATCTCCT	179
	RP	GCATTACACACCACTCCAC	
<i>Nrf2</i>	FP	CCTTCCTCTGCTGCCATTAG	150
	RP	GTTCAAGTGAATGCCGGAGT	
<i>Nqo-1</i>	FP	GAAGCTGCAGACCTGGTGAT	170
	RP	GTGGTGATGGAAAGCAAGGT	
<i>Gapdh</i>	FP	CTCATGACCACAGTCCATGC	155
	RP	TTCAGCTCTGGGATGACCTT	

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Chapter 3

Evaluating the effect of a mixture of
pyrethroids administered at doses
relevant to human consumption on the
male reproductive system

3.1. Introduction

Pesticide usage continues to increase over a period of time. Synthetic pyrethroids are preferentially used because of their high insecticidal activity and biodegradability and thus account for almost one-fourth of the world's insecticide market (1). In general, a mixture of insecticides is used for improving crop production which contaminates the agricultural land and water (2). An impending result of this is that they enter the food chain, which is evident from recent studies that many pyrethroids can be detected in food items such as rice, wheat, vegetables, fruits, and nuts (3,4). This is also true in the Indian context (5,6). Thus, humans get constantly exposed to more than one pesticide at a time. Though many studies reported the effects of individual pesticides (including pyrethroids) on different organ systems, active investigations on the toxic effects of mixed pesticides have gained importance in the recent past because of the potential of human exposure via the food chain. In humans poisoned with a mixture of cypermethrin and chlorpyrifos, the ventilator-free days were shorter when compared with humans exposed to one of the compounds (7).

Mixed pesticides are known to cause synergistic toxicity in animal models (8-10). A large number of studies reported that exposure to pesticides and insecticides alters biochemical, histological, and reproductive parameters. In a recent study, it is observed that oxidant and antioxidant status is altered in cypermethrin exposed pregnant rats and their newborns (11). Similarly, we previously demonstrated alterations in oxidant and antioxidant status in adult rats and offspring born to female rats exposed to allethrin (12-15). Pyrethroid toxicity also disrupts the steroidogenesis by influencing the activities of the steroidogenic enzymes, especially 3β - and 17β -hydroxysteroid dehydrogenase (HSD) (14,16-19). Histopathological changes induced by a combination of pesticides are reported (20-22)). A recent study indicates that malformations in the reproductive tract are evident in rats exposed to mixed pesticides (23). A combination of chlorpyrifos and cypermethrin contributes to reproductive toxicity in male albino mice (22). Further, a combined low dose of cyromazine, 4-(2-methyl-4-chlorophenoxy) butyric acid (MCPB), pirimicarb, quinochloramine, thiram, and ziram caused decreased birth weight in rats (24). A mixture of pesticides at a dose equivalent to the no-observed-effect-level (NOEL) caused impairment in spermatogenesis in rats (25).

Previous studies were conducted on the effect of different pesticides at concentrations equivalent to lowest-observed-adverse-effect level (LOAEL), lowest-observed-effect level

(LOEL), lowest effect level (LEL), and no-observed-effect level (NOEL). However, the toxic effects of a mixture of pyrethroids are not well studied. It is evident that food items consumed by humans contain pyrethroids. The annual report published by the Indian Government agencies presents the levels of a variety of pyrethroids in different vegetables, rice, wheat, and other foods being sold in markets across the country (5,6). But studies that use doses of a mixture of pyrethroids that are relevant to food consumed by humans are not yet reported. In this study, we referred to the annual reports (2013-2014 and 2014-2015) published by “Monitoring of Pesticide Residues at National Level” prepared by Project Coordinating Cell, All India Network Project on Pesticide Residues, Indian Agricultural Research Institute, New Delhi – 110 012 on the residual levels of pyrethroids in food items (5,6); and the report published by National Institute of Nutrition, Hyderabad, India (26), on the average food consumption by Indians, to determine the dose of a mixture of pyrethroids to be administered in the experimental setup. The common food items consumed in the Indian context are rice and vegetables. These food items are thoroughly washed before cooking and thus a majority of the residual pyrethroids may be washed off. Hence, we used doses that are five and twenty-five times less than the residual levels present in the quantity of uncooked rice and vegetables consumed by an individual in the Indian context. The long-term effect of these doses was tested in a rat model system. In the real human setting, exposure to the mixture of pyrethroids happens over a period of time (every day) through food. Animal experimentation using such a setting would be the best. However, in this study, the mixture of pyrethroids was delivered through oral gavage. This route of administration was preferred over the administration through food because of practical problems (no control over the amount of food consumed by each rat; and the stability of pyrethroids during preparation of rat feed, which involves high temperature processing).

In light of previous studies that indicate the multi-dimensional effects of pyrethroids, we analyzed the oxidative and antioxidant status, biochemical profiling, histopathological changes, and fecundity in adult rats exposed to a mixture of pyrethroids. To the best of our knowledge, this is the first study that attempts to investigate the possible toxic effects of a dosage of pyrethroids that is relevant to the residual levels of pyrethroids in food consumed by the average Indian population.

3.2. RESULTS

PART I: Effect of a mixture of pyrethroids on the male reproductive function when exposed to doses relevant to human consumption (Experimental protocol -I).

3.2.1. Fecundity

To determine whether pyrethroid toxicity leads to decreased fecundity, we noted the litter size of pups born to control or pyrethroid (LD or HD) treated rats for 9 or 12 months (**Table 5**). A decrease in the litter size was observed in rats (F0 generation) that received LD pyrethroids for 9 to 15 months. However, such an effect was not observed in the HD group. To determine whether the litter size is affected in further generations, pups born to F0 generation rats were allowed to reach adulthood and put for mating. A similar protocol was followed up to F3 generation pups. Litter size was found to be reduced in the later generation rats that arose from F0 generation rats that were treated with LD of pyrethroids. Such an effect was mostly not observed in the descendants of F0 rats treated with HD of pyrethroids.

3.2.2. Sperm count

Sperm count was significantly reduced in LD-treated rats for 9 and 12 months compared to the controls. On the contrary, increases sperm count was observed in HD treated rats for 9 and 12 months. Rats that received HD or LD pyrethroid treatments for 15 months had significantly decreased sperm count compared to the controls (**Table 6**).

3.2.3. 3 β - and 17 β -HSD activity

To determine whether the enzymes involved in steroidogenesis are affected due to long-term pyrethroid toxicity, we measured 3 β - and 17 β -HSD activity in the testes of all the experimental animals in this study. The activities of both 3 β - and 17 β -HSD were significantly reduced in the testes of low or high-dose treated rats for 9 or 12 months. In the 15 months treated rats, decreased activity of both 3- β HSD and 17- β HSD were evident in the testes obtained from rats of LD or HD groups (**Table 6**).

Table 5. Fecundity in rats treated with pyrethroids.

Group	9 months			12 months			15 months		
	F0	F1	F2	F0	F1	F2	F0	F1	F2
Control	12.7 ± 1.6	11.6 ± 1.5	11.3 ± 1.8	13.0 ± 0.7	8.8 ± 1.6	10.1 ± 1.4	7 ± 1.4	8.5 ± 0.7	9.5 ± 1.2
LD	9.5 ± 1.2*	7.4 ± 2.8*	7.8 ± 3.3*	9.5 ± 1.8*	5.8 ± 1.6*	2.0 ± 2.0*	5.5 ± 0.7*	8.5 ± 2.1	8.5 ± 1.2
HD	9.5 ± 2.2	10.6 ± 1.6	9.2 ± 3.5	8.5 ± 3.1*	7.6 ± 1.6	10.8 ± 2.1	6.5 ± 0.7	9.3 ± 1.5	8.75 ± 1.7

*indicates $p < 0.05$ compared to respective control.

Table 6. Sperm count and hydroxysteroid dehydrogenase activities in pyrethroid treated rats.

Sperm count/ HSD's activity	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Sperm count (x 10⁴ / ml)	105.81 ± 4.33	59.24 ± 9.53*	168.18 ± 8.03*	129.29 ± 12.31	81.16 ± 6.36*	168.4 ± 6.97*	143 ± 17	81 ± 18*	91 ± 4.6*
3-β HSD[#]	0.237 ± 0.040	0.086 ± 0.022*	0.112 ± 0.043*	0.200 ± 0.020	0.036 ± 0.019*	0.113 ± 0.014*	0.374 ± 0.066	0.241 ± 0.16*	0.181 ± 0.004*
17-β HSD^{\$}	0.870 ± 0.284	0.288 ± 0.013*	0.361 ± 0.033*	0.304 ± 0.040	0.237 ± 0.022*	0.228 ± 0.019*	0.317 ± 0.065	0.160 ± 0.039*	0.181 ± 0.032*

*indicates $p < 0.05$ compared to respective control; [#]nano moles of NAD reduced/min/mg protein; ^{\$}nano moles of NADPH oxidized/min/mg protein

3.2.4. Acrosome reaction

Measurement of intracellular calcium in the spermatozoa as a function of acrosome reaction was performed using the fluorescent dye Fluo-3-AM. Calcium levels increase during acrosome reaction due to its release from intracellular stores. The binding of calcium to Fluo-3-AM enhances the fluorescence. We observed increased mean fluorescence intensity in spermatozoa of control rats when the ionophore was added to induce acrosome reaction (**Figure 10**). Such an increase was not observed in the spermatozoa obtained from rats treated with LD or HD of pyrethroids for 9 or 12 months. (**Figure 10**). In rats treated for 15 months, the effect on acrosome reaction was analyzed in vitro by measuring the fluorescence intensity of and PNA-FITC that specifically binds to glycoproteins on the acrosome. A significant decrease in fluorescence intensity of FITC was observed in spermatozoa obtained from control rats indicating the occurrence of acrosome reaction (**Figure 10**). Such a decrease was not observed in the spermatozoa obtained from the LD or HD treated groups.

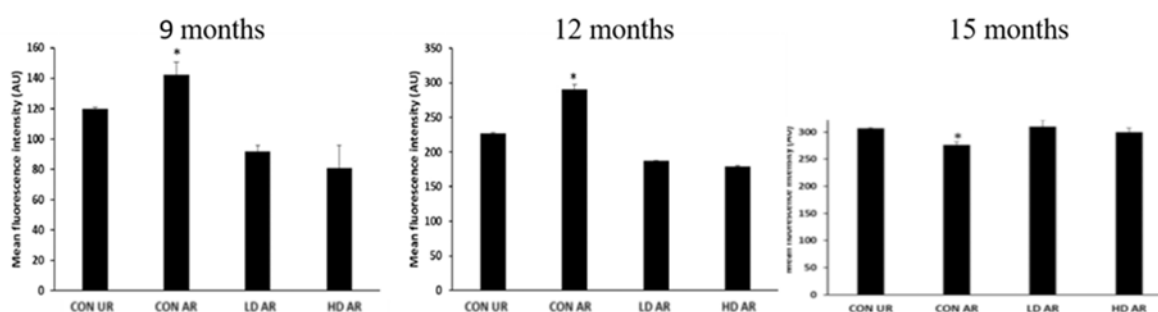


FIGURE 10. Acrosome reaction in the spermatozoa of pyrethroid treated rats. Spermatozoa were collected from control and pyrethroid treated rats ($n=10$ from each group) were allowed to undergo ionophore induced acrosome reaction and the fluorescence intensity of specific dyes measured. The mean fluorescence intensity presented on y-axis is expressed in arbitrary units (AU). Values shown are mean $\pm$ S.D of independent experiments. * indicates $p < 0.05$. CON UR – unreacted spermatozoa; CON AR – acrosome reacted spermatozoa; LD AR – low dose acrosome reacted; HD AR – high dose acrosome reacted.

3.2.5. Relative organ weights

Except for the prostate, no significant changes in the weights of testes, caput, and cauda were observed at all the time points analyzed when treated for 12 months. An increase in the relative organ weight of prostate was observed in animals treated with HD or LD dose for 9 or 12 months when compared to the respective controls. In the 15 months treated animals, a significant decrease in the body weight of animals was evident in the HD group compared to the control (**Table 7**). Though there was also a decrease in the body weight of rats in the LD group, it was not significantly different from the control group. An increase in the relative organ weight was observed for all the tissues in the HD group. Similarly, increased relative organ weight of testis, caput, prostate, and vas deferens was observed in the LD group. Though

an increase in relative organ weight was also observed for other tissues in the LD group, statistical significance was not evident (**Table 7**).

3.2.6. Lipid peroxidation and nitric oxide levels

In general, lipid peroxidation (LPO) levels were not altered in all the tissues analyzed at all the time points in rats treated for 9 to 12 months. In rats exposed for 15 months, lipid peroxidation products were increased in the caput, cauda, and prostate obtained from rats of HD group (**Table 8**). Nitric oxide levels were not altered in all the tissues obtained from rats treated with low or high dose pyrethroids for 9 months. However, a significant increase in the levels of nitric oxide was observed in the testes and cauda obtained from rats treated with HD and LD for 12 months. However, no changes in the levels of NO were observed in all the tissues obtained from both the treatment groups (**Table 9**).

3.2.7. Antioxidant enzymes

Catalase activity was not altered in all the tissues obtained from rats treated with LD or HD pyrethroids for 9 months. Its activity was found to be significantly increased in the testes, caput, and cauda, whereas the same was decreased in the prostate obtained from rats treated with HD or LD of pyrethroids for 12 months. In the 15 months treated animals, catalase activity was significantly increased in the caput, cauda, and prostate of both LD and HD groups. Catalase activity was significantly decreased in the testes obtained from both the treatment groups (**Table 10**). Glutathione peroxidase activity (GPx) was significantly increased in the testes, caput, cauda, and prostate of rats that received LD or HD pyrethroid treatment for 9 or 12 months (**Table 11**). GPx activity was not altered in the reproductive organs (testis, caput, cauda, and prostate) of both the treatment for 15 months (**Table 11**). Glutathione- S- transferase (GST) activity was significantly increased in the testis and caput of rats that received LD pyrethroid treatment for 9 months. No significant changes in GST activity were observed in all the tissues of rats that received LD or HD pyrethroid treatment for 12 months. In the cauda obtained from both LD and HD groups, GST activity was significantly increased compared to the control. Similarly, increased activity was evident in the testes and caput obtained from LD group. On the contrary, the activity of GST was found to be significantly decreased in the prostate and testes of LD and HD treated rats respectively (**Table 12**).

Table 7. Relative organ weights (grams per 100 g body weight) in the pyrethroid treated rats.

	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Testis	0.36 ± 0.10	0.41 ± 0.02	0.39 ± 0.01	0.42 ± 0.028	0.41 ± 0.038	0.42 ± 0.026	0.326 ± 0.033	0.375 ± 0.030*	0.406 ± 0.022*
Caput	0.078 ± 0.005	0.063 ± 0.017	0.083 ± 0.007	0.066 ± 0.001	0.065 ± 0.003	0.075 ± 0.007	0.054 ± 0.006	0.100 ± 0.005*	0.072 ± 0.003*
Cauda	0.055 ± 0.014	0.060 ± 0.012	0.075 ± 0.011	0.046 ± 0.008	0.053 ± 0.002	0.06 ± 0.007	0.044 ± 0.009	0.037 ± 0.008	0.059 ± 0.008*
Prostate	0.11 ± 0.01	0.14 ± 0.01*	0.19 ± 0.03*	0.11 ± 0.02	0.15 ± 0.01*	0.19 ± 0.01	0.111 ± 0.015	0.166 ± 0.037*	0.161 ± 0.009*

*indicates $p < 0.05$ compared to respective control

Table 8. Lipid peroxidation# in various tissues of rats treated with a mixture of pyrethroids.

	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Testis	0.078 ± 0.017	0.152 ± 0.008*	0.048 ± 0.012	0.184 ± 0.024	0.230 ± 0.009	0.267 ± 0.098	0.085 ± 0.019	0.089 ± 0.008	0.091 ± 0.001
Caput	0.175 ± 0.078	0.163 ± 0.052	0.185 ± 0.002	0.269 ± 0.029	0.342 ± 0.043	0.277 ± 0.085	0.153 ± 0.008	0.225 ± 0.021	0.207 ± 0.028*
Cauda	0.173 ± 0.001	0.187 ± 0.021	0.196 ± 0.006	0.346 ± 0.055	0.380 ± 0.043	0.394 ± 0.127	1.141 ± 0.002	0.187 ± 0.016	0.165 ± 0.011*
Prostate	0.017 ± 0.006	0.014 ± 0.011	0.014 ± 0.001	0.025 ± 0.005	0.154 ± 0.032	0.019 ± 0.003	0.040 ± 0.002	0.065 ± 0.002	0.057 ± 0.001*

nano moles MDA/ mg protein. *indicates $p < 0.05$ compared to respective control.

Table 9. Nitric oxide levels# in various tissues of rats treated with a mixture of pyrethroids.

	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Testis	0.622 ± 0.080	0.588 ± 0.029	0.734 ± 0.080	0.472 ± 0.073	0.878 ± 0.264*	0.803 ± 0.058*	0.403 ± 0.067	0.581 ± 0.063	0.424 ± 0.023
Caput	1.844 ± 0.120	1.919 ± 0.240	2.597 ± 0.100	1.430 ± 0.103	1.667 ± 0.312	1.867 ± 0.292	2.840 ± 0.146	2.524 ± 0.206	2.490 ± 0.068
Cauda	4.132 ± 0.170	5.444 ± 0.130	6.253 ± 2.860	1.034 ± 0.111	2.125 ± 0.050*	2.095 ± 0.032*	2.417 ± 0.526	2.288 ± 0.824	2.971 ± 0.851
Prostate	1.572 ± 0.660	1.513 ± 0.360	1.329 ± 0.320	0.976 ± 0.125	0.601 ± 0.083*	0.555 ± 0.177*	2.886 ± 0.151	2.974 ± 0.063	3.065 ± 0.501

#μmoles nitrite/mg protein. *indicates $p < 0.05$ compared to respective control

Table 10. Catalase activity# in various tissues of rats treated with a mixture of pyrethroids.

	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Testis	0.036 ± 0.009	0.050 ± 0.020	0.037 ± 0.005	0.046 ± 0.018	0.075 ± 0.001*	0.064 ± 0.019	0.079 ± 0.001	0.035 ± 0.001*	0.026 ± 0.010*
Caput	0.034 ± 0.016	0.046 ± 0.009	0.027 ± 0.002	0.063 ± 0.009	0.083 ± 0.012*	0.098 ± 0.005*	0.040 ± 0.003	0.069 ± 0.014*	0.071 ± 0.012*
Cauda	0.022 ± 0.003	0.036 ± 0.009	0.019 ± 0.003	0.029 ± 0.003	0.100 ± 0.028*	0.042 ± 0.001*	0.032 ± 0.009	0.051 ± 0.002*	0.074 ± 0.017*
Prostate	0.016 ± 0.008	0.013 ± 0.005	0.014 ± 0.005	0.036 ± 0.009	0.017 ± 0.004*	0.026 ± 0.002*	0.022 ± 0.005	0.044 ± 0.012*	0.050 ± 0.011 *

$K30 = (2.303/30) \times \log (A1/A2)$ where A1 and A2 are the initial and final absorbance respectively of hydrogen peroxide for enzyme assay. *indicates $p < 0.05$ compared to respective control.

Table 11. Glutathione peroxidase activity# in various tissues of rats with a mixture of pyrethroids

Name of the tissue	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Testis	3.740 ± 0.100	4.930 ± 1.093*	4.230 ± 0.619*	1.282 ± 0.149	3.484 ± 0.454*	2.621 ± 0.275*	2.309 ± 0.280	3.034 ± 0.640	1.903 ± 0.200
Caput	3.010 ± 0.100	3.540 ± 0.100*	4.530 ± 0.028*	1.976 ± 0.203	4.321 ± 0.928*	2.553 ± 0.675*	6.030 ± 2.436	5.819 ± 2.437	4.635 ± 0.450
Cauda	2.940 ± 0.100	5.680 ± 0.058*	6.140 ± 0.100*	2.631 ± 0.559	4.114 ± 0.288*	4.232 ± 0.143*	7.127 ± 0.331	6.808 ± 0.611	5.933 ± 1.079
Prostate	2.180 ± 0.100	6.150 ± 0.100	12.520 ± 0.100*	1.612 ± 0.107	1.982 ± 0.194*	3.446 ± 0.331*	2.595 ± 0.291	3.231 ± 0.258	1.808 ± 0.575

#μmoles NADPH oxidized/ min/ mg of protein. *indicates $p < 0.05$ compared to respective control.

Table 12. Glutathione S- transferase activity# in various tissues of rats with a mixture of pyrethroids

Name of the tissue	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Testis	0.260 ± 0.070	0.480 ± 0.050*	0.310 ± 0.020	0.259 ± 0.023	0.208 ± 0.044	0.204 ± 0.003	0.452 ± 0.033	0.923 ± 0.029*	0.304 ± 0.085*
Caput	0.610 ± 0.100	0.900 ± 0.090*	0.550 ± 0.090	0.454 ± 0.056	0.546 ± 0.152	0.412 ± 0.092	0.723 ± 0.060	1.362 ± 0.485*	0.743 ± 0.095
Cauda	0.770 ± 0.080	0.800 ± 0.040	0.670 ± 0.070	0.508 ± 0.137	0.526 ± 0.060	0.476 ± 0.158	1.159 ± 0.148	1.596 ± 0.140*	1.580 ± 0.182*
Prostate	0.330 ± 0.050	0.228 ± 0.030*	0.159 ± 0.010*	0.216 ± 0.024	0.149 ± 0.023*	0.102 ± 0.022*	0.247 ± 0.050	0.159 ± 0.003*	0.262 ± 0.042

#μmoles of GST-GSH conjugate formed / min /mg protein. *indicates $p < 0.05$ compared to respective control.

3.2.8. Histopathological analyses

The effect of mixture of pyrethroids on the anatomical changes in different tissues of rats exposed for 9 months was examined. In the testes of rats treated with the LD, severe or complete degeneration of spermatogonial cells in seminiferous tubules with an absence of sperms was observed (**Figure 11**), whereas HD treatment resulted in infiltration of inflammatory cells or giant cells, and edema or fluid accumulation are noticed in seminiferous tubules (Figure 2). In the caput obtained from LD treated rats, mild to moderate mucosal epithelial hyperplasia was noticed, whereas in the HD treated animals, severe mucosal degeneration in epithelial layers of tubules, less volume or number of lysed sperms stored inside the tubules is evident (**Figure 11**). In the LD treated rats, cauda epididymis displayed moderate degeneration in mucosal epithelial cells and moderate proliferation of fibrous tissue in between the tubules and oligospermia are noticed. Moderate deposition of pink material [glycogen or lipids or fibrous], was evident in the cauda obtained from HD treated animals. Prostate from the LD and HD treated rats displayed moderate mucosal epithelial hyperplasia and translucent storage in the lumen (**Figure 11**).

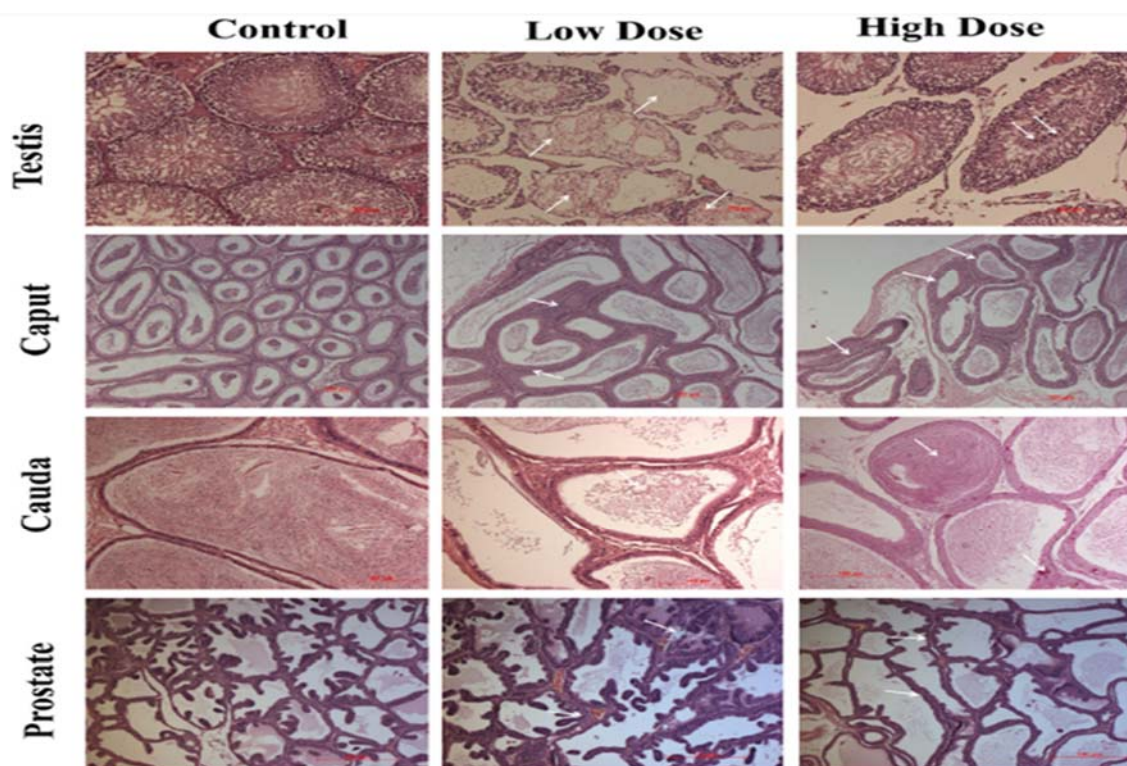


FIGURE 11. Histopathology of male reproductive tract tissues in rats treated with pyrethroid mixture. Tissue sections obtained from rats that were treated with a mixture of pyrethroids for 9 months were stained with hematoxylin and eosin. Arrows indicate the histopathological change observed in the LD and HD treated groups (TESTIS: degeneration of spermatogonial cells, absence of sperms, infiltration of inflammatory cells or giant cells and edema or fluid accumulation; CAPUT: mucosal epithelial hyperplasia, mucosal degeneration, lysed sperms; CAUDA: degeneration in mucosal epithelial cells, proliferation of fibrous tissue, deposition of glycogen or lipids; PROSTATE: mucosal epithelial hyperplasia and translucent storage). Scale bar: 100 μ m.

In the 12 months treated group, moderate to severe degeneration [vacuolar type] in seminiferous tubules and oligospermia was observed in testes of LD treated rats (**Figure 12**). In the HD treated rats, severe seminiferous tubular degeneration and fibrosis or atrophy along with aspermia [absence of sperms] was noticed in the seminiferous tubules (Figure 3). Epithelial vacuolization and spermatid presence was also observed in the testes of control rats (**Figure 12**). In the caput obtained from rats treated with LD and HD of pyrethroids, moderate degeneration, and hyperplasia in mucosal epithelial cells (**Figure 12**). Mild mucosal degeneration, mucosal hyperplasia, and hemorrhages were observed in the cauda of rats treated with LD and HD of pyrethroid mixture (**Figure 12**). LD treatment resulted in mild fibrous tissue proliferation and degenerative changes in the prostate whereas HD treatment resulted in moderate thickening or fibrosis along with hemorrhages noticed (**Figure 12**).

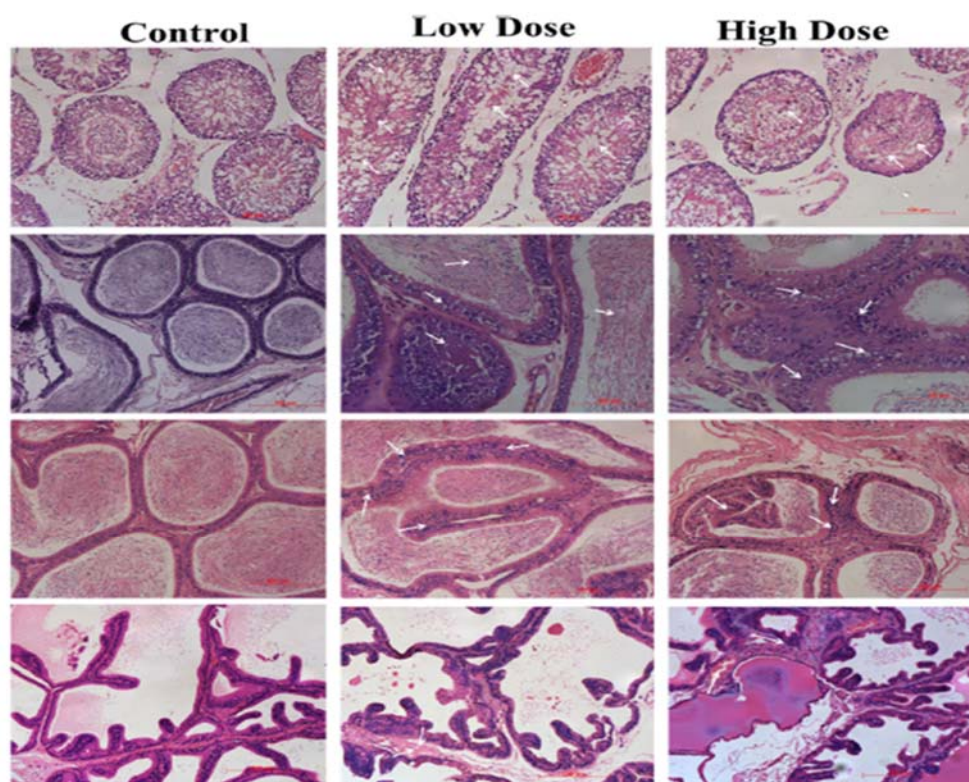


FIGURE 12. *Histopathology of male reproductive tract tissues in rats treated with pyrethroid mixture. Tissue sections obtained from rats that were treated with a mixture of pyrethroids for 12 months were stained with hematoxylin and eosin. Arrows indicate the histopathological change observed in the LD and HD treated groups Scale bar: 100 μ m.*

In the 15 months treated rats, testes obtained from rats of LD group displayed severe seminiferous tubular degeneration, accumulation of edematous fluid, and fragmented necrotic materials in between the seminiferous tubules, and atrophy or necrosis of spermatogonial cells with aspermia was noticed (**Figure 13**). Severe seminiferous tubular degeneration of vacuolar type with atrophy or necrosis and fragmentation of spermatogonial cells with aspermia was

evident in the testes of HD treated rats (**Figure 13**). In the caput of rats from LD, group oligospermia and aspermia and moderate degeneration of mucosal epithelial cells along with mucosal hyperplasia are noticed. Mild degeneration of mucosal epithelial cells along with oligospermia was also noted in the caput of rats in HD group. Moderate vacuolar degeneration of mucosal epithelial cells of tubules, moderate fibrosis in between tubules, and oligospermia was observed in the cauda of LD treated rats (**Figure 13**). Cauda from rats of HD group displayed moderate oligospermia or aspermia and severe fibrosis in between tubules (**Figure 13**). Mild hyperplasia of mucosal epithelial folds along with homogenous fluid accumulation was noticed in the prostate of rats from the LD and HD groups (**Figure 13**).

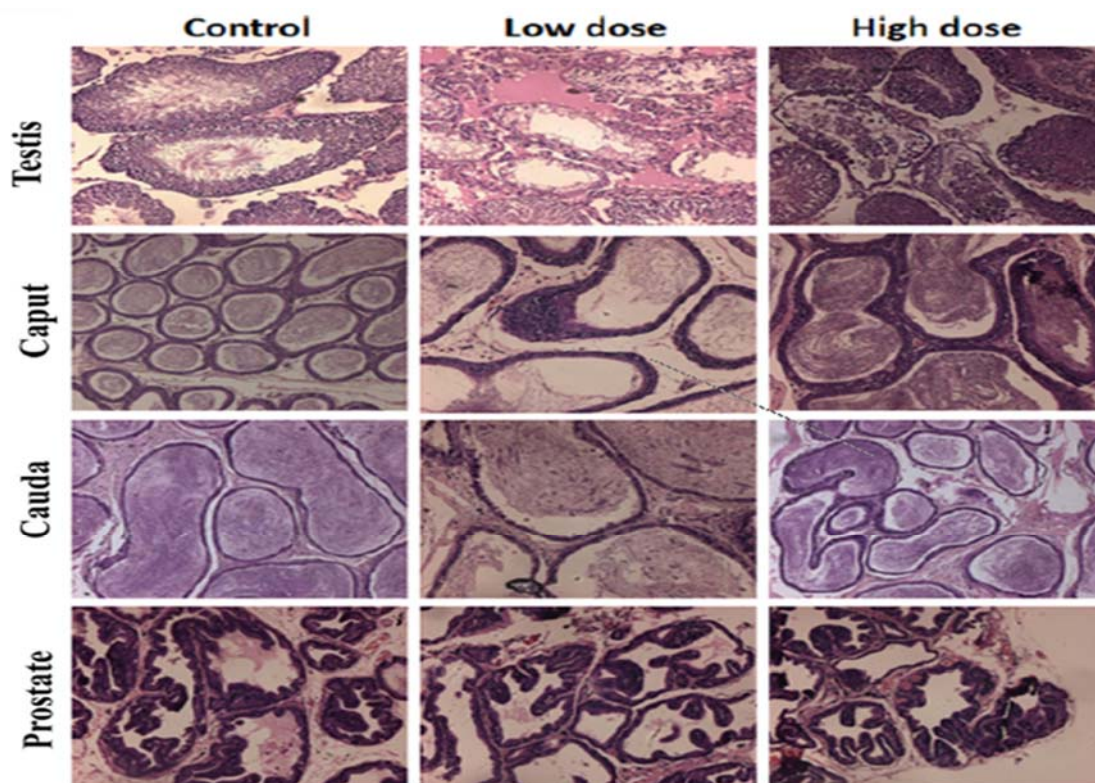


FIGURE 13. *Histopathological evaluation of reproductive tissues in rats treated with a mixture of pyrethroids. Testes, caput, cauda, and prostate of rats administered with a mixture of pyrethroids for 15 months were stained with hematoxylin and eosin.*

PART II: Effect of a mixture of pyrethroids on the male reproductive function when exposed to doses equivalent to human consumption (Experimental protocol –II).**3.2.9. Fecundity**

The effect of long-term exposure to pyrethroid mixture on fecundity was analyzed in the control and treated rats by mating studies. Further, fecundity was also analyzed in the males of F1 and F2 generations born to respective control and pyrethroid treated forefathers. The litter size in the pyrethroid treated rats (exposed for 1, 3, 6, 9, 12, and 15 months) was significantly lower compared to the respective control at all the time points analyzed (**Table 13**). Similarly, litter size was found to be significantly decreased in the F1 and F2 generation male rats that were born to forefathers exposed to pyrethroids for 1, 3, 6, 9, 12, and 15 months.

3.2.10. Sperm count

To determine whether the litter size observed could be due to impaired spermatogenesis, sperm count was evaluated in the pyrethroid-treated rats. No significant changes in the sperm count were observed in rats of 1 month treatment group (**Table 14**), whereas a significant decline was observed in rats that were treated with a mixture of pyrethroids for 3, 6, 9, 12, and 15 months (**Table 14**).

3.2.11. 3 β - and 17 β -HSD activity

A decline in sperm count is a manifestation of disturbed spermatogenesis, a process governed by a variety of testicular factors, hormones, and enzymes that catalyze the production of steroid hormones. In line with this, we estimated the activity of 3 β -HSD and 17 β -HSD in the testicular homogenates. The activities of both these enzymes were significantly decreased in the testes of rats treated with a mixture of pyrethroids for 3, 6, 9, 12, and 15 months, while such a decline was not observed after 1 month treatment (**Table 14**).

Table 13. Transgenerational fecundity in pyrethroid treated rats.

		1 month	3 months	6 months	9 months	12 months	15 months
F0	Control	12.5 ± 0.97	10.1 ± 1.34	10.7 ± 1.38	11.1 ± 1.72	10.4 ± 1.14	10.2 ± 1.70
	Treated	8.0 ± 1.29*	7.5 ± 1.39*	5.8 ± 1.46*	6.6 ± 1.03*	7.8 ± 1.30*	7.5 ± 2.12*
F1	Control	10.2 ± 1.29	10.2 ± 1.89	9.5 ± 1.29	10.2 ± 1.92	11.5 ± 1.29	--
	Treated	8.33 ± 1.15*	7.5 ± 1.29*	6.25 ± 1.50*	9.0 ± 1.00*	8.4 ± 1.51*	--
F2	Control	10.0 ± 2.94	11.0 ± 1.82	10.0 ± 1.82	8.7 ± 2.21	9.5 ± 1.29	--
	Treated	7.0 ± 1.00*	6.5 ± 2.51*	6.3 ± 1.15*	5.5 ± 1.91*	7.5 ± 1.29*	--

*indicates $p < 0.05$ compared to respective control

Table 14. Sperm count and hydroxysteroid dehydrogenase activities in pyrethroid treated rats

	Group	1 month	3 months	6 months	9 months	12 months	15 months
Sperm count (x 10⁴/ml)	Control	124.01 ± 9.09	297.69 ± 16.99	188.14 ± 20.76	253.30 ± 28.15	226.15 ± 12.62	198.63 ± 15.65
	Treated	125.55 ± 7.53	205.34 ± 14.51*	120.85* ± 22.84	180.50* ± 13.95	128.39* ± 27.68	133.96* ± 30.90
3β-HSD activity[#]	Control	0.071 ± 0.009	0.064 ± 0.003	0.047 ± 0.005	0.061 ± 0.005	0.068 ± 0.010	0.041 ± 0.006
	Treated	0.075 ± 0.019	0.040 ± 0.007*	0.035 ± 0.005*	0.028 ± 0.011*	0.028 ± 0.003*	0.024 ± 0.006*
17β-HSD activity^{\$}	Control	0.427 ± 0.051	0.562 ± 0.023	0.456 ± 0.031	0.454 ± 0.014	0.474 ± 0.019	00
	Treated	0.418 ± 0.085	0.395 ± 0.039*	0.344 ± 0.034	0.365 ± 0.030*	0.285 ± 0.020*	00

*indicates $p < 0.05$ compared to respective control; [#]nano moles of NAD reduced/min/mg protein; ^{\$}nano moles of NADPH oxidized/min/mg protein.

3.2.12. Capacitation and acrosome reaction

Sperm function in terms of capacitation and acrosome reaction is very crucial for successful fertilization, which in turn affects fecundity. The possibility of improper sperm function to influence fecundity in pyrethroid treated rats was assessed by evaluating capacitation and acrosome reaction by fluorescence-based flow cytometry.

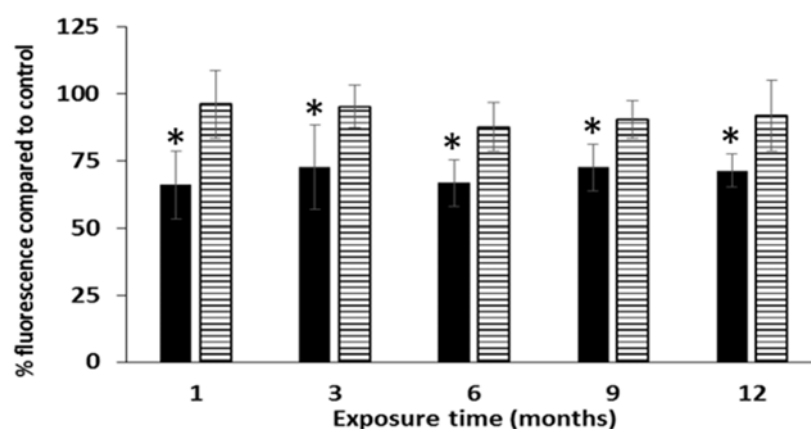


FIGURE 14. Capacitation in the spermatozoa of pyrethroid treated rats. Spermatozoa were collected from control and pyrethroid treated rats ($n=10$ from each group) were allowed to undergo capacitation in M 2 medium and the fluorescence intensity of filipin was measured. The mean fluorescence intensity presented on y-axis is expressed in percentage. Values shown are mean $\pm$ S.D of independent experiments. *indicates $p < 0.05$. ■ - Control capacitated; ▨ - Pyrethroid treated capacitated.

The fluorescence intensity exhibited by the uncapacitated spermatozoa of control rats was considered as 100 % and accordingly the same in the capacitated spermatozoa of control and pyrethroid treated rats were expressed as percent fluorescence compared to uncapacitated control. Capacitation was assessed by monitoring the fluorescence intensity of filipin (cholesterol-binding dye in the capacitated and uncapacitated spermatozoa. A significant decrease in percent fluorescence intensity was observed in the capacitated sperm of control rats when compared to their uncapacitated counterparts at all the time points (1 to 12 months) tested (**Figure 14**). Such a decrease was not observed in the spermatozoa of all the groups of rats that were treated with a mixture of pyrethroids for 1 to 12 months, indicating that capacitation is not happening.

The fluorescence intensity of FITC labelled PNA (that binds to glycoproteins on sperm surface) was monitored to assess acrosome reaction. A decrease in percent fluorescence intensity was observed in the acrosome reacted spermatozoa of control rats when compared to their unreacted counterparts (**Figure 15**). The percent fluorescence intensity was also significantly lower in the spermatozoa obtained from all the treatment endpoints (**Figure 15**),

suggesting that acrosome reaction is taking place. However, the percent fluorescence intensity in the spermatozoa of pyrethroid-treated rats appears to be more than that is observed in the spermatozoa of control rats at all the time points. From these results, it appears that long-term treatment with pyrethroids appears to affect capacitation and not acrosome reaction to a certain extent.

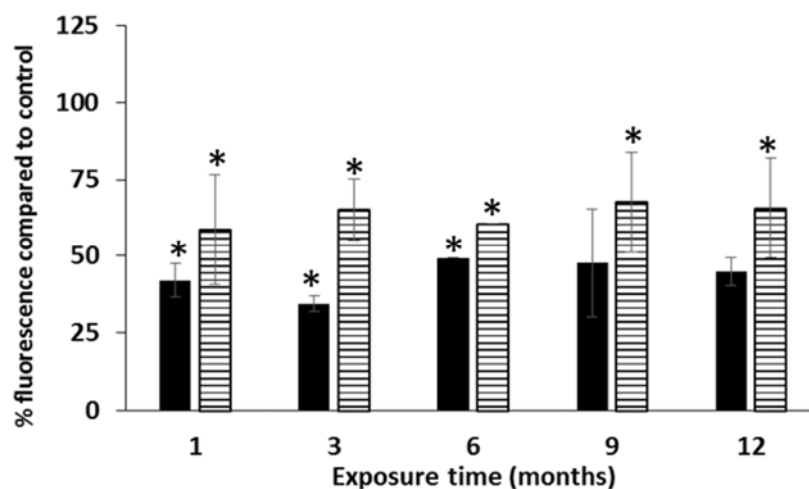


FIGURE 15. Acrosome reaction in the spermatozoa of pyrethroid treated rats. Spermatozoa were collected from control and pyrethroid treated rats ($n=10$ from each group) were allowed to undergo capacitation in M 2 medium. Capacitated spermatozoa were subjected to acrosome reaction with the ionophore A23187 and stained with PNA-FITC. The extent of acrosome reacted spermatozoa was assessed by recording the fluorescence intensity of the membrane-bound lectin PNA-FITC. The mean fluorescence intensity presented on y-axis is expressed in percentage. Values shown are mean $\pm$ S.D of independent experiments. * indicates $p < 0.05$. ■ - Control capacitated; ▨ - Pyrethroid treated capacitated

3.2.13. Hormonal profile

FSH levels were significantly increased in the serum of rats treated with a mixture of pyrethroids for 12 or 15 months (**Table 15**) However, GnRH levels were significantly increased only at 1, 3, 6, and 15 month treatment time points. A significant increase at the 1st and 3rd month time points followed by a decrease at the 9th, 12th, and 15th months was observed for INHB. The serum levels of KISS1 were significantly increased at the 1, 3, and 15 month time points whereas a decrease was observed in the samples obtained after 12 months of treatment with pyrethroids. LH levels were significantly decreased in the sera obtained from rats treated for 1, 9, and 15 months. Exposure to a mixture of pyrethroids for 3, 6, and 9 months resulted in a significant decrease in the levels of serum PRL, while an increase was observed at the 12 month time point. Testosterone (T) levels were significantly lower in the serum of rats exposed to a mixture of pyrethroids for 6, 9, 12, and 15 months. A significant increase in the serum levels of TSH was observed only in rats treated for 9 and 12 months (**Table 15**).

3.2.14. Relative organ weights

The final body weights of control and pyrethroid-treated rats were noted at the end of each treatment period. No significant change in body weights between the treated and control rats was observed at any of the time points analyzed (**Table 16**). Similarly, no differences in the relative organ weights were observed for all the tissues at all the time points analyzed (**Table 12**).

3.2.15. Lipid peroxidation and nitric oxide

The extent of lipid peroxidation in various tissues of control and pyrethroid-treated rats was estimated as a function of the levels of lipid peroxidation products (**Table 17**). In the testes of rats obtained from 12 and 15 months, significantly higher levels of lipid peroxidation were observed. A significant increase in the levels of lipid peroxidation was observed in the caput of pyrethroid treated rats at all the time points analyzed, whereas in the cauda and prostate such an increase was observed in rats treated for 3 to 15 months and 9 to 15 months, respectively. Nitric oxide levels were found to be increased in all the tissues analyzed. In the case of testes and prostate, the significant increase was evident in rats treated for 6 to 15 months, while such as increase for caput was evident in rats treated for 3 to 15 months. Significantly increased levels of nitric oxide were observed in the cauda of rats obtained from all the treatment points (**Table 18**).

Catalase activity was in general was significantly increased in all the tissues analyzed with variations in the duration of treatment (**Table 19**). While its activity was increased in the caput at all the time points analyzed, such a change in the testes was observed only in rats treated for 3 to 15 months. Cauda obtained from rats treated for 6 to 15 months and prostate from rats treated for 9 to 15 months also displayed a significant increase in catalase activity when compared to the respective controls (**Table 19**). GPx activity was significantly increased in the cauda, of rats obtained from all the treatment points (**Table 16**), which such an increase in the testes was restricted to rats treated for 6 to 15 months. In the case of prostate, a significant decrease was observed in rats treated for 1 to 6 months, whereas a significant increase was observed in rats treated for 9 to 15 months (**Table 20**). GST activity was not altered in any of the tissues obtained from all the time points (**Table 21**). SOD activity was found to be significantly increased in the cauda of rats treated with pyrethroids for 3 to 6 months, while such an increase was observed for cauda in rats treated for 6 to 15 months (**Table 22**). On the other hand, SOD activity was significantly decreased in the cauda of 6 to 15 month treated rats (**Table 22**).

Table 15. Hormonal profile in pyrethroid treated rats.

Hormone	Units	Group	1 month	3 months	6 months	9 months	12 months	15 months
FSH	mIU/ ml	Control	35.28 ± 2.59	32.10 ± 2.84	35.01 ± 1.93	34.82 ± 0.54	22.61 ± 0.24	36.43 ± 4.68
		Treated	32.77 ± 5.19	36.04 ± 1.95	35.59 ± 1.50	36.26 ± 1.16	31.08* ± 2.19	45.98 ± 1.33*
GnRH	pg/ml	Control	384.04 ± 22.05	488.43 ± 21.01	541.09 ± 28.01	614.79 ± 61.84	806.07 ± 61.40	449.47 ± 30.06
		Treated	456.43 ± 35.59*	553.40 ± 10.98*	636.30 ± 16.77*	519.67 ± 61.02	720.70 ± 115.92	499.12 ± 12.64
INHB	pg/ml	Control	494.82 ± 7.33	366.31 ± 61.44	576.32 ± 71.49	570.93 ± 44.56	520.73 ± 4.31	784.15 ± 3.28
		Treated	580.34 ± 43.33*	535.16 ± 84.18*	523.27 ± 6.17	519.82 ± 19.028	501.37 ± 13.93*	769.72 ± 9.41*
KISS1	pg/ml	Control	692.90 ± 9.41	895.26 ± 9.41	1058.69 ± 176.53	1071.60 ± 143.43	991.12 ± 288.41	885.86 ± 48.36
		Treated	753.53 ± 31.49*	938.35 ± 3.32	858.10 ± 95.51	1078.06 ± 137.05	531.44 ± 22.42*	1123.00 ± 113.34*
LH	mIU/ml	Control	29.55 ± 8.50	19.20 ± 1.60	18.67 ± 3.04	19.22 ± 1.30	17.72 ± 7.69	25.70 ± 4.46
		Treated	14.48 ± 1.97*	20.10 ± 2.52	16.79 ± 3.05	15.29 ± 0.48*	14.74 ± 5.96	15.18 ± 1.61*
PRL	ng/ml	Control	39.60 ± 19.66	23.73 ± 3.54	46.52 ± 0.24	20.50 ± 5.58	15.28 ± 1.62	28.71 ± 2.85
		Treated	27.35 ± 6.29	16.07 ± 2.37*	29.49 ± 1.50*	31.43 ± 1.29*	40.43 ± 2.31*	26.87 ± 2.52
T	ng/ml	Control	4.22 ± 0.25	3.62 ± 0.86	6.70 ± 0.54	4.99 ± 0.57	5.38 ± 1.01	4.22 ± 0.15
		Treated	3.77 ± 0.59	4.53 ± 0.62	4.68 ± 0.06*	2.85 ± 0.08*	4.01 ± 0.01*	3.77 ± 0.09*
TSH	ng/ml	Control	17.60 ± 3.38	17.21 ± 0.79	13.42 ± 0.17	15.91 ± 2.46	20.80 ± 4.46	20.54 ± 1.71
		Treated	16.68 ± 2.88	19.00 ± 1.93	20.40 ± 2.16*	22.41 ± 2.17*	16.88 ± 0.94	24.15 ± 3.16

*indicates $p < 0.05$ compared to the respective control.

Table 16. Relative organ weights of various tissues in rats treated with a mixture of pyrethroids for 1 to 15 months.

		1 month	3 months	6 months	9 months	12 months	15 months
Testis	Control	0.00569 ± 0.00088	0.00507 ± 0.00087	0.00378 ± 0.00036	0.00395 ± 0.00028	0.00374 ± 0.00032	0.00458 ± 0.00122
	Treated	0.00564 ± 0.00105	0.00466 ± 0.00048	0.00424 ± 0.00044	0.00557 ± 0.00041	0.00361 ± 0.00036	0.00400 ± 0.00073
Caput	Control	0.00093 ± 0.00022	0.00090 ± 0.00013	0.00074 ± 0.00004	0.00082 ± 0.00011	0.00086 ± 0.00017	0.00131 ± 0.00074
	Treated	0.00114 ± 0.00018	0.00071 ± 0.00013	0.00078 ± 0.00013	0.00295 ± 0.00536	0.00073 ± 0.00013	0.00080 ± 0.00018
Cauda	Control	0.00076 ± 0.00017	0.00084 ± 0.00018	0.00070 ± 0.00004	0.00072 ± 0.00013	0.00070 ± 0.00010	0.00091 ± 0.00021
	Treated	0.00086 ± 0.00024	0.00077 ± 0.00010	0.00074 ± 0.00008	0.00284 ± 0.00541	0.00062 ± 0.00008	0.00114 ± 0.00107
Prostate	Control	0.00115 ± 0.00045	0.00110 ± 0.00041	0.00126 ± 0.00027	0.00146 ± 0.00048	0.00148 ± 0.00028	0.00146 ± 0.00031
	Treated	0.00310 ± 0.00161	0.00113 ± 0.00019	0.00102 ± 0.00030	0.00328 ± 0.00521	0.00146 ± 0.00029	0.00111 ± 0.00053

*indicates $p < 0.05$.**Table 17. Lipid peroxidation levels# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.**

		1 month	3 months	6 months	9 months	12 months	15 months
Testis	Control	0.023 ± 0.016	0.017 ± 0.004	0.012 ± 0.003	0.014 ± 0.003	0.086 ± 0.026	0.033 ± 0.009
	Treated	0.030 ± 0.011	0.021 ± 0.006	0.019 ± 0.005	0.014 ± 0.005	0.376 ± 0.143*	0.087 ± 0.046*
Caput	Control	0.030 ± 0.011	0.034 ± 0.006	0.018 ± 0.004	0.013 ± 0.003	0.065 ± 0.017	0.025 ± 0.002
	Treated	0.149 ± 0.018*	0.054 ± 0.006*	0.043 ± 0.013*	0.029 ± 0.006*	0.096 ± 0.006*	0.062 ± 0.011*
Cauda	Control	0.221 ± 0.060	0.086 ± 0.011	0.063 ± 0.007	0.025 ± 0.006	0.083 ± 0.023	0.123 ± 0.004
	Treated	0.247 ± 0.018	0.211 ± 0.125*	0.079 ± 0.003*	0.054 ± 0.008*	0.230 ± 0.044*	0.206 ± 0.066*
Prostate	Control	0.008 ± 0.002	0.016 ± 0.002	0.004 ± 0.001	0.006 ± 0.002	0.017 ± 0.008	0.012 ± 0.003
	Treated	0.006 ± 0.001	0.011 ± 0.004	0.005 ± 0.003	0.011 ± 0.001*	0.037 ± 0.004*	0.021 ± 0.006*

- nanomoles MDA / min / mg protein; *indicates $p < 0.05$ compared to the respective control.

Table 18. Nitric oxide levels# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

		1 month	3 months	6 months	9 months	12 months	15 months
Testis	Control	0.639 ± 0.144	0.811 ± 0.602	0.177 ± 0.007	0.313 ± 0.093	0.494 ± 0.082	0.428 ± 0.148
	Treated	0.704 ± 0.075	1.486 ± 0.462	0.386 ± 0.081*	0.701 ± 0.263*	1.253 ± 0.542*	0.755 ± 0.183*
Caput	Control	0.941 ± 0.174	1.439 ± 0.376	0.492 ± 0.028	0.716 ± 0.220	1.272 ± 0.576	1.493 ± 0.160
	Treated	1.262 ± 0.235	2.465 ± 0.596*	1.080 ± 0.269*	0.863 ± 0.096*	3.588 ± 1.045*	2.114 ± 0.283*
Cauda	Control	1.107 ± 0.103	2.402 ± 0.386	1.469 ± 0.363	0.809 ± 0.164	4.028 ± 0.856	1.331 ± 0.142
	Treated	1.922 ± 0.006*	4.868 ± 0.757*	4.143 ± 1.104*	1.156 ± 0.194*	6.666 ± 0.075*	2.173 ± 0.438
Prostate	Control	2.404 ± 0.196	2.903 ± 0.805	1.130 ± 0.110	0.821 ± 0.102	2.040 ± 0.714	1.404 ± 0.155
	Treated	2.642 ± 0.160	3.127 ± 0.844	1.416 ± 0.132*	1.870 ± 0.065*	5.690 ± 1.572*	2.484 ± 0.215

- μ moles nitrite / mg protein; *indicates $p < 0.05$ compared to the respective control.

Table 19. Catalase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

		1 month	3 months	6 months	9 months	12 months	15 months
Testis	Control	0.017 ± 0.006	0.021 ± 0.008	0.019 ± 0.005	0.023 ± 0.006	0.033 ± 0.003	0.032 ± 0.015
	Treated	0.017 ± 0.004	0.040 ± 0.009*	0.037 ± 0.008*	0.035 ± 0.001*	0.075 ± 0.010*	0.134 ± 0.079*
Caput	Control	0.026 ± 0.003	0.019 ± 0.001	0.020 ± 0.001	0.043 ± 0.004	0.033 ± 0.007	0.035 ± 0.017
	Treated	0.041 ± 0.003*	0.055 ± 0.002*	0.037 ± 0.010*	0.071 ± 0.001*	0.073 ± 0.013*	0.081 ± 0.018*
Cauda	Control	0.029 ± 0.012	0.043 ± 0.004	0.033 ± 0.004	0.038 ± 0.008	0.021 ± 0.010	0.045 ± 0.007
	Treated	0.040 ± 0.006	0.051 ± 0.002	0.111 ± 0.011*	0.072 ± 0.025*	0.057 ± 0.007*	0.069 ± 0.023*
Prostate	Control	0.021 ± 0.012	0.018 ± 0.004	0.030 ± 0.009	0.022 ± 0.012	0.022 ± 0.003	0.012 ± 0.001
	Treated	0.019 ± 0.007	0.021 ± 0.003	0.038 ± 0.017	0.056 ± 0.011*	0.036 ± 0.007*	0.027 ± 0.003*

- K30 / min / mg protein; *indicates $p < 0.05$ compared to the respective control.

Table 20. Glutathione peroxidase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

		1 month	3 months	6 months	9 months	12 months	15 months
Testis	Control	29.28 ± 6.88	32.83 ± 5.71	31.93 ± 2.03	21.49 ± 2.79	8.41 ± 2.13	43.85 ± 6.38
	Treated	28.00 ± 4.43	32.30 ± 2.10	57.34 ± 2.43*	31.76 ± 4.60*	25.71 ± 1.14*	63.47 ± 3.11*
Caput	Control	30.01 ± 0.39	29.32 ± 2.48	74.93 ± 14.01	25.65 ± 8.05	7.74 ± 2.94	92.09 ± 10.89
	Treated	32.39 ± 6.49	36.87 ± 1.63	71.10 ± 6.53	28.65 ± 5.50	10.26 ± 2.81	82.57 ± 11.95
Cauda	Control	32.53 ± 4.61	36.05 ± 0.59	66.05 ± 4.15	46.70 ± 9.22	5.69 ± 2.18	80.68 ± 33.49
	Treated	55.42 ± 10.06*	53.44 ± 20.20*	99.78 ± 8.05*	82.84 ± 1.18*	15.34 ± 5.79*	160.25 ± 16.61*
Prostate	Control	35.16 ± 3.88	33.80 ± 5.80	67.79 ± 11.31	33.88 ± 1.72	10.79 ± 4.75	58.49 ± 21.54
	Treated	21.10 ± 3.15*	19.22 ± 3.45*	53.33 ± 1.31*	56.43 ± 1.33*	15.12 ± 1.24*	82.55 ± 2.07*

- μ moles NADPH oxidized / min / mg protein; *indicates $p < 0.05$ compared to the respective control.

Table 21. Glutathione-S-transferase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

		1 month	3 months	6 months	9 months	12 months	15 months
Testis	Control	0.080 ± 0.033	0.132 ± 0.040	0.148 ± 0.022	0.087 ± 0.025	0.098 ± 0.031	0.096 ± 0.026
	Treated	0.084 ± 0.024	0.187 ± 0.077	0.115 ± 0.042	0.078 ± 0.032	0.220 ± 0.079	0.120 ± 0.028
Caput	Control	0.083 ± 0.033	0.110 ± 0.017	0.184 ± 0.046	0.114 ± 0.032	0.184 ± 0.021	0.127 ± 0.035
	Treated	0.087 ± 0.035	0.159 ± 0.066	0.168 ± 0.045	0.098 ± 0.033	0.098 ± 0.051	0.129 ± 0.034
Cauda	Control	0.067 ± 0.016	0.167 ± 0.038	0.131 ± 0.020	0.222 ± 0.141	0.143 ± 0.013	0.102 ± 0.003
	Treated	0.094 ± 0.035	0.126 ± 0.044	0.183 ± 0.050	0.167 ± 0.076	0.154 ± 0.034	0.110 ± 0.014
Prostate	Control	0.021 ± 0.007	0.042 ± 0.024	0.039 ± 0.009	0.040 ± 0.015	0.023 ± 0.004	0.066 ± 0.028
	Treated	0.025 ± 0.011	0.048 ± 0.030	0.085 ± 0.051	0.048 ± 0.018	0.053 ± 0.011	0.127 ± 0.060

- μ moles CDNB-GSH conjugate formed / min / mg protein; *indicates $p < 0.05$ compared to the respective control.

Table 22. Superoxide dismutase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

		1 month	3 months	6 months	9 months	12 months	15 months
Testis	Control	301.07 ± 100.83	313.69 ± 51.38	262.44 ± 20.51	258.96 ± 58.23	253.83 ± 65.34	108.13 ± 45.92
	Treated	260.87 ± 68.69	262.49 ± 48.46	348.41 ± 44.22	239.21 ± 23.75	297.14 ± 55.69	160.15 ± 41.81
Caput	Control	310.10 ± 86.59	138.82 ± 54.58	348.05 ± 77.21	157.44 ± 30.46	252.47 ± 42.07	372.33 ± 22.55
	Treated	277.59 ± 35.63	244.03 ± 56.23	156.48 ± 84.08*	64.90 ± 6.82*	87.33 ± 6.981*	54.88 ± 4.62*
Cauda	Control	339.71 ± 51.75	329.77 ± 9.52	345.43 ± 29.21	368.26 ± 42.62	176.22 ± 31.66	264.46 ± 19.59
	Treated	467.38 ± 132.97	279.13 ± 14.15	448.67 ± 19.43*	501.14 ± 72.21*	264.05 ± 20.87*	330.22 ± 21.83*
Prostate	Control	385.64 ± 79.00	276.37 ± 46.17	240.63 ± 78.13	286.61 ± 111.91	269.41 ± 78.18	170.20 ± 16.76
	Treated	283.74 ± 55.62	206.07 ± 37.84	242.60 ± 21.89	313.87 ± 117.85	283.09 ± 67.63	272.26 ± 27.40*

- activity / mg protein; *indicates $p < 0.05$ compared to the respective control.

3.2.16. Histopathological analyses

The anatomical changes that may occur in vital organs due to long-term pyrethroid exposure was assessed by subjecting the testes, caput, cauda, prostate to histopathological examination. In general, all the tissues analyzed displayed anatomical damage at all the time points tested and the severity of damage appears to increase with the time of exposure. The anatomical changes observed are presented in (Figure 16). In the testes, mild degenerative changes in the spermatogonial cells were observed up to 6 months of treatment), while severe degeneration, edema, and fluid accumulation are evident in rats treated for 9 to 15 months. Mild to moderate degenerative changes, hyperplasia, intratubular fibrosis, and connective tissue proliferation between tubules were evident in the caput of rats treated for 3 to 15 months, while no anatomical damage was observed in the rats treated for 1 month (Figure 16). No anatomical damage was observed in the caput.

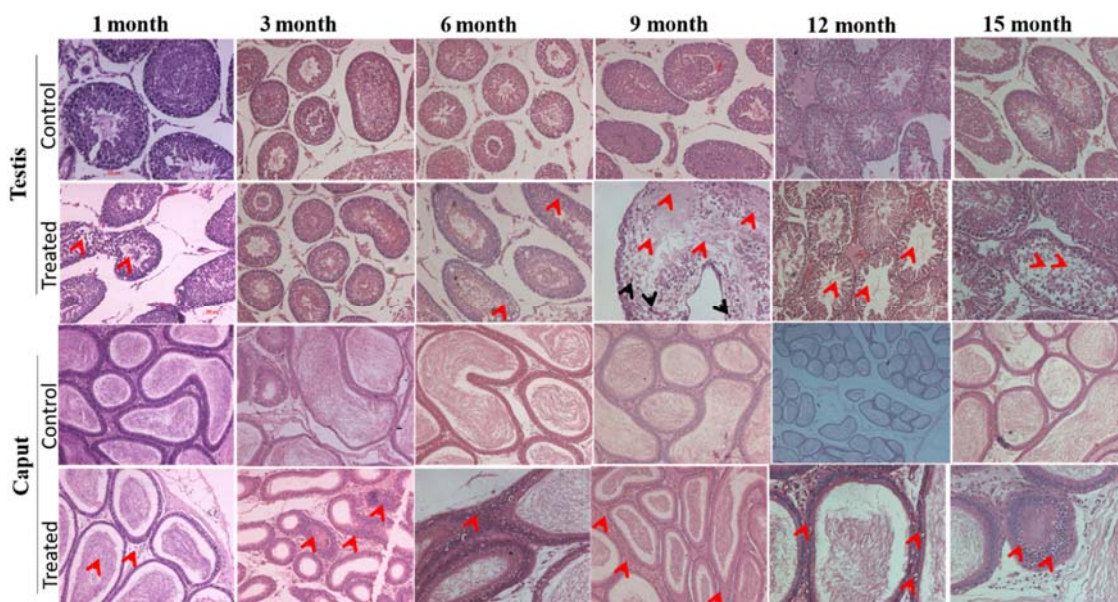


FIGURE 16. *Histopathological evaluation of reproductive tissues in rats treated with a mixture of pyrethroids.* Testes and caput of rats administered with a mixture of pyrethroids were stained with hematoxylin and eosin. The red arrow indicates degenerative change, and the block arrow indicates atrophy of seminiferous tubules.

Anatomical damage was observed in the cauda, and prostate of 1 month treated rats. In the caput obtained from rats treated with pyrethroids for 3 to 15 months, mild degenerative changes, hyperplasia in mucosal epithelial cells, and proliferation in between tubules were observed. Moderate to severe degeneration and hyperplasia of mucosal epithelial cells was observed in the cauda of pyrethroid treated rats for 3 to 15 months. In the prostate obtained from rats treated with pyrethroids for 3 to 15 months, moderate fibrosis in between mucosal glands, edema, hyperplasia of mucosal cells, accumulation of inflammatory exudates and inflammatory cells was observed (Figure 17).

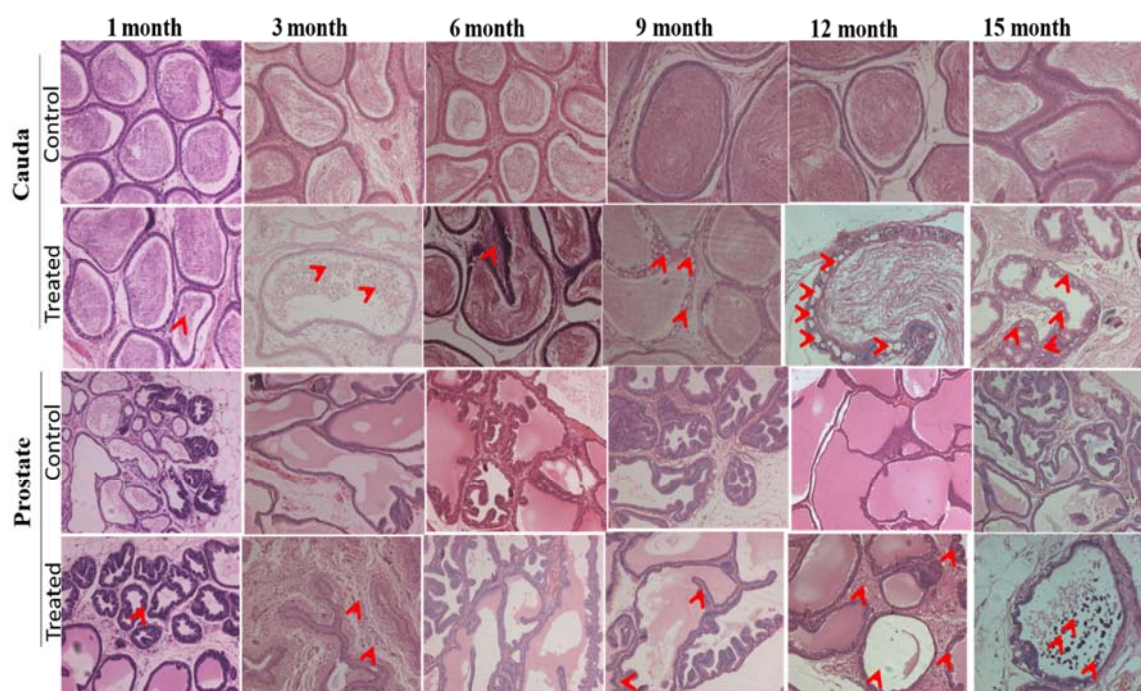


FIGURE 17. *Histopathological evaluation of reproductive tissues in rats treated with a mixture of pyrethroids. cauda and prostate of rats administered with a mixture of pyrethroids were stained with hematoxylin and eosin. The Red arrow indicates degenerative changes in the cauda and degenerative changes, fibrosis, and hyperplasia in the prostate gland.*

3.3. Discussion

Usage of pyrethroids for better agricultural production resulted in their entrapment into the food chain as well as occupational exposure. Several studies on the consequences of occupational exposure to pyrethroids indicate loss of function in different organ systems including that of the male reproductive system (27,28). Reports clearly indicate the presence of pyrethroids in a variety of foods that are routinely consumed (29,30). In such a scenario, the effects of long-term exposure to pyrethroids at doses that are relevant to human consumption attain significance. For the first time, this study reports the effects of a mixture of pyrethroids on various parameters in rats exposed to doses that are relevant to human consumption. In animal experimentation wherein human equivalent dose (HED) is to be studied, it has been a standard practice to use doses based on body surface area and metabolism (31,32). In our study, the dosage used in HD treatment was equal to HED.

We observed that long-term exposure to a mixture of pyrethroids did not significantly alter the body and relative organ weight in the rats. Alterations in the bodyweight during pyrethroid exposure are documented (33). Changes in the relative tissue weight may depend on the dose used. While previous studies used doses that are on the higher side (1.5 mg/kg/day), we have used a very low dose (0.0030794 mg/ kg/day), which may not have a serious effect on the body and relative organ weight. Among the tissues analysed, an increase in weight was only observed in the prostate. The dose-dependent change in prostate relative weight appears to be absolute since there was no change in the body weight. Increased weight of the prostate was observed in rats exposed to the insecticide Parastar (a combination of lambda-cyhalothrin and imidacloprid) (34). In utero exposure of male rats to DDT or a mixture of DDT and pyrethroid resulted in increased prostate mass (35). Cypermethrin administration resulted in epithelial degenerative changes and stromal alterations in the prostate of rats (36). In this study, prostate obtained from pyrethroid treated rats displayed mucosal epithelial hyperplasia, mild to moderate fibrous tissue proliferation, and degenerative changes. The increase in prostate weight could be due to the histopathological changes observed and may predispose to prostatic hyperplasia or cancer in the long run. However, detailed analyses on the possibility of developing prostatic hypertrophy or cancer need an in-depth investigation. It is also interesting to note that among the androgen-dependent tissues, only the weight of the prostate was increased, suggesting the high susceptibility of this organ to pyrethroid exposure. Testes, also an androgen-dependent organ is protected by the blood-testis barrier, and this could have offered a certain level of protection and thus could be more resistant to pyrethroid toxicity

when compared to the prostate). In this study, we observed that rats exposed to a mixture of pyrethroids displayed alterations in the activity of antioxidant enzymes in the male reproductive tract tissues. Exposure to pyrethroids and the effects on the status of lipid peroxidation, nitric oxide, and antioxidant enzyme status is reported. We previously reported that exposure to allethrin-based mosquito coil resulted in disturbances in increased lipid peroxidation and disturbed antioxidant status (14,37).

Histopathological changes were evident in the testes, caput, and cauda of rats exposed to a mixture of pyrethroids. Recent studies indicate that exposure to cypermethrin, chlorpyrifos, and deltamethrin caused histopathological alterations in various tissues (38). In this study, the histopathological changes observed in all the tissues analyzed could be due to alterations in the direct tissue-damaging ability of pyrethroids or due to the changes in redox status. Though histopathological observations were made in all the tissues analyzed, their effects were evident only in the liver at the biochemical level. An increase in prostate weight could be a result of histopathological changes observed. Caput, cauda, and testes also displayed altered histology and we observed decreased fecundity and sperm function in these animals. The presence of epithelial vacuolization observed in the testes of control rats could be due to age-related effects. Further investigations are required at the molecular level to correlate the relation between histopathological observation of caput, cauda, and testis to the decreased fecundity and sperm function. The histopathological changes observed in this study assume significance because of the fact that the doses used were very low and for a long period of time. These results indicate that exposure to pyrethroids whose levels are less than the levels present in regular food consumption can also cause serious damages to many tissues in the long run.

3.4. References

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Chapter 4

Evaluating the effect of a mixture of pyrethroids administered at doses relevant to human consumption on the factors that affect male reproduction at the transcriptomic and translational level

4.1. Introduction

Pesticide usage is rampant throughout the world for domestic and agricultural purposes. Indiscriminate use of pesticides caused approximately 3,000,000 instances of poisoning and 220,000 deaths annually (1). In India, the agricultural sector mainly depends on spraying pesticides to protect the crop to achieve a better yield. As per the Food and Agriculture Organization of United Nations, 52,750 metric tons of pesticide are used in India annually with 13th position on the global pesticide usage index (2). The presence of residual levels of a variety of pesticides including pyrethroids in cereals, vegetables, fruits, and milk products are reported in India (3,4).

Among the pesticides used, pyrethroid-based ones are more popular because of their high biodegradability and insecticidal activity. They primarily target the nervous system to cause malfunctioning of the voltage-sensitive sodium channels (5). In humans, pyrethroid toxicity is implicated to induce motor deficiency associated with the central nervous system and Parkinson's disease (6-8). In murine models, pyrethroids cause cognitive defects (9) alterations in the levels of GABA, 3, 4- dihydroxyphenylacetic acid (DOPAC) and noradrenaline (NA) (10), dopaminergic neurodegeneration (11), and alterations in xenobiotic enzyme levels of the brain (12). Pyrethroid toxicity in other organ systems is reported as well. Pyrethroids damage the immune system (13,14). Immunotoxicity is characterized by increased oxidative stress (15), deficiency in granulocyte differentiation (16) and myelotoxicity (17). The respiratory system toxicity caused by pyrethroids is effected by impairment in the nociceptor functioning (18). The carcinogenic capacity of pyrethroids in animal models (19,20) and the risk of developing Non-(H) hodgkin lymphoma (NHL), acute leukemia, and brain tumours in humans were demonstrated recently (21-23).

Pyrethroid induced male reproductive toxicity continues to be an active area of research because of the fact that the majority of the human population is exposed to these chemicals on a routine basis i.e., intake of the residual pyrethroids present in the food, use of insect repellents for household pest management and spraying on crops. Research reports in the last five years indicate that exposure to pyrethroids resulted in delayed Leydig cell development (24), endocrine disruption, reproductive dysfunction (25-27), and alterations in the epithelial and stromal cells of prostate (28). We demonstrated that exposure to allethrin caused reproductive dysfunction in male rats and disruption of expression of genes involved in germ cell production (29-31). Majority of the studies conducted for many decades focused on the effect of single pyrethroid using doses that are not relevant to human exposure settings. Because of the

presence of residual amounts of multiple pyrethroids in the staple foods, it is important to determine the effect of a mixture of pyrethroids at doses that are actually present in the general food items consumed by an average individual. Towards this direction, in our recent study, male rats were subjected to long-term administration of a mixture of pyrethroids (six pyrethroids) at doses that are relevant to the levels of pyrethroids present in food consumed by an average Indian (32). We observed perturbations in the oxidant status of epididymis, testis, and prostate, decreased fertility, and inability of sperm to undergo capacitation and acrosome reaction (32).

Germ cell production in males is accomplished by the coordinated processes that occur in spermatogenesis, steroidogenesis, and genetic reprogramming of the gametes. Spermatogenesis is a complex and asynchronous process that is regulated by a variety of genes. The differential expression of genes at specific stages both in time and space provides a highly controlled process for the generation of male gametes (33). Any aberrations in the expression pattern of the molecular machinery that lead to compromised male germ cell production and differentiation. It is also well known that androgens, especially testosterone play a critical role in spermatogenesis, thus making the process of steroidogenesis closely associated with spermatogenesis. Genomic reprogramming by epigenetic modifications in the developing spermatozoa allows the formation of highly specialized structures and to facilitates) totipotency of the zygote (34). Chromatin remodeling, DNA methylation, histone acetylation/methylation, and production of protamines are the hallmarks of genomic reprogramming (35). Aberrations in the activity and expression levels of the enzymes that carry out methylation/acetylation and factors that control epigenetic modifications may result in male infertility (36,37).

In studies mentioned under objective 1, a decrease in litter size was observed in rats administered with a mixture of pesticides (32) and it is anticipated that it could be due to alterations in the molecular machinery of processes that govern male germ cell production. It is important to determine whether pyrethroid exposure will affect the factors involved in these processes and thereby cause a reduction in fertility. Hence, we analyzed the effect of long-term administration of a mixture of pyrethroids on the transcriptomic and proteomic dynamics that play an important role in the processes that govern male germ cell production and development.

4.2. RESULTS

PART – I: Effect of a mixture of pyrethroids on the male reproductive function when exposed to doses relevant to human consumption (Experimental protocol –I).

4.2.1. Expression of genes related to spermatogenesis

To gain insight into the molecular mechanisms that may have contributed to the reduction in litter size, the mRNA expression pattern of genes involved in spermatogenesis was analyzed (Figures 1, 2, and 3).

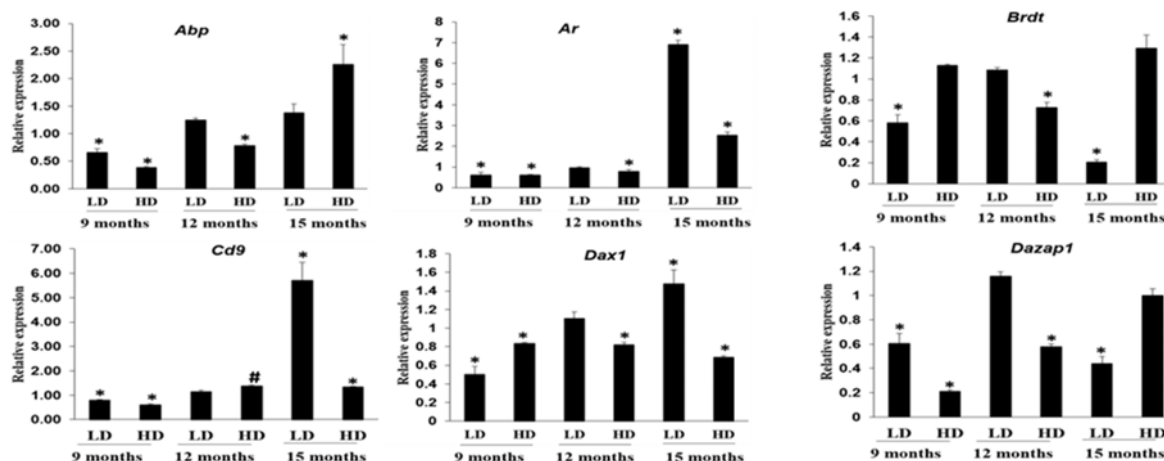


FIGURE 18. Expression pattern of spermatogenesis-related genes. RNA isolated from the testes of rats treated with a mixture of pyrethroids for 9 to 15 months was reverse-transcribed and real-time polymerase chain reaction performed using gene-specific primers. Relative expression of the specific gene in the treated group, when compared to the respective control is presented. Values shown are mean $\pm$ SD. *, # $p < 0.05$ for downregulation and upregulation, respectively. HD, high dose; LD, low dose.

In the testes obtained from pyrethroid mixture treated rats for 9 months, majority of the genes were significantly downregulated in both the treatment groups. The mRNA levels of *Abp*, *Ar*, *Cd9*, *Dax1*, *Dazap1*, *Ddx3y*, *Gdnf*, *Gfra1*, *Grth*, *Inhb*, *Ovol1*, *P1*, *Plzf*, *Pygo2*, *Scf*, *Tgfb1*, *Tp1*, *Tp2* and *Vim1* were significantly down-regulated in the testes obtained from LD and HD treatment groups, whereas *Ybx2* levels were significantly lower in the HD group. *Brdt*, *Fshr*, *Hsf2*, *Pcna*, and *Rhox5* were down-regulated only in the LD group but not in the HD group (Figures 18-19). No change in the expression levels was observed for *P2*, *Thy1*, *Wt1*, and *Wt4* in both the treatment groups (Figures 18, 19 and 20). In the testes obtained from rats treated with pyrethroid mixture for 12 months, the expression of *Gdnf*, *Hsf2*, *Inhb*, *Tgfb1*, *Thy1*, and *Ybx2* were significantly decreased in both LD and HD groups when compared to their control groups (Figures 18, 19 and 20).

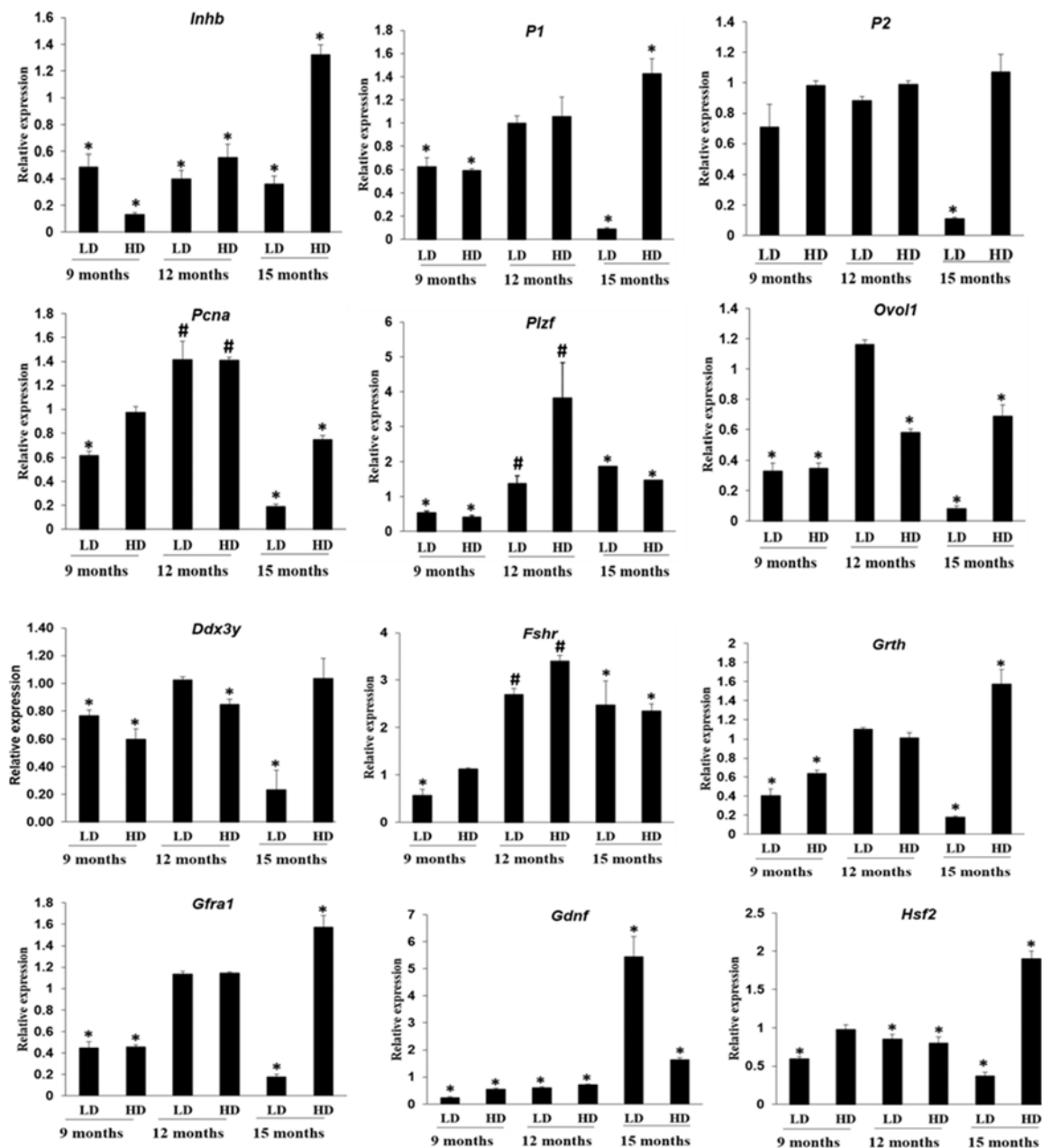


FIGURE 19. Expression pattern of spermatogenesis-related genes. RNA isolated from the testes of rats treated with a mixture of pyrethroids for 9 to 15 months was reverse-transcribed and real-time polymerase chain reaction performed using gene-specific primers. Relative expression of the specific gene in the treated group when compared to the respective control is presented. Values shown are mean $\pm$ SD. *, # $p < 0.05$ for downregulation and upregulation, respectively. HD, high dose; LD, low dose.

Abp, Ar, Brdt, Dax1, Dazap1, Ddx3y, and Ovl1 expression was significantly reduced only in the HD treatment group. Contrary to this, the significant increase in the expression of Fshr, Pcna, Plzf, P2, Vim1, and Wt4 was observed in LD and HD groups, whereas such an increase only in the HD group was observed for Cd9 and Rhox1 genes. No change in the expression levels was observed for Gfra1, Grth, P1, P2, Pygo2, Scf, Tp1, and Wt1 (**Figures 18-20**).

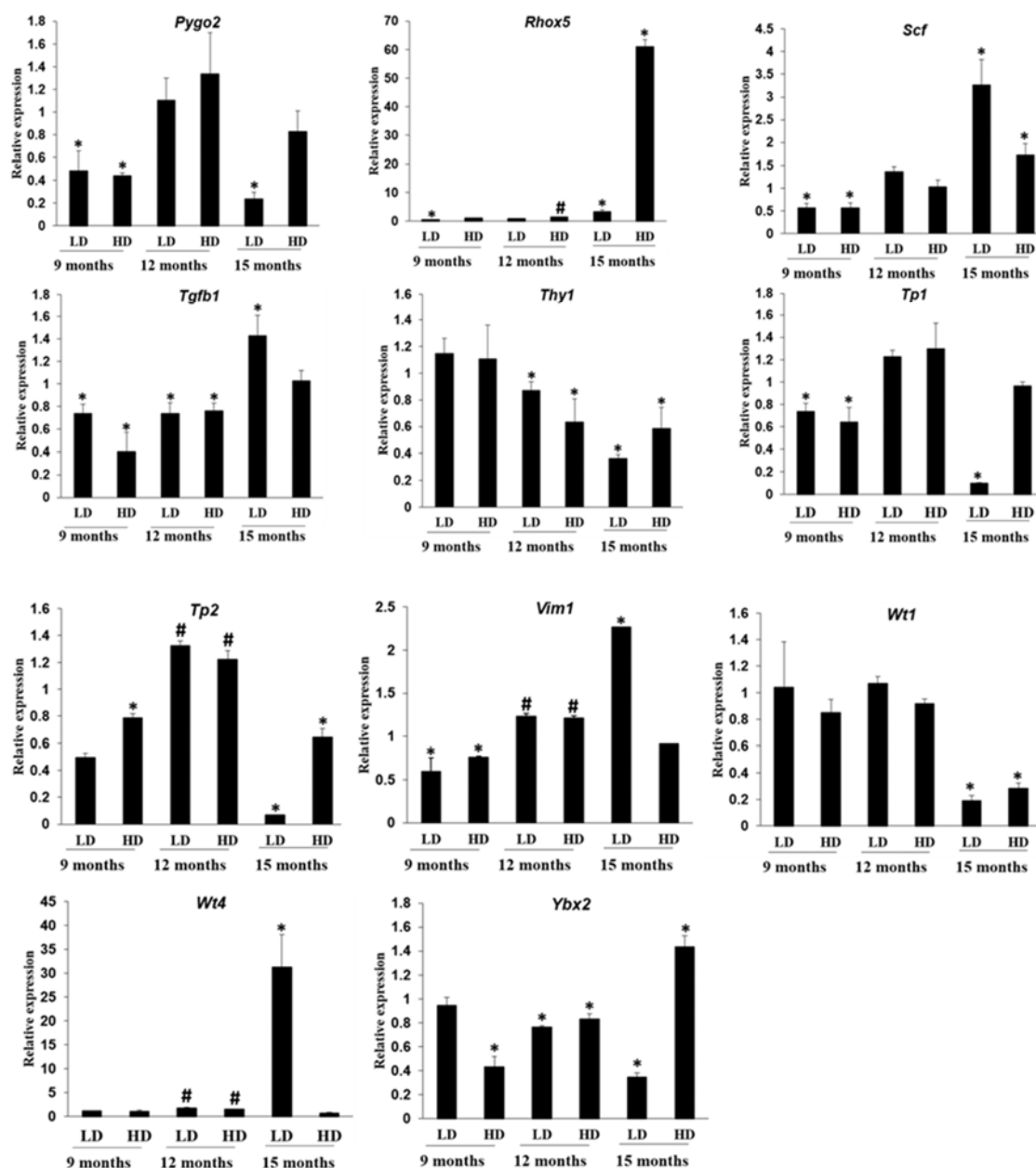


FIGURE 20. Expression pattern of spermatogenesis-related genes. RNA isolated from the testes of rats treated with a mixture of pyrethroids for 9 to 15 months was reverse-transcribed and real-time polymerase chain reaction performed using gene-specific primers. Relative expression of the specific gene in the treated group when compared to the respective control is presented. Values shown are mean $\pm$ SD. *, # $p < 0.05$ for downregulation and upregulation, respectively. HD, high dose; LD, low dose.

In the testes obtained from rats treated with pyrethroid mixture for 15 months, among the genes that are involved in spermatogenesis, the mRNA levels of *Fshr*, *Plzf*, *Rhox5* and *Scf* were significantly increased in the testes of both LD and HD treated rats compared to the control group. Increased expression of *Abp*, *Gfra1*, *Grth*, *Hsf2*, *Inhb*, *P1*, and *Ybx2* was evident only in the HD group, whereas such an increase only the LD group was observed for *Ar*, *Cd9*,

Dax1, Gdnf, Tgfb1, Vim1, and Wt4. Decreased expression in both the groups was observed for Klf4, Oval1, Pcna, Thy1, Tp2, and Wt1. Significantly lower levels of Brdt, Dazap1, Ddx3y, Gfra1, Grth, Hsf2, Inhb, P1, P2, Pygo2, Tp1, and Ybx2 was evident in the testes of LD group, whereas such a decrease in the HD group was observed for Cd9, Dax1, and Gdnf.

4.2.2. Expression of genes related to steroidogenesis

The effect of pyrethroid treatment on the expression of genes associated with steroidogenesis was analyzed. In general, majority of the genes were downregulated in the testes of rats treated with pyrethroid mixture for 9 months (**Figures 21 and 22**). 17- β -Hsd, Gata4, Hmgcr, Hmgcs1, Pde4b, and Tspo gene expression was significantly reduced in both LD and HD treated groups, whereas the expression of 3- β -Hsd, Creb1, Cyp17a1, Cyp19a1, Lhr, Srb1, Star, and Trpm2 were significantly decreased only in the HD treated group. On the contrary, increased expression of Cyp11a1 was observed in both the groups, whereas increased expression of 3- β -Hsd, Lhr, and Star was observed only in the LD group (**Figures 21 and 22**). No change in the expression of Crem and Sox9 was noted in both the treatment groups. A mixed response was observed in the testes of rats treated for 12 months (**Figures 21 and 22**). Creb1 mRNA levels were significantly decreased in both the treatment groups, whereas Gata4, Hmgcr, Hmgcs1, Pde4b, and Srb1 gene expression was decreased only in the HD group. Significant increase in the expression levels of Cyp11a1, Cyp19a1, Lhr, and Tspo was observed in both the treatment groups (**Figures 21 and 22**). Cyp17a1 expression was increased only in the HD group. The expression of 3- β -Hsd, Gata4, Sox9, and Star was significantly increased in the LD group and not in the HD group. 17- β -Hsd, Crem and Trpm2 mRNA levels remained unaltered in both the treatment groups (**Figures 4 and 5**). In the testes obtained from rats treated with pyrethroid mixture for 15 months, a significant increase in the mRNA levels of Cyp11a1, Lhr, Star, and Trpm2 was evident in testes obtained from both LD and HD groups. 3- β -Hsd, 17- β -HSD, Creb1, Sf1, Sox9, Srb1, and Tspo expression was significantly increased in the LD group, whereas such an increase in the HD group was evident for Cyp19a1. Significant decrease in both LD and HD groups was observed for Crem and Hmgcs1. While the expression of Cyp19a1, Hmgcr, and Pde4b was significantly reduced in LD group, 17- β -HSD, Creb1,

Cyp11a1, Cyp17a1, Sf1, and Sox9 levels were lowered in the HD group. No significant change was observed in both the groups for Gata4 (Figures 21 and 22)

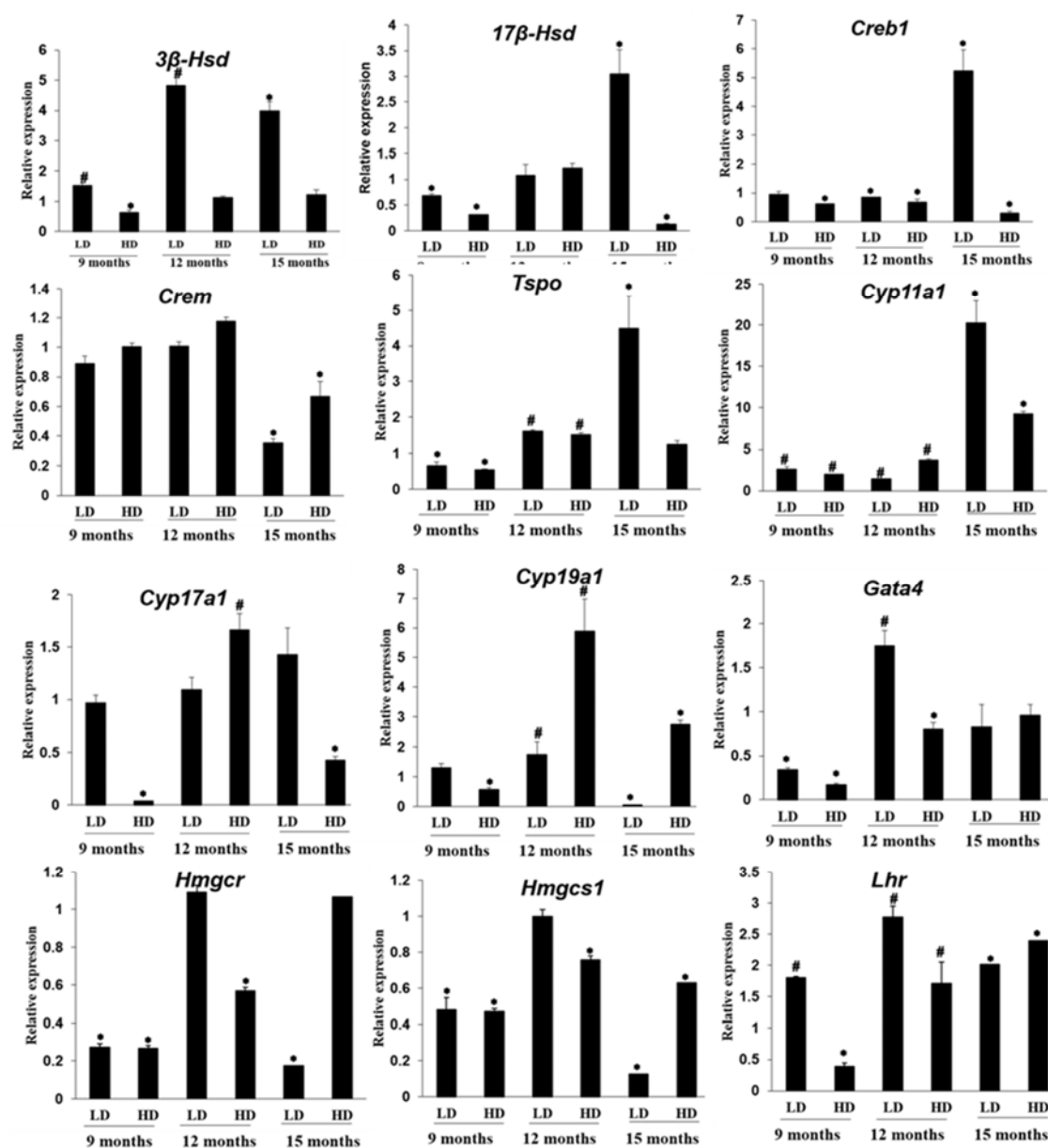


FIGURE 21. Expression pattern of steroidogenesis related genes. RNA isolated from the testes of rats treated with a mixture of pyrethroids for 9 to 15 months was reverse-transcribed and real-time polymerase chain reaction performed using gene-specific primers. Relative expression of the specific gene in the treated group when compared to the respective control is presented. Values shown are mean $\pm$ SD. *, # $p < .05$ for downregulation and upregulation, respectively. HD, high dose; LD, low dose.

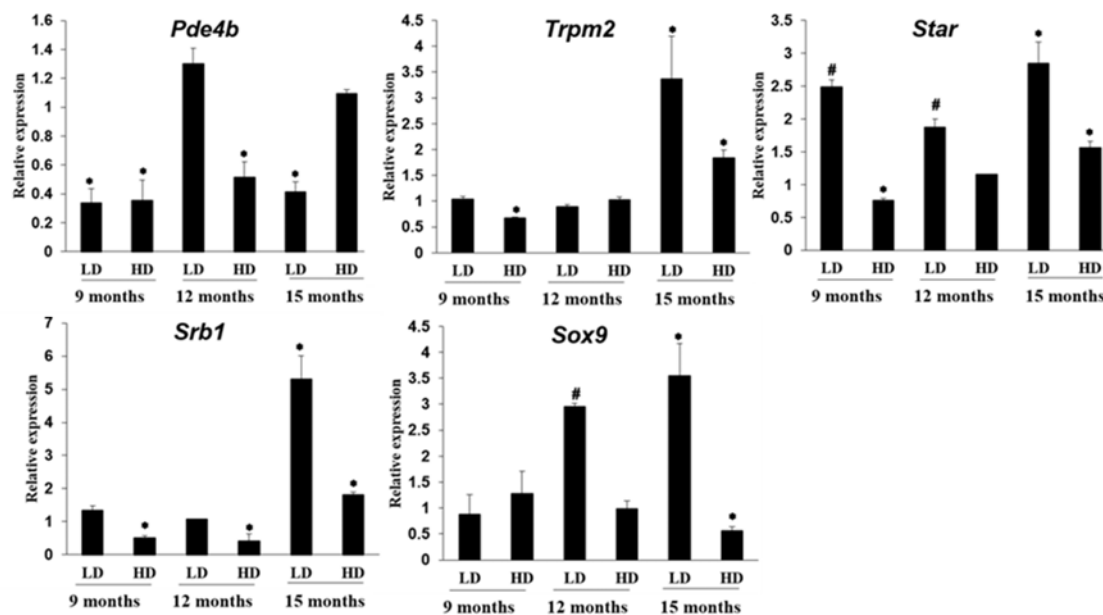


FIGURE 22. Expression pattern of steroidogenesis-related genes. RNA isolated from the testes of rats treated with a mixture of pyrethroids for 9 to 15 months was reverse-transcribed and real-time polymerase chain reaction performed using gene-specific primers. Relative expression of the specific gene in the treated group when compared to the respective control is presented. Values shown are mean $\pm$ SD. *, # $p < .05$ for downregulation and upregulation, respectively. HD, high dose; LD, low dose.

4.2.3. Expression of genes related to epigenetic modifications

The expression pattern of genes that code for crucial factors involved in epigenetic modifications was evaluated in the testes of pyrethroid administered rats. Majority of the genes were downregulated in the testes of rats treated for 9 months (**Figures 23 and 24**). Dnmt1, Dnmt3a, Dnmt3b, Hdac10, Hp1bp3, Kat3a Kat3b, Mch2ta, Ncoa7 and Sirt1 expression was significantly reduced in both HD and LD groups (**Figures 23 and 24**). Decreased expression only in the HD group was observed for Dnmt2, Hdac1, and Hdac4. Increased expression of Ezh2 was evident in the testes of HD group. Tet1 gene expression levels remained unaltered in both the treatment groups (**Figures 23 and 24**). In the testes obtained from rats treated for 12 months, Hdac10, Mch2ta, Ncoa7 and Sirt1 mRNA expression levels were significantly decreased in both the treatment groups.

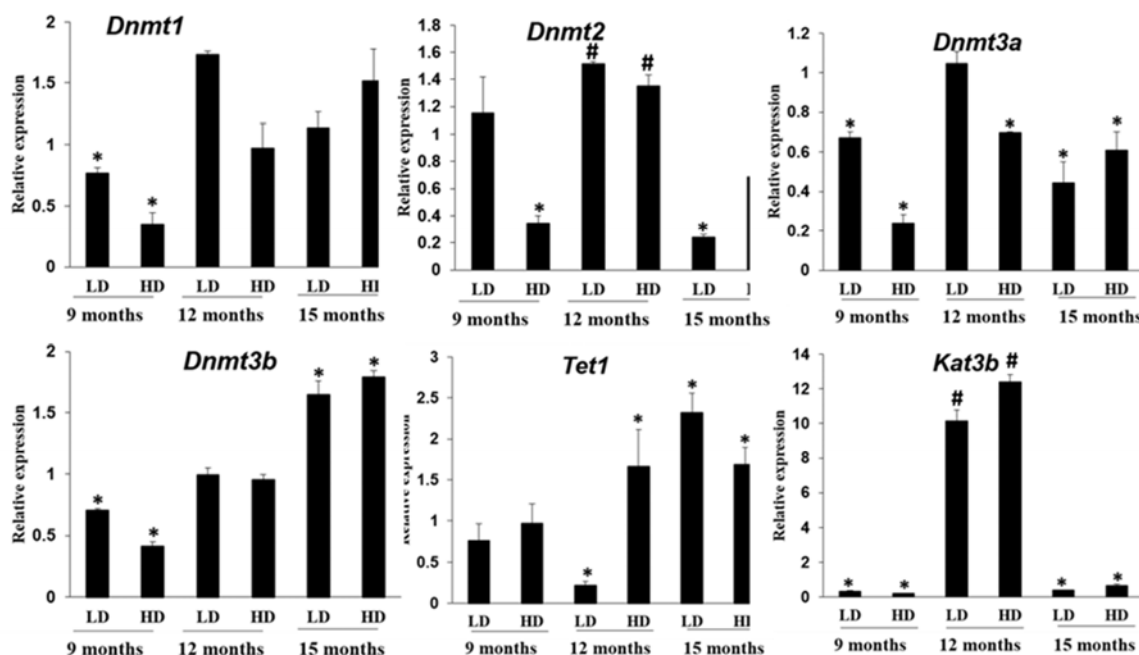


FIGURE 23. Expression pattern of epigenetic modification-related genes. RNA isolated from the testes of rats treated with a mixture of pyrethroids for 9 to 15 months was reverse-transcribed and real-time polymerase chain reaction performed using gene-specific primers. Relative expression of the specific gene in the treated group when compared to the respective control is presented. Values shown are mean $\pm$ SD. *, # $p < .05$ for downregulation and upregulation, respectively. HD, high dose; LD, low dose.

While Dnmt3a mRNA levels were reduced only in the HD group, Hdac4 and Tet1 exhibited such a pattern in only in the LD group. Increased expression of Dnmt2, Hdac1, Kat3a, and Kat3b was observed in both LD and HD groups, Tet1 in HD group and Dnmt1 in LD was evident. No change in the expression levels of Dnmt3b, Ezh2, and H1bp3 was observed for both the treatment groups (**Figures 23 and 24**). In the testes obtained from rats treated for 12 months, Dnmt3b and Tet1 mRNA levels were significantly increased in both LD and HD groups, while such an increase in LD group was observed for Hdac4 and similarly for Ezh2, Mch2ta, and Sirt1 in the HD group. Significant decrease in both LD and HD groups was observed for Dnmt2, Dnmt3a, Kat3b, and Ncoa7. In the LD group, Ezh2 and Hdac1 gene expression was significantly reduced, and such a trend was observed for Hdac10, Hp1bp3, and Kat3a. No change in Dnmt1 expression was observed in both the treatment groups (**Figures 23 and 24**).

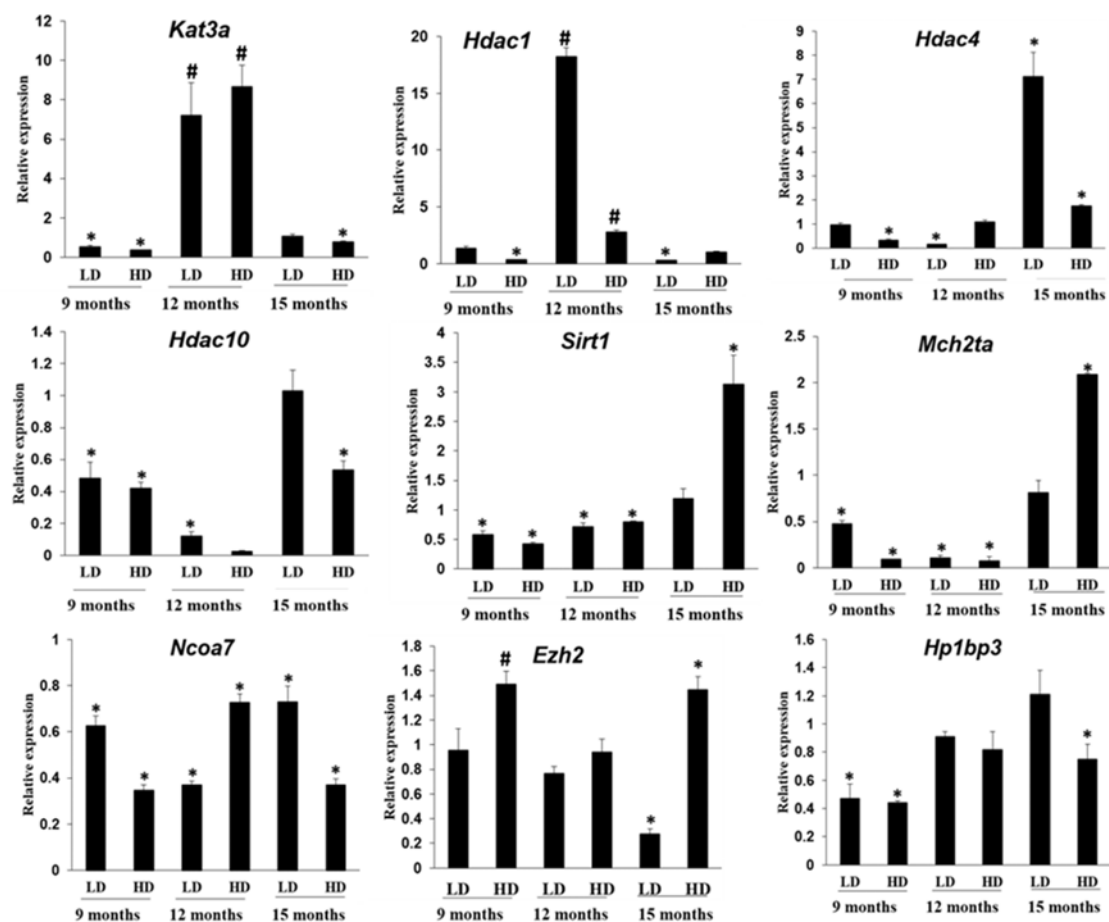


FIGURE 24. Expression pattern of epigenetic modification-related genes. RNA isolated from the testes of rats treated with a mixture of pyrethroids for 9 to 15 months was reverse-transcribed and real-time polymerase chain reaction performed using gene-specific primers. Relative expression of the specific gene in the treated group when compared to the respective control is presented. Values shown are mean $\pm$ SD. *, # $p < .05$ for downregulation and upregulation, respectively. HD, high dose; LD, low dose.

PART – II: Effect of a mixture of pyrethroids on the male reproductive function when exposed to doses equivalent to human consumption (Experimental protocol –II).

4.3.1 Expression of genes related to spermatogenesis

Perturbations in the hormonal profile prompted us to further investigate whether the mRNA expression pattern of molecular factors that govern spermatogenesis, steroidogenesis, sperm epigenetic modulators, and germ cell apoptotic markers are affected in the testis of rats treated with a mixture of pyrethroids. Differential expression of genes involved in spermatogenesis is presented in **Table 23**. In the testes of rats treated for one-month, significant decrease in the mRNA levels of Abp, Amh, Ar, Brdt, Cd9, Dax2, Ddx3y, Gdnf, Grth, Hsf2, Oval4, Scf, Sox9, Vim and Wt1 was evident. On the contrary, up-regulation of Dazapa1, Fshr, Pcnal, Rox5, Tp2, and Ybx2 genes were observed. When treated for 3 months, the expression pattern of Ar, Gfra1, P2, Rox5, Scf, Sox9, and Ybx2 mRNA levels were significantly down-regulated, whereas up-regulation of Abp, Amh, Cd9, Hsf2, Oval4, and Tgfb1 were up regulated. At the end of 6 months of treatment, the testicular mRNA expression of Cd9, Oval4, P1, Sox9, and Ybx2 were downregulated whereas an opposite trend was observed for Grth, P2, Rox5, Scf, Sox, and Rgfb1. Pyrethroid exposure to 9 months resulted in the down regulation of Dazapa1, Ddx3y, Hsf2, P2, Scf, Sox0, Tgfb1, Tp2, and Ybx2 genes, whereas significant up-regulation was observed only for Amh. In the testes of rats treated for 12 months, the mRNA levels of Amh, Dax2, Dazapa1, Ddx3y, Gfra1, Hsf2, Rox5, Sox9, Tgfb1, Tp2, Vim, Wt1, and Ybx2 were significantly down-regulated, whereas up-regulation of Amh, Brdt, Gdnf and Grth mRNA were evident. In the testes of rats treated for 15 months, Amh, Fshr, Pcnal, Sox9, Tgfb1, Tp1, Tp2, and Ybx2 were significantly downregulated. The expression of all other genes that are not mentioned at each time point remained unchanged.

Table 23. Expression pattern of spermatogenesis-related genes in the testes of pyrethroid treated rats.

Gene	Treatment in months					
	1	3	6	9	12	15
<i>Abp</i>	▼	▲	—	—	▲	—
<i>Amh</i>	▼	▲	—	▲	▼	▼
<i>Ar</i>	▼	▼	—	—	—	—
<i>Brdt</i>	▼	—	—	—	▲	—
<i>Cd9</i>	▼	▲	▼	—	—	—
<i>Dax2</i>	▼	—	—	—	▼	—
<i>Dazapa1</i>	▲	—	—	▼	▼	—
<i>Ddx3y</i>	▼	—	—	▼	▼	—
<i>Fshr</i>	▲	—	—	—	—	▼
<i>Gdnf</i>	▼	—	—	—	▲	—
<i>Gfra1</i>	—	▼	—	—	▼	—
<i>Grth</i>	▼	—	▲	—	▲	—
<i>Hsf2</i>	▼	▲	—	▼	▼	—
<i>Klf4</i>	—	—	—	—	—	—
<i>Oval4</i>	▼	▲	▼	—	—	—
<i>P1</i>	—	—	▼	—	—	—
<i>P2</i>	—	▼	▲	▼	—	—
<i>Pcna</i>	▲	—	—	—	—	▼
<i>Rhox5</i>	▲	▼	▲	—	▼	—
<i>Scf</i>	▼	▼	▲	▼	—	—
<i>Sox9</i>	▼	▼	▼	▼	▼	▼
<i>Tgfb1</i>	—	▲	▲	▼	▼	▼
<i>Tp1</i>	—	—	—	—	—	▼
<i>Tp2</i>	▲	—	—	▼	▼	▼
<i>Vim</i>	▼	—	—	—	▼	—
<i>Wtl</i>	▼	—	—	—	▼	—
<i>Ybx2</i>	▲	▼	▼	▼	▼	▼

▲ and ▼ indicate up-regulation, down-regulation with $p < 0.05$ compared to the respective control; whereas — indicates no change in gene expression compared to respective control.

4.3.2 Expression of genes related to steroidogenesis

Steroidogenesis-related gene expression was evaluated in the testes of rats treated with a mixture of pyrethroids for 1 to 15 months and the changes observed are presented in **Table 24**. When treated for 1 month, the mRNA levels of 3β Hsd, 17β Hsd, Creb, Crem, Gata4, Hmgcr, Hmgcs1, and Star were significantly decreased, whereas that of Cyp17a1, Srb1, and Tspo were up regulated. Crem and Cyp17a1 genes were down-regulated, and that of 17β Hsd, Creb, and Cyp11a1 were up-regulated significantly in the testes obtained from rats treated for 3 months.

Table 24. Expression pattern of steroidogenesis-related genes in the testes of pyrethroid treated rats.

Gene	Treatment in months					
	1	3	6	9	12	15
<i>3βHsd</i>	▼	—	—	—	▲	▲
<i>17βHsd</i>	▼	▲	▼	—	▼	▼
<i>Creb</i>	▼	▲	▲	—	▼	—
<i>Crem</i>	▼	▼	▼	▲	▼	▲
<i>Cyp11a1</i>	—	▲	▲	▲	▲	▲
<i>Cyp17a1</i>	▲	▼	▲	▲	▼	▲
<i>Gata4</i>	▼	—	▼	▲	▼	▼
<i>Hmgcr</i>	▼	—	▲	—	▲	▲
<i>Hmgcs1</i>	▼	—	▲	—	▼	▲
<i>Lhr</i>	—	—	▼	▼	▼	▼
<i>Srb1</i>	▲	—	▲	—	▼	▲
<i>Star</i>	▼	—	▼	▲	▲	▲
<i>Trpm</i>	—	—	▲	—	—	—
<i>Tspo</i>	▲	—	▲	▼	▲	▼

▲ and ▼ indicate up-regulation, down-regulation with $p < 0.05$ compared to the respective control; whereas — indicates no change in gene expression compared to respective control.

6 months exposure to pyrethroids resulted in the downregulation of *17βHsd*, *Crem*, *Gata4*, *Lhr*, and *Star*; whereas up-regulation of *Creb*, *Cyp11a1*, *Cyp17a1*, *Hmgcr*, *Hmgcs1*, *Srb1*, *Trpm* and *Tspo* was evident. When treated for 9 months, the testicular expression of *Lhr* and *Tspo* was significantly reduced, while that of *Crem*, *Cyp11a1*, *Cyp17a1*, *Gata4*, and *Star* were up-regulated. *17βHsd*, *Creb*, *Crem*, *Cyp17a1*, *Gata4*, *Hmgcs1*, *Lhr*, and *Srb1* mRNA levels were significantly reduced and that of *3βHsd*, *Cyp11a1*, *Hmgcr*, *Star* and *Tspo* were increased in the testes of rats that were treated for 12 months. When treated for 15 months, *17βHsd*, *Gata4*, *Lhr*, and *Tspo* mRNA levels were significantly reduced, whereas an increase was observed for *3βHsd*, *Crem*, *Cyp11a1*, *Cyp17a1*, *Hmgcr*, *Hmgcs1*, *Srb1*, and *Star* genes. The mRNA expression levels remained unchanged for those genes not mentioned for each time point.

4.3.3. Expression of genes related to germ cell epigenetics.

The effect of long-term exposure to pyrethroids on the expression of genes that are involved in male germ cell epigenetic changes was analyzed in the testes and the observations are presented **Table 25**. Significantly decreased mRNA levels of *Dnmt2*, *Hdac4*, *Hdac10*, *Hp1bp3*, *Kat1a*, *Kat2a*, *Mch2ta*, *Ncoa7*, and *Tet1* and increased levels of *Dnmt3a*, *Dnmt3b*, *Hdac1* and *Sirt1* were observed in the 1 month treatment group.

Table 25. Expression pattern of epigenetic modulation related genes in the testes of pyrethroid treated rats.

Gene	Treatment in months					
	1	3	6	9	12	15
<i>Dnmt2</i>	▼	▼	▲	▼	▲	▼
<i>Dnmt3a</i>	▲	—	▲	▲	▲	▲
<i>Dnmt3b</i>	▲	—	▲	▼	▼	▼
<i>Ezh2</i>	—	▼	—	—	▼	▲
<i>Hdac1</i>	▲	—	—	—	—	—
<i>Hdac4</i>	▼	▼	▲	▼	▼	—
<i>Hdac10</i>	▼	▼	—	—	—	—
<i>Hp1bp3</i>	▼	▼	▲	▼	▼	—
<i>Kat3a</i>	▼	▼	—	▼	▼	▲
<i>Kat3b</i>	▼	—	▼	—	—	▼
<i>Mch2ta</i>	▼	▼	—	▼	—	▼
<i>Ncoa7</i>	▼	—	—	—	—	▼
<i>Sirt1</i>	▲	▼	—	—	▼	▼
<i>Tet1</i>	▼	▲	—	—	—	—

▲ and ▼ indicate up-regulation, down-regulation with $p < 0.05$ compared to the respective control; whereas — indicates no change in gene expression compared to respective control.

When treated for 3 months, the testicular mRNA levels of *Dnmt2*, *Ezh2*, *Hdac4*, *Hdac10*, *Hp1bp2*, *Kat3a*, *Mch2a*, and *Sirt1* were significantly decreased, while that of *Tet1* was increased. In the 6 months treatment group, *Kat3b* mRNA levels were decreased, while an increase was evident for *Dnmt2*, *Dnmt3a*, *Dnmt3b*, and *Hp1bp3*. *Dnmt2*, *Dnmt3b*, *Hdac4*, *Hp1bp3*, *Kat3a*, and *Mch2a* mRNA levels were significantly reduced in the testes of rats treated for 9 months; and that of *Dnmt3a* gene was increased. Pyrethroid treatment for 12 months resulted in significantly reduced mRNA levels of *Dnmt3b*, *Ezh2*, *Hdac4*, *Hp1bp3*, *Kat3a*, and *Sirt1*; and on the contrary *Dnmt2* and *Dnmat3a* mRNA levels were increased. In the 15 month treatment group, *Dnmt2*, *Dnmt3b*, *Kat3b*, *Mch2ta*, *Ncoa7*, and *Sirt1* mRNA levels were significantly reduced, while that of *Dnmt3a* and *Kat3a* were increased. For all other genes that were not specifically mentioned, their mRNA levels remain unchanged when compared with the respective control.

4.3.4. Expression of genes related to germ cell apoptosis

Changes in the expression pattern of genes involved in testicular apoptosis and maintenance of oxidative stress were analyzed in rats subjected to pyrethroid exposure for 1 to 15 months (Table 26). *Casp3*, *Casp9*, *Nqo1*, and *Nrf2* mRNA levels were significantly reduced while that of *Bcl2* and *Hif1 α* were increased in the rats treated for 1 month. In the 3 months treated group, *Casp3* and *Nrf2* mRNA levels were reduced and that of *Casp9*, *Hif1 α* , and *Nqo1*

were increased. While *Nrf2* expression was reduced in the testes of 6 months treated group, increased levels were observed for *Bcl2*, *Hif1 α* , and *Ho1*. In the rats treated for 9 months, *Casp3*, *Casp9*, and *Ho1* expression were decreased and that of *Bcl2* was increased. Decreased mRNA levels of *Bax* and *Nqo1* and increased expression of *Bcl2* were evident in rats treated for 12 months. In the rats treated with pyrethroids for 15 months, while *Bax2* mRNA levels were significantly decreased, an increase was observed for *Bcl2*, *Casp9*, and *Hif1 α* .

Table 26. Expression pattern of apoptosis and oxidative stress-related genes in the testes of pyrethroid treated rats.

Gene	Treatment in months					
	1	3	6	9	12	15
<i>Bax</i>	—	—	—	—	▼	▼
<i>Bcl2</i>	▲	—	▲	▲	▲	▲
<i>Casp3</i>	▼	▼	—	▼	—	—
<i>Casp9</i>	▼	▲	—	▼	—	▲
<i>Hif1α</i>	▲	▲	▲	—	—	▲
<i>Ho1</i>	—	—	▲	▼	—	—
<i>Nqo1</i>	▼	▲	—	—	▼	▼
<i>Nrf2</i>	▼	▼	▼	—	—	—

▲ and ▼ indicate up-regulation, down-regulation with $p < 0.05$ compared to the respective control; whereas — indicates no change in gene expression compared to respective control.

4.4. Transgenerational sperm proteome analyses

Proteins secreted into the testicular and epididymal lumen are added to the sperm surface to aid in spermatogenesis and sperm maturation. Further, a variety of factors produced by the developing spermatogonial also aid in gametogenesis. In order to determine whether the expression and addition of these factors to the sperm surface is affected due to pyrethroid exposure, spermatozoa from the control and treated rats were subjected to GC-MS analyses. Sperm lysates were prepared using gametes obtained from rats (n=3) treated with a pyrethroid mixture for 6 months. Lysates were also prepared from the spermatozoa collected from the matching control rats (n=3). GC-MS analyses were conducted on technical triplicates for each sample. A total of 506 proteins that were present in all the biological triplicates of control rats were identified and the levels of these proteins in the sperm lysates obtained from pyrethroid treated rats were analyzed. The complete list of proteins included for analysis is provided (Appendix -1). The differential expression for each protein was expressed as the ratio of the Protein Lynx Global Server (PLGS) score of treated to control (treated / control ratio; T/C ratio). Proteins that had a T/C ratio of < 0.5 and > 2.0 were considered to be down-regulated and up-regulated respectively. Among the 506 proteins analyzed, 153 had a ratio of 0; 41 had a ratio ranging from > 0 to < 0.5 ; and 10 had a ratio > 2.0 (Table 27).

In this study, we observed that fecundity was affected in pyrethroid-treated rats as well as in the males that were born to these rats. To determine whether the differential expression of proteins observed in the spermatozoa of pyrethroid treated could also be transgenerational, sperm lysates of pups (F1, F2, and F3 generations) born to pyrethroid exposed rats (F0) were subjected to GC-MS. The differentially expressed proteins identified in the sperm lysates of F0 rats were analyzed in the F1, F2, and F3 generations. We observed that the differential expression observed for a majority of proteins identified in the sperm lysates of F0 rats remained the same in the next generations (**Table 28**). 22 proteins that were downregulated in the F0 generation continued to remain under-expressed in the F1, F2, and F3 generations. Similarly, 45 proteins exhibited under expression up to F2 generation, while only 1 protein exhibited continued to be under-expressed up to F1 generation. 4 of the up-regulated proteins in the sperm lysates of F0 generation continued to be overexpressed up to F3 generation. 1 protein each was found to be overexpressed up to F2 and F1 generations. Some of the proteins that have a role in male reproduction continued to be differentially expressed in the later generations (**Table 28**). These results indicate that the reduced fecundity observed in the later generations could be due to the continued differential expression that was initiated due to pyrethroid treatment in the F0 rats.

Table 27. Differential expression of sperm proteome in rats treated with a mixture of pyrethroids.

(T/C ratio indicated in light green and light pink boxes indicate down regulation and up regulation respectively).

UNIPROT ENTRY	DESCRIPTION	RATIO (T/C)			
PK3CA_RAT	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform	0	EPHA3_RAT	Ephrin type-A receptor 3	0
AEBP1_RAT	Adipocyte enhancer-binding protein 1	0	TRPC4_RAT	Short transient receptor potential channel 4	0
S38A5_RAT	Sodium-coupled neutral amino acid transporter 5	0	LTBP2_RAT	Latent-transforming growth factor beta-binding protein 2	0
SOBP_RAT	Sine oculis-binding protein homolog	0	BRCA1_RAT	Breast cancer type 1 susceptibility protein homolog	0
TIGD5_RAT	Tigger transposable element derived 5	0	PPIG_RAT	Peptidyl-prolyl cis-trans isomerase G	0
MA1B1_RAT	Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase	0	ABCB6_RAT	ATP-binding cassette sub-family B member 6, mitochondrial	0
UBP4_RAT	Ubiquitin carboxyl-terminal hydrolase 4	0	PACS1_RAT	Phosphofurin acidic cluster sorting protein 1	0
PTSS2_RAT	Phosphatidylserine synthase 2	0	ARC1B_RAT	Actin-related protein 2/3 complex subunit 1B	0
CTL2_RAT	Choline transporter-like protein 2	0	ACKR3_RAT	Atypical chemokine receptor 3	0
TBD2A_RAT	TBC1 domain family member 2A	0	VTDB_RAT	Vitamin D-binding protein	0
S52A2_RAT	Solute carrier family 52, riboflavin transporter, member 2	0	A2MG_RAT	Alpha-2-macroglobulin	0
SMRCD_RAT	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D	0	MMEL1_RAT	Membrane metallo-endopeptidase-like 1	0
MYLIP_RAT	E3 ubiquitin-protein ligase MYLIP	0	SCG2_RAT	Secretogranin-2	0
SIDT2_RAT	SID1 transmembrane family member 2	0	CP17A_RAT	Steroid 17-alpha-hydroxylase/17,20 lyase	0
HTRA4_RAT	Serine protease HTRA4	0	TGFB1_RAT	Transforming growth factor beta-1 proprotein	0
PI51A_RAT	Phosphatidylinositol 4-phosphate 5-kinase type-1 alpha	0	KCNA5_RAT	Potassium voltage-gated channel subfamily A member 5	0
NLK_RAT	Serine/threonine-protein kinase NLK	0	GSH1_RAT	Glutamate-cysteine ligase catalytic subunit	0
WDR26_RAT	WD repeat-containing protein 26	0	GRIA3_RAT	Glutamate receptor 3	0
UNC5D_RAT	Netrin receptor UNC5D	0	MA2C1_RAT	Alpha-mannosidase 2C1	0
RPGF2_RAT	Rap guanine nucleotide exchange factor 2	0	CAC1C_RAT	Voltage-dependent L-type calcium channel subunit alpha-1C	0
TEX14_RAT	Inactive serine/threonine-protein kinase TEX14	0	MCR_RAT	Mineralocorticoid receptor	0
STX12_RAT	Syntaxin-12	0	OL226_RAT	Olfactory receptor 226	0
ZN335_RAT	Zinc finger protein 335	0	GRM1_RAT	Metabotropic glutamate receptor 1	0
RN157_RAT	E3 ubiquitin ligase Rnf157	0	GRN_RAT	Progranulin	0
MARK1_RAT	Serine/threonine-protein kinase MARK1	0	SL9A4_RAT	Sodium/hydrogen exchanger 4	0
LIPP_RAT	Pancreatic triacylglycerol lipase	0	ADCY2_RAT	Adenylate cyclase type 2	0
SCNNA_RAT	Amiloride-sensitive sodium channel subunit alpha	0	LRC8C_RAT	Volume-regulated anion channel subunit LRRC8C	0
ANXA6_RAT	Annexin A6	0	PSMD2_RAT	26S proteasome non-ATPase regulatory subunit 2	0
GUC2E_RAT	Retinal guanylyl cyclase 1	0	ERCC3_RAT	General transcription and DNA repair factor IIF helicase subunit XPB	0
VGFR1_RAT	Vascular endothelial growth factor receptor 1	0	GIMAB_RAT	GTPase IMAF family member 8	0
VAV_RAT	Proto-oncogene vav	0	RNF8_RAT	E3 ubiquitin-protein ligase RNF8	0
ATN1_RAT	Atrophin-1	0	RING2_RAT	E3 ubiquitin-protein ligase RING2	0
CA2D1_RAT	Voltage-dependent calcium channel subunit alpha-2/delta-1	0	MRC2_RAT	C-type mannose receptor 2	0
EPHA6_RAT	Ephrin type-A receptor 6	0	COL12_RAT	Collectin-12	0
PEX6_RAT	Peroxisome assembly factor 2	0	PAF1_RAT	RNA polymerase II-associated factor 1 homolog	0
S12A1_RAT	Solute carrier family 12 member 1	0	PELP1_RAT	Proline-, glutamic acid- and leucine-rich protein 1	0
PGCB_RAT	Brevican core protein	0	MED23_RAT	Mediator of RNA polymerase II transcription subunit 23	0
ANKH_RAT	Progressive ankylosis protein homolog	0	MLEC_RAT	Malectin	0
CSN2_RAT	COP9 signalosome complex subunit 2	0	GARS_RAT	Glycine-tRNA ligase	0
PHAR1_RAT	Phosphatase and actin regulator 1	0	NOC4L_RAT	Nucleolar complex protein 4 homolog	0
PLD1_RAT	Phospholipase D1	0	TBX5_RAT	T-box transcription factor TBX5	0
KAP1_RAT	cAMP-dependent protein kinase type I-beta regulatory subunit	0	ARMCS_RAT	Armadillo repeat-containing protein 5	0
ESM1_RAT	Endothelial cell-specific molecule 1	0	MELT_RAT	Ventricular zone-expressed PH domain-containing protein homolog 1	0
SMC3_RAT	Structural maintenance of chromosomes protein 3	0	RTKL1_RAT	Regulator of telomere elongation helicase 1	0
KKCC1_RAT	Calcium/calmodulin-dependent protein kinase kinase 1	0	TRI35_RAT	Tripartite motif-containing protein 35	0
ODO2_RAT	Dihydrodipicolyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	0	TAB2_RAT	TGF-beta-activated kinase 1 and MAP3K7-binding protein 2	0
CAC1S_RAT	Voltage-dependent L-type calcium channel subunit alpha-1S	0	SLN13_RAT	Schlafen family member 13	0
LOX15_RAT	Polyunsaturated fatty acid lipooxygenase ALOX15	0	CARL3_RAT	Capping protein, Arp2/3 and myosin-I linker protein 3	0
GEPH_RAT	Gephyrin	0	LPP_RAT	Lipoma-preferred partner homolog	0
SSRP1_RAT	FACT complex subunit SSRP1	0	MCMD2_RAT	Minichromosome maintenance domain-containing protein 2	0
SRBP2_RAT	Sterol regulatory element-binding protein 2	0	CNTN3_RAT	Contactin-3	0
			EXOC3_RAT	Exocyst complex component 3	0

M3K1_RAT	Mitogen-activated protein kinase kinase kinase 1	0	CORA1_RAT	Collagen alpha-1(XVII) chain	0
LEPR_RAT	Leptin receptor	0	IP3KC_RAT	Inositol-trisphosphate 3-kinase C	0
ADDA_RAT	Alpha-adducin	0	MAST1_RAT	Microtubule-associated serine/threonine-protein kinase 1	0
KCNB2_RAT	Potassium voltage-gated channel subfamily B member 2	0	RN22_RAT	Zinc phosphodiesterase ELAC protein 2	0
GRIK5_RAT	Glutamate receptor ionotropic, kainate 5	0	BRNP3_RAT	BMP/retinoic acid-inducible neural-specific protein 3	0
KDM3A_RAT	Lysine-specific demethylase 3A	0	LPAR3_RAT	Lysophosphatidic acid receptor 3	0
ECHA_RAT	Trifunctional enzyme subunit alpha, mitochondrial	0	LIPA4_RAT	Liprin-alpha-4 (Fragment)	0
AT2A1_RAT	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	0	PALM_RAT	Paralemm-1	0
PTPR5_RAT	Receptor-type tyrosine-protein phosphatase 5	0	S26A3_RAT	Chloride anion exchanger	0
INSRR_RAT	Insulin receptor-related protein	0	ABCG5_RAT	ATP-binding cassette sub-family G member 5	0
INT14_RAT	Integrator complex subunit 14	0	CCN1_RAT	CCN family member 1	0
NPL_RAT	N-acetylneuraminase lyase	0	ECEL1_RAT	Endothelin-converting enzyme-like 1	0
C2D1A_RAT	Coiled-coil and C2 domain-containing protein 1A	0	HIF3A_RAT	Hypoxia-inducible factor 3-alpha	0
FHL5_RAT	Four and a half LIM domains protein 5	0	S4A4_RAT	Electrogenic sodium bicarbonate cotransporter 1	0
RECQ1_RAT	ATP-dependent DNA helicase Q1	0	RIMB2_RAT	RIMS-binding protein 2	0
ODFP2_RAT	Outer dense fiber protein 2	0	TRPC3_RAT	Short transient receptor potential channel 3	0
GNL1_RAT	Guanine nucleotide-binding protein-like 1	0	TRPC1_RAT	Short transient receptor potential channel 1 OS=Rattus norvegicus OX=10116 GN	0
ORC4_RAT	Origin recognition complex subunit 4	0	LEF1_RAT	Lymphoid enhancer-binding factor 1	0
TRPA1_RAT	Transient receptor potential cation channel subfamily A member 1	0	PLK3_RAT	Serine/threonine-protein kinase PLK3	0
TRPA1_RAT	Transient receptor potential cation channel subfamily A member 1	0	SEM6C_RAT	Semaphorin-6C	0
SC16B_RAT	Protein transport protein Sec16B	0	AVIL_RAT	Advillin	0
PGAP1_RAT	GPI inositol-deacylase	0	ITSN1_RAT	Intersectin-1	0
IQEC3_RAT	IQ motif and SEC7 domain-containing protein 3	0	FBLN5_RAT	Fibulin-5 OS=Rattus norvegicus	0
SCN4B_RAT	Sodium channel subunit beta-4	0	PK3CB_RAT	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform	0
RIPR2_RAT	Rho family-interacting cell polarization regulator 2	0	ACTN1_RAT	Alpha-actinin-1	0
WASC2_RAT	WASH complex subunit 2	0	STAB2_RAT	Stabilin-2 (Fragment)	0.212713243
TUTLA_RAT	Protein turtle homolog A	0.233780462	WDR81_RAT	WD repeat-containing protein 81	0.439126907
BRDT_RAT	Bromodomain testis-specific protein	0.244948387	VGFR3_RAT	Vascular endothelial growth factor receptor 3	0.443415583
CP4F4_RAT	Cytochrome P450 4F4	0.25017834	ERC2_RAT	ERC protein 2	0.448389384
CK5P2_RAT	CDK5 regulatory subunit-associated protein 2	0.27707502	KCNT1_RAT	Potassium channel subfamily T member 1	0.453239609
ACBG2_RAT	Long-chain-fatty-acid--CoA ligase ACSBG2	0.302162905	LPH_RAT	Lactase-phlorizin hydrolase	0.461436762
ANPRB_RAT	Atrial natriuretic peptide receptor 2	0.319759053	DUOX2_RAT	Dual oxidase 2	0.47678014
FARP1_RAT	FERM, ARHGEF and pleckstrin domain-containing protein 1	0.32591555	UN13B_RAT	Protein unc-13 homolog B	0.488441165
MCM9_RAT	DNA helicase MCM9	0.326984311	DDR1_RAT	Epithelial discoidin domain-containing receptor 1	0.490031805
KCNH3_RAT	Potassium voltage-gated channel subfamily H member 3	0.339176665	TGM1_RAT	Protein-glutamine gamma-glutamyltransferase K	0.49080124
PLCB2_RAT	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-2	0.342600606	NRX2A_RAT	Neurexin-2	0.492569052
MAGI2_RAT	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein	0.342600823	SNUT1_RAT	U4/U6.U5 tri-snRNP-associated protein 1	0.495516177
GTF2I_RAT	General transcription factor II-I	0.355552887	CFTR_RAT	Cystic fibrosis transmembrane conductance regulator	0.507130876
PDS5A_RAT	Sister chromatid cohesion protein PDS5 homolog A	0.364479206	INSR_RAT	Insulin receptor OS=Rattus norvegicus	0.508614831
LMBL2_RAT	Lethal(3)malignant brain tumor-like protein 2	0.369221703	PLOD3_RAT	Multifunctional procollagen lysine hydroxylase and glycosyltransferase LH3	0.509737641
DLG2_RAT	Disks large homolog 2	0.372956678	FEM1B_RAT	Protein fem-1 homolog B	2.02274075
CAD22_RAT	Cadherin-22	0.377312398	CAMP1_RAT	Calmodulin-regulated spectrin-associated protein 1	2.053420077
KBTB8_RAT	Kelch repeat and BTB domain-containing protein 8	0.381153175	ATS7_RAT	A disintegrin and metalloproteinase with thrombospondin motifs 7	2.078889526
SNED1_RAT	Sushi, nidogen and EGF-like domain-containing protein 1	0.389384342	NBR1_RAT	Next to BRCA1 gene 1 protein	2.0939692
ZN148_RAT	Zinc finger protein 148	0.396430685	OAS3_RAT	2'-5'-oligoadenylate synthase 3	2.135913828
DHX30_RAT	ATP-dependent RNA helicase DHX30	0.401708248	HOIL1_RAT	RanBP-type and C3HC4-type zinc finger-containing protein 1	2.184342902
MYO1A_RAT	Unconventional myosin-1a (Fragment)	0.409450493	PGH1_RAT	Prostaglandin G/H synthase 1	2.209897659
DDX46_RAT	Probable ATP-dependent RNA helicase DDX46	0.41631713	TGON3_RAT	Trans-Golgi network integral membrane protein	2.352062564
SPAG1_RAT	Sperm-associated antigen 1	0.425517267	ERBB3_RAT	Receptor tyrosine-protein kinase erbB-3 OS=Rattus norvegicus	2.460513763
SYNRG_RAT	Synergism gamma	0.426987026	AEGP_RAT	Apical endosomal glycoprotein OS=Rattus norvegicus	2.561234817
GRM5_RAT	Metabotropic glutamate receptor 5	0.432309166			
REXOS_5	RNA exonuclease 5	0.435407442			

Table 28. Transgenerational analyses for differential expression of proteins in the sperm lysates of control and pyrethroid treated rats.
(T/C ratio indicated in light green and light pink boxes indicate down-regulation and up-regulation, respectively. Proteins with known functions related to male reproduction are indicated in blue font.)

	GENERATION					
ENTRY	F0	F1	F2	F3	PROTEIN DESCRIPTION	FUNCTION IN MALE REPRODUCTION
	RATIO	RATIO	RATIO	RATIO		
PK3CA_RAT	0	0.443	0	1.347	PK3A catalytic subunit alpha isoform	Role in capacitation and acrosome reaction
APBP2_RAT	0.753	0	0	1.068	Amyloid protein-binding protein 2	SLY1 interacts with the acrosomal protein DKKL1
SOBP_RAT	0	0	0	1.663	Sine oculis-binding protein homolog	Gene expression altered in DEHP exposure
SMRCD_RAT	0	0	0	0	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1	Role in male fertility
MYLIP_RAT	0	0	0	0	E3 ubiquitin-protein ligase	Required for the progression of mature spermatozoa
WDR26_RAT	0	0.314	0	0	WD repeat-containing protein 26	--
UNC5D_RAT	0	0	0	0	Netrin receptor UNC5D	--
STX12_RAT	0	0	0	0	Syntaxin-12	--
RN157_RAT	0	0	0	1.607	E3 ubiquitin ligase Rnf157	--
PPIG_RAT	0	0	0	2.333	Peptidyl-prolyl cis-trans isomerase G	--
ABCB6_RAT	0	0	0	0.962	ATP-binding cassette sub-family B member 6, mitochondrial	Expressed both in Sertoli cells, peritubular cells, and in the germline
PACS1_RAT	0	0.317	0	2.951	Phosphofurin acidic cluster sorting protein 1	--
ARC1B_RAT	0	0	0	5.006	Actin-related protein 2/3 complex subunit 1B	Role in spermiation
TUTLA_RAT	0.234	0	0	0	Protein turtle homolog A	--
KCNB1_RAT	0.518	0.67	0	0.807	Potassium voltage-gated channel subfamily B member 1	--
TGFB1_RAT	0	0	0	0	Transforming growth factor beta-1 proprotein	--
THRB_RAT	0.728	0	0	0.251	Prothrombin	--
OL226_RAT	0	0	0	1.557	Olfactory receptor 226	--
SL9A4_RAT	0	0	0	1.966	Sodium/hydrogen exchanger 4	--
LIPP_RAT	0	0	0	1.02	Pancreatic triacylglycerol lipase	Role in postnatal development of rats
GRM5_RAT	0.432	0.4	0.113	0.989	Metabotropic glutamate receptor 5	--

ANXA6_RAT	0	0	0	0	Annexin A6	Regulates the release of Ca ²⁺ from intracellular stores
PEX6_RAT	0	0	0	4.222	Peroxisome assembly factor 2	--
S12A1_RAT	0	0	0	2.097	Solute carrier family 12 member 1	--
MDGA2_RAT	0.616	0	0	2.049	MAM domain-containing glycosylphosphatidylinositol anchor protein 2	--
PHAR1_RAT	0	0	0	1.512	Phosphatase and actin regulator 1	--
KAP1_RAT	0	0	0	4.392	cAMP-dependent protein kinase type I-beta regulatory subunit	--
ESM1_RAT	0	0	0	1.95	Endothelial cell-specific molecule 1	--
SMC3_RAT	0	0.616	0.424	0.816	Structural maintenance of chromosomes protein 3	Role in sister chromatid cohesion during meiosis in human oocytes
KKCC1_RAT	0	0	0	3.371	Calcium/calmodulin-dependent protein kinase kinase 1	Regulation of Chicken Sperm Functions
SRBP2_RAT	0	0	0.136	2.583	Sterol regulatory element-binding protein 2	Spermatogenesis
LRC8C_RAT	0	0.43	0	1.661	Volume-regulated anion channel subunit LRRC8C	Spermatid development
PSMD2_RAT	0	0.644	0	0.671	26S proteasome non-ATPase regulatory subunit 2	Sperm Capacitation
GIMA8_RAT	0	0	0	1.006	GTPase IMAP family member 8	--
RNF8_RAT	0	0	0	0	E3 ubiquitin-protein ligase RNF8	Spermatid development
RING2_RAT	0	0	0	0	E3 ubiquitin-protein ligase RING2	Germ cell development
NBR1_RAT	2.094	2.168	0	1.086	Next to BRCA1 gene 1 protein	--
TBX5_RAT	0	0	0	2.348	T-box transcription factor TBX5	--
TAB2_RAT	0	0	0	0	TGF-beta-activated kinase 1 and MAP3K7-binding protein 2	--
CARL3_RAT	0	0	0	0	Capping protein, Arp2/3, and myosin-I linker protein 3	--
ERBB3_RAT	2.461	5.349	4.582	3.965	Receptor tyrosine-protein kinase erbB-3	--
AEGP_RAT	2.561	10.5	8.275	7.118	Apical endosomal glycoprotein	--
CAD22_RAT	0.377	0	0	0.715	Cadherin-22	Capacitation of sperm and gamete contact during fertilization.
ABCC9_RAT	1.969	3.657	2.4	2.056	ATP-binding cassette sub-family C member 9	--
PGH1_RAT	2.21	4.814	1.966	0	Prostaglandin G/H synthase 1	--
ECHA_RAT	0	0	0	0.73	Trifunctional enzyme subunit alpha, mitochondrial	--
RED_RAT	0	0	0	1.363	Protein Red	--
NPL_RAT	0	0	0	0.844	N-acetylneuraminate lyase	--

C2D1A_RAT	0	0	0	0	Coiled-coil and C2 domain-containing protein 1A	--
C2D1A_RAT	1.654	2.484	3.48	2.117	Coiled-coil and C2 domain-containing protein 1A	--
ORC4_RAT	0	0	0	0	Origin recognition complex subunit 4	--
TRPA1_RAT	0	0	0	0	Transient receptor potential cation channel subfamily A member 1	--
SCN4B_RAT	0	0	0	0	Sodium channel subunit beta-4	--
IP3KC_RAT	0	0	0	2.014	Inositol-trisphosphate 3-kinase C	Sperm capacitation and the acrosome reaction
BRNP3_RAT	0	0	0	5.941	BMP/retinoic acid-inducible neural-specific protein 3	--
ERC2_RAT	0.448	0.636	1.193	1.103	ERC protein 2	--
PALM_RAT	0	0	0	3.865	Paralemmin-1	--
S26A3_RAT	0	0	0	0.98	Chloride anion exchanger	Sperm capacitation
LONM_RAT	0.534	0.234	0	1.777	Lon protease homolog, mitochondrial	Degradation of StAR
ABCG5_RAT	0	0	0	1.144	ATP-binding cassette sub-family G member 5	--
CCN1_RAT	0	0	0	2.718	CCN family member 1	--
C1QR1_RAT	0.793	0	0	2.7	Complement component C1q receptor	Sperm-egg interaction
ECEL1_RAT	0	0	0	0	Endothelin-converting enzyme-like 1	Acquisition of androgen-independent prostate cancer
HIF3A_RAT	0	0	0	1.364	Hypoxia-inducible factor 3-alpha	--
S4A4_RAT	0	0	0	2.675	Electrogenic sodium bicarbonate cotransporter 1	--
SYNRG_RAT	0.427	0.289	0	1.704	Synergin gamma	--
TRPC3_RAT	0	0.288	0	0	Short transient receptor potential channel 3	Sperm motility and capacitation
TRPC1_RAT	0	0.394	0	1.13	Short transient receptor potential channel 1	Calcium signalling during fertilization
LEF1_RAT	0	0	0	0	Lymphoid enhancer-binding factor 1	Estrogen receptor binding
MYO5A_RAT	0.671	0.726	0	1.43	Unconventional myosin-Va	--
SEM6C_RAT	0	0	0	1.513	Semaphorin-6C	Development of hormone systems and hormonal regulation
AVIL_RAT	0	0.397	0	0	Advillin	--
FBLN5_RAT	0	0	0	5.282	Fibulin-5	--

4.5. Discussion

One of the serious interventions that continue to affect human and animal health is the use of pyrethroid-based pesticides. Research reports in the last 2 years indicate the effect of pyrethroid-based pesticides in male reproductive physiology. Fenvalerate induced apoptosis and testicular damage (38), disruption of ERK/COX-2 signaling pathway in the testes by permethrin (39), alterations in the expression of testicular nucleotidase gene by lambda-cyhalothrin (40), the antiandrogenic activity of cypermethrin in Sertoli cells (41), disruption of steroidogenesis in the male rats by deltamethrin, permethrin, esfenvalerate, and cypermethrin (42) and lambda-cyhalothrin delayed pubertal development in rats (24) are some of the manifestations observed due to pyrethroid toxicity. This study was conducted to provide additional evidence on the toxic effects of pyrethroids when administered at doses relevant to human exposure through food. Transcriptome changes were reported in zebrafish under conditions of deltamethrin or cypermethrin or niclosamide exposure (43). In this study, the transcriptomic profile of genes associated with male germ cell production (spermatogenesis, steroidogenesis, and epigenetic modifications) was analyzed. We observed perturbations in the expression of genes related to spermatogenesis, steroidogenesis, and epigenetic modulators; and thus, contribute to the growing evidence on the manifestations of pyrethroid toxicity.

The hallmark of spermatogenesis is the stage-specific expression of genes. Though the effect of pyrethroids on spermatogenesis is reported in general, a thorough analysis of the expression pattern of genes involved in this process is lacking. The majority of the genes analyzed were downregulated in the testes obtained from LD or HD groups at all the time points. Alterations in the expression of *Sal4* and *Nano3* genes responsible for male germ cell differentiation were observed in the mice exposed to glyphosate (44). Leydig cell-specific gene expression was decreased in rats treated with triphenyltin (45). Cypermethrin-induced downregulation of *Cyp19a*, *Nanos2*, *Piwil1*, *Dazl*, and *Sycp3* genes; and upregulation of *Odf3b*, *Igf3*, *Ins13*, and *Dmrt1* genes to affect spermatogenesis was reported in the zebrafish model (46). Endosulfan treatment resulted in downregulation of *Sohlh1*, a spermatogenesis and oogenesis-specific transcription factor (47). Altered expression of genes related to germ cell production was observed in rats subjected to allethrin toxicity (30,48). We previously reported a decrease in litter size in rats exposed to a mixture of pyrethroids (32). Thus, the downregulation of spermatogenesis-related genes observed in this study could be one of the critical events to cause reduced fecundity.

The association of pyrethroid toxicity with perturbed steroidogenesis via alterations in the expression pattern of the molecular components is not well reported. Maternal exposure to cis-bifenthrin decreased the expression of the steroidogenic acute regulatory protein (StAR), cytochrome P450-17 α -hydroxysteroid dehydrogenase (P450-17 α), scavenger receptor class B type 1 (Srb1), and cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) in the fetal testes (49). Similarly, testosterone production-related gene expression was decreased in pups born to dams exposed to cypermethrin during gestation (50). We previously reported decreased levels of StAR mRNA levels and activities of 3 β -HSD and 17 β -HSD in allethrin-treated rats (30,48). On the other hand, no effect on the expression pattern of steroidogenesis-related genes was reported in offspring of female rats treated with deltamethrin or cypermethrin or esfenvalerate or permethrin (51). In this study, we observed that majority of the steroidogenesis-related genes were downregulated in the testes of pyrethroid-treated rats. When compared to previous reports, our study differs in three aspects namely, using doses that are relevant to human consumption, the duration of exposure, and analysis in the exposed rats rather in their offspring. This explains the differential results obtained in our study when compared to other studies.

Genomic reprogramming via epigenetic modifications occurs during spermatogenesis (52). Any aberrations in these processes are considered to be potential aspects of male infertility (53). The effect of pyrethroids on epigenetic modifications is reported in the brain of mice (54). Very limited information is available regarding pyrethroid toxicity and its relation to alterations in epigenetic modifications of the gametes and the molecular components that affect these processes. Epigenetic transgenerational inheritance of diseases and epimutations in the sperm was reported in pups born to rats exposed to a mixture of permethrin and N,N-diethyl-metatoluamide (55). In this study, we observed a decrease in the majority of the factors that contribute to the epigenetic modifications in the testes of rats exposed to a mixture of pyrethroids. Such perturbations clearly indicate that pyrethroid toxicity could affect the whole epigenome reprogramming of the spermatozoa during development. An in-depth analysis of the nature and extent of disrupted epigenetic modifications will be of potential interest.

An interesting aspect that needs special attention in this study is that the pattern of expression for some genes was not similar within the two dosage groups (LD and HD). Brdt, Fshr, Hsf2, PcnA, and Rhox5 mRNA levels were decreased only in the LD group, but not in the HD groups treated for 9 months. On the same lines, in the 12-month-treated rats, the expression pattern was observed for 3- β -Hsd, Gata4, Sox9, Star, Dnmt1, Hdac4, and Tet1 LD

group was reflected in the HD group. Further, for some genes, the effect observed at 9 months was not necessarily the same at the 12-month time point. Conventionally, a dose and time-dependent response is expected, wherein the response observed at a lower dose or at an earlier time point should be compounded at a higher dose or at a later time point. However, such a compounding effect on gene expression was not observed for some genes in our study. This could be due to the nonmonotonic dose responses, a concept that is widely reported in toxicological studies (56).

The proteomic composition of the male gamete determines their fertilization ability. During spermatogenesis and sperm maturation, which occur in the testes and epididymis respectively, the sperm membrane acquires a large number of proteins from the luminal fluid. These proteins display a wide range of functions during gamete formation and through their journey towards successful fertilization (57,58). Alterations in the sperm proteome by environmental toxicants and the implications on fertility are not well studied. In this study, proteomic analyses of sperm lysates obtained from rats treated with a mixture of pyrethroids indicated differential expression of a large number of proteins, majority of which were down-regulated or completely absent when compared to the control. A recent report suggests that in rats exposed to low doses of fenvalerate orally, sperm proteins are differentially expressed and majority of them are involved in spermatogenesis and p53-related processes (59). The emerging concept of alterations in sperm proteome due to pyrethroid toxicity is further strengthened by the results presented in this study.

Evidence on the transgenerational effects at the physiological and molecular level in generations that are born to the pesticide/pyrethroid exposed forefathers is redefining the extent of toxicological studies that are to be conducted to determine the deleterious effects in the long run. Recent studies have shown that perinatal exposure to cypermethrin impairs reproductive function in F1 generation rats (60,61). Sperm epimutations and epigenetic transgenerational inheritance of disease are reported in rats exposed to permethrin and DEET (55,62). We demonstrate that some of the sperm proteins that were differentially regulated due to pyrethroid toxicity, continued to be maintained at the altered levels in the spermatozoa of rats in later generations (up to F3). Among those that were transgenerationally altered included proteins that are implicated to have essential roles in sperm function and male fertility as a whole. The differential expression of these proteins in the pyrethroid treated rats and in later generations could have contributed to the decreased fecundity in the parental males and their grandchildren rats born up to F2 generation.

Appendix -1. List of proteins that were commonly present in the sperm lysates of biological triplicates of control rats and their differential expression pattern in sperm of pyrethroid treated rats.

(T/C ratio indicated in light green and light pink boxes indicate down-regulation and up-regulation respectively)

ID	UNIPROT ENTRY	DESCRIPTION	RATIO
A0A0G2K344	PK3CA_RAT	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform	0
A1A5R7	REXO5_RAT	RNA exonuclease 5	
A1L1J6	ZN652_RAT	Zinc finger protein 652	
A1L1K7	ACBG2_RAT	Long-chain-fatty-acid--CoA ligase ACSBG2	0.30216291
A2RRT9	CP4V2_RAT	Cytochrome P450 4V2	1.00236691
A2RUV9	AEBP1_RAT	Adipocyte enhancer-binding protein 1	0
A2VCW5	S38A5_RAT	Sodium-coupled neutral amino acid transporter 5	0
A4L9P7	PDS5A_RAT	Sister chromatid cohesion protein PDS5 homolog A	0.36447921
A5HK05	APBP2_RAT	Amyloid protein-binding protein 2	0.75313278
A7XYI6	SOBP_RAT	Sine oculis-binding protein homolog	0
B1H285	KBTB8_RAT	Kelch repeat and BTB domain-containing protein 8	0.38115317
B1WC15	ZCHC7_RAT	Zinc finger CCHC domain-containing protein 7	0.60049181
B1WC39	TIGD5_RAT	Tigger transposable element derived 5	0
B2GUV7	IF2P_RAT	Eukaryotic translation initiation factor 5B	1.55584864
B2GUY0	MA1B1_RAT	Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase	0
B2GUZ1	UBP4_RAT	Ubiquitin carboxyl-terminal hydrolase 4	0
B2GV22	PTSS2_RAT	Phosphatidylserine synthase 2	0
B4F777	HMGN5_RAT	High mobility group nucleosome-binding domain-containing protein 5	0.77772577
B4F795	CTL2_RAT	Choline transporter-like protein 2	0
B5DEL3	TTC17_RAT	Tetratricopeptide repeat protein 17	0.68332086
B5DFA1	TBD2A_RAT	TBC1 domain family member 2A	0
B5MEV3	S52A2_RAT	Solute carrier family 52, riboflavin transporter, member 2	0
C0HL12	AGRB1_RAT	Adhesion G protein-coupled receptor B1	0.90048194

D3Z8E6	CAMP1_RAT	Calmodulin-regulated spectrin-associated protein 1	2.05342008
D3Z8N4	KLH20_RAT	Kelch-like protein 20	0.95249475
D3Z9Z9	SMRCD_RAT	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1	0
D3ZD32	CHD5_RAT	Chromodomain-helicase-DNA-binding protein 5	0.85604572
D3ZDI6	MYLIP_RAT	E3 ubiquitin-protein ligase MYLIP	0
D3ZEF4	CUL7_RAT	Cullin-7	0.78941765
D3ZEH5	SIDT2_RAT	SID1 transmembrane family member 2	0
D3ZEN0	MILK2_RAT	MICAL-like protein 2	0.65621003
D3ZFD0	MY18A_RAT	Unconventional myosin-XVIIIa	0.60443042
D3ZG52	DNA2_RAT	DNA replication ATP-dependent helicase/nuclease DNA2	0.88391002
D3ZGX1	NEXMI_RAT	Neurite extension and migration factor	1.33363712
D3ZJP6	MYO10_RAT	Unconventional myosin-X	0.98504308
D3ZKF5	HTRA4_RAT	Serine protease HTR4	0
D3ZLH5	PLXB3_RAT	Plexin-B3	0.98509373
D3ZPX4	PLXA3_RAT	Plexin-A3	0.86523599
D3ZQX2	NILR1_RAT	Neutrophil immunoglobulin-like receptor 1	1.0672329
D3ZS74	OMA1_RAT	Metalloendopeptidase OMA1, mitochondrial	1.70736413
D3ZSI8	PI51A_RAT	Phosphatidylinositol 4-phosphate 5-kinase type-1 alpha	0
D3ZSP7	TTC3_RAT	E3 ubiquitin-protein ligase TTC3	1.19837295
D3ZSZ3	NLK_RAT	Serine/threonine-protein kinase NLK	0
D3ZUC6	TOPZ1_RAT	Protein TOPAZ1	0.66757597
D3ZZ80	OBSL1_RAT	Obscurin-like protein 1	0.68165388
D3ZZL9	GCC2_RAT	GRIP and coiled-coil domain-containing protein 2	0.80586162
D3ZZN9	SYDE1_RAT	Rho GTPase-activating protein SYDE1	1.2720453
D4A0X3	K1107_RAT	AP2-interacting clathrin-endocytosis protein	0.78820233
D4A1F2	MICA2_RAT	[F-actin]-monooxygenase MICAL2	1.45077431
D4A631	BIG1_RAT	Brefeldin A-inhibited guanine nucleotide-exchange protein 1	0.64490493

D4A666	UBN2_RAT	Ubinuclein-2	0.91793117
D4A6L0	GP158_RAT	Probable G-protein coupled receptor 158	1.57378933
D4A7T3	BRDT_RAT	Bromodomain testis-specific protein	0.24494839
D4A7V9	E2AK4_RAT	eIF-2-alpha kinase GCN2	0.67131825
D4A929	WDR81_RAT	WD repeat-containing protein 81	0.43912691
D4AE41	RMXL1_RAT	RNA binding motif protein, X-linked-like-1	0.78305518
D4AEC2	CAMP2_RAT	Calmodulin-regulated spectrin-associated protein 2	0.62077816
E9PSL7	CTRO_RAT	Citron rho-interacting kinase	0.7564166
E9PTA2	TRPM2_RAT	Transient receptor potential cation channel subfamily M member 2	0.72685303
E9PU17	ABCAH_RAT	ATP-binding cassette sub-family A member 17	1.22357971
F1LNJ2	U520_RAT	U5 small nuclear ribonucleoprotein 200 kDa helicase	0.776549
F1LP64	TRIPC_RAT	E3 ubiquitin-protein ligase TRIP12	0.97777278
F1LRS8	CD2AP_RAT	CD2-associated protein	0.52217555
F1LTE0	TANC2_RAT	Protein TANC2	0.82539945
F1LTR1	WDR26_RAT	WD repeat-containing protein 26	0
F1LW30	UNC5D_RAT	Netrin receptor UNC5D	0
F1LYQ8	FARP1_RAT	FERM, ARHGEF and pleckstrin domain-containing protein 1	0.32591555
F1M386	RPGF2_RAT	Rap guanine nucleotide exchange factor 2	0
F1M4A4	KIF1A_RAT	Kinesin-like protein KIF1A	0.94738588
F1M5F3	MCM9_RAT	DNA helicase MCM9	0.32698431
F1M5M3	TEX14_RAT	Inactive serine/threonine-protein kinase TEX14	0
F1M775	DIAP1_RAT	Protein diaphanous homolog 1	0.86207787
F1MAD2	INADL_RAT	InaD-like protein	1.33599425
F8WLE0	KIF28_RAT	Kinesin-like protein KIF28P	1.39167522
G3V7P1	STX12_RAT	Syntaxin-12	0
G3V7Q0	DEN5A_RAT	DENN domain-containing protein 5A	1.24366084
G3V893	ZN335_RAT	Zinc finger protein 335	0

G3V9R8	HNRPC_RAT	Heterogeneous nuclear ribonucleoprotein C	0.57537332
M0R5D6	RN157_RAT	E3 ubiquitin ligase Rnf157	0
M0RD54	CEFIP_RAT	Cardiac-enriched FHL2-interacting protein	1.58599971
O08562	SCN9A_RAT	Sodium channel protein type 9 subunit alpha	0.70979604
O08628	PCOC1_RAT	Procollagen C-endopeptidase enhancer 1	1.68891776
O08662	PI4KA_RAT	Phosphatidylinositol 4-kinase alpha	0.61484923
O08678	MARK1_RAT	Serine/threonine-protein kinase MARK1	0
O08680	EPHA3_RAT	Ephrin type-A receptor 3	0
O08775	VGFR2_RAT	Vascular endothelial growth factor receptor 2	0.73977158
O08815	SLK_RAT	STE20-like serine/threonine-protein kinase	0.61450878
O08873	MADD_RAT	MAP kinase-activating death domain protein	0.97225556
O08874	PKN2_RAT	Serine/threonine-protein kinase N2	0.68187638
O08961	ZN423_RAT	Zinc finger protein 423	0.74156246
O08962	KCNH2_RAT	Potassium voltage-gated channel subfamily H member 2	1.32089872
O35119	TRPC4_RAT	Short transient receptor potential channel 4	0
O35132	CP27B_RAT	25-hydroxyvitamin D-1 alpha hydroxylase, mitochondrial	1.02387609
O35412	SIIL1_RAT	Signal-induced proliferation-associated 1-like protein 1	0.84675603
O35413	SRBS2_RAT	Sorbin and SH3 domain-containing protein 2	0.56858148
O35787	KIF1C_RAT	Kinesin-like protein KIF1C	1.75686402
O35789	B3GA1_RAT	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1	0.9343814
O35806	LTBP2_RAT	Latent-transforming growth factor beta-binding protein 2	0
O35821	MBB1A_RAT	Myb-binding protein 1A	0.63853533
O54852	KCNH7_RAT	Potassium voltage-gated channel subfamily H member 7	0.94703686
O54874	MRCKA_RAT	Serine/threonine-protein kinase MRCK alpha	0.95837352
O54952	BRCA1_RAT	Breast cancer type 1 susceptibility protein homolog	0
O55005	ROBO1_RAT	Roundabout homolog 1	0.60663591
O55007	RGPA1_RAT	Ral GTPase-activating protein subunit alpha-1	1.18889119

O55035	PPIG_RAT	Peptidyl-prolyl cis-trans isomerase G	0
O55156	CLIP2_RAT	CAP-Gly domain-containing linker protein 2	0.57714624
O55164	MPDZ_RAT	Multiple PDZ domain protein	1.03725579
O70196	PPCE_RAT	Prolyl endopeptidase	1.42643235
O70595	ABCB6_RAT	ATP-binding cassette sub-family B member 6, mitochondrial	0
O70608	SYCP2_RAT	Synaptonemal complex protein 2	0.96926261
O88279	SLIT1_RAT	Slit homolog 1 protein	0.63169255
O88280	SLIT3_RAT	Slit homolog 3 protein	1.14928525
O88281	MEGF6_RAT	Multiple epidermal growth factor-like domains protein 6	0.78341494
O88382	MAGI2_RAT	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 2	0.34260082
O88420	SCN8A_RAT	Sodium channel protein type 8 subunit alpha	0.59182965
O88457	SCNBA_RAT	Sodium channel protein type 11 subunit alpha	1.56350472
O88480	CABIN_RAT	Calcineurin-binding protein cabin-1	0.5334178
O88588	PACS1_RAT	Phosphofurin acidic cluster sorting protein 1	0
O88656	ARC1B_RAT	Actin-related protein 2/3 complex subunit 1B	0
O88658	KIF1B_RAT	Kinesin-like protein KIF1B	1.19716709
O88902	PTN23_RAT	Tyrosine-protein phosphatase non-receptor type 23 (Fragment)	0.902525
O88944	MOGS_RAT	Mannosyl-oligosaccharide glucosidase	1.1197448
O89039	ACKR3_RAT	Atypical chemokine receptor 3	0
O89040	PLCB2_RAT	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-2	0.34260061
O89042	DPOLA_RAT	DNA polymerase alpha catalytic subunit (Fragment)	0.96081065
O89047	KCNH3_RAT	Potassium voltage-gated channel subfamily H member 3	0.33917666
P01026	CO3_RAT	Complement C3	0.87219481
P02563	MYH6_RAT	Myosin-6	1.30177862
P02564	MYH7_RAT	Myosin-7	1.10198203
P04276	VTDB_RAT	Vitamin D-binding protein	0
P04774	SCN1A_RAT	Sodium channel protein type 1 subunit alpha	1.43846622

P04775	SCN2A_RAT	Sodium channel protein type 2 subunit alpha	1.01762397
P06238	A2MG_RAT	Alpha-2-macroglobulin	0
P06494	ERBB2_RAT	Receptor tyrosine-protein kinase erbB-2	0.7598903
P07897	PGCA_RAT	Aggrecan core protein	0.84140065
P08104	SCN3A_RAT	Sodium channel protein type 3 subunit alpha	0.82279493
P0C1T0	MMEL1_RAT	Membrane metallo-endopeptidase-like 1	0
P0C5H6	TUTLA_RAT	Protein turtle homolog A	0.23378046
P0C6C0	SPKAP_RAT	A-kinase anchor protein SPHKAP	0.80654953
P0C6P5	ARG28_RAT	Rho guanine nucleotide exchange factor 28	0.84002836
P0C6P7	FEM1B_RAT	Protein fem-1 homolog B	2.02274075
P0CB49	YLPM1_RAT	YLP motif-containing protein 1	0.84800568
P10362	SCG2_RAT	Secretogranin-2	0
P11442	CLH1_RAT	Clathrin heavy chain 1	1.09694038
P11506	AT2B2_RAT	Plasma membrane calcium-transporting ATPase 2	0.64796105
P11654	PO210_RAT	Nuclear pore membrane glycoprotein 210	1.20331863
P11715	CP17A_RAT	Steroid 17-alpha-hydroxylase/17,20 lyase	0
P12847	MYH3_RAT	Myosin-3	0.63927621
P14046	A1I3_RAT	Alpha-1-inhibitor 3	0.70333859
P15127	INSR_RAT	Insulin receptor	0.50861483
P15146	MTAP2_RAT	Microtubule-associated protein 2	0.84494007
P15387	KCNB1_RAT	Potassium voltage-gated channel subfamily B member 1	0.51764716
P15389	SCN5A_RAT	Sodium channel protein type 5 subunit alpha	0.97569683
P15390	SCN4A_RAT	Sodium channel protein type 4 subunit alpha	0.82109926
P16067	ANPRB_RAT	Atrial natriuretic peptide receptor 2	0.31975905
P17246	TGFB1_RAT	Transforming growth factor beta-1 proprotein	0
P18292	THRB_RAT	Prothrombin	0.72800141
P19024	KCNA5_RAT	Potassium voltage-gated channel subfamily A member 5	0

P19468	GSH1_RAT	Glutamate--cysteine ligase catalytic subunit	0
P19490	GRIA1_RAT	Glutamate receptor 1	0.7533517
P19492	GRIA3_RAT	Glutamate receptor 3	0
P19814	TGON3_RAT	Trans-Golgi network integral membrane protein TGN38	2.35206256
P21139	MA2C1_RAT	Alpha-mannosidase 2C1	0
P21263	NEST_RAT	Nestin	0.51193162
P22002	CAC1C_RAT	Voltage-dependent L-type calcium channel subunit alpha-1C	0
P22199	MCR_RAT	Mineralocorticoid receptor	0
P22756	GRIK1_RAT	Glutamate receptor ionotropic, kainate 1	1.70268988
P22985	XDH_RAT	Xanthine dehydrogenase/oxidase	1.2016516
P23270	OL226_RAT	Olfactory receptor 226	0
P23347	B3A2_RAT	Anion exchange protein 2	0.95731614
P23348	B3A3_RAT	Anion exchange protein 3	1.6132827
P23385	GRM1_RAT	Metabotropic glutamate receptor 1	0
P23606	TGM1_RAT	Protein-glutamine gamma-glutamyltransferase K	0.49080124
P23739	SUIS_RAT	Sucrase-isomaltase, intestinal	0.77854894
P23785	GRN_RAT	Progranulin	0
P23897	GUC2C_RAT	Heat-stable enterotoxin receptor	0.74317115
P24135	PLCG2_RAT	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2	0.9934561
P25109	ACHB_RAT	Acetylcholine receptor subunit beta	0.99668794
P26434	SL9A4_RAT	Sodium/hydrogen exchanger 4	0
P26769	ADCY2_RAT	Adenylate cyclase type 2	0
P26770	ADCY4_RAT	Adenylate cyclase type 4	1.04646753
P27657	LIPP_RAT	Pancreatic triacylglycerol lipase	0
P31424	GRM5_RAT	Metabotropic glutamate receptor 5	0.43230917
P34158	CFTR_RAT	Cystic fibrosis transmembrane conductance regulator	0.50713088
P35000	VIPR2_RAT	Vasoactive intestinal polypeptide receptor 2	0.78854148

P35444	COMP_RAT	Cartilage oligomeric matrix protein	1.30275641
P35446	SPON1_RAT	Spondin-1	1.11437206
P35952	LDLR_RAT	Low-density lipoprotein receptor	1.28287944
P37089	SCNNA_RAT	Amiloride-sensitive sodium channel subunit alpha	0
P39052	DYN2_RAT	Dynamin-2	0.6555042
P40146	ADCY8_RAT	Adenylate cyclase type 8	1.73243297
P41413	PCSK5_RAT	Proprotein convertase subtilisin/kexin type 5	0.7279028
P41516	TOP2A_RAT	DNA topoisomerase 2-alpha	1.54801439
P41777	NOLC1_RAT	Nucleolar and coiled-body phosphoprotein 1	1.2682688
P42260	GRIK2_RAT	Glutamate receptor ionotropic, kainate 2	0.67423038
P48037	ANXA6_RAT	Annexin A6	0
P49797	RGS3_RAT	Regulator of G-protein signaling 3	0.61222214
P49816	TSC2_RAT	Tuberin	0.86863991
P51578	P2RX5_RAT	P2X purinoceptor 5	0.89468226
P51839	GUC2D_RAT	Guanylate cyclase 2D	1.33063731
P51840	GUC2E_RAT	Retinal guanylyl cyclase 1	0
P51842	GUC2F_RAT	Retinal guanylyl cyclase 2	1.73708339
P51869	CP4F4_RAT	Cytochrome P450 4F4	0.25017834
P52590	NU107_RAT	Nuclear pore complex protein Nup107	0.7292561
P53767	VGFR1_RAT	Vascular endothelial growth factor receptor 1	0
P54100	VAV_RAT	Proto-oncogene vav	0
P54258	ATN1_RAT	Atrophin-1	0
P54290	CA2D1_RAT	Voltage-dependent calcium channel subunit alpha-2/delta-1	0
P54758	EPHA6_RAT	Ephrin type-A receptor 6	0
P54777	PEX6_RAT	Peroxisome assembly factor 2	0
P55016	S12A1_RAT	Solute carrier family 12 member 1	0
P55068	PGCB_RAT	Brevican core protein	0

P56741	MYPC_RAT	Myosin-binding protein C, cardiac-type	0.95581801
P58366	ANKH_RAT	Progressive ankylosis protein homolog	0
P60756	MDGA2_RAT	MAM domain-containing glycosylphosphatidylinositol anchor protein 2	0.61649876
P61203	CSN2_RAT	COP9 signalosome complex subunit 2	0
P62024	PHAR1_RAT	Phosphatase and actin regulator 1	0
P62813	GBRA1_RAT	Gamma-aminobutyric acid receptor subunit alpha-1	1.08648228
P68907	PZRN3_RAT	E3 ubiquitin-protein ligase PDZRN3	0.55513207
P70475	MYT1L_RAT	Myelin transcription factor 1-like protein	0.7933729
P70496	PLD1_RAT	Phospholipase D1	0
P70531	EF2K_RAT	Eukaryotic elongation factor 2 kinase	1.30404508
P70539	ACV1C_RAT	Activin receptor type-1C	1.65134464
P70569	MYO5B_RAT	Unconventional myosin-Vb	1.22939965
P81128	RHG35_RAT	Rho GTPase-activating protein 35	1.18601641
P81377	KAP1_RAT	cAMP-dependent protein kinase type I-beta regulatory subunit	0
P86410	RLGPB_RAT	Ral GTPase-activating protein subunit beta	1.02003292
P86411	RGPA2_RAT	Ral GTPase-activating protein subunit alpha-2	0.84062798
P97608	OPLA_RAT	5-oxoprolinase	0.52799645
P97609	HAIR_RAT	Lysine-specific demethylase hairless	0.85785176
P97682	ESM1_RAT	Endothelial cell-specific molecule 1	0
P97690	SMC3_RAT	Structural maintenance of chromosomes protein 3	0
P97756	KKCC1_RAT	Calcium/calmodulin-dependent protein kinase kinase 1	0
P97837	DLGP2_RAT	Disks large-associated protein 2	0.79244483
P98166	VLDLR_RAT	Very low-density lipoprotein receptor	1.55208103
Q00918	LTBP1_RAT	Latent-transforming growth factor beta-binding protein 1	0.67964201
Q00959	NMDE1_RAT	Glutamate receptor ionotropic, NMDA 2A	1.13900347
Q00960	NMDE2_RAT	Glutamate receptor ionotropic, NMDA 2B	0.91531314
Q01062	PDE2A_RAT	cGMP-dependent 3',5'-cyclic phosphodiesterase	1.01060276

Q01205	ODO2_RAT	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	0
Q01728	NAC1_RAT	Sodium/calcium exchanger 1	1.74731315
Q02401	LPH_RAT	Lactase-phlorizin hydrolase	0.46143676
Q02485	CAC1S_RAT	Voltage-dependent L-type calcium channel subunit alpha-1S	0
Q02759	LOX15_RAT	Polyunsaturated fatty acid lipoyxygenase ALOX15	0
Q03555	GEPH_RAT	Gephyrin	0
Q04400	ADCY5_RAT	Adenylate cyclase type 5	0.70015164
Q04931	SSRP1_RAT	FACT complex subunit SSRP1	0
Q05546	TENR_RAT	Tenascin-R	0.69235161
Q07310	NRX3A_RAT	Neurexin-3	1.3410158
Q09429	ABCC8_RAT	ATP-binding cassette sub-family C member 8	0.60714119
Q0V8T3	CTP5D_RAT	Contactin-associated protein like 5-4	0.85235937
Q10728	MYPT1_RAT	Protein phosphatase 1 regulatory subunit 12A	1.08217953
Q10743	ADA10_RAT	Disintegrin and metalloproteinase domain-containing protein 10	0.7284945
Q1EHB3	ATS7_RAT	A disintegrin and metalloproteinase with thrombospondin motifs 7	2.07888953
Q1JU68	EIF3A_RAT	Eukaryotic translation initiation factor 3 subunit A	1.12830631
Q29RW1	MYH4_RAT	Myosin-4	1.53164473
Q2IBD4	CTTB2_RAT	Cortactin-binding protein 2	0.55697971
Q2Q0I9	FNDC1_RAT	Fibronectin type III domain-containing protein 1	0.59400999
Q2WEA5	TRPM1_RAT	Transient receptor potential cation channel subfamily M member 1	0.69177539
Q3MHU3	TDRD9_RAT	ATP-dependent RNA helicase TDRD9	1.14960177
Q3MIF2	LMBL2_RAT	Lethal(3)malignant brain tumor-like protein 2	0.3692217
Q3SWT4	IWS1_RAT	Protein IWS1 homolog	1.17826557
Q3T1I5	SRBP2_RAT	Sterol regulatory element-binding protein 2	0
Q3T1I9	RPAP1_RAT	RNA polymerase II-associated protein 1	0.74553095
Q498S6	ZCHC2_RAT	Zinc finger CCHC domain-containing protein 2	0.6333382
Q498T9	LRC8C_RAT	Volume-regulated anion channel subunit LRRC8C	0

Q499R0	Z518A_RAT	Zinc finger protein 518A	0.97630035
Q4FZT9	PSMD2_RAT	26S proteasome non-ATPase regulatory subunit 2	0
Q4G005	ERCC3_RAT	General transcription and DNA repair factor IIIH helicase subunit XPB	0
Q4G017	NISCH_RAT	Nischarin	0.83619569
Q4G033	PIWL4_RAT	Piwi-like protein 4	1.75734856
Q4KLG2	GIMA8_RAT	GTPase IMAF family member 8	0
Q4KLH6	CE162_RAT	Centrosomal protein of 162 kDa	0.89894908
Q4KLJ8	PDCL3_RAT	Phosducin-like protein 3	0.9076632
Q4KLM4	KLH25_RAT	Kelch-like protein 25	1.08002158
Q4KLN8	RNF8_RAT	E3 ubiquitin-protein ligase RNF8	0
Q4KLY4	RING2_RAT	E3 ubiquitin-protein ligase RING2	0
Q4TU93	MRC2_RAT	C-type mannose receptor 2	0
Q4V7B1	CF20D_RAT	Protein CFAP20DC	0.63255069
Q4V885	COL12_RAT	Collectin-12	0
Q4V886	PAF1_RAT	RNA polymerase II-associated factor 1 homolog	0
Q501R9	NBR1_RAT	Next to BRCA1 gene 1 protein	2.0939692
Q56B11	PELP1_RAT	Proline-, glutamic acid- and leucine-rich protein 1	0
Q5BJS0	DHX30_RAT	ATP-dependent RNA helicase DHX30	0.40170825
Q5BK26	PLPL7_RAT	Patatin-like phospholipase domain-containing protein 7	1.67940388
Q5BK82	TRI69_RAT	E3 ubiquitin-protein ligase TRIM69	0.66468158
Q5D006	UBP11_RAT	Ubiquitin carboxyl-terminal hydrolase 11	0.89011969
Q5EB59	MED23_RAT	Mediator of RNA polymerase II transcription subunit 23	0
Q5EXX3	ZBT38_RAT	Zinc finger and BTB domain-containing protein 38	0.94418615
Q5FVC7	ACAP2_RAT	Arf-GAP with coiled-coil, ANK repeat, and PH domain-containing protein 2	0.85467528
Q5FVQ4	MLEC_RAT	Malectin	0
Q5I0G4	GARS_RAT	Glycine--tRNA ligase	0
Q5I0I8	NOC4L_RAT	Nucleolar complex protein 4 homolog	0

Q5I2P1	TBX5_RAT	T-box transcription factor TBX5	0
Q5I6B8	PI51C_RAT	Phosphatidylinositol 4-phosphate 5-kinase type-1 gamma	0.65151452
Q5JCS6	SIIL2_RAT	Signal-induced proliferation-associated 1-like protein 2	1.03605523
Q5MYT7	OAS3_RAT	2'-5'-oligoadenylate synthase 3	2.13591383
Q5PQJ5	RHG29_RAT	Rho GTPase-activating protein 29	0.8708364
Q5PQN1	HERC4_RAT	Probable E3 ubiquitin-protein ligase HERC4	1.05829579
Q5PQP9	ARMC5_RAT	Armadillo repeat-containing protein 5	0
Q5PQQ8	ITGBL_RAT	Integrin beta-like protein 1	0.90028718
Q5PQS3	MELT_RAT	Ventricular zone-expressed PH domain-containing protein homolog 1	0
Q5PQT2	MTMR3_RAT	Myotubularin-related protein 3	0.95081046
Q5QE78	AOXB_RAT	Aldehyde oxidase 2	1.3266637
Q5QE80	AOXC_RAT	Aldehyde oxidase 3	0.66307289
Q5RJZ1	RTEL1_RAT	Regulator of telomere elongation helicase 1	0
Q5RKG6	TRI35_RAT	Tripartite motif-containing protein 35	0
Q5TKR9	KAT6A_RAT	Histone acetyltransferase KAT6A	0.78141349
Q5U1Z0	RBGPR_RAT	Rab3 GTPase-activating protein non-catalytic subunit	1.08013175
Q5U2X2	SPAG1_RAT	Sperm-associated antigen 1	0.42551727
Q5U2Y1	GTF2I_RAT	General transcription factor II-I	0.35555289
Q5U2Y9	LCA5_RAT	Lebercilin	1.24273554
Q5U303	TAB2_RAT	TGF-beta-activated kinase 1 and MAP3K7-binding protein 2	0
Q5U311	SLN13_RAT	Schlafen family member 13	0
Q5U367	PLOD3_RAT	Multifunctional procollagen lysine hydroxylase and glycosyltransferase LH3	0.50973764
Q5XHY1	CARL3_RAT	Capping protein, Arp2/3 and myosin-I linker protein 3	0
Q5XI07	LPP_RAT	Lipoma-preferred partner homolog	0
Q5XI14	MCMD2_RAT	Minichromosome maintenance domain-containing protein 2	0
Q5XI78	ODO1_RAT	2-oxoglutarate dehydrogenase, mitochondrial	0.63936939
Q5XIW8	SNUT1_RAT	U4/U6.U5 tri-snRNP-associated protein 1	0.49551618

Q5ZQU0	SNED1_RAT	Sushi, nidogen and EGF-like domain-containing protein 1	0.38938434
Q62635	MUC2_RAT	Mucin-2 (Fragment)	0.62692492
Q62682	CNTN3_RAT	Contactin-3	0
Q62689	S6A18_RAT	Sodium-dependent neutral amino acid transporter B(0)AT3	0.66418749
Q62717	CAPS1_RAT	Calcium-dependent secretion activator 1	1.17950923
Q62768	UN13A_RAT	Protein unc-13 homolog A	0.89070596
Q62769	UN13B_RAT	Protein unc-13 homolog B	0.48844116
Q62774	MYO1A_RAT	Unconventional myosin-Ia (Fragment)	0.40945049
Q62780	DDX46_RAT	Probable ATP-dependent RNA helicase DDX46	0.41631713
Q62799	ERBB3_RAT	Receptor tyrosine-protein kinase erbB-3	2.46051376
Q62806	ZN148_RAT	Zinc finger protein 148	0.39643068
Q62812	MYH9_RAT	Myosin-9	1.00356666
Q62825	EXOC3_RAT	Exocyst complex component 3	0
Q62901	RERE_RAT	Arginine-glutamic acid dipeptide repeats protein	0.70512667
Q62919	NELL1_RAT	Protein kinase C-binding protein NELL1	1.29709476
Q62921	HOIL1_RAT	RanBP-type and C3HC4-type zinc finger-containing protein 1	2.1843429
Q62924	AKA11_RAT	A-kinase anchor protein 11	0.78124886
Q62925	M3K1_RAT	Mitogen-activated protein kinase kinase kinase 1	0
Q62956	ERBB4_RAT	Receptor tyrosine-protein kinase erbB-4	0.83632153
Q62959	LEPR_RAT	Leptin receptor	0
Q62968	SCNAA_RAT	Sodium channel protein type 10 subunit alpha	0.71707434
Q63028	ADDA_RAT	Alpha-adducin	0
Q63041	AIM_RAT	Alpha-1-macroglobulin	0.80359051
Q63099	KCNB2_RAT	Potassium voltage-gated channel subfamily B member 2	0
Q63120	MRP2_RAT	Canalicular multispecific organic anion transporter 1	0.60015237
Q63148	CHRD_RAT	Chordin	1.91561491
Q63180	ADAM7_RAT	Disintegrin and metalloproteinase domain-containing protein 7	0.92333056

Q63191	AEGP_RAT	Apical endosomal glycoprotein	2.56123482
Q63199	TNR6_RAT	Tumor necrosis factor receptor superfamily member 6	1.09920913
Q63258	ITA7_RAT	Integrin alpha-7	0.54745219
Q63272	JAK3_RAT	Tyrosine-protein kinase JAK3	0.88817585
Q63273	GRIK5_RAT	Glutamate receptor ionotropic, kainate 5	0
Q63315	CAD22_RAT	Cadherin-22	0.3773124
Q63358	MYO9B_RAT	Unconventional myosin-IXb	0.86821433
Q63374	NRX2A_RAT	Neurexin-2	0.49256905
Q63376	NRX2B_RAT	Neurexin-2-beta	0.68058549
Q63416	ITIH3_RAT	Inter-alpha-trypsin inhibitor heavy chain H3	1.18679803
Q63474	DDR1_RAT	Epithelial discoidin domain-containing receptor 1	0.49003181
Q63505	TF3C1_RAT	General transcription factor 3C polypeptide 1	0.57534034
Q63548	SEM3A_RAT	Semaphorin-3A	1.02898652
Q63560	MAP6_RAT	Microtubule-associated protein 6	0.8059308
Q63563	ABCC9_RAT	ATP-binding cassette sub-family C member 9	1.27558057
Q63622	DLG2_RAT	Disks large homolog 2	0.37295668
Q63632	S12A4_RAT	Solute carrier family 12 member 4	1.89275209
Q63633	S12A5_RAT	Solute carrier family 12 member 5	0.821435
Q63644	ROCK1_RAT	Rho-associated protein kinase 1	0.92014698
Q63679	KDM3A_RAT	Lysine-specific demethylase 3A	0
Q63722	JAG1_RAT	Protein jagged-1	1.18026982
Q63744	RHG07_RAT	Rho GTPase-activating protein 7	1.58215724
Q63755	PRDM2_RAT	PR domain zinc finger protein 2	0.94238648
Q63862	MYH11_RAT	Myosin-11 (Fragments)	0.60818994
Q63921	PGH1_RAT	Prostaglandin G/H synthase 1	2.2098
Q64060	DDX4_RAT	Probable ATP-dependent RNA helicase DDX4	0.5758864
Q641X2	LEO1_RAT	RNA polymerase-associated protein LEO1	0.89958432

Q64428	ECHA_RAT	Trifunctional enzyme subunit alpha, mitochondrial	0
Q64542	AT2B4_RAT	Plasma membrane calcium-transporting ATPase 4	0.71305765
Q64568	AT2B3_RAT	Plasma membrane calcium-transporting ATPase 3	0.53403176
Q64578	AT2A1_RAT	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	0
Q64604	PTPRF_RAT	Receptor-type tyrosine-protein phosphatase F	0.53613898
Q64605	PTPRS_RAT	Receptor-type tyrosine-protein phosphatase S	0
Q64612	PTPRV_RAT	Receptor-type tyrosine-protein phosphatase V	0.75944983
Q64716	INSRR_RAT	Insulin receptor-related protein	0
Q66H58	INT14_RAT	Integrator complex subunit 14	0
Q66H59	NPL_RAT	N-acetylneuraminate lyase	0
Q66HA5	C2D1A_RAT	Coiled-coil and C2 domain-containing protein 1A	0
Q66HG8	RED_RAT	Protein Red	0.97699053
Q66X93	SND1_RAT	Staphylococcal nuclease domain-containing protein 1	1.65422644
Q68FW3	ST7L_RAT	Suppressor of tumorigenicity 7 protein-like	0.77297808
Q6AXT1	FHL5_RAT	Four and a half LIM domains protein 5	0
Q6AYF4	ITB6_RAT	Integrin beta-6	0.9873462
Q6AYJ1	RECQ1_RAT	ATP-dependent DNA helicase Q1	0
Q6AYX5	ODFP2_RAT	Outer dense fiber protein 2	0
Q6ED65	EMAL5_RAT	Echinoderm microtubule-associated protein-like 5	1.13172852
Q6F6B3	TANC1_RAT	Protein TANC1	1.11351242
Q6GVH5	GGNB2_RAT	Gametogenetin-binding protein 2	1.10248592
Q6IE24	UBP54_RAT	Inactive ubiquitin carboxyl-terminal hydrolase 54	0.99498921
Q6IE52	MUG2_RAT	Murinoglobulin-2	0.91534048
Q6IV68	FACD2_RAT	Fanconi anemia group D2 protein homolog	0.51580685
Q6MG06	GNL1_RAT	Guanine nucleotide-binding protein-like 1	0
Q6MG48	PRC2A_RAT	Protein PRRC2A	0.91868228
Q6P3V7	TTC41_RAT	Tetratricopeptide repeat protein 41	1.4395435

Q6P9Z8	ORC4_RAT	Origin recognition complex subunit 4	0
Q6Q760	NALCN_RAT	Sodium leak channel non-selective protein	0.64563184
Q6QI44	NAA25_RAT	N-alpha-acetyltransferase 25, NatB auxiliary subunit	1.05740995
Q6RI86	TRPA1_RAT	Transient receptor potential cation channel subfamily A member 1	0
Q6RI86	TRPA1_RAT	Transient receptor potential cation channel subfamily A member 1	0
Q6TQE1	ZCH18_RAT	Zinc finger CCCH domain-containing protein 18	1.00762032
Q6TRW4	PDS5B_RAT	Sister chromatid cohesion protein PDS5 homolog B	0.85063477
Q6UVM4	KCNT2_RAT	Potassium channel subfamily T member 2	0.81238111
Q75N33	SC16B_RAT	Protein transport protein Sec16B	0
Q765A7	PGAP1_RAT	GPI inositol-deacylase	0
Q76M68	IQEC3_RAT	IQ motif and SEC7 domain-containing protein 3	0
Q7M6Z5	KIF27_RAT	Kinesin-like protein KIF27	0.97525078
Q7M730	SCN4B_RAT	Sodium channel subunit beta-4	0
Q7TNJ2	ABCA7_RAT	ATP-binding cassette sub-family A member 7	1.36018497
Q7TP54	RIPR2_RAT	Rho family-interacting cell polarization regulator 2	0
Q7TSU1	BIG2_RAT	Brefeldin A-inhibited guanine nucleotide-exchange protein 2	0.9273792
Q80U96	XPO1_RAT	Exportin-1	0.76926475
Q80X08	WASC2_RAT	WASH complex subunit 2	0
Q80YN4	CORIN_RAT	Atrial natriuretic peptide-converting enzyme	1.03047728
Q80ZF0	CORA1_RAT	Collagen alpha-1(XXVII) chain	0
Q80ZG2	IP3KC_RAT	Inositol-trisphosphate 3-kinase C	0
Q810W7	MAST1_RAT	Microtubule-associated serine/threonine-protein kinase 1	0
Q811A3	PLOD2_RAT	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	0.60058254
Q811M5	CO6_RAT	Complement component C6	0.98195889
Q811R2	PRGC2_RAT	Peroxisome proliferator-activated receptor gamma coactivator 1-beta	0.62327577
Q811U3	RB6I2_RAT	ELKS/Rab6-interacting/CAST family member 1	1.00484642
Q8CF82	ABCA5_RAT	ATP-binding cassette sub-family A member 5	1.16347208

Q8CFM6	STAB2_RAT	Stabilin-2 (Fragment)	0.21271324
Q8CG09	MRP1_RAT	Multidrug resistance-associated protein 1	0
Q8CGS5	RNZ2_RAT	Zinc phosphodiesterase ELAC protein 2	0
Q8CGU9	TPH2_RAT	Tryptophan 5-hydroxylase 2	1.1525919
Q8CHI5	PER1_RAT	Period circadian protein homolog 1	0.55404261
Q8CIY2	DUOX1_RAT	Dual oxidase 1	0.72499276
Q8K1M7	BRNP3_RAT	BMP/retinoic acid-inducible neural-specific protein 3	0
Q8K1P7	SMCA4_RAT	Transcription activator BRG1	0.96110276
Q8K3M6	ERC2_RAT	ERC protein 2	0.44838938
Q8K5E0	LPAR3_RAT	Lysophosphatidic acid receptor 3	0
Q8VHK2	CSKI1_RAT	Caskin-1	0.64831259
Q8VHU4	ELP1_RAT	Elongator complex protein 1	1.09519913
Q8VHZ8	DSCAM_RAT	Down syndrome cell adhesion molecule homolog	0.76498421
Q8VIG2	MARF1_RAT	Meiosis regulator and mRNA stability factor 1	0.84520244
Q91Z80	LIPA4_RAT	Liprin-alpha-4 (Fragment)	0
Q91ZT1	VGFR3_RAT	Vascular endothelial growth factor receptor 3	0.44341558
Q920Q0	PALM_RAT	Paralemm-1	0
Q924C9	S26A3_RAT	Chloride anion exchanger	0
Q924S5	LONM_RAT	Lon protease homolog, mitochondrial	0.53381888
Q925B3	TRPM7_RAT	Transient receptor potential cation channel subfamily M member 7	0.73384361
Q99J86	ATRN_RAT	Attractin	0.57196962
Q99JE4	RGRF2_RAT	Ras-specific guanine nucleotide-releasing factor 2	0.98929897
Q99JE6	PLCB3_RAT	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-3	1.34594933
Q99M76	STAG3_RAT	Cohesin subunit SA-3	1.09231078
Q99PE7	ABCG5_RAT	ATP-binding cassette sub-family G member 5	0
Q9EPZ7	SO1C1_RAT	Solute carrier organic anion transporter family member 1C1	0.76809952
Q9EQG6	KDIS_RAT	Kinase D-interacting substrate of 220 kDa	0.89299679

Q9ERC5	OTOF_RAT	Otoferlin	1.39889583
Q9ERH3	WDR7_RAT	WD repeat-containing protein 7	0.79005936
Q9ES45	DUOX2_RAT	Dual oxidase 2	0.47678014
Q9ES72	CCN1_RAT	CCN family member 1	0
Q9ESV1	LUZP1_RAT	Leucine zipper protein 1	0.65591218
Q9ET61	C1QR1_RAT	Complement component C1q receptor	0.79301928
Q9JHL3	ECEL1_RAT	Endothelin-converting enzyme-like 1	0
Q9JHS2	HIF3A_RAT	Hypoxia-inducible factor 3-alpha	0
Q9JI66	S4A4_RAT	Electrogenic sodium bicarbonate cotransporter 1	0
Q9JIL8	RAD50_RAT	DNA repair protein RAD50	0.58592752
Q9JIR0	RIMB1_RAT	Peripheral-type benzodiazepine receptor-associated protein 1	0.94536475
Q9JIR1	RIMB2_RAT	RIMS-binding protein 2	0
Q9JIS1	RIMS2_RAT	Regulating synaptic membrane exocytosis protein 2	0.61183384
Q9JJ25	MEFV_RAT	Pyrin	0.70055435
Q9JK25	CLIP1_RAT	CAP-Gly domain-containing linker protein 1	1.69187942
Q9JK71	MAGI3_RAT	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 3	1.15783375
Q9JKC9	SYNRG_RAT	Synergin gamma	0.42698703
Q9JKL7	SREK1_RAT	Splicing regulatory glutamine/lysine-rich protein 1	1.31663217
Q9JKU3	IF172_RAT	Intraflagellar transport protein 172 homolog	0.62891528
Q9JLA3	UGGG1_RAT	UDP-glucose:glycoprotein glucosyltransferase 1	1.37567948
Q9JLH5	CK5P2_RAT	CDK5 regulatory subunit-associated protein 2	0.27707502
Q9JLT0	MYH10_RAT	Myosin-10	0.87937361
Q9JMI9	TRPC3_RAT	Short transient receptor potential channel 3	0
Q9QW07	PLCB4_RAT	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-4	0.53220096
Q9QX01	TRPC1_RAT	Short transient receptor potential channel 1	0
Q9QXN1	LEF1_RAT	Lymphoid enhancer-binding factor 1	0
Q9QYF3	MYO5A_RAT	Unconventional myosin-Va	0.67141523

Q9QYM0	MRP5_RAT	Multidrug resistance-associated protein 5	1.21151892
Q9QYM2	PARG_RAT	Poly(ADP-ribose) glycohydrolase	1.2228867
Q9QYP1	LRP4_RAT	Low-density lipoprotein receptor-related protein 4	0.93600346
Q9QYP2	CELR2_RAT	Cadherin EGF LAG seven-pass G-type receptor 2 (Fragment)	0.91166927
Q9R011	PLK3_RAT	Serine/threonine-protein kinase PLK3	0
Q9R085	UBP15_RAT	Ubiquitin carboxyl-terminal hydrolase 15	0.9413775
Q9R095	SPEF2_RAT	Sperm flagellar protein 2	0.55665297
Q9WTL3	SEM6C_RAT	Semaphorin-6C	0
Q9WU06	AVIL_RAT	Advillin	0
Q9WV48	SHAN1_RAT	SH3 and multiple ankyrin repeat domains protein 1	0.83335012
Q9WVE9	ITSN1_RAT	Intersectin-1	0
Q9WVH8	FBLN5_RAT	Fibulin-5	0
Q9Z0U5	AOXA_RAT	Aldehyde oxidase 1	1.18145484
Q9Z0Y8	CAC11_RAT	Voltage-dependent T-type calcium channel subunit alpha-1I	1.05297864
Q9Z1L0	PK3CB_RAT	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform	0
Q9Z1M9	SMC1A_RAT	Structural maintenance of chromosomes protein 1A	0.9322201
Q9Z1P2	ACTN1_RAT	Alpha-actinin-1	0
Q9Z1Z1	E2AK3_RAT	Eukaryotic translation initiation factor 2-alpha kinase 3	0.91092144
Q9Z258	KCNT1_RAT	Potassium channel subfamily T member 1	0.45323961
Q9Z2K3	ZN394_RAT	Zinc finger protein 394	1.58415483
Q9Z2Q4	METH_RAT	Methionine synthase	0.9015676
Q9Z301	PER2_RAT	Period circadian protein homolog 2	0.61430364
Q9Z330	DNMT1_RAT	DNA (cytosine-5)-methyltransferase 1	0.86662516
Q9Z340	PARD3_RAT	Partitioning defective 3 homolog	1.37954687

4.6. References

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Chapter 5

Evaluating the effect of a mixture
of pyrethroids administered at
doses relevant to human
consumption on the general
physiological processes

5.1. Introduction

Environmental factors play a key role in the well-being of an individual. Besides the lifestyle factors, the quality of air, food, and water that one consumes is also a crucial factor in maintaining good health and avoiding susceptibility to various diseases. Exposure to pesticides by humans in agricultural, domestic, and industrial settings has contributed to the onset of a number of health hazards, which ultimately affected the general physiological functions. Food contamination by pesticides has been a matter of concern because of the percolation of these compounds into the food chain when used for agricultural purposes. The presence of residual levels of pesticides in agricultural products is reported globally (1,2). Among the pesticides used for agricultural purposes, synthetic pyrethroid-based ones are the preferred choice, because of their high specificity and low toxicity to vertebrates (3). In line with the global statistics, in the Indian scenario, pyrethroid-based pesticide use is highest among the choices available (3) and residual levels of these were also detected in the agricultural products. The residual levels of pyrethroids in different agricultural food products were reported by the Project Coordinating Cell, All India Network Project on Pesticide Residues, Indian Agricultural Research Institute, New Delhi (4,5). Thus, it is pertinent that the Indian consumers are routinely exposed to pyrethroid-based insecticides through the food on a daily basis and this could lead to impairment in the function of many organ systems and also to the development of general and organ-specific pathologies.

Scientific literature survey reveals that a large number of studies reported on the physiological, biochemical, and molecular aspects of pyrethroid toxicity in multiple organ systems using different animal models. Concepts that link pyrethroid toxicity with the dysfunctioning of different organ systems backed by analyses at the biochemical and molecular level are emerging in the last few years, suggesting that studying pyrethroid toxicity continues to an active area of investigation. The nephron-, hepato- and reproductive toxicity have been reported besides the effect on general biochemical parameters of the blood. Bifenthrin induced IL-1ss in the liver and kidney of mice (6), cypermethrin induced hepatotoxicity that involved ROS mediated crosstalk between Nrf2/Keap1 and NF- κ B/ κ B- α (7), reduction in red and white blood cell counts and increased IL-1b production in the liver of mice exposed to sub lethal dose of λ -cyhalothrin (8), induction of hyperthyroidism by cypermethrin via the PI3K/Akt-FOXA1 cascade (9), perturbations in the biochemical parameters of lung, liver, brain, heart and testis of rats

by deltamethrin (10), permethrin induced hepatotoxicity modulated by oxidative stress and mitochondrial dysfunction (11), genotoxicity of fenpropathrin (12), multi organ toxicity of pyrethroid based formulations (13), liver fibrosis in quails by activation of TGF-beta1/Smad pathway by (14), nephrotoxicity of fenpropathrin in rats (15), nephrotoxicity of rabbits by (16), alterations in the sperm proteome of fenvalerate treated rats (17), permethrin induced DNA methylation epimutation markers in sperm of rats (18), male reproductive toxicity of deltamethrin (19), impairment of Leydig and Sertoli cell function and reproductive toxicity of bifenthrin (20,21) and loss of male reproductive function due to cypermethrin (22) are some of the studies published in the last one and half years.

Whilst majority of the studies have reported on the toxic effects of pyrethroids, rarely the doses used were of relevance to human exposure. Keeping in view the way humans are exposed to pyrethroids on a regular basis, either due to occupation or domestic use or via food, it is very important to conduct studies that reflect the real-time settings. To further explore the effects of pyrethroids on the functioning of various organ systems (hepatic, renal, and male reproductive) at a biochemical level, in this study, we treated rats with a mixture of pyrethroids (that are found to be present in rice and vegetables available at Indian markets) at doses that are equivalent to residual levels of these present in the quantity of rice and vegetables consumed by an average Indian. Thus, this study reflects the real-time settings of human exposure. To the best of our knowledge, this is the first study that describes the toxic effects of pyrethroids at doses that is equivalent to the amount consumed by humans on a routine basis.

5.2. RESULTS

PART-I: Effect of a mixture of pyrethroids on the liver, kidney, and lung of rats the male reproductive function when exposed to doses relevant to human consumption (Experimental protocol –I).

5.2.1. Body and relative organ weights

No significant changes in the weights of liver, kidney, and lung were observed at all the time points analyzed in rats treated with pyrethroids for 9 to 12 months. In the 15 months treated animals, a significant decrease in the body weight of animals was evident in the HD group compared to the control (Table 1). Though there was also a decrease in the body weight of rats in the LD group, it was not significantly different from the control group. Except for the kidney, an increase in the relative organ weight was observed for all the other tissues in the HD group. Similarly, increased relative organ weight of liver and lung were observed in the LD group. Though an increase in relative organ weight was also observed for other tissues in the LD group, statistical significance was not evident (Table 29).

5.2.2. Lipid peroxidation and antioxidant status

In general, lipid peroxidation (LPO) levels were not altered in all the tissues analyzed at all the time points. (Table 30). Nitric oxide levels were not altered in all the tissues obtained from rats treated with low or high dose pyrethroids for 9 months. However, a significant increase in the levels of nitric oxide was observed in the liver and kidney obtained from rats treated with HD and LD for 12 months (Table 31). In rats treated for 15 months, the levels of lipid peroxides and nitric oxide, and activities of catalase, GPx, and GST were analyzed. Levels of lipid peroxidation products and NO remained unchanged in all the tissues obtained from both the treatment groups (Table 31).

5.2.3. Antioxidant enzymes

Catalase activity was not altered in all the tissues obtained from rats treated with LD or HD pyrethroids for 9 months. In rats exposed to pyrethroids for 15 months, catalase activity was significantly increased in the kidney of HD group (Table 32). Glutathione peroxidase activity was significantly increased in the liver, kidney, and lungs of rats that

received LD or HD pyrethroid treatment for 9 or 12 months. In rats exposed to pyrethroids for 15 months, GPx activity was observed in the lungs of both treatment groups, and kidneys obtained from HD group (**Table 33**). GST activity was significantly increased in the liver of these animals and the increase was evident at both the dosages. No significant changes in GST activity were observed in all the tissues of rats that received LD or HD pyrethroid treatment for 12 months. In the liver obtained from both LD and HD groups treated for 15 months, GST activity was significantly increased compared to the control (**Table 34**).

Table 29. Body and elative organ weights in the pyrethroid treated rats.

Name of the tissue	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Liver	2.86 ± 0.28	3.51 ± 0.42	3.25 ± 0.52	2.72 ± 0.07	2.54 ± 0.15	2.84 ± 0.13	2.201 ± 0.564	2.985 ± 0.180*	3.270 ± 0.332*
Lung	0.71 ± 0.07	0.083 ± 0.06	0.69 ± 0.08	0.64 ± 0.08	0.72 ± 0.04	0.74 ± 0.09	0.568 ± 0.031	0.759 ± 0.089*	0.980 ± 0.544*
Kidney	0.65 ± 0.08	0.67 ± 0.02	0.58 ± 0.11	0.31 ± 0.03	0.33 ± 0.02	0.33 ± 0.03	0.310 ± 0.026	0.248 ± 0.66	0.353 ± 0.069
Body weight	381 ± 7.63	386 ± 5.77	401 ± 17.55	416 ± 12.5	408 ± 11.15	428 ± 16.07	461.66 ± 36.17	417.66 ± 38.55	378.6 ± 27.02*

Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

Table 30. Lipid peroxidation# in various tissues of rats treated with a mixture of pyrethroids.

Name of the tissue	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Liver	0.071 ± 0.013	0.057 ± 0.020	0.057 ± 0.020	0.107 ± 0.015	0.262 ± 0.038	0.116 ± 0.006	0.160 ± 0.050	0.134 ± 0.027	0.131 ± 0.021
Kidney	0.054 ± 0.011	0.058 ± 0.022	0.051 ± 0.013	0.226 ± 0.006	0.262 ± 0.038	0.279 ± 0.043	0.272 ± 0.022	0.252 ± 0.039	0.234 ± 0.031
Lung	0.029 ± 0.001	0.018 ± 0.002	0.061 ± 0.011	0.061 ± 0.011	0.055 ± 0.009	0.071 ± 0.017	0.050 ± 0.007	0.040 ± 0.010	0.066 ± 0.012

nano moles MDA/ mg protein. Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

Table 31. Nitric oxide levels# in various tissues of rats treated with a mixture of pyrethroids.

Name of the tissue	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Liver	0.637 ± 0.050	0.793 ± 0.150	0.873 ± 0.090	0.254 ± 0.039	0.663 ± 0.106*	0.453 ± 0.037*	0.568 ± 0.119	0.791 ± 0.084	0.61 ± 0.049
Kidney	0.492 ± 0.090	0.628 ± 0.070	0.665 ± 0.040	0.715 ± 0.115	0.849 ± 0.001	0.725 ± 0.106	0.110 ± 0.069	1.08 ± 0.081	0.984 ± 0.001
Lung	0.504 ± 0.100	0.475 ± 0.090	0.667 ± 0.270	0.536 ± 0.004	0.588 ± 0.053	0.562 ± 0.077	0.692 ± 0.117	0.766 ± 0.073	0.709 ± 0.002

#µmoles nitrite/mg protein. Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

Table 32. Catalase activity (K30#) in various tissues of rats with a mixture of pyrethroids.

Name of the tissue	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Liver	0.239 ± 0.157	0.252 ± 0.104	0.203 ± 0.065	0.345 ± 0.114	0.498 ± 0.057	0.421 ± 0.158	0.251 ± 0.033	0.380 ± 0.089*	0.421 ± 0.039*
Kidney	0.369 ± 0.001	0.396 ± 0.070	0.437 ± 0.010	0.239 ± 0.039	0.249 ± 0.013	0.316 ± 0.114	0.358 ± 0.048	0.285 ± 0.061	0.568 ± 0.004 *
Lung	0.060 ± 0.042	0.068 ± 0.056	0.100 ± 0.008	0.135 ± 0.011	0.149 ± 0.070	0.178 ± 0.028	0.194 ± 0.068	0.210 ± 0.023	0.172 ± 0.027

#K30 = $(2.303/30) \times \log (A1/A2)$ where A1 and A2 are the initial and final absorbance respectively of hydrogen peroxide for enzyme assay. Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

Table 33. Glutathione peroxidase activity# in various tissues of rats with a mixture of pyrethroids.

Name of the tissue	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Liver	3.050 ± 0.100	6.150 ± 0.100	4.820 ± 0.100	1.690 ± 0.131	2.570 ± 0.434*	2.560 ± 0.351*	1.453 ± 0.835	1.586 ± 0.392	1.808 ± 0.112
Kidney	1.409 ± 0.107	1.040 ± 0.100	6.520 ± 0.100	1.476 ± 0.134	2.462 ± 0.547*	2.301 ± 0.559*	2.448 ± 0.435	2.494 ± 0.314	3.798 ± 0.097*
Lung	2.820 ± 0.100	3.960 ± 0.100	3.890 ± 0.100	1.580 ± 0.246	5.000 ± 1.288*	2.970 ± 0.179*	2.401 ± 0.520	3.754 ± 0.530*	3.553 ± 0.798*

#μmoles NADPH oxidized/ min/ mg of protein. Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

Table 34. Glutathione S- transferase activity# in various tissues of rats with a mixture of pyrethroids.

Name of the tissue	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Liver	0.056 ± 0.014	0.100 ± 0.016*	0.100 ± 0.004*	0.088 ± 0.004	0.103 ± 0.010	0.080 ± 0.005	0.056 ± 0.010	0.121 ± 0.027*	0.120 ± 0.021*
Kidney	0.026 ± 0.009	0.033 ± 0.007	0.031 ± 0.008	0.016 ± 0.004	0.017 ± 0.001	0.019 ± 0.006	0.028 ± 0.004	0.024 ± 0.002	0.033 ± 0.018
Lung	0.023 ± 0.004	0.031 ± 0.017	0.026 ± 0.002	0.026 ± 0.006	0.029 ± 0.004	0.018 ± 0.005	0.018 ± 0.012	0.027 ± 0.002	0.028 ± 0.006

#μmoles of CDNB-GSH conjugate formed / min/ mg protein. Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

5.2.4. Serum lipid profile, liver, and kidney function

In the serum obtained from rats treated with pyrethroids levels of SGPT, SGOT and ALP were estimated as a function of liver performance. Significant increase in the levels of SGPT, SGOT, and ALP was observed in the serum of rats that received LD or HD pyrethroid treatment for 9 or 12 months (**Table 35**), with no changes in the total protein levels. When treated for 15 months, the activities of SGPT, SGOT, ALP, and concentration of bilirubin were significantly increased in both the treatment groups compared to control. No change in the total protein concentration was evident (**Table 35**). Serum creatinine and urea levels, measured as a function of kidney performance were not altered in both the treatment groups at both the time points (**Table 36**). In rats treated for 15 months, only the levels of BUN were found to be decreased in rats of HD and LD groups (**Table 36**), whereas all the other parameters tested were not altered (**Table 36**). The levels of total cholesterol, triglycerides, HDL, and VLDL were not altered in all the treatment groups, suggesting no changes in the lipid profile (**Table 37**). When treated for 15 months, concentrations of triglycerides, and VLDL were significantly increased in the serum collected from both LD and HD treated rats (**Table 37**). No change in the levels of cholesterol and HDL was observed (**Table 37**).

Table 35. Liver function in rats treated with a mixture of pyrethroids.

Parameter name	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
SGPT/ALT (Units/litre serum)	65 ± 8.48	94.5 ± 3.53*	95.5 ± 14.84*	65.5 ± 10.60	79.0 ± 5.19*	88 ± 14.14*	105 ± 16	217 ± 3.5*	244 ± 7.0*
SGOT/ALT (Units/litre serum)	139 ± 19.799	170.5 ± 6.364*	191 ± 22.627*	158.5 ± 6.36	179 ± 9.00*	180 ± 10.00*	196 ± 1.7	525 ± 6.4*	539 ± 7.0*
ALP (Units/litre serum)	141.5 ± 3.53	208.5 ± 30.40*	322 ± 87.68*	358 ± 11.00	670.66 ± 107.93*	813 ± 285.67*	240 ± 27	307 ± 8.5*	306 ± 19*
Total protein (g/dl)	6.8 ± 0.100	7.267 ± 0.551	7.3 ± 0.200	7.867 ± 0.20	7.233 ± 0.153	7.367 ± 0.416	7.65 ± 0.68	7.08 ± 0.87	7.18 ± 1.01

Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

Table 36. Kidney function in rats treated with a mixture of pyrethroids.

Parameter name	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Creatinine (mg/dl)	0.467 ± 0.058	0.500 ± 0.100	0.500 ± 0.10	0.70 ± 0.012	0.733 ± 0.053	0.733 ± 0.058	0.715 ± 0.07	0.733 ± 0.02	0.735 ± 0.021
Urea (mg/dl)	30.50 ± 4.590	38.000 ± 1.732	36.00 ± 2.82	37.33 ± 7.76	34.333 ± 6.807	36.33 ± 4.93	44 ± 3.46	36.33 ± 2.08	39 ± 7.11
Uric acid (mg/dl)	4.63 ± 1.35	6.26 ± 4.70	4.1 ± 0.9	5 ± 1.734	6.56 ± 4.801	4.63 ± 1.209	5.83 ± 1.63	6.90 ± 1.20	5.68 ± 0.44

Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

Table 37. Lipid profile in rats treated with a mixture of pyrethroids.

Parameter name	9 months			12 months			15 months		
	Control	LD	HD	Control	LD	HD	Control	LD	HD
Total Cholesterol (mg/dl)	51.66 ± 5.50	56.33 ± 3.05	51.50 ± 3.53	83.0 ± 9.53	74.33 ± 10.017	72.33 ± 21.00	59 ± 1.52	58 ± 1.15	60 ± 1.15
Triglycerides (mg/dl)	92.66 ± 16.28	81.66 ± 16.2	105.00 ± 11.31	82.66 ± 13.05	85.33 ± 24.78	59.66 ± 17.61	57.66 ± 3.46	79.5 ± 6.36*	88.35 ± 2.3*
HDL (mg/dl)	54.333 ± 3.21	56.33 ± 3.05	49.50 ± 2.12	30.00 ± 3.0	26.66 ± 4.16	27.00 ± 7.81	31.12 ± 8.44	29.14 ± 3.49	28.57 ± 7.97
VDL (mg/dl)	20.50 ± 0.70	18.00 ± 1.41	25.00 ± 2.82	16.6 ± 2.51	17.00 ± 5.29	12.00 ± 3.46	11.53 ± 0.68	17.67 ± 2.60*	17.93 ± 0.75*

*Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.*

5.2.5. Histopathology

Histopathological analyses of livers obtained from rats treated for 9 months showed foci of infiltration of inflammatory cells (mononuclear) in the centrilobular region was observed in the liver of rats that received LD or HD pyrethroid treatment. In the lungs of LD and HD treated rats, severe peri-bronchiolar lymphoid tissue hyperplasia and peri-bronchiolar inflammation, alveolar inflammation and fibrosis, and alveolar hemorrhage was noticed (**Figure 25**).

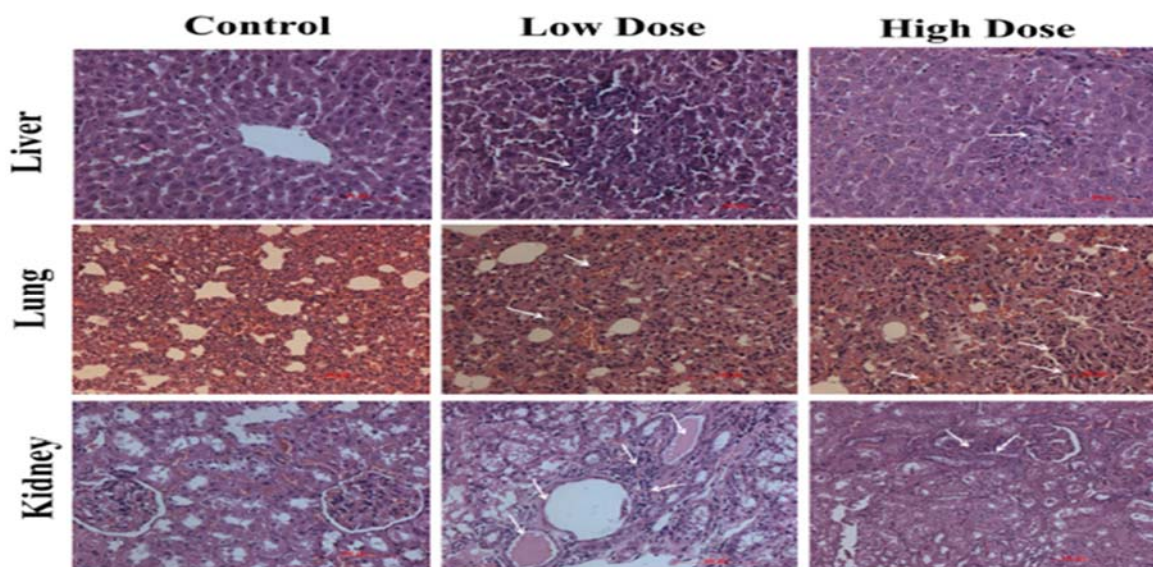


FIGURE 25. Histopathology of liver, kidney, and lung in rats treated with pyrethroid mixture. Tissue sections obtained from rats that were treated with a mixture of pyrethroids for 9 months were stained with hematoxylin and eosin. Arrows indicate the histopathological change observed in the LD and HD treated groups Scale bar: 100 μ m.

Kidneys obtained from LD and HD treated rats showed moderate cystic degeneration of tubules, tubular inflammation, mild foci of infiltration of inflammatory cells in the interstitial region between the tubules was observed (**Figure 25**). Histopathological analyses of livers obtained from rats treated for 12 months showed moderate peri-portal and peri-biliary inflammation with infiltration of inflammatory cells and mild foci of necrosis in hepatocytes of centrilobular region of liver was observed in LD and HD pyrethroid treated rats. Peri-bronchiolar lymphoid tissue hyperplasia, moderate inflammation of alveolar region of lungs with alveolar fibrosis, and hemorrhages was observed in both the treatment groups. In the kidney obtained from LD and HD treated rats, moderate tubular inflammation along with infiltration of inflammatory cells and hemorrhage were observed are noticed (**Figure 26**).

In the kidney obtained from LD and HD treated rats, moderate tubular inflammation along with infiltration of inflammatory cells and hemorrhage were observed are noticed (**Figure 26**).

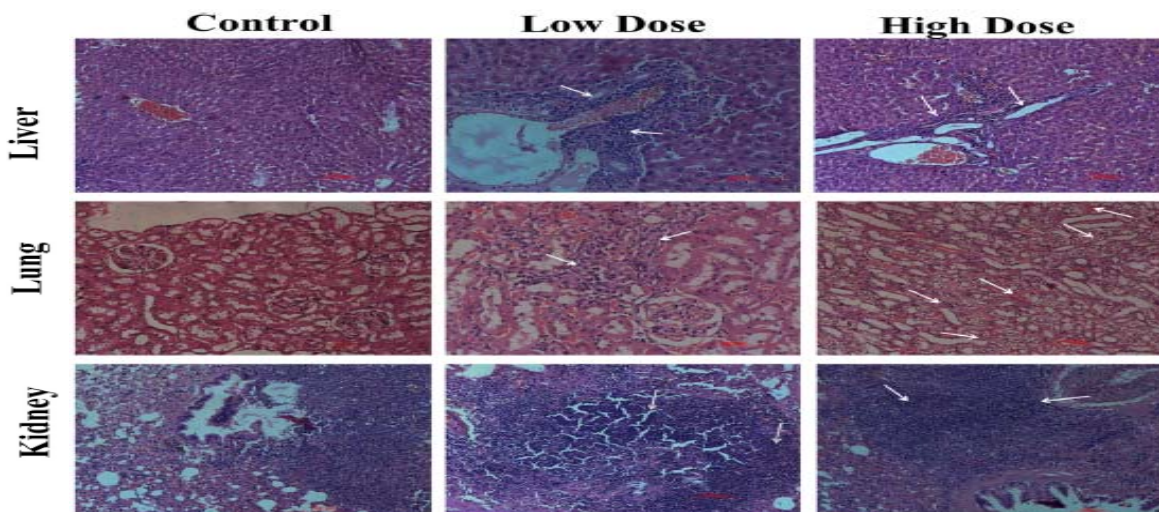


FIGURE 26. Histopathology of liver, kidney, and lung in rats treated with pyrethroid mixture. Tissue sections obtained from rats that were treated with a mixture of pyrethroids for 12 months were stained with hematoxylin and eosin. Arrows indicate the histopathological change observed in the LD and HD treated groups Scale bar: 100 μ m.

Histopathological analyses of livers obtained from rats treated for 15 months showed periportal fibrosis, mild peri biliary infiltration of inflammatory cells and centrilobular necrosis was evident in the liver of HD and LD treated rats (**Figure 27**).

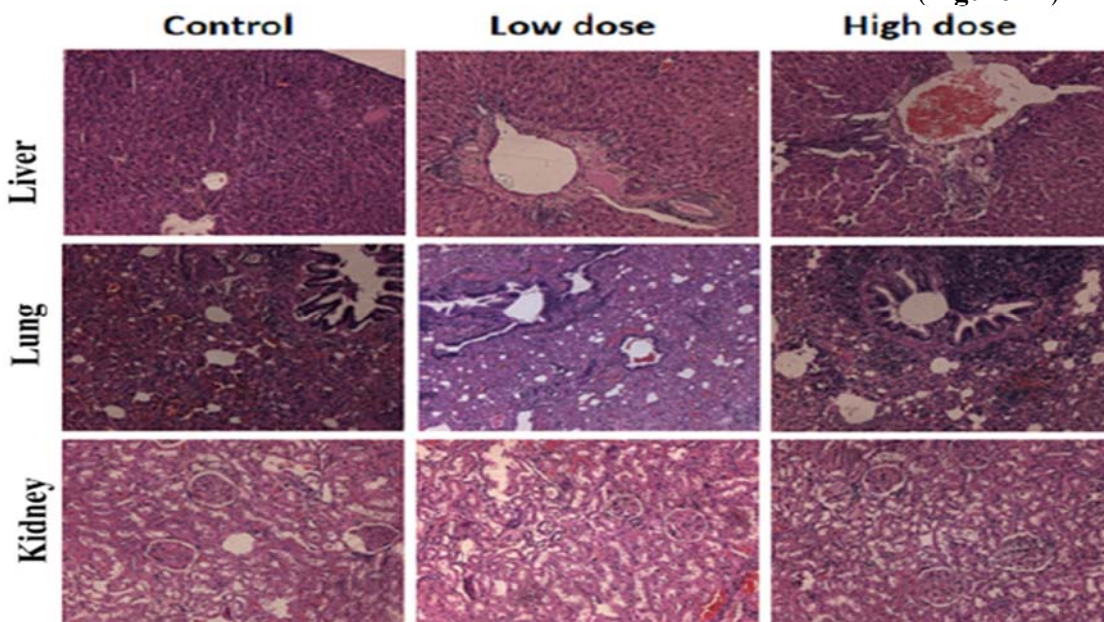


FIGURE 27. Histopathology of liver, kidney, and lung in rats treated with pyrethroid mixture. Liver, kidney, lung of rats administered with a mixture of pyrethroids for 15 months was stained with hematoxylin and eosin. Arrows indicate the anatomical damages. All images are with a scale bar of 100 μ m.

Moderate tubular hemorrhages were evident in the kidneys of both LD and HD treated rats. In the lungs of HD and LD treated rats, peribronchiolar lymphoid hyperplasia moderate alveolar were observed (**Figure 27**).

PART- II: Effect of a mixture of pyrethroids on the liver, kidney, and lung of rats exposed to doses equivalent to human consumption (Experimental protocol –II).

5.2.6. Body and relative organ weights

The final body weights of control and pyrethroid-treated rats were noted at the end of each treatment period. No significant change in body weights between the treated and control rats was observed at any of the time points analyzed. Similarly, no differences in the relative organ weights were observed for all the tissues at all the time points analyzed (**Table 38**).

5.2.7. Lipid peroxidation and nitric oxide

While a significant increase in the levels of lipid peroxidation products was observed in the kidneys of pyrethroid treated rats obtained at all the time points, such an effect in the liver and lung was observed in rats treated for 3 to 15 months (**Table 39**).

Nitric oxide levels in general were significantly increased in all the tissues obtained from rats treated with pyrethroids. In the case of lung, liver, and kidney the significant increase was evident in rats treated for 6 to 15 months (**Table 40**).

5.2.8. Antioxidant enzyme activities

Catalase activity was in general was significantly increased in all the tissues analyzed with variations in the duration of treatment (**Table 41**). While its activity was increased in the kidney at all the time points analyzed, such a change in the liver and lung was observed only in rats treated for 3 to 15 months. GPx activity was significantly increased in the liver, lung, and kidney of rats obtained from all the treatment points (**Table 42**). GST activity was not altered in any of the tissues obtained from all the time points (**Table 43**). In the kidney, increased SOD levels were observed in rats treated for 12 to 15 months (**Table 44**).

Table 38. Body and relative organ weights in rats treated with a mixture of pyrethroids for 1 to 15 months.

Name of the tissue		1 month	3 months	6 months	9 months	12 months	15 months
Liver	Control	0.03308 ± 0.004	0.03636 ± 0.008	0.02866 ± 0.003	0.02895 ± 0.002	0.02813 ± 0.002	0.03239 ± 0.005
	Treated	0.03532 ± 0.007	0.0361 ± 0.004	0.03087 ± 0.004	0.02592 ± 0.006	0.03066 ± 0.002	0.03129 ± 0.002
Lung	Control	0.00836 ± 0.001	0.00748 ± 0.001	0.00648 ± 0.002	0.00676 ± 0.000	0.00658 ± 0.003	0.00973 ± 0.002
	Treated	0.00821 ± 0.001	0.00721 ± 0.001	0.00801 ± 0.001	0.00818 ± 0.002	0.00686 ± 0.001	0.00801 ± 0.001
Kidney	Control	0.00397 ± 0.001	0.00383 ± 0.003	0.00320 ± 0.001	0.00324 ± 0.005	0.00340 ± 0.001	0.00416 ± 0.001
	Treated	0.00411 ± 0.001	0.00377 ± 0.001	0.00326 ± 0.001	0.00517 ± 0.004	0.0032 ± 0.0001	0.00334 ± 0.001
Body weight (grams)	Control	242.66 ± 33.73	282.60 ± 39.06	418.83 ± 18.77	414.83 ± 50.38	442.83 ± 50.04	352.00 ± 25.55
	Treated	260.83 ± 42.35	313.80 ± 29.78	342.27 ± 27.06	400.33 ± 50.03	438.00 ± 54.18	372.14 ± 48.52

Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

Table 39. Lipid peroxidation# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

Name of the tissue		1 month	3 months	6 months	9 months	12 months	15 months
Liver	Control	0.054 ± 0.014	0.032 ± 0.001	0.019 ± 0.006	0.015 ± 0.001	0.017 ± 0.004	0.034 ± 0.009
	Treated	0.050 ± 0.025	0.041 ± 0.010*	0.037 ± 0.006*	0.033 ± 0.004*	0.060 ± 0.007*	0.128 ± 0.019*
Lung	Control	0.015 ± 0.001	0.014 ± 0.004	0.012 ± 0.004	0.009 ± 0.003	0.032 ± 0.011	0.019 ± 0.009
	Treated	0.014 ± 0.004	0.038 ± 0.008*	0.028 ± 0.010*	0.019 ± 0.001*	0.341 ± 0.012*	0.197 ± 0.120
Kidney	Control	0.123 ± 0.001	0.076 ± 0.001	0.058 ± 0.001	0.050 ± 0.001	0.117 ± 0.010	0.060 ± 0.014
	Treated	0.151 ± 0.020*	0.081 ± 0.001*	0.070 ± 0.004*	0.071 ± 0.002*	0.291 ± 0.088*	0.180 ± 0.022*

nano moles MDA/ mg protein. Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

Table 40. Nitric oxide levels# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

Name of the tissue		1 month	3 months	6 months	9 months	12 months	15 months
Liver	Control	0.529 ± 0.063	0.820 ± 0.224	0.426 ± 0.033	0.505 ± 0.101	0.673 ± 0.116	0.375 ± 0.012
	Treated	0.573 ± 0.088	1.416 ± 0.477	0.552 ± 0.012*	0.792 ± 0.111*	0.987 ± 0.114*	0.723 ± 0.114*
Lung	Control	0.759 ± 0.113	1.101 ± 0.024	0.253 ± 0.027	0.329 ± 0.084	0.404 ± 0.100	0.435 ± 0.112
	Treated	0.872 ± 0.050	0.990 ± 0.181	0.584 ± 0.035*	0.764 ± 0.065*	0.979 ± 0.088*	0.661 ± 0.094*
Kidney	Control	0.885 ± 0.081	1.386 ± 0.176	0.683 ± 0.043	0.591 ± 0.036	0.590 ± 0.043	0.541 ± 0.033
	Treated	0.734 ± 0.152	1.478 ± 0.218	0.831 ± 0.012*	0.712 ± 0.052*	0.844 ± 0.056*	0.618 ± 0.031*

#μmoles nitrite/mg protein. Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

Table 41. Catalase activity (K30#) in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

		1 month	3 months	6 months	9 months	12 months	15 months
Liver	Control	0.099 ± 0.004	0.075 ± 0.018	0.084 ± 0.011	0.090 ± 0.015	0.132 ± 0.051	0.119 ± 0.004
	Treated	0.139 ± 0.015	0.138 ± 0.018*	0.156 ± 0.018*	0.161 ± 0.018*	0.329 ± 0.085*	0.173 ± 0.015
Lung	Control	0.047 ± 0.011	0.046 ± 0.003	0.093 ± 0.007	0.072 ± 0.011	0.041 ± 0.013	0.058 ± 0.005
	Treated	0.045 ± 0.006	0.081 ± 0.016*	0.187 ± 0.006*	0.118 ± 0.020*	0.105 ± 0.015*	0.094 ± 0.015
Kidney	Control	0.078 ± 0.042	0.090 ± 0.007	0.155 ± 0.046	0.117 ± 0.014	0.106 ± 0.034	0.069 ± 0.025
	Treated	0.191 ± 0.010*	0.148 ± 0.023*	0.220 ± 0.011*	0.183 ± 0.008*	0.220 ± 0.031*	0.121 ± 0.008*

$K30 = (2.303/30) \times \log (A1/A2)$ where $A1$ and $A2$ are the initial and final absorbance respectively of hydrogen peroxide for enzyme assay. Values shown are Mean ± S.D. *indicates $p < 0.05$ compared to respective control.

Table 42. Glutathione peroxidase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

		1 month	3 months	6 months	9 months	12 months	15 months
Liver	Control	11.90 ± 1.41	11.68 ± 1.06	13.71 ± 4.08	5.62 ± 0.17	10.40 ± 2.20	16.23 ± 1.37
	Treated	20.58 ± 1.24*	16.62 ± 0.29*	18.97 ± 0.83*	11.68 ± 1.72*	27.70 ± 6.48*	24.00 ± 0.95*
Lung	Control	27.95 ± 1.78	43.74 ± 1.58	39.80 ± 4.12	30.34 ± 9.62	8.85 ± 3.30	38.71 ± 2.42
	Treated	46.34 ± 2.60*	69.32 ± 10.29*	95.41 ± 6.32*	48.42 ± 8.14*	22.45 ± 4.83*	52.58 ± 6.03*
Kidney	Control	12.21 ± 3.05	14.04 ± 0.58	18.38 ± 3.45	9.99 ± 3.85	7.11 ± 4.79	16.56 ± 1.09
	Treated	17.11 ± 0.64*	20.91 ± 1.12*	33.70 ± 0.42*	14.85 ± 0.24*	16.08 ± 4.18*	24.66 ± 2.35*

μ moles NADPH oxidized/ min/ mg of protein. Values shown are Mean $\pm$ S.D. *indicates $p < 0.05$ compared to respective control.

Table 43. Glutathione S- transferase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

		1 month	3 months	6 months	9 months	12 months	15 months
Liver	Control	0.034 ± 0.013	0.065 ± 0.014	0.037 ± 0.019	0.056 ± 0.032	0.038 ± 0.013	0.052 ± 0.009
	Treated	0.050 ± 0.018	0.079 ± 0.020	0.054 ± 0.024	0.055 ± 0.021	0.253 ± 0.134	0.044 ± 0.009
Lung	Control	0.011 ± 0.001	0.028 ± 0.008	0.031 ± 0.023	0.014 ± 0.006	0.005 ± 0.001	0.017 ± 0.005
	Treated	0.015 ± 0.011	0.027 ± 0.003	0.015 ± 0.006	0.021 ± 0.011	0.023 ± 0.015	0.019 ± 0.010
Kidney	Control	0.006 ± 0.002	0.015 ± 0.003	0.015 ± 0.004	0.011 ± 0.002	0.008 ± 0.003	0.014 ± 0.006
	Treated	0.008 ± 0.004	0.021 ± 0.002	0.015 ± 0.008	0.008 ± 0.004	0.022 ± 0.020	0.011 ± 0.004

μ moles of CDNB-GSH conjugate formed / min /mg protein. Values shown are Mean $\pm$ S.D. *indicates $p < 0.05$ compared to respective control.

Table 44. Superoxide dismutase activity# in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

		1 month	3 months	6 months	9 months	12 months	15 months
Liver	Control	101.56 ± 21.48	103.55 ± 7.54	52.72 ± 27.60	84.56 ± 7.74	145.50 ± 62.29	58.30 ± 7.21
	Treated	138.50 ± 42.06	120.17 ± 6.00*	101.23 ± 5.92*	135.76 ± 26.49*	277.47 ± 46.41*	97.39 ± 3.87*
Lung	Control	240.28 ± 23.54	242.68 ± 18.22	246.92 ± 23.38	184.75 ± 52.27	228.87 ± 53.02	230.69 ± 25.10
	Treated	183.80 ± 23.76	279.23 ± 22.94	204.06 ± 22.40	186.02 ± 32.99	174.62 ± 13.81	244.59 ± 106.12
Kidney	Control	162.13 ± 84.44	197.34 ± 88.50	128.15 ± 35.40	131.74 ± 35.10	154.14 ± 5.16	137.99 ± 44.88
	Treated	192.09 ± 62.32	186.91 ± 26.66	139.48 ± 49.64	122.62 ± 21.32	266.75 ± 28.74*	226.61 ± 0.68*

- activity / mg protein; *indicates $p < 0.05$ compared to the respective control.

5.2.9. Liver and Kidney function

In the liver, total bilirubin and unconjugated bilirubin levels were significantly reduced at all the time points analyzed (**Table 45**), whereas such a reduction for conjugated bilirubin was observed only in rats treated for 9 to 15 months. The activities of ALP, AST, and LDH were significantly reduced in rats treated for 1 to 3 months, whereas the same was significantly increased in rats treated for 6 to 15 months. Similarly, ALT activity was decreased in the liver of rats obtained from 1 to 6 month treatment, while an increase in its activity was observed in rats treated for 9 months and beyond (**Table 17**). None of the parameters analyzed to assess kidney function test was altered at all the time points tested (**Table 46**).

5.2.10. Lipid profile and complete blood picture

The levels of total cholesterol, triglycerides, and VLDL were decreased in the initial treatment time points but were increased in the later time points (**Table 47**). A significant decrease in total cholesterol levels was evident in rats treated with pyrethroids for 6 and 9 months, while 12 and 15 month treatment resulted in a significant increase. In the case of triglycerides, the decrease was observed in rats obtained from 1 to 9 month treatment groups, whereas the increase was observed in 12 and 15 month treatment groups. In the rats treated for 1 to 6 months, VLDL levels were significantly decreased, though this trend was reversed in rats treated for 9 to 15 months (**Table 47**). Of all the parameters analyzed for complete blood picture, only the platelet count was found to be significantly increased in rats treated with pyrethroids for 3 to 15 months (**Table 48**).

Table 45. Liver function in rats treated with a mixture of pyrethroids for 1 to 15 months.

Parameter	Units	Group	1 month	3 months	6 months	9 months	12 months	15 months
Total Bilirubin	(mg/dL)	Control	0.055 ± 0.007	0.050 ± 0.001	0.070 ± 0.017	0.150 ± 0.071	0.200 ± 0.001	0.15 ± 0.071
		Treated	0.037 ± 0.006*	0.060 ± 0.010*	0.043 ± 0.015*	0.047 ± 0.006*	0.020 ± 0.001*	0.02 ± 0.001*
Conjugated Bilurubin	(mg/dL)	Control	0.013 ± 0.006	0.035 ± 0.007	0.035 ± 0.007	0.105 ± 0.007	0.100 ± 0.001	0.10 ± 0.000
		Treated	0.013 ± 0.006	0.030 ± 0.017	0.030 ± 0.010	0.027 ± 0.015*	0.010 ± 0.001*	0.01 ± 0.004*
Unconjugated Bilurubin	(mg/dL)	Control	0.040 ± 0.001	0.040 ± 0.014	0.060 ± 0.014	0.050 ± 0.000	0.100 ± 0.001	0.08 ± 0.014
		Treated	0.023 ± 0.006*	0.013 ± 0.006*	0.013 ± 0.006*	0.020 ± 0.017*	0.010 ± 0.001*	0.00 ± 0.003*
Total protein	(g/dL)	Control	7.900 ± 0.173	7.433 ± 0.321	8.000 ± 0.200	8.167 ± 0.404	8.100 ± 0.529	7.66 ± 1.007
		Treated	7.233 ± 0.416	7.467 ± 0.493	7.433 ± 0.153	7.800 ± 0.755	7.600 ± 0.458	7.33 ± 0.404
Albumin	(g/dL)	Control	4.333 ± 0.208	4.000 ± 0.100	3.867 ± 0.058	4.033 ± 0.404	3.967 ± 0.115	3.36 ± 0.451
		Treated	4.033 ± 0.306	3.633 ± 0.115	3.900 ± 0.265	3.933 ± 0.115	3.967 ± 0.462	3.23 ± 0.231
Globulin	(g/dL)	Control	3.567 ± 0.306	3.433 ± 0.404	4.133 ± 0.153	4.133 ± 0.153	4.133 ± 0.503	4.30 ± 0.557
		Treated	3.200 ± 0.200	3.833 ± 0.379	3.833 ± 0.115	3.867 ± 0.751	3.633 ± 0.153	4.10 ± 0.624
A/G Ratio	--	Control	1.243 ± 0.125	1.200 ± 0.173	0.967 ± 0.058	1.000 ± 0.100	1.050 ± 0.071	0.80 ± 0.000
		Treated	1.287 ± 0.103	0.933 ± 0.058	1.100 ± 0.100	1.067 ± 0.208	1.120 ± 0.159	0.80 ± 0.173
ALT/SGPT	(U/L)	Control	107.333 ± 9.609	83.000 ± 1.414	81.000 ± 5.568	78.333 ± 6.429	62.500 ± 3.536	73.66 ± 11.930
		Treated	59.000 ± 2.000*	65.000 ± 1.732*	59.333 ± 3.214*	96.000 ± 4.243*	93.500 ± 4.950*	116.5 ± 24.74*
AST/SGOT	(U/L)	Control	459.850 ± 36.55	242.000 ± 9.899	139.66 ± 22.811	134.50 ± 19.092	107.950 ± 8.556	145.50 ± 23.33
		Treated	176.00 ± 38.03*	137.33 ± 11.93*	190.00 ± 33.46*	180.50 ± 10.30*	195.96 ± 11.51*	258.0 ± 77.35*
ALP	(U/L)	Control	483.500 ± 26.16	454.00 ± 106.06	257.000 ± 6.557	216.50 ± 16.263	221.50 ± 6.8589	325.66 ± 41.63
		Treated	201.00 ± 12.72*	324.000 ± 5.65*	463.50 ± 74.24*	299.00 ± 29.69*	249.00 ± 2.828*	444.33 ± 9.27*
LDH	(U/L)	Control	2878.0 ± 231.93	1049.00 ± 346.4	454.66 ± 23.921	308.00 ± 21.213	352.00 ± 77.350	479.50 ± 72.83
		Treated	531.5 ± 152.02*	446.00 ± 33.94*	543.66 ± 36.92*	441.00 ± 26.47*	528.66 ± 30.82*	714.0 ± 98.39*

*indicates $p < 0.05$ compared to respective control.

Table 46. Kidney function in rats treated with a mixture of pyrethroids for 1 to 15 months

Parameter	Units	Group	1 month	3 months	6 months	9 months	12 months	15 months
Blood Urea Nitrogen	(mg/dL)	Control	19.333 ± 2.082	15.333 ± 1.155	15.000 ± 1.000	16.667 ± 1.528	16.000 ± 1.000	18.66 ± 2.51
		Treated	20.667 ± 1.528	14.000 ± 2.000	17.333 ± 4.163	16.333 ± 0.577	17.000 ± 0.000	17.66 ± 1.52
Uric Acid	(mg/dL)	Control	6.800 ± 0.283	5.700 ± 0.283	5.367 ± 1.582	5.700 ± 0.265	5.833 ± 0.503	6.233 ± 2.61
		Treated	9.650 ± 2.475	6.700 ± 2.100	8.533 ± 1.007	6.300 ± 1.212	5.500 ± 1.273	4.467 ± 0.68
Creatinine	(mg/dL)	Control	0.553 ± 0.171	0.497 ± 0.067	0.550 ± 0.070	0.437 ± 0.021	0.503 ± 0.032	0.520 ± 0.07
		Treated	0.500 ± 0.040	0.530 ± 0.060	0.563 ± 0.025	0.430 ± 0.046	0.470 ± 0.028	0.487 ± 0.07
Sodium (Na)	mmol/L	Control	145.00 ± 0.000	144.66 ± 1.155	147.33 ± 0.577	143.66 ± 0.577	147.66 ± 1.155	146.0 ± 2.64
		Treated	143.33 ± 1.155	143.66 ± 1.155	145.00 ± 1.732	146.33 ± 2.517	153.00 ± 2.828	144.3 ± 3.05
Potassium (K)	mmol/L	Control	9.007 ± 0.436	8.470 ± 1.455	7.167 ± 0.729	7.650 ± 0.604	7.863 ± 0.525	8.257 ± 0.85
		Treated	8.560 ± 1.683	7.767 ± 1.396	9.043 ± 0.561	7.503 ± 0.359	6.765 ± 0.247	8.883 ± 1.24
Chloride (Cl)	mmol/L	Control	95.733 ± 1.617	96.667 ± 0.577	100.00 ± 1.000	97.333 ± 2.082	96.333 ± 2.082	102.6 ± 5.50
		Treated	98.267 ± 1.102	96.333 ± 0.577	99.300 ± 2.524	100.33 ± 2.082	102.40 ± 1.980	105.0 ± 0.00
Calcium	mg/L	Control	12.867 ± 3.252	13.267 ± 0.643	12.033 ± 0.252	12.700 ± 0.557	12.767 ± 0.404	11.60 ± 0.65
		Treated	11.520 ± 2.563	12.800 ± 0.346	12.533 ± 0.289	12.833 ± 0.252	12.215 ± 0.304	10.63 ± 0.41

*indicates $p < 0.05$ compared to respective control.

Table 47. Lipid profile in rats treated with a mixture of pyrethroids for 1 to 15 months.

Parameter	Units	Group	1 month	3 months	6 months	9 months	12 months	15 months
Total cholesterol	(mg/dL)	Control	57.500 ± 7.77	61.000 ± 6.245	68.000 ± 8.185	72.000 ± 4.243	56.667 ± 9.074	57.667 ± 10.116
		Treated	55.950 ± 7.14	63.667 ± 9.292	51.000 ± 7.211*	62.667 ± 3.512*	72.700 ± 6.120*	82.000 ± 2.000*
Triglycerides	(mg/dL)	Control	135.40 ± 20.5	217.500 ± 20.50	171.500 ± 7.07	72.000 ± 7.071	71.667 ± 5.508	76.500 ± 12.021
		Treated	75.70 ± 21.6*	80.500 ± 12.02*	118.66 ± 17.21*	113.00 ± 14.93*	102.50 ± 13.61*	120.50 ± 27.57*
VLDL	(mg/dL)	Control	27.000 ± 4.00	43.500 ± 3.536	34.000 ± 0.001	16.000 ± 3.606	14.000 ± 1.000	16.667 ± 2.517
		Treated	15.000 ± 4.2*	16.000 ± 2.828*	23.667 ± 3.215*	22.333 ± 3.215*	26.667 ± 2.887*	24.000 ± 5.657*
LDL	(mg/dL)	Control	11.933 ± 2.90	17.333 ± 2.082	17.667 ± 2.517	23.000 ± 3.000	16.000 ± 1.732	19.000 ± 2.000
		Treated	13.850 ± 0.21	21.333 ± 3.055	21.000 ± 1.414	19.667 ± 2.887	20.333 ± 2.082	13.000 ± 2.000
HDL	(mg/dL)	Control	36.767 ± 7.64	33.667 ± 4.619	34.667 ± 6.028	44.000 ± 2.828	25.00 ± 1.414	27.667 ± 7.572
		Treated	29.867 ± 7.15	31.333 ± 5.508	24.000 ± 5.568	35.667 ± 3.215	42.800 ± 16.974	23.667 ± 4.163
Cholesterol/HDL Ratio	--	Control	1.630 ± 0.234	1.813 ± 0.081	2.165 ± 0.615	1.693 ± 0.101	1.960 ± 0.225	2.200 ± 0.436
		Treated	1.670 ± 0.052	2.067 ± 0.115	2.187 ± 0.220	1.733 ± 0.058	1.790 ± 0.312	2.200 ± 0.361

*indicates $p < 0.05$ compared to respective control.

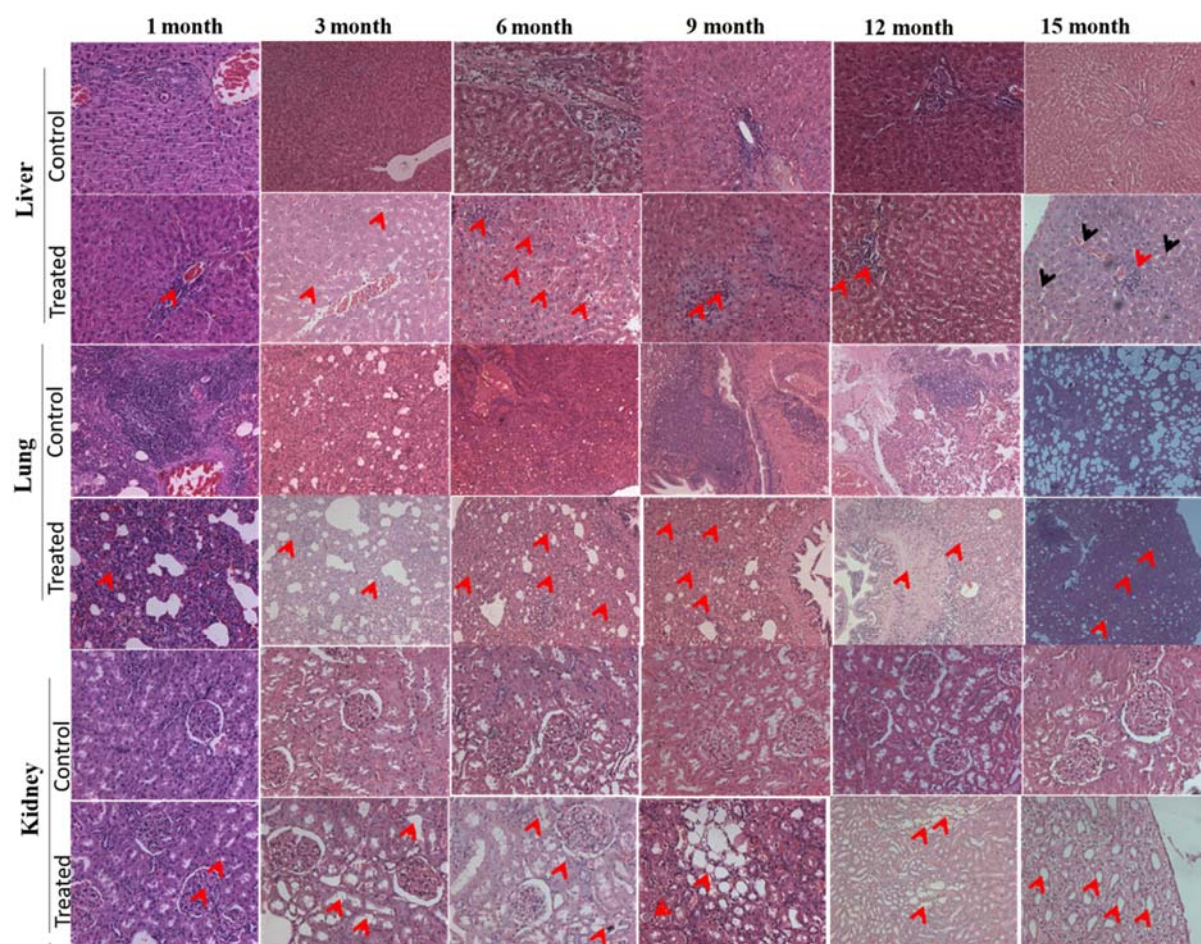
Table 48. Complete blood picture profile in rats treated with a mixture of pyrethroids for 1 to 15 months.

Parameter	Units	Group	1 month	3 months	6 months	9 months	12 months	15 months
Glucose	mg/dL	Control	105.33 ± 16.3	107.33 ± 8.64	99.33 ± 5.82	116.83 ± 9.62	109.00 ± 10.90	108.86 ± 9.30
		Treated	101.50 ± 11.9	109.67 ± 15.11	108.50 ± 9.83	112.33 ± 8.14	117.17 ± 8.01	113.86 ± 8.36
Glycated hemoglobin	%	Control	5.600 ± 0.283	6.033 ± 0.451	5.600 ± 0.400	7.533 ± 1.436	3.300 ± 0.361	2.400 ± 0.300
		Treated	7.000 ± 1.400	6.033 ± 0.208	5.367 ± 0.808	10.550 ± 0.071	3.567 ± 0.987	2.167 ± 0.351
Total leukocyte count	10 ³ /μl	Control	15.900 ± 4.38	15.600 ± 5.274	16.100 ± 2.546	12.833 ± 6.643	10.367 ± 2.631	18.067 ± 7.803
		Treated	20.100 ± 0.42	23.433 ± 8.563	17.700 ± 1.572	20.600 ± 2.987	13.733 ± 5.950	20.67 ± 2.274
RBC count	10 ⁶ /μl	Control	9.133 ± 0.709	9.000 ± 0.500	8.733 ± 1.021	8.100 ± 0.093	8.900 ± 0.600	7.950 ± 0.212
		Treated	8.467 ± 0.208	8.167 ± 0.404	8.600 ± 0.964	8.433 ± 0.115	8.533 ± 0.493	7.700 ± 0.424
Hemoglobin	g/dL	Control	15.833 ± 1.33	14.900 ± 0.529	14.700 ± 2.252	12.167 ± 2.593	14.433 ± 1.150	13.600 ± 1.273
		Treated	14.133 ± 0.30	13.733 ± 0.929	14.167 ± 1.415	13.667 ± 0.208	13.867 ± 1.002	13.050 ± 0.495
Hematocrit	%	Control	49.533 ± 3.95	46.800 ± 1.74	46.600 ± 5.919	42.500 ± 1.131	46.000 ± 4.104	39.550 ± 0.636
		Treated	45.667 ± 1.33	43.300 ± 2.816	45.133 ± 3.573	42.633 ± 0.586	44.700 ± 3.132	40.700 ± 1.697
Mean corpuscular volume	fL	Control	54.333 ± 0.83	51.933 ± 1.415	53.167 ± 0.702	51.433 ± 2.212	51.667 ± 1.115	48.933 ± 1.531
		Treated	54.033 ± 2.13	52.867 ± 1.060	52.533 ± 1.343	50.533 ± 0.473	52.367 ± 0.874	51.033 ± 3.338
Mean corpuscular hemoglobin	Pg	Control	17.333 ± 0.11	16.500 ± 0.436	16.700 ± 0.700	16.900 ± 0.566	16.200 ± 0.200	17.200 ± 2.121
		Treated	16.733 ± 0.58	16.733 ± 0.416	16.467 ± 0.289	16.233 ± 0.306	16.267 ± 0.321	16.950 ± 0.212
Mean corpuscular hemoglobin concentration	g/dL	Control	31.900 ± 0.60	31.833 ± 0.115	31.400 ± 0.900	30.700 ± 4.897	31.433 ± 0.321	33.333 ± 3.362
		Treated	30.967 ± 0.20	31.667 ± 0.208	31.367 ± 0.651	32.067 ± 0.321	31.033 ± 0.208	31.700 ± 0.520
Red cell distribution width	%	Control	15.100 ± 0.81	15.200 ± 0.656	14.267 ± 0.321	14.900 ± 0.849	15.700 ± 0.794	13.967 ± 1.629
		Treated	15.233 ± 0.37	14.467 ± 0.058	14.033 ± 0.153	15.200 ± 0.346	14.867 ± 0.757	14.133 ± 1.159
Platelet count	10 ³ /μl	Control	878.0 ± 129.7	951.66 ± 64.53	750.500 ± 24.749	808.500 ± 67.17	701.3 ± 100.72	718.5 ± 103.94
		Treated	858.5 ± 21.92	1254.0 ± 52.3*	1009.00 ± 25.45*	979.00 ± 14.12*	919.6 ± 99.00*	932.0 ± 21.21*
Mean platelet volume	fL	Control	7.833 ± 0.814	8.667 ± 0.115	7.467 ± 0.666	8.900 ± 0.624	8.500 ± 0.265	7.600 ± 1.300
		Treated	7.767 ± 1.021	7.967 ± 1.185	7.467 ± 0.702	8.733 ± 0.115	8.467 ± 0.208	7.933 ± 0.987
Lymphocytes	%	Control	93.000 ± 3.00	93.333 ± 4.726	67.500 ± 3.536	94.667 ± 1.528	82.667 ± 21.36	74.333 ± 18.03
		Treated	88.667 ± 1.52	94.000 ± 6.083	69.667 ± 4.933	92.333 ± 7.234	83.667 ± 10.01	84.000 ± 11.53
Eosinophils	%	Control	1.667 ± 1.155	2.667 ± 1.155	1.667 ± 0.577	4.000 ± 1.000	3.000 ± 1.000	5.500 ± 0.707
		Treated	1.000 ± 0.001	1.333 ± 0.577	3.667 ± 1.155	2.667 ± 1.155	4.000 ± 1.000	1.500 ± 0.707

*indicates $p < 0.05$ compared to respective control.

5.2.11. Histopathological analyses

Mild to moderate peri portal infiltration of inflammatory cells, necrosis of hepatocytes, sinusoidal haemorrhages and inflammation of hepatocytes was evident in the livers of rats subjected to 1-to-15-month pyrethroid treatment (**Figure 28**). In the lungs of pyrethroid-treated rats, severe alveolar / interstitial inflammation with infiltration of inflammatory cells and fibrosis, interstitial edema, haemorrhages and infiltration of inflammatory cells and lymphoid tissue hyperplasia were observed. Mild tubular dilatation, degeneration and inflammation was observed



in the kidneys of pyrethroid-treated rats (**Table 49**).

FIGURE 28. Histopathological evaluation of organs of non-reproductive tissues in rats treated with a mixture of pyrethroids. Liver, kidney, lung of rats administered with a mixture of pyrethroids for 1 to 15 months were stained with hematoxylin and eosin. Arrows indicate the anatomical damages. All images are with a scale bar of 100 μ m.

Table 49. Histopathological changes in various tissues of rats treated with a mixture of pyrethroids for 1 to 15 months.

Tissue	1 month	3 months	6 months	9 months	12 months	15 months
Liver	Mild periportal infiltration of inflammatory cells and centrilobular necrosis of hepatocytes	Mild sinusoidal haemorrhages and mild periportal fibrosis	Moderate periportal / peribiliary inflammation and fibrosis along with sinusoidal haemorrhages	Moderate periportal / centrilobular inflammation in hepatocytes	Multi focalcentri lobular inflammation and periportal fibrosis	Moderate periportal inflammation and infiltration of inflammatory cells with sinusoidal hemorrhages
Lung	Severe alveolar / interstitial inflammation with infiltration of inflammatory cells and fibrosis	Severe alveolar / interstitial inflammation with infiltration of inflammatory cells and fibrosis	Severe alveolar / interstitial inflammation with peribronchiolar inflammation	Severe alveolar / interstitial edema, haemorrhages, and infiltration of inflammatory cells	Moderate peribronchiolar lymphoid tissue hyperplasia and alveolar / interstitial inflammation	Severe alveolar / interstitial inflammation with peribronchiolar lymphoid hyperplasia
Kidney	NAD	Mild tubular dilatation and degeneration	Mild tubular degeneration	Mild tubular degeneration and inflammation	Mild tubular degeneration and dilatation	Moderate tubular dilatation / degeneration in collecting ducts

NAD- No abnormality detected

5.3. Discussion

Pesticide usage for agricultural purposes has become a necessary evil, because of the demand for more food as the global population keeps rising. Consequent to this, many of the agricultural food products ended up having residual levels of these toxic compounds, and the consumers are exposed to these chemicals on a routine basis. In view of such an exposure, it is pertinent to conduct studies to determine how the general and organ-specific physiological functions are affected by designing experiments that mimic human exposure settings. In this study, animal experimentation was conducted to determine the effect of long-term exposure to a mixture of pyrethroids that are commonly present in rice and vegetables at doses equivalent to the amount consumed by an average Indian individual. Analyses of the parameters that define the hepato-, nephro-, pulmonary- and male reproductive toxicity indicated disruption in the functioning of the vital organ systems, which is evident by increased oxidative stress, disrupted antioxidant enzyme activity, damage to the anatomical architecture, and altered levels of biochemical clinical markers in the serum. A recent interesting study revealed that in the 176 adults, serum metabolome was altered due to long-term pesticide exposure (including pyrethroids) and these changes could be due to oxidative stress, inflammatory reactions, and mitochondrial dysfunction (23). Alterations in plasma profile and lipid homeostasis were evident in rats exposed to tetramethrin, and prallethrin via the nasal route (24). Retention of native and oxidized LDL in the aorta, altered levels of cholesterol and HDL due to permethrin or bifenthrin induced toxicity in rats (25,26), disturbances in the plasma biomarkers (inflammatory cytokines and that of liver function) by deltamethrin (27), increased levels of plasma cholesterol and triglycerides in rats exposed to cypermethrin (28) and DNA damage in the peripheral lymphocytes of farmers who sprayed pesticides (deltamethrin) (29) are some recent reports related to the effects of pyrethroids on the hematological parameters. In this study, we observed disturbances in the levels of serum biomarkers that define liver function and lipid profile. Increased platelet count in the pyrethroid-treated rats indicated the development of thrombocytosis. The relation between thrombocytosis and pyrethroid toxicity is not yet reported. An in-depth analysis to understand the molecular mechanisms will be very interesting. Overall, it indicates that exposure to pyrethroids at doses used in this study alters the serum profile of many biomarkers that indicate multi-organ function.

To gain further insight on the possible effects of pyrethroids on liver function, standard markers were evaluated in the serum. Decreased levels of total bilirubin, though

indicates the normal liver function, are potential risk of deep white matter lesions (DWMLs). The correlation between lower bilirubin levels and DWMLs was reported in apparently healthy subjects in Japan (30). Further, lower bilirubin levels were found to be potential markers of coronary artery disease in the Korean population (31). The role of bilirubin as a metabolic hormone and its relation to multiple health disorders is extensively reviewed (32). The risk of cardiovascular disease and DWMLs due to pyrethroid toxicity was never reported and thus, we for the first time, provide an indirect evidence on the association between pyrethroid toxicity and metabolic diseases. We observed that the activity of liver enzymes SGOT/AST, SGPT/ALT, ALP, and LDH were lower in the earlier treatment time points, while an increased level was observed in the later time points. Interestingly, low levels of ALP may serve as an independent marker for increased mortality in middle-aged adults (33). Low levels of ALT are associated with cardiovascular diseases and diabetes (34-38). Lower levels of AST are associated with chronic kidney disease (39,40). LDH deficiency was first reported in patients with exertional myoglobinuria and has been implicated in a variety of other disorders (41). Thus, it is possible that pyrethroid toxicity could contribute to the risk of cardiac and renal diseases associated with diabetic complications. On the other hand, the four enzyme biomarkers of liver function were found to be elevated at later time points of treatment, suggesting damage to the liver. This is corroborated with the anatomical abnormalities observed in the liver. This is supported by the histopathological damage observed in this organ system. Hepatorenal damage associated with genotoxicity in rats was reported due to subacute exposure to the new pyrethroid, imiprothrin (42). Alterations in the liver function enzymes by deltamethrin (26,42-44), hepatotoxicity of lambda-cyhalothrin not sure may be it is Cyhalothrin (45), liver and kidney damage by bifenthrin (46), altered biochemical parameters in liver by cypermethrin (47), were reported. The hepatotoxicity observed in this study agrees with the previously reported studies. Changes in the lipid profile (increased levels of cholesterol, triglycerides, and VLDL) observed in the rats treated for longer durations (9 to 15 months) were evident in this study. It is well accepted that altered carbohydrate metabolism manifests the disturbed lipid profile (48,49). Thus, it is possible that pyrethroid toxicity induces disturbances in the carbohydrate metabolism which in turn affects the lipid profile.

Perturbations in the oxidant to antioxidant ratio and damage to the anatomical structure of vital organs is a hallmark of environmental toxicants. In this study, we observed increased levels of lipid peroxidation and nitric oxide in all of the tissues

studied, though the increase varied with the duration of treatment in different tissues. Further, alterations in the activities of antioxidant enzymes (except GST) were also observed in all the tissues analysed. Loss of oxidant to antioxidant balance is implicated in many pathological conditions and the disturbance in this ratio by pyrethroids is a direct evidence of the possibility of developing multiple health hazards due to exposure to these chemicals. Liver damage by lambda-cyhalothrin (50), and deltamethrin (14,51), hepatotoxicity by cypermethrin (7), renal toxicity by deltamethrin (10), multi-organ damage by pyrethroid based insecticides (13), nephrotoxicity of deltamethrin (52-54), severe pulmonary toxicity by pyrethroids (55), bifenthrin induced damage to lungs (46) and promotion of lung cancer by cypermethrin and permethrin (56,57) were found to be associated with oxidative stress and disturbances in the antioxidant status. The histopathological damage observed in this study could be a manifestation of the increased levels of reactive oxygen species and the failure of the antioxidant enzymes to maintain the balance that is required for the protection of anatomical structure.

We report that rats when treated with a mixture of pyrethroids relevant / equivalent to human consumption, results in decreased levels of liver function markers in the first few months of exposure, thus initiating a predisposition to cardiac and renal disorders. Increased levels of liver function biomarkers indicated damage to this vital organ. Higher platelet count in the pyrethroid treated rats points to the possibility of development of thrombocytosis. Increased levels of liver function markers increased oxidative stress, and disturbing activity of antioxidant enzymes in the liver, lung, and kidney indicate the multiorgan damage that can be caused due to long-term pyrethroid exposure. Impairment in the functioning of multiple organs is backed by damage to the anatomical architecture. To the best of our knowledge, we for the first time report the toxicity of pyrethroids when administered to rats at doses that are relevant /equivalent to that consumed through the major agricultural products (rice and vegetables) in the Indian context.

5.4. References

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Summary of the study

Objective 1: Evaluating the effect of a mixture of pyrethroids administered at doses relevant to human consumption on the male reproductive system.

PART – I: Effect of doses that are relevant to human consumption ($1/5^{\text{th}}$ (HD) or $1/25^{\text{th}}$ (LD) of actual exposure)

The physiological effects that may occur in the male reproductive system was analysed as a function of fecundity, sperm function, hormonal status, antioxidant status and histopathological changes. An increase in the relative organ weight of only the prostate was observed in animals treated with HD or LD dose for 9 or 12 months when compared to the respective controls, while such effect for testis was observed in animals treated with HD or LD dose for 15 months. A decrease in the litter size was observed in rats that received LD or HD pyrethroids for 9, 12 and 15 months. To determine the effects on sperm function, capacitation and acrosome reaction were analysed *in vitro* by measuring the fluorescence intensity of membrane bound filipin and PNA-FITC and FLUO-3-AM respectively. We observed the inability of spermatozoa obtained from pyrethroid mixture treated rats to undergo capacitation and acrosome reaction. Decreased activity of testicular hydroxysteroid dehydrogenase enzyme activities were evident, indicating an effect on steroidogenesis and spermatogenesis. The anatomical changes that may occur due to the toxic effects of a mixture of pyrethroids was evaluated by analysing the tissue sections stained with haematoxylin and Eosin (H & E staining). Severe anatomical changes in the testis, caput, cauda, and prostate were observed in pyrethroid treated rats. Further, perturbations in the oxidative stress markers (lipid peroxides and nitric oxide) and antioxidant enzyme (catalase, GST, GPX, and SOD) activities were observed in the male reproductive tissues and these changes depended on the dose and duration of exposure.

PART – II: Effect of doses that are equivalent to human consumption.

No differences in the relative organ weights were observed for all the reproductive tissues at all the time points analysed. The litter size in the pyrethroid treated rats (exposed for 1, 3, 6, 9, 12 and 15 months) was significantly lower compared to the respective control at all the time points analysed. Similarly, litter size was found to be significantly decreased in the F1 and F2 generation male rats that were born to forefathers exposed to pyrethroids for 1, 3, 6, 9 and 12 months. No significant changes in the sperm count were observed in rats of 1 month treatment group, whereas a significant decline was observed in rats that were treated with a mixture of pyrethroids for 3, 6, 9, 12 and 15 months. We

observed that long term treatment with pyrethroids causes significant impairment in capacitation and to a certain degree of acrosome reaction. The activities of hydroxysteroid dehydrogenases in the testes of pyrethroid treated rats were lower suggesting an effect on steroidogenesis and spermatogenesis. Hormonal profile that dictates reproductive function was analysed. Disturbances in the levels of FSH, GnRH, INHB, KISS1, LH, TSH, PTL and testosterone was evident in the pyrethroid treated rats. With regards to anatomical damage due to pyrethroid exposure, all the reproductive tissues analysed displayed severe damage at all the time points tested and the severity of damage appears to increase with the time of exposure. In line with this, increased lipid peroxidation and disrupted activity of antioxidant enzymes (catalase, GST, GPx, and SOD) was evident in all the tissues obtained from pyrethroid treated rats.

Objective 2: Evaluating the effect of a mixture of pyrethroids administered at doses relevant to human consumption on the factors that affect male reproduction at the transcriptomic and proteomic level.

PART – I: Effect of doses that are relevant to human consumption ($1/5^{\text{th}}$ *h=HD) or $1/25^{\text{th}}$ (LD) of actual exposure)

To provide further evidence on the role of factors that contribute to spermatogenesis, steroidogenesis, germ cell epigenetic changes and apoptosis, which ultimately define sperm function, the expression pattern of genes that govern these processes were analysed in the testes by real time PCR. The expression pattern of majority of the genes involved in these pathways were derailed in the testes of rats treated with low doses of a mixture of pyrethroids for 9 to 15 months, suggesting a possibility of impaired male germ cell production and development. The disruption in gene expression depended on the dose and duration of exposure to pyrethroids.

PART – II: Effect of doses that are equivalent to human consumption.

Overall, we observed that perturbations in the expression profile of genes involved in various process of gametogenesis (spermatogenesis, steroidogenesis, germ cell epigenetic modulators and germ cell apoptosis), which depended on the duration of exposure.

Proteins secreted into the testicular and epididymal lumen are added on to the sperm surface to aid in spermatogenesis and sperm maturation. In order to determine whether the expression and addition of these factors to the sperm surface is affected due to pyrethroid exposure, spermatozoa from the control and treated rats were subjected to

GC-MS analyses. Sperm lysates were prepared using gametes obtained from rats treated with a pyrethroid mixture for 6 months. Lysates were also prepared from the spermatozoa collected from the matching control rats (n=3). A total of 506 proteins that were present in all the biological triplicates of control rats were identified and the levels of these proteins in the sperm lysates obtained from pyrethroid treated rats was analysed. Among the 506 proteins analysed, 153 had a ratio of 0; 41 had a ratio ranging from > 0 to <0.5; and 10 had a ratio > 2.0.

To determine whether the differential expression of proteins observed in the spermatozoa of pyrethroid treated could also be transgenerational, the differentially expressed proteins identified in the sperm lysates of F0 rats were analysed in the F1, F2 and F3 generations. We observed that the differential expression observed for a majority of proteins identified in the sperm lysates of F0 rats remained the same in the next generations. 22 proteins that were down regulated in the F0 generation continued to remain under expressed in the F1, F2 and F3 generations. Similarly, 45 proteins exhibited under expression up to F2 generation, while only 1 protein exhibited continued to be under expressed up to F1 generation. 4 of the up regulated proteins in the sperm lysates of F0 generation continued to be over expressed up to F3 generation. 1 protein each was found to be over expressed up to F2 and F1 generations. Among the proteins that continued to be differentially expressed in the later generations, some of them are reported to have critical roles in male reproduction. These results indicate that the reduced fecundity observed in the later generations could be due to the continued differential expression that was initiated due to pyrethroid treatment in the F0 rats.

Objective 3: Evaluating the effect of a mixture of pyrethroids administered at doses relevant to human consumption on the general physiological processes.

PART – I: Effect of doses that are relevant to human consumption (1/5th (HD) or 1/25th (LD) of actual exposure)

No significant changes in the relative weights of all the tissues (liver, kidney, and lung) were observed at all the time points analysed in animals treated with HD or LD dose for 9 or 12 months when compared to the respective controls, except for a few variations. Oxidative stress and changes in the activity of antioxidant activities (catalase, GST, GPx, and SOD) were evident in the lung, kidney, and liver of pyrethroid treated rats. Damage to the anatomical architecture in these organs was also evident. Alterations in the biomarkers that define liver function (bilirubin, AST, ALP and LDH) and lipid

profile (Cholesterol, LDL, HDL and VLDL) was observed, with no change in kidney function (BUN, creatinine, and electrolytes).

PART – II: Effect of doses that are equivalent to human consumption.

No significant change in body weight and relative organ weights between the treated and control rats were observed at any of the time points analysed. Increased oxidative stress and perturbed activity of catalase, GPX and SOD was evident in the liver, lung, and kidney of pyrethroid treated rats. Damage to the internal anatomical architecture was also evident as observed by histopathological analyses. Liver and kidney function were analysed by estimating standard parameters that define the normal functioning of these important organs. Alterations in these parameters indicated impaired liver function. Lipid profile was significantly altered, while kidney function was not affected.

OVERALL SUMMARY

In general, long term pyrethroid exposure at doses relevant / equivalent to human consumption causes systemic toxicity and can affect the functioning of vital organs (liver, kidney, and lung) in the body. Reproductive function (in terms of sperm function and fertility) was severely affected in exposed rats and reduction in fertility could be due to the anatomical damages of this organ system. Transcriptomic and proteomic changes in the machinery involved in steroidogenesis, spermatogenesis, epigenetic modulation, and apoptosis in the testes of pyrethroid exposed rats indicates that damages at the molecular level will have far reaching implications. Transgenerational effects on fertility are a serious concern, which will have effects on the generations to come. Results of this study will provide first-hand information that may happen in human settings due to prolonged exposure to pyrethroids. Observations in this study strongly suggest a need to revisit agricultural practices and to come up with revised policies that will lead to better food processing technologies to reduce pesticide content.

Publications



Long term oral administration of a mixture of pyrethroids affects reproductive function in rats

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ABSTRACT

Pyrethroid toxicity using dosages that are relevant to the human settings are not reported. Male Wistar rats were treated for 9 or 12 months daily with a mixture of pyrethroids equivalent to a fifth or a twenty-fifth of that is present in cereals and vegetables consumed by an average Indian adult. Altered oxidant and antioxidant status, severe anatomical damage in the testis, caput, cauda, prostate, liver, lung and kidney and increased serum SGPT, SGOT and ALP activity was evident in all the treatment groups. Decreased levels of 3 β - and 17 β -HSD activity, litter size and impaired acrosome reaction was observed in all the treatment groups. To the best of our knowledge, for the first time, we demonstrate that exposure to even very low levels of pyrethroids (relevant to human consumption) for longer durations could cause damage to tissues that are important in general and male reproductive physiology.

1. Introduction

Pyrethroid usage to control pests in agricultural and domestic set ups continues to increase over a period of time. Synthetic pyrethroids are preferentially used because of their high insecticidal activity and biodegradability and thus accounts for almost one fourth of the world's insecticide market [1]. In general, a mixture of insecticides are used for improving crop production which contaminates the agricultural land and water [2]. An impending result of this is that they enter the food chain, which is evident from recent studies that many pyrethroids can be detected in food items such as rice, wheat, vegetables, fruits and nuts [3,4]. This is also true in the Indian context [5,6]. Thus, humans get constantly exposed to more than one pesticide at a time. Though many studies reported the effects of individual pesticides (including pyrethroids) on different organ systems, active investigations on the toxic effects of mixed pesticides have gained importance in the recent past because of the potential of human exposure via the food chain. In humans poisoned with a mixture of cypermethrin and chlorpyrifos, the ventilator free days were shorter when compared with humans exposed to one of the compounds [7].

Mixed pesticides are known to cause synergistic toxicity in animal models [8–10]. A large number of studies reported that exposure to pesticides and insecticides alters biochemical, histological and reproductive parameters. In a recent study, it is observed that oxidant and antioxidant status is altered in cypermethrin exposed pregnant rats and

their new borns [11]. Similarly, we previously demonstrated alterations in oxidant and antioxidant status in adult rats and in off spring born to female rats exposed to allethrin [12–15]. A mixture of propoxur (PR) and permethrin (PE) when administered to rats contribute to disrupted redox status in the liver of rats [16]. Biochemical profiling related to liver and kidney function tests are reported in animal studies to evaluate pyrethroid toxicity. Measuring aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), total bilirubin (TBIL), total proteins (TPs), cholesterol (CHOL), urea, and creatinine provide vital information on the damage caused to vital organs during pyrethroid exposure. Aberrations in ALKP, AST, ALT, TBIL, TPs, cholesterol, urea and creatinine was observed in mice treated with deltamethrin [17]. Pyrethroid toxicity also causes disruption in the steroidogenesis by influencing the activities of the steroidogenic enzymes, especially 3 β - and 17 β -hydroxy steroid dehydrogenase (HSD) [14,18–21]. Histopathological changes induced by a combination of pesticides is reported [16,22,23]. A recent study indicates that malformations in the reproductive tract is evident in rats exposed to mixed pesticides [24]. A combination of chlorpyrifos and cypermethrin contributes to reproductive toxicity in male albino mice [23]. Further, a combined low dose of cyromazine, 4-(2-methyl-4-chlorophenoxy) butyric acid (MCPB), pirimicarb, quinoctamine, thiram, and ziram caused decreased birth weight in rats [25]. A mixture of pesticides at dose equivalent to the no-observed-effect-level (NOEL) caused impairment in spermatogenesis in rats [26].

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Effect of long-term treatment with a mixture of pyrethroids on the expression of genes that govern male germ cell production in rats

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Abstract

Humans are exposed to pyrethroid-based pesticides through agricultural produce. In this study, male Wistar rats were orally treated for 9 to 12 months with a mixture of pyrethroids that is equivalent to one-fifth (high dose; HD) or one-twenty fifth (low dose; LD) of the amount of pyrethroids present in the cereals and rice consumed by an average Indian. In rats treated for 9 months, the spermatogenesis-associated genes *Abp*, *Ar*, *Cd9*, *Dax1*, *Dazap1*, *Ddx3y*, *Gdnf*, *Gfra1*, *Grth*, *Inhb*, *Ovol1*, *P1*, *Plzf*, *Pygo2*, *Scf*, *Tgfb1*, *Tp1*, *Tp2*, and *Vim1* were downregulated in both LD and HD groups. In rats treated for 12 months *Gdnf*, *Hsf2*, *Inhb*, *Tgfb1*, *Thy1*, and *Ybx2* expression was downregulated in both LD and HD groups. Steroidogenesis-associated genes *17-β-Hsd*, *Gata4*, *Hmgcr*, *Hmgcs1*, *Pde4b*, and *Tspo* gene expression were reduced in both LD- and HD-treated groups treated for 9 months. In 12-month-treated rats, *Creb1* expression decreased in both LD and HD groups. The epigenetic reprogramming-associated genes, *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Hdac10*, *Hp1bp3*, *Kat3a*, *Kat3b*, *Mch2ta*, *Ncoa7*, and *Sirt1* were downregulated in both HD and LD groups of 9-months-treated rats. In rats treated for 12 months, *Hdac10*, *Mch2ta*, *Ncoa7*, and *Sirt1* messenger RNA levels decreased in both the HD and LD groups. Thus, we demonstrate that long-term exposure to a mixture of pyrethroids caused aberrations in the transcriptome of factors involved in sperm production and development.

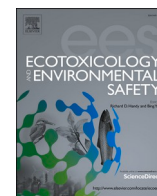
KEYWORDS

genetic reprogramming, pyrethroids, spermatogenesis, steroidogenesis, testis

1 | INTRODUCTION

Pesticide usage is rampant throughout the world for domestic and agricultural purposes. Indiscriminate use of pesticides caused approximately 3 000 000 instances of poisoning and 220 000 deaths annually.^[1] In India, the agricultural sector mainly depends on spraying pesticides to protect the crop to achieve a better yield. As per the Food and Agriculture Organization of the United Nations, 52 750 metric tons of pesticide is used in India annually with the 13th position on the global pesticide usage index.^[2] The presence of residual levels of a variety of pesticides including pyrethroids in cereals, vegetables, fruits, and milk products is reported in India.^[3,4]

Among the pesticides used, pyrethroid-based ones are more popular because of their high biodegradability and insecticidal activity. They primarily target the nervous system to cause malfunctioning of the voltage-sensitive sodium channels.^[5] In humans, pyrethroid toxicity is implicated to induce the motor deficiency associated with the central nervous system and Parkinson's disease.^[6–8] In murine models, pyrethroids cause cognitive defects,^[9] alterations in the levels of GABA, 3, 4-dihydroxyphenylacetic acid, and noradrenaline^[10] dopaminergic neurodegradation^[11] and alterations in xenobiotic enzyme levels of the brain.^[12] Pyrethroid toxicity in other organ systems is reported as well. Pyrethroids damage the immune system.^[13,14] Immunotoxicity is characterized



Research paper

Effect of oral administration of a mixture of pyrethroids at doses relevant to human exposure on the general and male reproductive physiology in the rat

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ABSTRACT

Studies on the effects of unintentional intake of pyrethroid pesticides that are akin to actual human exposure settings are very rare. Such an exposure is primarily by consuming the food products as routine diet that contain residual levels of pyrethroids. In this study, rats were orally administered for 15 months with a mixture of pyrethroids at a dose that is one-fifth (high dose; HD) or one-twenty fifth (low dose; LD) of the residual levels commonly present in the average amount of rice and vegetables consumed by Indian population. Lipid profile, kidney and liver function were assessed. Lipid peroxidation, nitric oxide, antioxidant enzyme activities and histopathological changes were analyzed in the liver, lung, kidney, pancreas, testes, caput, cauda and prostate. The effect on the male reproductive system as a function of sperm count, enzyme activity of 3 β -HSD and 17 β -HSD and the expression profile of genes involved in spermatogenesis, steroidogenesis, genetic reprogramming and apoptosis of male gametes were evaluated. Significant increase in the relative organ weight, perturbations in the activities of antioxidant enzymes, lipid profile and liver function were observed in both LD and HD groups. Damage to the anatomical architecture was evident in all the tissues due to pyrethroid toxicity. Exposure to LD and HD of pyrethroid mixture resulted in decreased sperm count, activities of 3 β -HSD and 17 β -HSD, impaired capacitation and acrosome reaction and perturbations in the expression of genes that govern male gamete production. Results of our study indicate that exposure to pyrethroids for longer durations even at doses that are far below the residual levels present in the food consumed will result in severe damage to general physiological processes as well as reproductive function.

1. Introduction

Pesticide usage to increase agriculture production resulted in the percolation of these chemicals into water bodies, landscape and the food chain thereby posing a threat to all life forms. Further, use of pesticides in households for pest management compounded the exposure in humans. Among the pesticides used, pyrethroid based ones are more popular because of their low toxicity on mammals, high toxicity to insects and biodegradability. Hence, pyrethroid based ones constitute to one fourth of the global pesticide market (Saillenfait et al., 2015). The primary mechanism of action of pyrethroids is based on their ability to target the voltage gated channels of the nervous system (Field et al., 2017). Other effects of pyrethroids that were recently reported include toxicity to the immune and respiratory systems and carcinogenicity (Alavanja et al., 2014; Ambwani et al., 2018; Kawamoto et al., 2020; Pauluhn, 2018; Yamada et al., 2019). Defective liver and kidney function, alterations in the hematological and lipid profile and imbalance in

the oxidant-antioxidant status have been recently reported to be associated with pyrethroid toxicity (Feriani et al., 2020; Jaremek et al., 2020; Martínez et al., 2018). Evaluation of the biochemical parameters that define these functions is crucial, especially in studies that involve long term exposure settings. Hence, in this study, we determined the effect of a mixture of pyrethroids on liver, kidney and hematological biochemical parameters along with the status of oxidative stress and the antioxidant enzymes.

Reproductive toxicity of pyrethroid based insecticides continues to be an active area of investigation. It is being recognized globally that increased risk of reproductive cancers and falling fertility rates is associated with pesticide exposure. Germ cell production in the males (spermatogenesis) is a continuous process that is tightly articulated by androgens and testicular factors. Differential gene expression of factors that contribute to spermatogenesis, steroidogenesis, genetic reprogramming via epigenetic modifications (DNA methylation, histone methylation and acetylation) and apoptotic pathways occur in a

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REVIEW ARTICLE



Pyrethroid based pesticides – chemical and biological aspects

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ABSTRACT

Human and animal welfare primarily depends on the availability of food and surrounding environment. Over a century and half, the quest to identify agents that can enhance food production and protection from vector borne diseases resulted in the identification and use of a variety of pesticides, of which the pyrethroid based ones emerged as the best choice. Pesticides while improved the quality of life, on the other hand caused enormous health risks. Because of their percolation into drinking water and food chain and usage in domestic settings, humans unintentionally get exposed to the pesticides on a daily basis. The health hazards of almost all known pesticides at a variety of doses and exposure times are reported. This review provides a comprehensive summation on the historical, epidemiological, chemical and biological (physiological, biochemical and molecular) aspects of pyrethroid based insecticides. An overview of the available knowledge suggests that the synthetic pyrethroids vary in their chemical and toxic nature and pose health hazards that range from simple nausea to cancers. Despite large number of reports, studies that focused on identifying the health hazards using doses that are equivalent or relevant to human exposure are lacking. It is high time such studies are conducted to provide concrete evidence on the hazards of consuming pesticide contaminated food. Policy decisions to decrease the residual levels of pesticides in agricultural products and also to encourage organic farming is suggested.

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KEYWORDS

Pesticides; pyrethroids; allethrin; cypermethrin; permethrin; fenvalerate; deltamethrin; λ -cyhalothrin; neurotoxicity; reproductive toxicity; oxidative stress; apoptosis; epigenetics; carcinogenicity

Table of contents

1. Introduction	1	4.7. Epigenomic toxicity	16
1.1. Literature review methodology	1	5. Conclusions and future prospects	16
1.2. Historical perspective	2	Acknowledgements	17
1.3. Usage and exposure	2	Declaration of interest	17
2. Classification of pesticides	3	Supplemental material	17
2.1. Natural pesticides	3	References	17
2.2. Synthetic pesticides	3		
2.2.1. Organochlorines (OCs)	3		
2.2.2. Organophosphates (OPs)	3		
2.2.3. Carbamates (CAs)	4		
2.2.4. Pyrethrins and pyrethroids (PYs)	4		
3. Pyrethroids	6		
3.1. Chemistry, classification and properties	6		
3.2. Routes of exposure, absorption, metabolism and excretion	7		
3.3. Epidemiology of exposure	8		
3.4. Mechanism of action	10		
3.5. Degree of toxicity	10		
4. Health hazards of pyrethroids	11		
4.1. Reproductive toxicity	11		
4.2. Immunotoxicity	13		
4.3. Respiratory toxicity	14		
4.4. Carcinogenicity	14		
4.5. Neurotoxicity	15		
4.6. Oxidative stress, inflammation and apoptosis	15		

1. Introduction

1.1. Literature review methodology

This review is a compilation of scientific material on the physical, chemical and biological aspects of pesticides to benefit the researchers working in the area of pesticide toxicology. Thus, it is to be noted that this is not a systematic review prepared keeping a specific aspect in focus. Information required to prepare this review was collected by searching multiple sources (PubMed, Google Scholar, Google and SCOPUS. The review was prepared by consulting different kinds of scientific material that are related to pesticide chemistry and toxicity. The methodology used to collect scientific material and the inclusion and exclusion criteria adopted in selecting the requisite material to compile this review is presented in [Supplementary Table 1](#). The inclusion criteria was to select research articles (animal, human and *in vitro* studies), book chapters, commentaries, reports of

RESEARCH COMMUNICATION



Evaluation of the reproductive toxicity of antiretroviral drug loaded lactoferrin nanoparticles

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ABSTRACT

Multiple prevention therapy has gained importance for the prevention and treatment of sexually transmitted diseases, especially HIV/AIDS. Antiretroviral drugs encapsulated in nanoparticles have been developed for efficient delivery of the drugs to the vaginal surface. Lactoferrin nanoparticles (LFNPs) encapsulating anticancer or antiretroviral drugs are found to be promising agents to specifically deliver drugs at the target sites. Recent studies indicate that the bioavailability is higher for antiretroviral drugs delivered by LFNPs than when the drugs are administered alone. Although LFNP-mediated drug delivery via the oral or vaginal route for the treatment of HIV/AIDS is promising, the effect of such administrations is not well studied. Drug-loaded LFNPs when administered to rats by the vaginal route did not show any effect on the reproductive performance, fertility, and postnatal development. Oral administration of drug-loaded LFNPs caused a significant decrease in litter size, whereas the reproductive performance and postnatal development remained normal. In our model system, the results indicate that vaginal administration of drug-loaded LFNPs appears safer and can be projected for the delivery of antiretroviral agents via the vaginal route.

Abbreviations: LFNPs: lactoferrin nanoparticles; STIs: sexually transmitted diseases infections; NPs: nanoparticles; LF: lactoferrin; DL-LFNPs: drug loaded lactoferrin nanoparticles; MPT: multiple prevention techniques

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Nanoparticles; lactoferrin; antiretroviral drugs; reproductive toxicity

Introduction

Globally, the incidence of sexually transmitted disease infections (STIs)-related morbidity and mortality is a major concern and there is always an urgent need to develop technologies by which drug delivery is specific and effective (Hill et al. 2007; Singh et al. 2010). To accomplish this, vaginal microbicide-based strategies are gaining importance (Liu et al. 2005; Thurman et al. 2011; Kizima et al. 2014), but with limitations. For example, the microbicide PRO 2000, BufferGel and Carraguard failed at different phases of clinical trials due to efficacy issues such as release at optimum concentrations, bioavailability, and side effects in the target organ (D'Cruz and Uckun 2004; Tao et al. 2008; Veazey 2008; Abdool Karim et al. 2011; Nutan 2011). Delivery of Anti-HIV drugs such as Tenofovir by way of intra-vaginal rings, gels, and tablets resulted in sustained release (Darroch and Frost 1999; El-Kamel et al. 2002; Bilensoy et al. 2006; Woolfson et al. 2006). In view of the side effects of these drugs, use of natural compounds has gained importance. One such compound

is Curcumin, the active ingredient in the roots of the plant *Curcuma longa*. Side effects were not observed with curcumin because of its hydrophobic nature and low bioavailability (Hatcher et al. 2008). Although powerful drugs are available to treat many diseases, major obstacles in the treatment are the effective delivery of the drug to the target site and sustained release (Zhao et al. 2013). A recent development for the treatment of many infectious diseases including the sexually transmitted diseases is the specific delivery of drugs using protein-based nanoparticles (NPs).

NPs whose size is generally in the range of 10–100 nm can transport and release drugs (Mudshinge et al. 2011). Their use for biological and therapeutic purposes in the form of nano-carriers confers advantage in preventing drug degradation, increasing immune responses of the host, delivery of the drug to a specific target, enhanced bioavailability, delivery of drugs with poor cell membrane permeability, and also delivery of macromolecules such as peptides and nucleotides (Krishna et al. 2009; Mallipeddi and

Expression of SARS-CoV2 Infectivity Machinery in the Male Reproductive Tract: Possible Outcomes on Fertility

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Abstract

Emergence of the COVID-19 pandemic continues to rage and rattles the entire world causing multifaceted hardships. Though initially thought to be a disease that primarily affects the lungs, latest evidence suggests its possible Long-term effects on multiple organ systems. SARS-Cov-2, the virus responsible for this disease infects the cells through ACE2 receptor and the serine protease TMPRSS2. In light of the fact that ACE2 expression is very high in the testis and the expression of TMPRSS2 in other reproductive organs, there has been growing interest to determine the effect of SARS-Cov-2 infection on the male reproductive system, especially on fertility. Through bioinformatics analyses, *in vitro* and cohort studies, the effects on SARS-Cov-2 infection at the molecular to physiological levels are proposed. Perturbations in hormonal levels, damage to the anatomical structure and inflammation in reproductive organs, decline in sperm count and sperm function have been reported. Thus, the significance of COVID-19 on global reproductive health has gained importance. In this article, we summarize the reported facts related to SARS-Cov-2 infectivity on male reproductive system. Such a comprehensive summation herein will help the researchers to have an up to date knowledge in this area of research and to coronavirus newer studies to address the effects of the COVID-19 pandemic on male reproduction, especially fertility.

Keywords: COVID-19, Fertility, Male Reproductive System, SARS-Cov-2

1. Introduction

The emergence of COVID-19 pandemic affected almost all the countries in a number of ways. As on 24th April, 2020, the pandemic caused 3,101,540 deaths and 146,312,705 infections worldwide to cause severe hardships in terms of health and economic crisis (<https://www.worldometers.info/coronavirus/>). COVID-19 is caused by the novel corona virus, SARS-Corona virus 2 (SARS-Cov-2), which primarily affects the respiratory system to cause severe lung disease¹. The infections primarily originated from the Wuhan city of Hubei province, China and culminated to infect millions of people across the world. In the last one and half years after the first outbreak of this disease in

December 2019, vast number of research studies reported the etiology and molecular mechanisms of this infection in the primarily affected organ i.e. the lung². Further, the susceptibility of other organs and the risks of post infection were also reported^{3,4}. Over a period of time it is emerging that this disease is not only fatal, but can cause High-risk mortality and morbidity in patients who have recovered from the infection. Considerable interest has also been generated to identify the effect of SARS-Cov-2 infections on the male reproductive tract. The gender bias for higher incidence of COVID-19 in men evinced interest to study the relation between the etiology of this disease and male reproductive physiology⁵. The purpose of this review is to consolidate the known aspects on the

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Effect of a mixture of pyrethroids at the doses relevant to human consumption on the general and male reproductive physiology in rats

by Anandha Rao Ravula

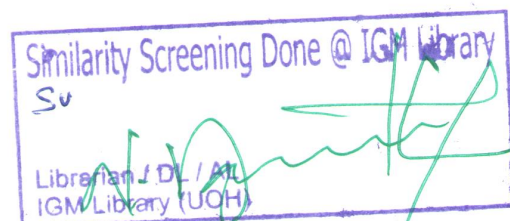
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