Study on lambda cyhalothrin-induced haematological, hepatic, and reproductive system dysfunction in rat and its modulation by taurine

Thesis submitted for the degree of

Doctor of Philosophy (Science)

of

Vidyasagar University

2016

By

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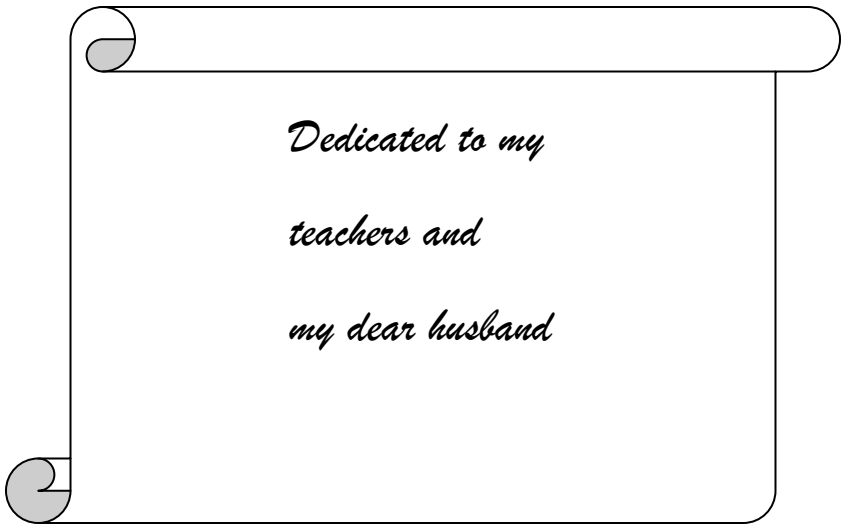
Dedicated to my
teachers and
my dear husband

CERTIFICATE

I, Dr. Sujata Maiti Choudhury hereby certify that this thesis entitled **"Study on lambda cyhalothrin-induced haematological, hepatic, and reproductive system dysfunction in rat and its modulation by taurine"**submitted by Rini Ghosh, is a record of bona fide research work under my supervision and I consider it worthy of consideration for the award of the degree of Doctor of Philosophy of the Department of Human Physiology with community Health, Vidyasagar University, West Bengal.

Date:

Place: West Bengal **Dr. Sujata Maiti Choudhury**

DECLARATION

I hereby declare that the thesis entitled "**Study on lambda cyhalothrin-induced haematological, hepatic, and reproductive system dysfunction in rat and its modulation by taurine** " submitted to Vidyasagar University , for the award of the degree of Doctor of Philosophy in Human Physiology with community Health, which is carried out by me in the Department of Human Physiology with community Health, Vidyasagar University, West Bengal, under the guidance of Dr. Sujata Maiti Choudhury.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

I have followed the guidelines provided by the Institute in writing the thesis.

I have conformed to the norms and guidelines given in the Institutional Animal Ethical Committee (IAEC).

Whenever I have used materials (data, theoretical analysis, and text) from other sources, I have given due credit to them by citing them in the text of the thesis and giving their details in the references.

Date:

Place: West Bengal **Rini Ghosh**

ACKNOWLEDGEMENT

I express my heartfelt gratitude and infinite immense to my respected supervisor, Dr. Sujata Maiti Choudhury, Associate Professor of Department of Human Physiology with Community Health, Vidyasagar University, who has been an excellent mentor throughout the period of my doctoral research. I am grateful for her critical comments, valuable advices, continuous monitoring, encouragement and motivation. I always enjoyed all the discussions with her on academic as well as other aspects of life. She is always there for me, being patient, amenable and supportive. Without her guidance and help this work could not have been done.

I sincerely acknowledge to Prof. Chandradipa Ghosh, head of the Department of Human Physiology of Community Health for her perceptive comments and kind co-operation in the submission of my thesis work.

I express my sincere gratitude to Prof. Somenath Roy, for helping me in carrying out this research work. I wish to express my honest gratitude to Prof. Prakash Dhara, for his valuable advice and comments. I owe my sincere gratitude to Dr. Sandip Sinha, for his continuous encouragement and co-operation during my work. I extend my sincere gratitude to Dr. Sumana Sorkhel, for her kind co-operation and advice during my work.

My earnest thanks are extended to the staffs of the Department of Human Physiology, Mr. Anup Barman, Mr. Malay Nayek, Mr. Sabyasachi Bera for allowing me avail the laboratory facilities.

I am grateful to University authorities for giving me administrative facilities.

I extend my sincere gratitude to Dr. Kuladip Jana, Department of Molecular Medicine, Bose Institute, Kolkata for giving me his time and toil for the preparation of my study.

I am thankful to my seniors and lab mates Tuhina Das, Saswata Sanyal, Pralay Maity, Madhubanti Bepari, Anurag Paramanik, Prasanta Sabud, Chaitali Ghosh, Ananya Pradhan, Surya Kanta Dey, Tamanna Roy, Debjani Chatterjee for their kind co-operation and important advice for the completion of the research work.

My earnest gratitude goes to Dr. Subhasis Das for his constant and enormous help in planning of experimental works.

I am indebted to all my respected teachers who show me the path of knowledge.

I am deeply indebted to my parents, in laws and my husband, brothers, sisters, friends for their love, encouragement, and continuous support that enabled me to cross all the hurdles. Their best wishes have always been a source of inspiration throughout my academic career.

Rini Ghosh

LIST OF ABBREVIATIONS AND SYMBOLS

ACP- Acid phosphatase

ALP- Alkaline phosphate

ATP Adenosine tri phosphate

BSA- Bovine serum albumin

CAT- Catalase

CDNB- 1-Chloro- 2,4-dinitrochlorobenzene

$CoCl_2$- Cobalt chloride

$CuSO_4$- Copper sulphate

DHEA- Dehydroepiandrosterone

DNPH- 2,4 Dinitrophenylhydrazane

DTNB- 5,5'-Dithiobis-(2-nitrobenzoic acid)

EDTA-Ethylenediaminetetraacetic acid

GPx- Glutathaione peroxidase

GR- Glutathaione reductase

GSH- Reduced glutathaione

GSSG- Oxidized glutathaione

GST- Glutathaione-s-tnrasferase

H_2O_2- Hydrogen peroxide

H_2SO_4- Sulphuric acid

HCl- Hydrochloric acid

HSD- Hydroxysteroid dehydrogenase

KH_2PO_4-Potasium di hydrogen phosphate

LCT-Lambda cyhalothrin

LOAEL -Lowest observed adverse effect level

MDA- Malondialdehyde

MMP-Mitochondrial membrane potential

Na_2CO_3- Sodium bicarbonate

NAD- Nicotinamide adenine dinucleotide

NaF- Sodium fluride

NaOH- Sodium hydroxide

NOEL- No observed effects level

PBS- Pehosphate buffer

PNP- Para nitro phenol

PNPP- Paranitro phenol phospthate

PVDF- Polyvinylidene difluoride

ROS-Reactive oxygen species

SDS- Sodium dodecyl sulfate

SEM- Scanning electron microscope

SGOT- Serum glutamate-oxaloacetate transaminase

SGPT- Serum glutamate-pyruvate transaminase

SOD- Super oxide dismutase

SSA- Sulfo salicylic acid

TA-Taurine

TBA- Thiobarbaturic acid

TCA- Trichloroacetic acid

TNAPP- Tetra sodium pyrophosphate

µg- Microgram

µl- Microliter

µM- Micro molar

< - Less than

>- More than

°C- Degree centigrade

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Abstract

Pyrethroids, synthetic derivatives of natural insecticide pyrethrins, are used for many applications and as possible replacements for organophosphates may not be a benign alternative because of their potential for neurotoxicity, reproductive toxicity and other systemic toxicity. Lambda cyhalothrin(LTC), a synthetic type II α-cyano-pyrethroid, is used worldwide to control a broad spectrum of insects and ectoparasites in agricultural production, forestry, animal husbandry and public health applications but reported to produce serious health problems. So, in this study lambda cyhalothrin induced haematological, hepatic, male and female reproductive toxicity were investigated in rat and alleviation of these toxicities by taurine was also examined. Lambda cyhalothrin (10.83 and 15.17mg/kg body wt. for male and 6.29 mg/kg body wt. and 11.33mg/ kg body wt.for female)was orally administered alone or combined with taurine (50mg/kg body wt.) for 14 consecutive days. Intoxication of lambda cyhalothrin caused haematological alterations with an increase in oxidative stress and morphological change in erythrocytes. Lambda cyhalothrin treatment generated excess reactive oxygen species (ROS) which leads to DNA damage and a decrease in mitochondrial membrane potential in hepatic cells. Hepatic dysfunction was reflected by significant changes in lipid profile, liver biomarker enzymes and hepatic histo-architecture in lambda cyhalothrin intoxicated rats. Spermatogenic and steroidogenic disorders were observed by a significant decrease in sperm count, viability, motility, hypo-osmotic swelling, mitochondrial membrane potential, seminal fructose concentration, serum testosterone and pituitary gonadotrophins and increase in testicular oxidative stress, sperm DNA fragmentation

in lambda cyhalothrin intoxicated rats. LCT treatment also altered testicular histology, reduced testicular $\Delta^5$3β and 17β-hydroxy steroid dehydrogenase activities and their protein expression along with steroidogenic acute regulatory protein (StAR) and cholesterol side chain cleavage enzyme (P450scc) expression.Increased ovarian malondialdehyde level, decreased ovarian reduced glutathione and antioxidant enzyme activity, high ovarian cholesterol, diminished activity of ovarian $\Delta^5$3β- and 17β-hydroxysteroid dehydrogenase (HSD) and significant reduction in estradiol, progesterone and gonadotropic level with suppressed expression of 17β-HSD and StAR and altered ovarian histo-architecture in LCT-treated rat were observed as a result of LCT exposure. Pretreatment with taurine significantly restored the haematological parameters, mitigated the hepatic abnormalities and exhibited the protective effect on male and female reproductive toxicity by normalizing the hypothalamo-pituitary gonadal axis in LCT induced rat. All these attenuating effects of taurine are mediated by its antioxidant action to scavenge ROS that are responsible for lambda cyhalothrin induced systemic toxicity.

Chapter-1

Introduction

1. Introduction

In 1962, publication of Rachel Carson's *Silent Spring* (Carson, 1962*)* first focussed the public attention towards the potential nontarget effects of pesticides (Moore et al., 2009). Carson highlighted the problems created from the intensive use of organochlorine insecticides. Due to much accumulation of organochlorines in the environment and reproductive toxicity in people, in most developed countries their use was ultimately phased out in the late 1970s and early 1980s (Moore et al., 2009). Since the late 1970s, organophosphate (OPs) and carbamate pesticides were extensively used substituting the organochlorines. The U.S. Environmental Protection Agency (EPA) prscribed certain uses of both OP and carbamate insecticides as part of the Food Quality Protection Act (FQPA) of 1996 due to their potential toxic effects on people. This decision has led to gradual replacement of OPs and carbamates with another class of insecticides, the pyrethroids (Oros and Werner, 2005).

1.1. Pyrethroids

Pyrethroids are synthetic derivatives of pyrethrins, natural insecticides obtained from the flowers of certain species of the chrysanthemum plant (Chrysanthemum cinerariaefolium).Pyrethroids are more effective than natural pyrethrins (WHO, 1990). Crops treated with synthetic pyrethroids not only give more yields but also provide better quality. They have high insecticidal activity at very low doses and keep up with their insecticidal action over a prolonged period of time which helps to control overlapping generations of pests (Rehman et al., 2014). They are extremely

toxic to insects and fishes but less toxic to mammals (Joy, 1994; Rehman et al., 2014)

1.1.1. Mode of action and classification of pyrethroids

Pyrethroids damage the peripheral nervous system of exposed subjects while the organophosphates disrupt the central nervous system. Pyrethroids keep the voltage-gated sodium channels open for an extended period of time, causing an alteration in nerve function. Pyrethroids are classified into type I and type II depending on their mode of action on sodium channels and on their produced symptoms. Type I pyrethroids are allethrin, d-phenothrin, bifenthrin, resmethrin, permethrin and tetramethrin and they result in restlessness, prostration, hyperexcitation, and body tremors as it creates repetitive nerve discharges. Cypermethrin, deltamethrin, cyfluthrin, fenvalerate, esfenvalerate, fluvalinate, and lambda-cyhalothrin are type II pyrethroids and they create stimulus-dependent nerve depolarization and blockage, which leads to hyperactivity, in coordination, convulsions and writhing (Ecobichon, 1996).

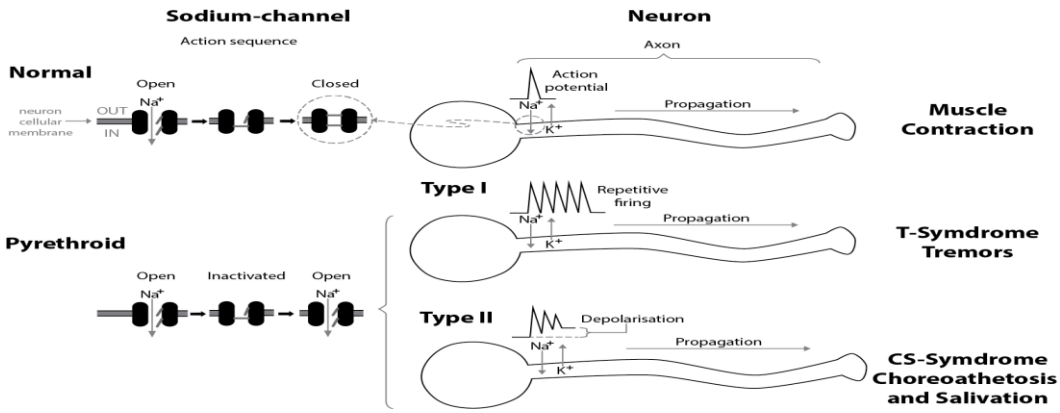

Figure 1.1: Mode of action of pyrethroids. The top diagram shows the normal functioning of sodium channels which open, allowing sodium to pass, but then close after the action potential. This single

action potential propagates through the nerve tail (axon) and triggers muscle contraction. Upon exposure to pyrethroids, the sodium channels go wrong, and may remain open. This will lead to repetitive firing (in type I pyrethroids) or depolarization (in type II pyrethroids) leading to tremors or involuntary movements (choreoathetosis) depending on the type of pyrethroid.

Source: Executive summary and Scientific Literature Review (Soderlund et al.,2002)

1.1.2 Uses of pyrethroids

After restrictions on the usage of cholinesterase-inhibiting insecticides (Feo et al., 2010), synthetic pyrethroids have become increasingly popular and are extensively used for last five decades to control insect pests in agriculture, horticulture, forestry, public health sectors and are active constituents of many insect-control products for indoor home use (Maund et al., 2001; Feo et al., 2010). Due to the high effectiveness, broad spectrum insecticidal activities, less mammalian and avian toxicity and biodegradability, pyrethroids have a high share of the insecticide market (Pap et al., 1996). WHO recommends synthetic pyrethroids for vector control in aircraft and certain other pyrethroids (cyfluthrin, bifenthrin, deltamethrin, lambda cyhalothrin) against malaria vectors (Walker, 2000). Pyrethroids are also regularly used in developing countries for disease vector abolition and pest control. Pyrethroids are now extensively used as termiticides, for landscape application, or as perimeter treatments to keep pests out of structures by professional pest control applicators.

1.1.3. Pyrethroids in the environment and its fate

Pyrethroids contaminate the terrestrial environment, are introduced largely from both agricultural and non-agricultural spray drift, accidental spills and from direct application to soil surfaces. Pyrethroids have strong tendency to adsorb to soils and

organic matter and significant migration does not occur from the place of direct application. Photolysis degrades pyrethroids in the soil.

Pyrethroids generally enter into aquatic systems during rainstorm events via sprayed field, lawn, parking lot runoff and a little through spray drift. As sediments are a sink for pyrethroids, aquatic species may be persistently exposed to sediment-associated pyrethroids. Pyrethroids show extremely toxicity to non-target invertebrates and non-target aquatic vertebrates such as fish and amphibians (Ali et al., 2011). Pyrethroids are strongly hydrophobic (Oros and Werner, 2005). So, water-soluble fractions of pyrethroids introduced into an aquatic system are short-lived and become quickly reduced.

1.1.4. Pyrethroids toxicity

Pyrethroids are growingly being used in public health and animal husbandry and are claimed to pose relatively low human toxicity. However, about two hundred cases of acute occupational pyrethroid poisoning resulting from inappropriate handling were first reported in China in 1982 (He et al., 1989) majority of which occurred due to the exposure to deltamethrin, fenvalerate, cypermethrin, fenpropathrin and cyfluthrin. The Pesticide Poisoning Prevention Programme in the years 2000-2002, reported that largest number of pesticide illness was associated with pyrethrins and synthetic pyrethroids exposure (IPCS, 1990).Now pyrethroid induced neurotoxicity and other toxic effects ranging from whole body tremors to convulsions and death are well documented**(**Timothy et al., 2005; Huedorf et al.,2004).

Pyrethroids at the LC_{50} values less than 1.0 parts per billion (ppb) are used to vector control for mosquito, black fly and fly larvae as well as for aquatic invertebrates

(Sayeed et al., 2003). Pyrethroids are highly toxic to fish. Deltamethrin is the most toxic for fishes, fenvalerate, permethrin and cypermethrin have intermediate toxicity (Tandon et al., 2005). Pyrethroids indirectly affect birds through their food supply. The pyrethroids are effective neurotoxin in vertebrates and invertebrates. They show low mammalian toxicity due to their rapid biotransformation (Kranthi et al., 2001).

1.2. Lambda cyhalothrin

Lambda cyhalothrin (LCT), a synthetic pyrethroid insecticide was first reported by Robson and Crosby and was introduced in Central America and the Far East in 1985 by ICI Agrochemicals (Robson and Crosby, 1984). It is applied for controlling a variety of pests including aphids, Colorado beetles, butterfly larvae and crops like cotton, cereals, hops, ornamentals, potatoes, vegetables or others and also used in the management of structural pest or in public health sectors, to control insects such as cockroaches, mosquitoes, ticks and flies (Royal Society of Chemistry, 1991). It has been observed that temperature influences the toxicity of lambda cyhalothrin and LCT-induced insect paralysis (Toth and Sparks, 1990).Lambda cyhalothrin is a commercially available synthetic pyrethroid that has different brand name, such as, Warrior, Karate, ICON etc.

1.2.1. Physico-chemical properties of lambda cyhalothrin

Lambda cyhalothrin is a 1:1 mixture of two stereoisomers, (S)-α-cyano-3-phenoxybenzyl-(Z)-(1R,3R)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylyclopropane carboxylate (fig.1.2a) and (R)-α-cyano-3-phenoxybenzyl-(Z) -

(1S,3S)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropane carboxylate(fig. 1.2b)(WHO, 1990)

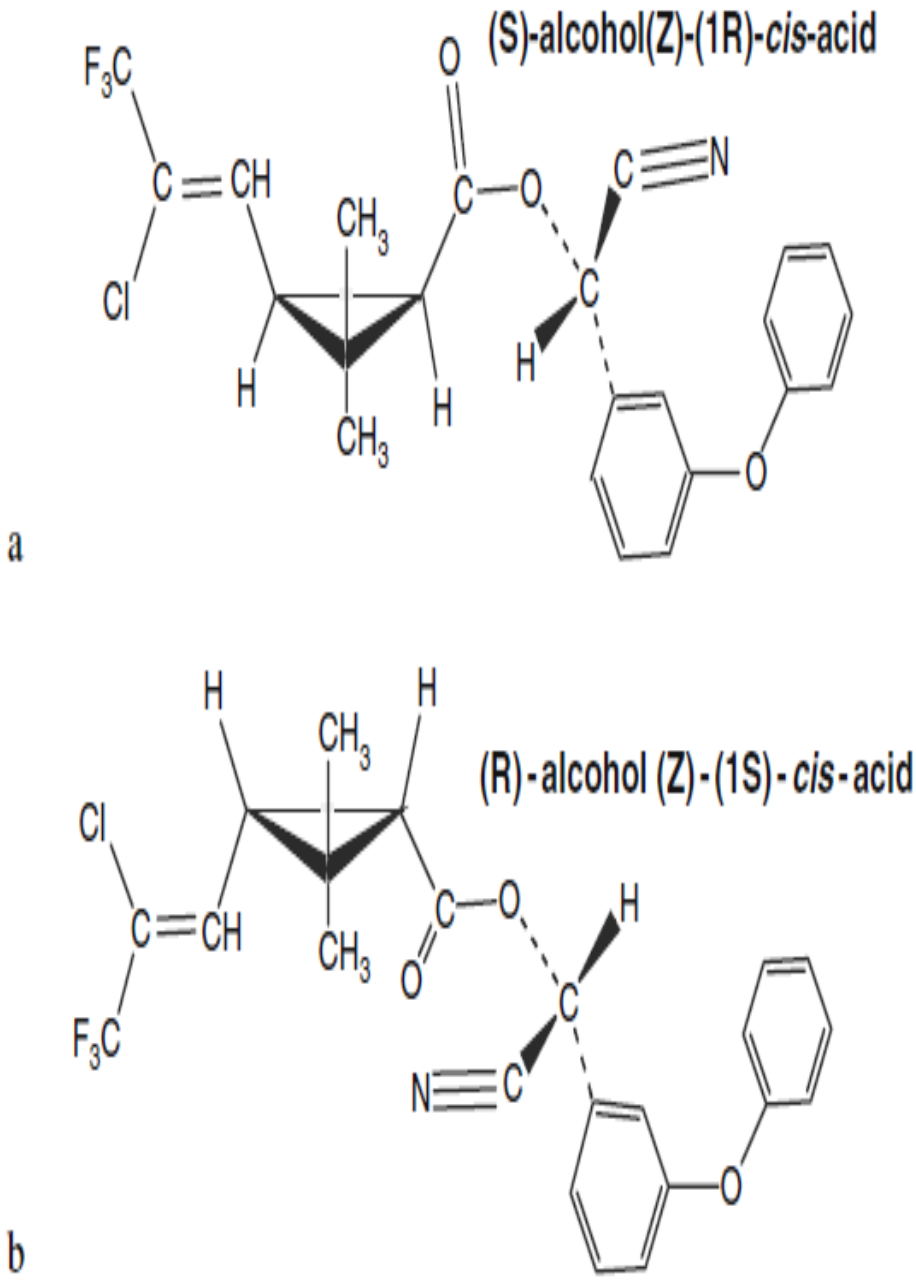

Figure. 1.2a, b. Structure of two isomers of lambda cyhalothrin.
Source: Environmental Chemistry, Ecotoxicity and Fate of Lambda Cyhalothrin

Table.1.1.Physico-chemical properties of LCT (Royal Society of Chemistry, 1991)

Chemical Name:	(RS)-alpha-cyano-3-phenoxybenzyl 3-(2-chloro-3,3,3-trifluoropropenyl)-2,2,-dimethylcyclopropane carboxylate
Molecular formula:	$C_{23}H_{19}ClF_3NO_3$
Molecular Weight:	449.9
Water solubility:	0.005 mg/L, pH 6.5 and 20 ºC
Solubility in other solvents:	acetone, methanol, toluene, hexane
Vapor Pressure:	negligible at 20 degrees C
Partition Coefficient (octanol/water):	10,000,000
Color	Yellowish in solution and remain colorless solid at room temperature.

1.2.2. Ecotoxicity

Lambda cyhalothrin is basically non-toxic to birds. It is confirmed that LCT does not accumulate in the eggs or tissues of birds. For many fishes and aquatic invertebrate species lambda cyhalothrin is acutely toxic.LCT shows its maximum toxicity on bees with an oral LD_{50} of 38 ng/bee and contact LD_{50} of 909 ng/bee (0.9µg/bee). LCT shows toxic effects especially on vertebrate non-target organisms, including mammals.

1.2.3. Environmental fate

Lambda cyhalothrin is moderately persists in the soil environment and its field half-life is close to thirty days in most soils (Wauchope, 1978). It displays a high affinity for soil. From the field studies it is observed that, leaching of lambda cyhalothrin

(Karate) and its degradation in the soil are almost minimal (Royal Society of Chemistry, 1991).

1.2.4. Toxicological effects

Based on several standard tests, lambda cyhalothrin is considered as an acute hazardous product. There was no clear literature regarding the sub-chronic, chronic toxicity of LCT. Lambda cyhalothrin caused a reduction in body weight gain and food consumption in rats after receiving oral dose of 1.5 mg/kg/day in a three-generation study (US Environmental Protection Agency, 1995).Lambda cyhalothrin caused reduced body weight gain at 15 mg/kg body wt. /day in pregnant rats (highest dose tested) and at 30 mg/kg body wt. /day in pregnant rabbits (also the highest dose tested). From a two year period of dietary administration of lambda cyhalothrin in rats and mice, it was observed that mice were more tolerant than rats. A dietary no observed effects level (NOEL) of 50 ppm (corresponding to a dose of 2.5 mg/kg body wt./day) with a corresponding lowest observed adverse effect level (LOAEL) of 250 ppm (12.5 mg/kg body wt./day) based on decreased body weight with no signs of neurotoxicity was found in rat study (US Environmental Protection Agency, 1995).

Lambda cyhalothrin causes no skin irritation in rabbits. In studies on guinea pigs, it did not cause skin sensitization (WHO, 1990).The Environmental Protection Agency classifies it as very low toxicant for skin effects. It also causes mild eye irritation in rabbits. ‘The US Environmental Protection Agency’ categorizes it as moderately toxic for eye. In a 4-hour inhalation study with a lambda cyhalothrin product, the

LC_{50} ranged from 0.315 to 0.175 mg/l indicating its moderate toxicity (US Environmental Protection Agency, 1988). In the three-generational rat study, the number of viable offspring reduced in the second and third generations at dose level of 50 mg/kg/day (US Environmental Protection Agency, 1995).

1.3. Taurine

1.3.1. Occurrence, structure and biosynthesis

Taurine, 2-aminoethanesulfonic acid is the major free amino acid, is found almost in all animal tissues, and is conditionally essential nutrient (Chesney, 1985).Taurine is available in much greater quantity in algae and other animal kingdom as well as in insects and arthropods as it is a phylogenetically ancient compound. Tissues that are excitable are generally rich in taurine (Huxtable, 1992).

Because of the lack of a carboxyl group, it is not a distinctive amino acid, and in its place a sulfonate group exists and so it may be called an amino-sulfonic acid.

Taurine, like the amino acid glycine does not polarize light and consequently it does not have an L- or D-configuration. It is synthesized from amino acids cysteine and methionine (Beetsch and Olson, 1998). Vitamin B-6 (pyridoxal-5' phosphate) is a key cofactor in this process.

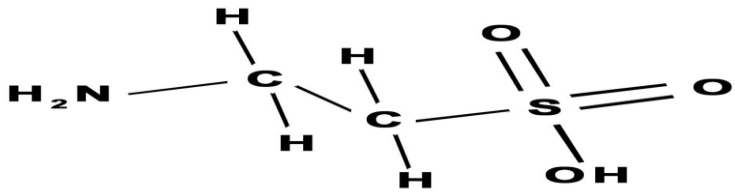

Figure 1.3: Chemical structure of taurine (Asha, 2010)

1.3.2. Physico-chemical properties

Table.1.2.Physico-chemical properties of taurine(Asha, 2010)

Molecular formula	$C_2H_7NO_3$ S
Molecular weight	125.15
Physical state	Large monoclinic prismatic rod shaped crystals.
Colour	White crystals
Odor	Odorless
Solubility	Soluble in water and insoluble in absolute alcohol.
Melting point	300^0C
pH (0.5M in water, 250C)	4.5-6
Optical rotation	Nil

1.3.3. Biological importance

Taurine plays a significant role in the functions of the body (Huxtable, 1992).Free radicals are highly reactive atoms that cause damage in the body by converting stable molecules into unstable ones (Harman, 1992). Recently, the role of hypotaurine, the precursor of taurine has been discovered as a potent antioxidant (Fontana et al., 2004). The sulfinyl group in the hypotaurine molecule works efficiently as a radical scavenger. Hypotaurine converts to taurine by a process to effectively scavenge free radicals and it increases cell viability.

Excitotoxicity is presence of excess amounts of the excitatory amino acids, especially glutamate and aspartate, such that they create an intracellular and extracellular toxic environment resulting in cell death (Chen et al., 1999). Taurine, buffers the dramatic changes caused by excitotoxicity (Wu et al., 2005). Cell volume is an important parameter in the cellular regulation of secretion, metabolism, cell growth and programmed cell death (Zonia and Munnik, 2007). Taurine plays a significant role as an organic osmolyte in cell volume control in mammalian cells and a change in the cellular taurine content marks a shift in the cell volume (Lambert, 2004).

With increase in age, the level of taurine is found to be reduced (EI Idrissi, 2008). Learning ability of the older rats gets reduced because of decrease in taurine level (Dawson et al., 1999). Interestingly 50% of the free amino acids in the heart cells are made up by taurine (Huxtable et al., 1980). Taurine's electrophysiological actions in cardiac cells are brought about by modulation of ion channels (Satoh, 1998). Taurine provides the antioxidant protective capacity which prevents alveolar macrophages on the surface of lung alveoli to become susceptible to oxidative stress (Minko et al., 2002). The decrease in taurine concentration may cause harmful to pulmonary tissue. In oxidative stress, endothelial cell apoptosis and necrosis is attenuated by taurine (Zhang et al., 2008) through intracellular calcium flux. Taurine is necessary for normal skeletal muscle functioning (Ito et al., 2008).

Bile synthesized in the liver, is essential for proper emulsification and digestion of fats. It is well known that taurine is conjugated with chenodeoxycholic acid and

cholic acid through its amino terminal group to form the bile salts sodium taurocheno deoxycholate and sodium taurocholate.

In case of high fat, high cholesterol diet consuming people taurine can attenuate the elevated total and LDL cholesterol (Wen et al., 2004) and help to maintain the favourable lipids ratio. Liver cholesterol content is also decreased by 19% by taurine. It may lower the cholesterol content and has the ability to promote the conversion of potentially harmful cholesterol to relatively harmless bile acids.

Proper kidney function is maintained by taurine. In the absence of taurine, renal capability to excrete toxic substances from the blood is grossly hampered.

Taurine is found in very high concentration among other amino acids in healthy eyes (Heinämäki et al., 1986). Taurine makes the outer segment of the retinal rod to become resistant to injury, through its osmoregulatory function (Militante and Lombardini, 1999).

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Chapter- 2
Review of literature and aim and objectives

2.1. Pyrethroids

2.2. Lambda cyhalothrin

2.3. Taurine

2.4. Aim and objectives

2.5. References

2.1. Pyrethroids

Pyrethroids, most effective group of pesticides used for many applications, are possible replacement for some of the organochlorine, organophosphate, or carbamate insecticides.

Pyrethroids stimulate nerves by producing prominent repetitive activity like dichlorodiphenyltrichloroethane. The effect is seen in the neuromuscular junction, motor neurons, sense organs, and central nervous system. Non-cyano pyrethroids produce nerve impulse trains of short duration whereas cyano pyrethroids persuaded long-lasting trains of repetitive nerve impulses.

In Chinese medical literature, 573 cases of pyrethroid poisoning, containing 344 cases of accidental and 229 cases of occupational poisoning were stated during 1983-1988(He et al., 1989).Most of the poisoning cases were triggered by deltamethrin followed by fenvalerate and cypermethrin. Indiscriminate use of pyrethroids results in a series of hematological, biochemical, reproductive and pathological changes in the body (Shah et al.,2007).

2.1.1. Haematological alterations by pyrethroids

Traces of pyrethroids are evident in several organs, tissues, and cells (Spiteller,1996). The increased activity of antioxidant enzymes in erythrocytes is perhaps an initial adaptive response to increased oxidative stress under the influence of pyrethroid in rats. Sub-lethal concentration (1/4th LC50) of fenvalerate (0.086ppm) for 96h to fish (*Channa marulius)* significantly decreased total erythrocytes count, haemoglobin %, packed cell volume, and mean corpuscular

haemoglobin concentration, with a minute increase of total leucocyte count and mean corpuscular haemoglobin, mean corpuscular volume (Patole et al.,2016). Reduction in erythrocytes count, haemoglobin %, packed cell volume and increase in WBCs by the lethal and sublethal effect of deltamethrin on fish (*C. mrigala*) showed its haematological toxic potential (David et al.,2015).Oral dosage of cypermethrin(60,150, 300mg/kg body wt.) on 8-week old male Wistar rat for 28days led to dose-dependent significant decrease in red blood cell counts, haematocrit, thrombocyte, and mean corpuscular volume (Sayim et al.,2005). Intraperitoneal administration of cypermethrin to female rabbits caused significant ($P<0.05$) reduction in erythrocyte counts, haemoglobin percentage and packed cell volume and significant ($P<0.05$) increase in total leucocyte count, lymphocytes and mean corpuscular volume (Shah et al., 2007).

2.1.2. Effects on hepatic system

Pyrethroids produce several hepatic abnormalities. Chronic (63 days) oral exposure of cypermethrin (6 and 12mg/kg body wt.) increased weight of liver, hepatic aspartate and alanine transaminase activities and decreased lactate dehydrogenase activity in the male Somali sheep (Adel et al., 2015).14day peritoneal administration of deltamethrin (2.5, 5 and 10 mg/kg/body wt.) in female rats caused significant increase in serum alanine amino transferase, aspartate aminotransferase and alkaline phosphatase and decrease in total protein. Fenvalerate treatment in rat model reflects the oxidative stress and liver impairment by elevating enzymatic, non-enzymatic hepatic markers along with altered malondialdehyde and significant reduction in antioxidant enzyme activities (Waheed and Mohammed, 2012).

2.1.3. Effects on reproductive system

The few studies carried out in this area reveal pyrethroid-induced changes in the male reproductive system. Most cases highlighting the effects of pyrethroid on sperm concentration, motility and morphology, conducted on animals. Different oral doses of cypermethrin (0, 7.5, 15, 30, or 60 mg/kg per day) for 15 days in male rat caused a decrease in sperm production, produced atrophic and distorted seminiferous tubules, reduced germ cells with deformed and disordered arrangement, reduced Sertoli cells producing vacuoles and disrupted Leydig cells and increased serum testosterone and decreased serum FSH level significantly (Fang et al., 2013). Expression of androgen receptor decreases by 15 days oral administration of beta cypermethrin(0, 15 and 30mg/kg body wt.) which also causes decrease in sperm production in male rat(Liu et al.,2010).Pyrethroid also disrupt endocrine system. Using primary cultured rat granulosa cells as model, fenvalerate decreased progesterone and 17 beta-estradiol production in a dose-dependent manner. Fenvalerate also enhanced the P450scc mRNA level as well as decreased 17 beta-HSD mRNA level after 24 h treatment (Chen et al., 2005).

2.2. Lambda cyhalothrin (LCT)

Acute toxicity (96 h period) study showed that lambda cyhalothrin was comparatively 50 times more toxic to the fish *Channa punctatus* than cypermethrin. Even at low concentrations, these pyrethroid compounds can modify behavioural pattern .

2.2.1. Effects on oxidative stress

Oxidative damage by significant increase ($p < 0.05$) in thiobarbituric acid reactive substances (TBARS with marked changes in antioxidant defense system in rat brain was exhibited by the mixture of fenitrothion 25%, lambda cyhalothrin2.5% and piperonylbutoxide 6% (El-Demerdash,2011). After exposure of a mixture of fenitrothion and lambda cyhalothrin to rat led to generation of lipid peroxidation and decreased levels of reduced glutathione, superoxide dismutase, catalase, glutathione S-transferase activities and protein content in kidney(El-Demerdash,2011).

2.2.2. Genotoxic potential of lambda cyhalothrin

In vivo genotoxic potential of lambda cyhalothrin (LCT) was assessed after 30 days sub-acute treatment of LCT in cultured lymphocytes. Significant clastogenic potential has been detected together with chromosome and chromatid gap and break and fragments (Sharma et al., 2010). *In vitro* exposure to Karate(a commercial formulation of lambda cyhalothrin) in rabbit blood peripheral lymphocyte revealed a dose-dependent increase in the frequency of chromosomal aberrations and showed the presence of satellite associations and gaps, as well as of aneuploid cells (Georgieva, 2006).

2.2.3. Effects of lambda cyhalothrin on haematological parameters

Orally administration of sub-lethal dose of lambda cyhalothrin for 28days revealed the significant alteration in erythrocyte and leukocyte counts, haemoglobin concentration, haematocrit value in male Wistar rats (Ramadhas et al., 2014).

2.2.4. Impact on hepatic system

4 weeks oral dosage of LCT to male rats caused significant increase in hepatic aspartate transaminase, alanine transaminase activity, and malondi aldehde content

with significant reduction in the activities of antioxidant enzymes in rat. In addition, LCT induced hepatotoxicity by causing liver DNA fragmentation seen by gel electrophoresis (Madkour,2012).Lambda cyhalothrin induced hepatopathological alterations in male Wistar rat exhibiting focal necrosis and inflammatory cell infiltration in hepatocytes and displaying disrupted, swollen and vacuolated hepatocytes (Ramadhas et al., 2014).Zebra fish intoxicated with lambda cyhalothrin and Neemgold in different concentrations altered the total liver protein, total free amino acids, and nucleic acids(Ahmad et al ,2012).

2.2.5. Effects of lambda cyhalothrin on females and their offspring

Long term exposure to low doses of LCT produced maximum level of lipid peroxidation on 14-21 days of pregnancy, which was accompanied by a reduction in activity of antioxidant enzymes (Tukhtaev et al., 2012).

2.3. Taurine

Taurine (2-aminoethanesulfonic acid),a ubiquitous free amino acid, present in most mammalian tissues, is known to have various physiological functions in cell metabolism including osmoregulation, membrane stabilization, detoxification and regulation of cellular calcium homeostasis (Huxtable, 1992).

2.3.1. Taurine in oxidative stress

Taurine shows a protective effect against cellular stress induced oxidation and acts as a free radical scavenger in different cells and tissues against oxidants produced toxic insult (Hagar, 2004). It has been revealed that taurine prevents lipid peroxidation and mitigated the activities of antioxidant enzymes after xenobiotics revelation (Tabassum et al., 2006; Dogru-Abbasoglu et al., 2001).

Jagadeesan and Pillai reported that mercury induced oxidative stress by inducing lipid peroxidation and alteration of the activity of glutathione (GSH), glutathione peroxidase (GPx),catalase (CAT) and superoxide dismutase (SOD) in the liver of rats were effectively attenuates by taurine treatment(Jagadeesan and Pillai, 2007). Literature also reported that taurine reduced hepatic oxidative stress and inhibited hepatocyte apoptosis in 13 weeks iron-overloaded mice (Zhang et al., 2014).

2.3.2. Defensive effect of taurine on haematological system

Increased haemoglobin %, erythrocytes count and packed cell volume and reduction in erythrocyte indices by sub-lethal copper induced stress was alleviated by taurine and garlic extract in fish *Clarias gariepinus*(Adil et al., 2013). Chlorpyrifos and lead (Pb) induced haematotoxicity in the rats reflected by reductions in packed cell volume, haemoglobin concentration, red blood cell count, erythrocyte indices, total white blood cell, lymphocyte and platelet counts was mitigated by taurine through its cytoprotective, osmoregulatory, and membrane stabilization properties(Akande et al.,2014).

2.3.3. Mitigating ability of taurine on hepatic system

Taurine administration improved liver functions against Cyclosporine A (CsA) induced hepatotoxicity, as evidenced by reduction in serum transaminases and gamma glutamyl transferase levels and elevation of serum total protein(Hagar, 2004).Taurine administration recovered the liver function in mercury intoxicated rats as indicated by the reduced levels of aspartate and alanine transaminase activities and alkaline phosphate in serum (Jagadeesan and Pillai, 2007).Taurine was reported to reduce methotrexate (an anti-rheumatic drug) induced hepatotoxicity produced by

elevated serum aspartate and alanine transaminase and gamma glutamyl transferase levels ($p<0.001$) and total protein level (Issabeagloo et al., 2011).

2.3.4. Reproductive efficacy of taurine

Taurine shows a protective effect against gonadal hypercholesterolemia in male rat by the elevation of reduced Follicular-stimulating hormone and luteinizing hormone in addition to the testosterone (Alzubaidi and AL Diwan, 2013).

2.3.5. Ameliorative effect of taurine against pesticides

Pretreatment of vitamin E and taurine showed a protective effect by resulting in a significant decrease in lipid peroxidation, alleviating effects on GSH and the activity of antioxidant enzymes in rat after administration of methiocarb (Ozden et al., 2012).Taurine through its antioxidant, hepatoprotective and nephroprotective properties protects pesticide and metal (chlorpyrifos and lead) induced toxicity in rat (Okeke et al., 2014).

2.4. Aim and objectives

Lambda cyhalothrin is predominantly used in the agricultural production in different districts of West Bengal, India. Attempts were made to investigate lambda cyhalothrin induced haematological, hepatic and reproductive system toxicity in rat and also to find out whether there is any ameliorative role of taurine in these toxicities.

The objectives of the present study are:

To find out the exact toxic dose levels of lambda cyhalothrin which can produce haematological, hepatic and gonadal toxicity in male, female Wistar rat.

To evaluate lambda cyhalothrin induced haematological alterations in experimental rats and the role of taurine to modulate it.

To explore lambda cyhalothrin induced hepatotoxicity in rats and the attenuating effect of taurine in this situation.

To investigate lambda cyhalothrin induced reproductive damage in both male and female rats and its amelioration by taurine.

To investigate whether there is any correlation between lambda cyhalothrin toxicity and oxidative stress in rat.

To detect lambda cyhalothrin elicited histopathological changes in rat hepatic and reproductive tissues and to find out the alleviative effect of taurine.

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Chapter-3

Lambda cyhalothrin elicited dose response toxicity on haematological, hepatic and gonadal biomarkers in rat

Abstract

Extensive application of pesticides is usually accompanied with serious problems of pollution and health hazards. Lambda cyhalothrin (LCT), a type II synthetic pyrethroid is widely used in agriculture, home pest control and protection of foodstuff. This study designed to evaluate the dose dependent haematological, hepatic and gonadal toxicity of LCT at different dose levels in Wistar rat. Rats were exposed to different doses of lambda cyhalothrin over a period of 14 consecutive days. Exposure to LCT produced ataxia, agitation, rolling and also tremors which were considered as the signs of toxicity. Significant decrease in erythrocyte count, haemoglobin percentage, seminal fructose concentration, hepatic and testicular reduced glutathione content was observed. Increase in leukocyte count, serum aspartic and alanine transaminase, hepatic and testicular malondialdehyde, testicular and ovarian cholesterol after LCT treatment were seen in male rats at the dose level of 10.83 mg/kg body wt. (1/7th LD_{50}). Elevated ovarian cholesterol and MDA and reduced 3β-hydroxy steroid dehydrogenase and reduced glutathione level were also observed in lambda cyhalothrin exposed female rat at the dose level of 6.29 mg/kg body wt. (1/9th LD_{50}). The present study showed that LCT exposure produced haematological, hepatic and gonadal toxicity at 10.83 mg/kg body wt. (1/7th LD_{50} dose) in male and at 6.29 mg/kg body wt. (1/9th LD_{50} dose) in female rat and also on above dose levels.

Rini Ghosh, Tuhina Das, Anurag Paramanik, Sujata Maiti Choudhury. Lambda cyhalothrin elicited dose response toxicity on haematological, hepatic, gonadal and lipid metabolic biomarkers in rat and possible modulatory role of taurine. Toxicology and forensic medicine. 2016; 1(2):42-51.

3.1. Introduction

Toxicology is concerned with toxicity by any chemical or compound by intentional or accidental exposure to living organisms. Toxicity is the degree to which a substance can harm an individual. Substances are potentially toxic depending on the quantity. This is why toxicity determination is primarily focused on determining the type and degree of harm, caused by different amounts of a substance. Dose-time-dependent tests form the basis of the experimental toxicology. Excess of any compound is harmful to life and regarded under toxicity studies. In modern times, use of chemicals and compounds that will gather or daily exposed to humans, are harmful in different ways. The contact of xenobiotics with the biological system is a multifaceted incident, which comprises of interplay between the environment, the host and the chemical substance. Chemical pollution as a result of pesticide introduction has been considered as one of the factors for the decline of natural fauna. Unsystematic application of pesticides is generally accompanied with serious issues regarding pollution and health hazards .

3.1.1. Principal types of animal-based toxicity tests

Effects of toxicity may arise in short term (acute effects), or after repeated exposure over a long period (sub-acute or chronic effects). For finding of toxicity, acute toxicity tests (single dose), sub-acute toxicity test (daily dose 14 to 28 days), sub-chronic toxicity test (daily dose – up to 90 days), chronic toxicity test (daily

dose – up to 12 months) are performed using rodent models(Bhardwaj and Gupta).

3.1.2. Acute toxicity test

First, the acute toxicity tests are conducted which provide critical data on the relative toxicity likely to derive from a single or brief exposure. To determine the therapeutic index the acute toxicity test was performed, which is the ratio between the lethal dose and the pharmacologically effective dose, in the same species and strains (LD_{50}/ED_{50}). The compound is safe when the index is higher. The acute test may provide initial information on the mode of toxic action of a substance and to establish the LD_{50} value that provide indices of potential types of drug activity(Bhardwaj and Gupta,2012).

3.1.3. Sub-acute and chronic toxicity test

The data from these studies offer important information on the cumulative exposure of target organs, and on general health hazards likely to occur as a result of repeated low-dose exposure to a chemical (Bhardwaj and Gupta,2012).

3.1.4. Repeated-dose toxicity studies

These studies are performed to determine the side effects, arise from repeated administration of a drug at lower doses. The main objectives are (i) to determine the toxicity that build up only after a certain length of continuous exposure (ii) to identify which organs are mostly affected and (iii) to decide the doses at which each one effect occurs.

3.1.5. Pyrethroid induced different toxicity studies

Despite the claims of low mammalian toxicity of pyrethroid, several researches showed the toxicological evidence of pyrethroid among various species of animals (Khan et al., 2012). Because of the lipophilic nature, pyrethroids are easily absorbed through gastrointestinal and respiratory tract and this also make them easier to be stored in the lipid rich internal tissues like body fat, skin, liver, kidney, central and peripheral nervous systems. For the analysis of the functional status of animals to suspected toxic agents, haemato-biochemical parameters are required. It may act as strong evidence against toxicity of contaminated pyrethroid insecticides. Recent reports have displayed that exposure to pyrethroid causes significant modifications in haematological findings (Khan et al., 2009).When toxicants enter the body; the liver is the first organ that encountered them. The liver accumulates vast amount of pyrethroid residues as it is the primary site for pyrethroid metabolism. A huge number of man-made chemicals such as pesticides is responsible for liver damage (Jaeschke et al., 2002) A number of investigations also told us that in experimental animals, pesticides badly affect the testicular functions **(**Song et al., 2008; Lifeng et al., 2006; Yousef et al.,2003**)** as well as they are potent endocrine disruptors (Zhang et al.,2007).Pesticides cause oxidative stress that leads to free radicals generation and DNA fragmentation (Anadon et al.,2006).

3.1.6. Toxic impact of lambda cyhalothrin

It has showed that lambda cyhalothrin (LCT), a type II pyrethroid pesticide is fairly toxic for mammals (Anadon et al.,2006) and extremely toxic for fish,

aquatic invertebrates and bees. LCT at low concentrations can lead to death in these species (Schroer et al.,2004). The degree of concentration and nature of solvent are significant for the toxicity of lambda cyhalothrin (Meister, 1992). Study was carried out to calculate the median lethal dose (LD_{50}) of LCT. Sharma et al. reported that Oral LD_{50} of male and female rats were found to be 75.85-mg/kg body wt. and 56.695-mg/kg body wt. respectively (Sharma et al.,2010).

For any pesticide, the exposure concentration is important in the alteration of its toxicity. So in the present study, LCT were administered at different concentrations to find out the exact toxic dose levels of LCT which can produce haematological, hepatic and gonadal toxicity in male and female Wistar rat.

3.2. Materials and methods

3.2.1. Chemicals and reagents

Lambda cyhalothrin 5% emulsifiable concentrate (EC) was procured from RPC Agro Industries, Kolkata. Taurine was purchased Sigma Aldrich Inc. USA.Hydrogen peroxide(H_2O_2), Red blood cell dilution fluid, White blood cell dilution fluid, Drabkin's diluents, Hydrochloric acid (HCl), Sulfo salicylic acid, Dithionitrobenzoic acid(DTNB), Tris-HCl, Pyrogallol, Thiobarbituric acid(TBA), *n*-Butanol-pyridine, Acetate buffer, Fructose,Zink sulphate($ZnSO_4$),Ferric chloride($FeCl_3$), Glacial acetic acid, Cholesterol,Sodium chloride(NaCl), Phosphate buffer(Pbs),Disodium hydrogen phosphate(Na_2HPO_4),Potasium di hydrogen phosphate (KH_2PO_4),Sodium di hydrogen phosphate(NaH_2PO_4), Sodium hydroxide(NaOH), DL-alanine, α-Ketoglutaric acid, Glycerol,

Ethylenediaminetetraacetic acid(EDTA), Pentobarbital sodium, Tetra sodium pyrophosphate(TNaPP), Nicotinamide adenine dinucleotide, Testosterone, Dehydroepiandrosterone (DHEA) and other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA; Himedia India, Ltd., Mumbai, India; and Merck India, Ltd., Mumbai, India for the experimentation.

3.2.2. Animal selection

For the current study, mature Wistar male and female albino rats (weighing 130-150 g) were taken. The laboratory rat belongs to the order Rodentia. The word rodent came from the Latin word *rodere*, which means "to gnaw". The laboratory rat is largely used in toxicological, genetic, nutritional, behavioural and environmental studies. The small size of rats and also the ease of housing and caring for them are the reasons behind the preference of making them pets as well as research animals. The use of human in experiments is rightfully restricted for ethical and economic reasons, respectively. Hence, rats have long been used as models of mammalian health and disease. Undoubtedly, the rat remains an excellent model for the studies of human embryo implantation and early pregnancy disorders.

3.2.3. Animal care

The animals were housed in polypropylene cages at an ambient temperature of 25°±2°C with 12 hrs light-dark cycle. The rats were acclimatized for one week prior to different treatments. The standard laboratory feed and water were

supplied throughout the period of experimentation. Experimental protocol was granted by the institution's animal ethical committee.

3.2.4. Experimental design

A commercial formulation of lambda cyhalothrin 5% emulsifiable concentrate (EC) 'Karate' was used for the study. Dilution of lambda cyhalothrin was done in distilled water to acquire the test concentrations. The test concentrations of lambda cyhalothrin were measured from the percentage of the active ingredient present in above mentioned commercial formulation of lambda cyhalothrin.

Healthy mature Wistar rats (n = 6) of either sex selected by random sampling technique were used for the study. The rats were kept fasting for overnight providing only sufficient water, after which lambda cyhalothrin were administered orally at the dose level of 6.89 mg /kg body wt (i.e. $1/11^{th}$ LD_{50} dose for male) and 5.15 mg/kg body wt.(i.e. $1/11^{th}$ LD_{50} dose for female) by oral feeding needle and observed for 14 days. Sharma et al. reported that oral LD_{50} dose of lambda cyhalothrin for mature male and female rats were 75.85 and 56.69 mg/kg body wt. respectively(Sharma et al.,2010). The procedure was repeated for the dose levels of 7.58($1/10^{th}$ LD_{50}), 8.42($1/9^{th}$ LD_{50}), 10.83($1/7^{th}$ LD_{50}), 15.17 ($1/5^{th}$ LD_{50}), 18.96($1/4^{th}$ LD_{50}), 25.28(1/3rd LD_{50}) mg/kg body wt. (Ecobichon, 1997) for male rats. For female rats, lambda cyhalothrin were administered orally at the dose levels of 5.66 ($1/10^{th}$ LD_{50}), 6.29 ($1/9^{th}$ LD_{50}), 8.09 ($1/7^{th}$ LD_{50}), 11.33 ($1/5^{th}$ LD_{50}), 14.17($1/4^{th}$ LD_{50}), 18.89(1/3rd LD_{50}) mg/kg body weight. Dose solutions were freshly prepared immediately before usage. At the end of the doses, the animals were fasted overnight, anesthetized with pentobarbital sodium

and sacrificed by cervical dislocation on 15th day. Samples were collected and stored at -80^{0}C until analysis.

3.2.5. Study on body weight

Body weight of individual overnight fasting male and female animals was taken regularly before the administration of lambda cyhalothrin. After 24 hrs of the treatment of the last dose, all animals were weighed and then sacrificed.

3.2.6. Measurement of haematological parameters in male rat

3.2.6.1. Erythrocyte count

Erythrocyte count was done by the dilution of blood with red blood cell dilution fluid (1:200) (Wintrobe,1967) and expressed as $\times 10^6 / mm^3$.

3.2.6.2. Estimation of haemoglobin percentage

The haemoglobin percentage was measured by cyanmethemoglobin method (Dacie and Lewis, 1975). Using Drabkin's diluents as a blank, the optical density was measured at 540 nm.

3.2.6.3. Total leukocyte count

Blood was diluted (1:20) with white blood cell dilution fluid and total leukocyte were counted using Neubaur haemocytometer (Wintrobe, 1967).

3.2.7. Study of hepatic biomarkers in male rat

3.2.7.1. Assay of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT)

For SGOT sample, 1 ml of buffer substrate (2.66gm aspartic acid, 60mg α-ketoglutaric acid and, 20.5ml of 1(N) NaOH and 100ml of volume was made by 0.1M phosphate buffer, pH7.4) and for SGPT sample, 1 ml of buffer substrate (1.78 gm DL-alanine, 30mg α-ketoglutaric acid, 20 ml of 0.1M phosphate buffer and 1.25ml of 0.4(N) NaOH) were taken and waited for 5min at 37^0C. Then 0.2ml of serum sample was mixed and incubated at 37^0C for 60 min. To prepare standard, 0.2 ml of working standard (200µM/100 ml) was taken in a test tube and 0.8ml of buffer substrate was added. For blank, 1.0 ml of buffer substrate was taken. In each of sample, standard and blank test tubes, 1ml of DNPH solution were added and waited for another 20 min. Then 10ml of 0.4(N) NaOH was mixed and waited for 10 minutes. Readings were taken at 520 nm in spectrophotometer (Goel, 1988) (UV-245 Shimadzu, Japan).

3.2.7.2. Assay of hepatic malondialdehyde (MDA)

Malondialdehyde was determined by the mixing of 1 ml of sample with 0.2ml of 8.1% sodium dodecyl sulfate, 1.5ml of acetate buffer (20%, pH-3.5) and 1.5 ml of aqueous solution of thiobarbituric acid (0.8%) and the mixtures were boiled for 60 min at 95˚C. After heating when the red pigment was produced, that was extracted with 5 ml of n-butanol-pyridine (15:1) and centrifuged at 5000 rpm for 10 min at room temperature. The optical density of supernatants was measured at 535nm (Ohkawa, 1979)

3.2.7.3. Estimation of hepatic reduced glutathione (GSH) content

Reduced glutathione was estimated by mixing of 100µl of sulfosalicylic acid with 200µl of sample. Then the mixture was allowed for centrifugation at 3000 rpm for 10 min. With the supernatant, 1.8 ml of DTNB was included and shaken well (Griffith, 1981). Final reading of the supernatant was noted at 412 nm.

3.2.8. Study of male reproductive parameters

3.2.8.1. Measurement of seminal fructose concentration

In a centrifuge tube, 1ml of diluted seminal plasma (five time dilution was done by mixing 0.1ml of seminal plasma with 4.9ml of distilled water) was added with 0.3ml of 1.8gm% $ZnSO_4$ and 2ml of 0.1M NaOH. After 15min, the mixture was centrifuged at 2000g to obtain the supernatant. Then seminal fructose concentration was measured by taking 0.5ml of supernatant as sample, 0.5ml of 0.14mM and 0.28mM fructose solutions as two standards and 0.5 ml of distilled water as blank. Then, 0.5 ml of indole reagent and 5 ml of concentrated HCl were added to each test tube. The test tubes were then incubated at 50^0C for 20 min and were cooled in ice water and then in room temperature (Karvonen and Malm,1955). The reading was taken at 470 nm in spectrophotometer (UV-245 Shimadzu, Japan).

3.2.8.2 Estimation of testicular cholesterol

Testicular tissue was homogenized with 0.5% $FeCl_3$ solution at a conc. of 20mg/ml. Supernatant was collected after centrifugation of the homogenized tissue at 2000rpm for 10 min. Then 0.1ml of supernatant was added with 6ml of

glacial acetic acid to prepare sample. Simultaneously 5.9 ml of glacial acetic acid was added with 0.1ml of working standard and 0.1ml of distilled water to prepare standard. Blank was prepared by mixing 6 ml of glacial acetic acid and 0.1ml of distilled water. Then 4ml of color reagents were added to each mixed vigorously and stand for 20 minutes for spectrophotometric reading at 570nm against blank (Zlatkis et al., 1953).

3.2.8.3. Assay of testicular malondialdehyde (MDA) and reduced glutathione (GSH) content

Testicular malondialdehyde (MDA) and reduced glutathione (GSH) content were measured by the respective above mentioned methods (Ohkawa et al,1979; Griffith, 1981).

3.2.9. Study of female reproductive parameters

3.2.9.1. Estimation of ovarian cholesterol and activity of ovarian Δ^5, 3β-hydroxy steroid dehydrogenase (Δ^5, 3β-HSD)

Ovarian cholesterol was estimated according to Zlatkis et al. (Zlatkis et al., 1953). To estimate the activity of ovarian Δ^5, 3β-HSD homogenizing media was prepared by 20ml of glycerol, 0.01M EDTA in 0.05M phosphate buffer in 100ml with redistilled water. Tissue homogenate (20mg/ml homogenizing media) was centrifuged at 10,000 rpm for 30 min at 4°C in a cold centrifuge. 1ml of supernatant was mixed with 0.9 ml distilled water,1 ml of sodium pyrophosphate buffer and 40 µl of dehydroepiandrosterone (DHEA). After addition of 0.1ml of

NAD, the activity of Δ^5,3β-HSD was measured at 340 nm against a blank (without NAD) (Talalay, 1962).

3.2.9.2. Assay of ovarian malondialdehyde (MDA) and reduced glutathione (GSH) content

Ovarian malondialdehyde (MDA) and reduced glutathione (GSH) content were measured by the respective above mentioned methods (Ohkawa, 1979; Griffith, 1981)

3.2.10. Statistical analysis

The data was expressed as Mean±SEM, The differences between the means of each group were tested using a one-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA). $P<0.05$ was considered to indicate a statistically significant difference.

3.3. Results

3.3.1. General observations

After each treatment of lambda cyhalothrin, the animals were kept under observation at least once in 30min interval. Special attention was given up to 4hrs. Rats those were exposed to different doses of lambda cyhalothrin over a period of 14 days produce ataxia, agitation, rolling and also tremors which were considered as the signs of toxicity up to eleventh day of treatment after which the symptoms were reduced.

3.3.2. Changes in body weight, food and water intake

Significant changes in final body weight were found at the dose level of 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50}) in male rats and at the dose level of 5.15 mg/kg body wt. ($1/11^{th}$ LD_{50}) in female rat (fig 3.1). No differences in food and water consumption were seen in last consecutive four days of treatment in the experimental schedule but there was little non-significant alterations observed in food consumption at the exposure dose levels of $1/4^{th}$ and $1/3^{rd}$ LD_{50}.

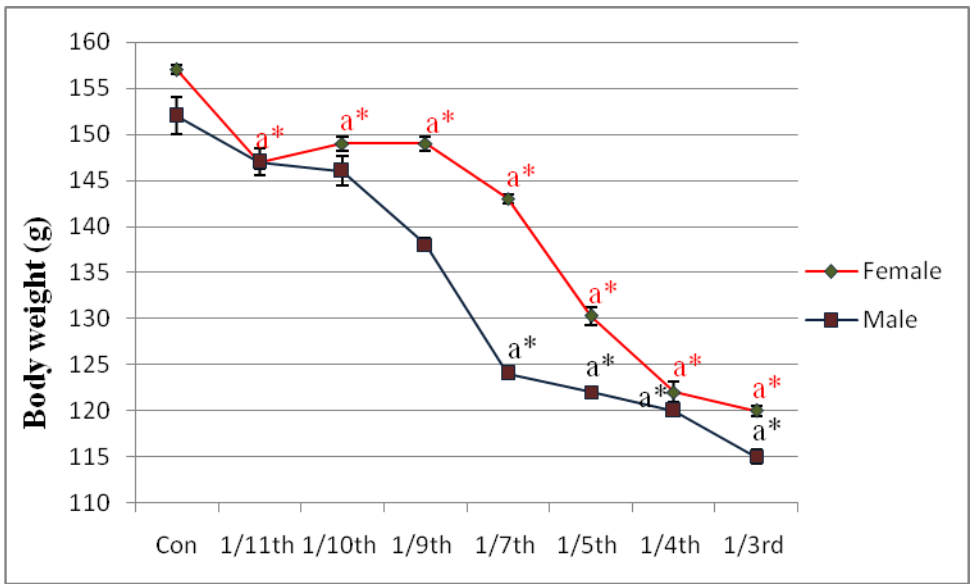

Figure- 3.1.effect of lambda cyhalothrin on body weight of male and female rat. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA. Superscript a control group versus all other groups (* indicates $p<0.001$). (For male and female the LD_{50} values are different which are mentioned in materials and methods section).

3.3.3. Effect on haematological parameters in male rat

To find out the dose dependent effect of LCT, some clinically significant biochemical parameters from each system were studied. From the study it was seen that total erythrocyte count was decreased significantly ($p<0.05$) from the dose level of 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50}) to 25.28 mg/kg body wt. ($1/3^{rd}LD_{50}$) in male rat (Table3.1). No significant changes were found below 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50} dose). Maximum toxic effect of LCT was exhibited at the 15.17 mg/kg body wt. ($1/5^{th}$ LD_{50} dose) without any mortality. Interestingly, the rate of mortality was increased from the dose level of 18.96 mg/kg body wt. ($1/4^{th}$ LD_{50}). Similar results were found in case of haemoglobin percentage ($p<0.01$). Table 3.1 displays the significant ($p<0.001$) increase in leukocyte count in lambda cyhalothrin treated male rats from the dose level of $1/7^{th}$ LD_{50} to $1/3^{rd}$ LD_{50}.

3.3.4. Effect on hepatic biomarkers in male rat

3.3.4.1. Activities of serum glutamate-oxaloacetate transaminase (SGOT) and serum glutamate-pyruvate transaminase (SGPT)

The effects of LCT on SGOT, SGPT are shown in fig. 3.2.(A,B) respectively. The activity of these two important hepatic transaminase enzymes were increased significantly ($p < 0.001$) with increase in the concentration of LCT as compared to the control rats. No significant alterations were noted below 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50} dose) in male rat.

Table 3.1. shows the effect of lambda cyhalothrin on haematological parameters in male rat.

Group	Erythrocyte count($\times 10^6$ / mm^3)	Hb percentage(gm/dl)	Leukocyte count($\times 10^6$/µl)
Control	7±0.3	14±0.5	6±0.17
$1/11^{th}$ LD_{50} dose	7.2±0.2	14±1.1	6±0.11
$1/10^{th}$ LD_{50} dose	7±0.1	13.3±1.4	6.03±0.14
$1/9^{th}$ LD_{50} dose	7±0.06	13.6±0.6	6.03±0.15
$1/7^{th}$ LD_{50} dose	6±0.2a*	10±0.6a**	8±0.11a***
$1/5^{th}$ LD_{50} dose	5.9±0.1a*	8±0.6a**	9.2±0.12a***
$1/4^{th}$ LD_{50} dose	5.9±0.2a*	8±0.6a**	9.1±0.17a***
1/3rd LD_{50} dose	5.9±0.1a*	8±1.1a**	9.2±0.12a***

Results are expressed as Mean±SEM. Analysis is done by one way ANOVA. Superscript a Control group versus all other groups (*indicates $p<0.05$,** indicates $p<0.01$, *** indicates $p<0.001$).

3.3.4.2. Hepatic lipid peroxidation and glutathione content

Hepatic malondialdehyde (MDA) and reduced glutathione content in the control and experimental groups of male rats are shown in figure 3.2(C and D). MDA was found to be significantly increased ($p<0.01$) whereas reduced glutathione content

was decreased significantly ($p<0.001$) at dose-dependent manner from the dose level of 10.83 mg/kg body wt. ($1/7^{th}$ LD50 dose) onwards.

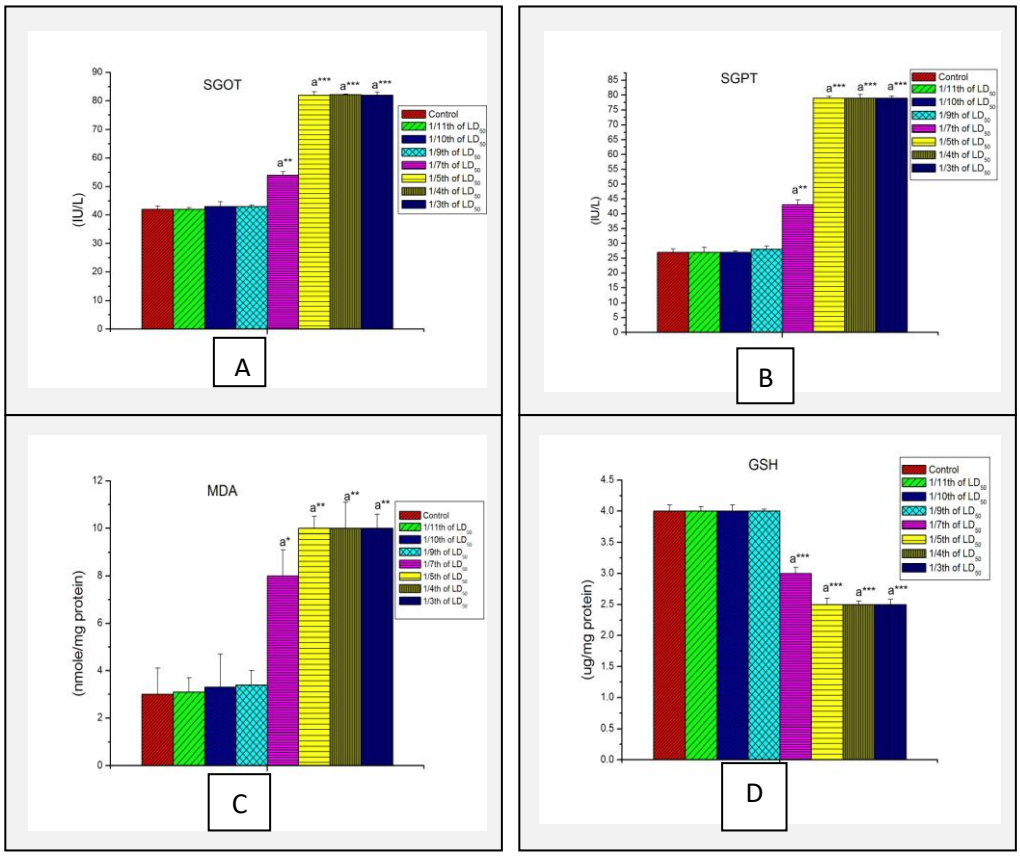

Figure-3.2. Effect of lambda cyhalothrin on some hepatic biomarkers in male rat. A= Effect on SGOT, B= Effect on SGPT, C= Effect on liver MDA, D= Effect on liver GSH. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA. Superscript a Control group versus all other groups (*indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$)

3.3.5. Effect on male reproductive system

3.3.5.1. Effect of LCT on seminal fructose concentration and testicular cholesterol.

As shown in table 3.2, the LCT induced reproductive toxicity by reducing seminal fructose concentration ($p<0.001$) and elevating testicular cholesterol level ($p<0.05$).

3.3.5.2. Impact of LCT on testicular oxidative stress and antioxidant status

Table 3.2, also shows the effect of LCT on MDA and GSH .Testicular MDA was found to be increased significantly ($p<0.01$) in response to LCT treatment whereas a significant decrease ($p<0.001$) in testicular GSH level was also noted. Significant changes were detected from the dose level of 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50} dose) onwards.

3.3.6. Effect on female reproductive function

3.3.6.1. Ovarian cholesterol content and activities of steroidogenic key enzyme

LCT induced ovarian toxicity was exhibited by significant elevation in ovarian cholesterol content and diminution in the activities of ovarian steroidogenic enzyme at the dose level of 6.29 mg/kg body wt. ($1/9^{th}$ LD_{50} dose) and were continued towards increased dose levels of LCT (figure 3.3A, B).

3.3.6.2. Effect of LCT on malondialdehyde (MDA) and reduce glutathione (GSH) level

LCT intoxicated rats shows a marked dose-dependent increase ($p<0.001$) in the lipid peroxidation, specified in term of MDA (fig-3.3C). Decline in GSH levels in LCT treated rat may also an indication of oxidative stress as GSH is used for the

detoxification of reactive toxic substances resulted from LCT exposure at the dose level of 6.29 mg/kg body wt. ($1/9^{th}$ LD_{50} dose) (fig-3.3D).

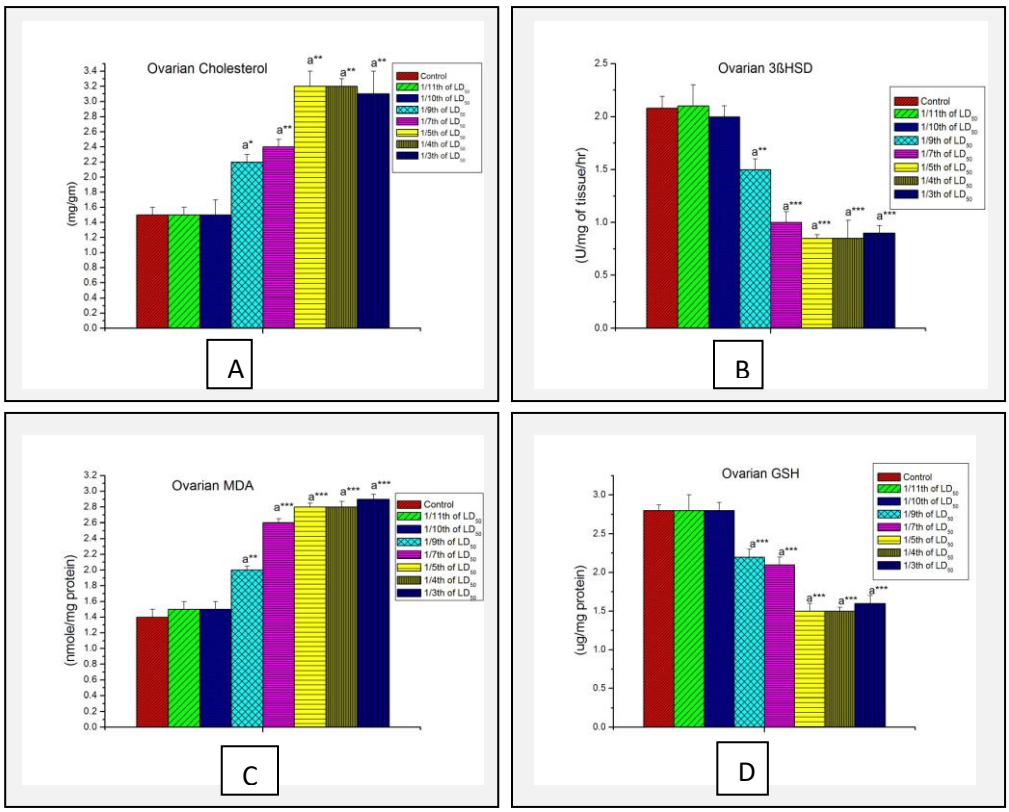

Figure-3.3. Effect of lambda cyhalothrin on female reproductive biomarkers. A= Effect on ovarian 3β HSD, B= Effect on ovarian cholesterol, C= Effect on ovarian MDA, D= Effect on ovarian GSH. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA. Superscript a Control group versus all other groups (*indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

Table 3.2. shows the effect of lambda cyhalothrin on some male reproductive parameters.

Group	**Seminal fructose concentratio** nmole of fructose/lit of seminal plasma	**Testicular Cholesterol (** mg/gm**)**	**Testicular MDA(** nmole/mg protein**)**	**Testicular GSH** (µg/mg protein)
Control	20±0.3	1.4±0.1	2.5±0.2	3.5±0.03
$1/11^{th}$ LD_{50}	20±0.6	1.4±0.2	2.6±0.2	3.4±0.12
$1/10^{th}$ LD_{50}	20±0.5	1.4±0.05	2.6±0.1	3.4±0.03
$1/9^{th}$ LD_{50}	19.5±0.2	1.4±0.1	2.6±0.3	3.4±0.09
$1/7^{th}$ LD_{50}	18±0.1a**	1.9±0.1a*	4±0.2a*	2.9±0.12a**
$1/5^{th}$ LD_{50}	15±0.3a***	2.4±0.3a*	5±0.3a**	2.36±0.22a***
$1/4^{th}$ LD_{50}	15±0.2a***	2.4±0.1a*	5±0.2a**	2.36±0.25a***
$1/3^{rd}$ LD_{50}	15±0.4a***	2.4±0.05a*	5±0.5a**	2.36±0.24a***

Results are expressed as Mean±SEM. Analysis is done by one way ANOVA. Superscript a Control group versus all other groups (*indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

3.3.7. Selection of experimental dose of lambda cyhalothrin

From these above findings for male rats, 10.83($1/7^{th}$ LD_{50} dose) and 15.17($1/5^{th}$ LD_{50} dose) mg/kg body wt and for female rats, 6.29 mg/kg body wt. (1/9th LD_{50}

dose) and 11.33($1/5^{th}LD_{50}$dose) mg/kg body wt. were selected as effective doses for our further studies. At the $1/7^{th}LD_{50}$ dose, significant alterations were seen in different systemic biochemical parameters of male rat. In female rat first significant toxic response was found at the dose level of 6.29 mg/kg body wt. (1/9th LD_{50} dose). So in case of male and female rat, $1/7^{th}LD_{50}$ dose and $1/9^{th}LD_{50}$ dose were considered as effective low dose. Better toxic response was found at $1/5^{th}LD_{50}$dose for both male and female rat after which animal mortality was increased. So this dose was considered as effective high dose for future studies.

3.4. Discussion

The current study was carried out to evaluate the toxic effects of LCT on the different system of male, female Wistar rats. In toxicological studies, body weight is a basic criterion for the assessment of organ toxicity. In the current study, oral administration of LCT brought about an important reduction in body weight of both male female rats.

A notable change in erythrocyte counts, haemoglobin percentage and leukocyte were found in lambda cyhalothrin exposed rats and these show the physiological disruption in the rat haemopoietic system. Haemolysis of blood cells (Mandal et al., 1986) is, perhaps, responsible for the decrease in erythrocyte counts in LCT intoxicated rats which in turn is responsible for the reduction in haemoglobin percentage. In the current study, decreased biosynthesis of haem in bone marrow may also cause significant reduction in haemoglobin percentage. Increased leukocyte count in lambda cyhalothrin treated group may go up because of the

immediate activation of the immune system of the body (Yousef et al.,2003) against lambda cyhalothrin.

SGOT, SGPT are two major hepatic enzyme biomarkers of hepatotoxicity. In the current study, a notable increase in SGOT, SGPT level after LCT treatment at different concentration shows active utilization of amino acids in energy-yielding metabolic processes like gluconeogenesis. Pyrethroids caused oxidative stress by the elevation of lipid peroxidation products (Banerjee et al.,1999; Goel et al.,2005). Elevated MDA level in LCT intoxicated rat liver was in an agreement with the above statement. This type of result has also indicated that LCT produces hepatic injury and pathogenesis through the generation of free radicals and by the alteration of antioxidant system. Decrease in cellular GSH concentrations may be through low production or non-enzymatic oxidation of GSH to glutathione disulfide (GSSG) because of oxidative stress in LCT treated rat liver at different dose levels.

Here the results also display that the male reproductive dysfunction after LCT exposure at different dose levels. The reduction in fructose content in seminal fluid gathered from LCT intoxicated rats drew attention towards the secretory ability of seminal vesicles as well as the nutritive potential for the semen. Previous studies (Mansour and Mossa, 2009; Mossa et al.,2013) stated that ROS were involved in the toxicity of various pesticides. ROS impedes steroidogenesis by disturbing cholesterol transport to mitochondria (Hales et al.,1999). LCT raised testicular cholesterol at the dose level of 10.83 mg/kg body wt. (1/7th LD_{50}

dose) and also on above dose levels. An increase in MDA, the mostly used biomarker of lipid peroxidation, shows serious cellular damage, inhibition of cellular functions and several enzymes (Thirunavukkarasu et al., 2001). GSH, one of the most potent biological molecules, plays a crucial role in the detoxification of the reactive toxic metabolites. A considerable decline in GSH levels in liver LCT treated rat may be because its use to challenge the common oxidative stress. The results display that the increase in ovarian cholesterol and significant decrease in ovarian steroidogenic enzyme activity pointed out towards the LCT induced ovarian toxicity through diminishing steroidogenesis. The maintenance of high redox potential is necessary for assuring the reproductive system functions in a healthy organism (Agarwal et al., 2012). Increased ovarian MDA and decreased ovarian GSH level in LCT intoxicated rats compared to control may be a sign of oxidative stress due to LCT exposure at the dose level of 6.29 mg/kg body wt. (1/9th LD_{50} dose) and also on above dose levels.

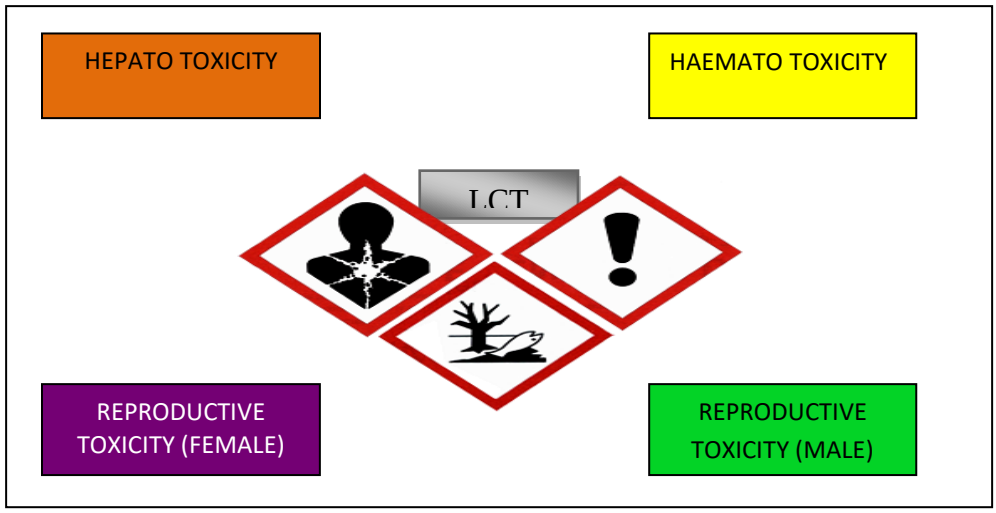

Figure 3.4. schematic representation of lambda cyhalothrin induced toxicity.

3.5. Conclusion

The above study showed that LCT exposure produced haematological, hepatic and gonadal toxicity at 10.83 mg/kg body wt (1/7th LD_{50} dose) in male and at 6.29 mg/kg body wt. (1/9th LD_{50} dose) in female rat and also on above dose levels.

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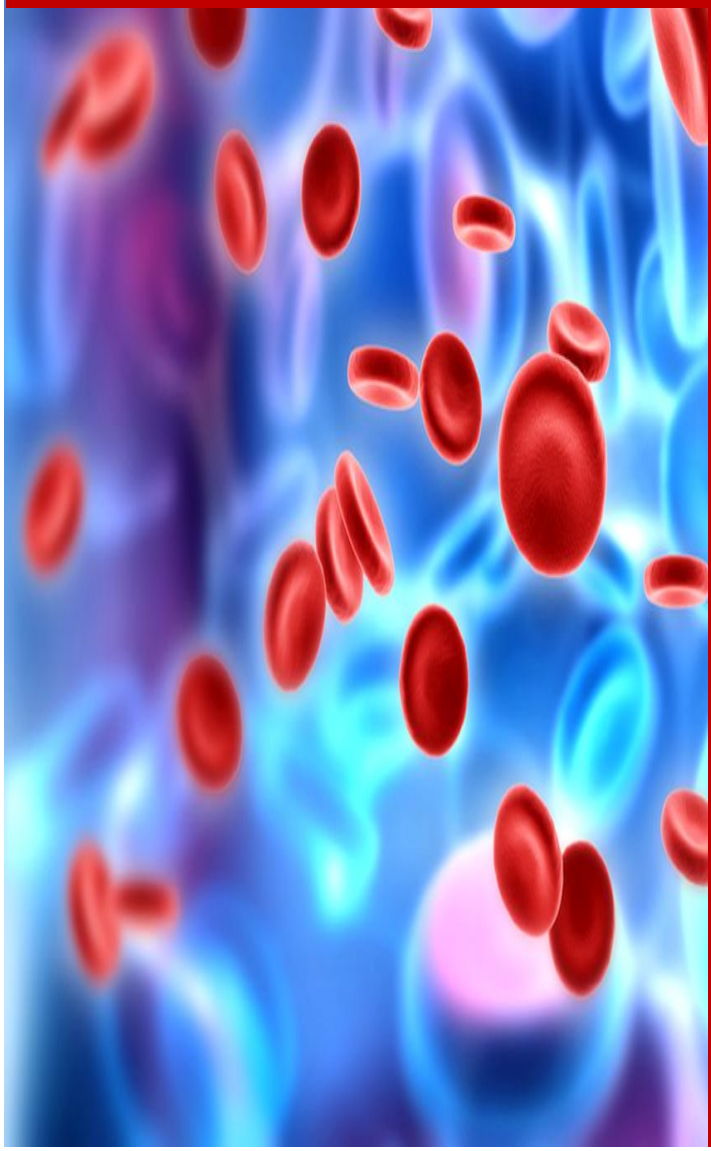
Chapter-4

Effect of lambda cyhalothrin on haematological parameters of rat, especially on alterations in morphology and oxidative stress in erythrocytes and the attenuating role of taurine



Abstract

Lambda cyhalothrin, a third generation type II pyrethroid, is used predominantly in agriculture production and animal husbandry. A study was conducted to investigate lambda cyhalothrin induced oxidative stress, morphological changes of erythrocytes and other hematological biomarkers in rat and its amelioration by taurine,2-amino ethane-sulfonic acid, a β-amino acid. Rats were randomly divided into six groups and lambda-cyhalothrin was orally administered at two dose levels (10.83 and 15.17mg/kg body wt), singly or combined with pretreated taurine (50mg/kg body wt) for 14 consecutive days. Treatment of lambda cyhalothrin resulted in an increase in malondialdehyde, oxidized glutathione level and depletion of reduced glutathione level, superoxide dismutase, catalase, glutathione-s-transferase and glutathione peroxidase activity in erythrocyte compared to control. Scanning electron microscopic studies showed a marked alteration in the morphology of lambda cyhalothrin treated erythrocytes. Lambda cyhalothrin exposure also showed a significant decrease in erythrocyte count, haemoglobin percentage, haematocrit and red cell indices, whereas a significant increase in white blood cells and lymphocyte count were observed. However, pretreatment with taurine significantly restored the above said parameters. These findings revealed that lambda cyhalothrin exposure produced oxidative stress, morphological changes of erythrocytes and other hematological biomarkers and its amelioration was accomplished by taurine through its ROS scavenging activity.

Rini Ghosh, Ananya Pradhan, Pralay Maity, Kuladip Jana, Sujata Maiti Choudhury. Lipid peroxidative damage, alterations in antioxidant status and morphology in rat erythrocytes on lambda-cyhalothrin exposure and its attenuation by taurine.Toxicology and environmental health science-ACCEPTED

4.1. Introduction

Haematology symbolizes the study of the numbers and morphology of the cellular components of the blood, the red cells (erythrocytes), white cells (leucocytes), and the platelets (thrombocytes) , a good indicators of the physiological status of animals (Khan and Zafar, 2005). Haematological studies are useful in the diagnosis of many diseases as well as the damage of blood cells, (Togun et al., 2007), also play a significant role in toxicological research (Siakpere et al., 2008). These are used to decide systemic relationship and physiological adaptations of animals and commonly measured variables. Many reports can be found regarding the pyrethroid-induced biochemical and physiological changes in target organs but not much attention has been paid to the effects of pesticides on non-target organisms. To understand the various status of the body and to determine stresses due to environmental, nutritional and/or pathological factors, haematological parameters are often used (Afolabi et al.,2011).

4.1.1. Haematological components and their functions

Haematological components consist of red blood cells that serve as a carrier of haemoglobin, white blood cells or leucocytes that fight against infections, platelets that are associated with blood clotting. Mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration are valuable in monitoring toxicity especially with feed constituents that affect blood as well as the health status of farm animals (Etim et al.,2014). Haemoglobin is the iron-containing oxygen-transport metalloprotein in the red blood cells of all vertebrates exception of

the fish family (Sidell and O' Brien, 2006). The percentage (%) of red blood cells in blood is known as packed cell volume (PCV) or haematocrit (Ht or Hct) or erythrocyte volume fraction (EVF) (Purves et al., 2003), involved in the transport of oxygen and absorbed nutrients.

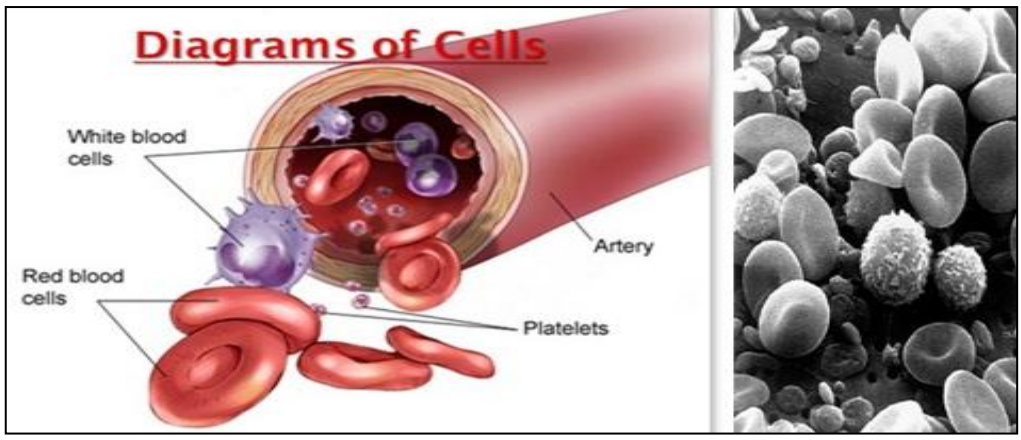


Figure 4.1.Haematological components

Source: American society of oncology,2004©

4.1.2. Haematological changes induced by pyrethroids in bird, fish and mammalian species

Pyrethroids are the major source of potential environmental hazards not only to birds, fish, and other animals but also to humans when they become part of food chains (Abd-Alla et al., 2002). Few workers reported non-significant changes in total erythrocyte count, haemoglobin percentage and haematocrite value in various animals treated with cypermethrin (Haratym-Maj, 2002; Sayim et al., 2005) whereas few studies reported that animals were suffering from anemia when treated with cypermethrin (Shah et al., 2007; Khan et al., 2009). In female mice, deltamethrin led to anemia which indicated downfall of erythropoiesis and haemoglobin synthesis.

4.1.3. Oxidant-antioxidant balance in erythrocyte

Oxidative stress is a condition that takes place by the disruption of oxidant-antioxidant balance which may lead to deleterious cellular damages (Ha et al., 2010). Antioxidants have protective role against free radicals and oxidative attack. By converting oxidants into non-toxic molecules it shields the organism from oxidative stress (Gate et al.,1999). It has been already confirmed that erythrocytes and their membranes are very much influenced by oxidative damage because of the presence of unsaturated fatty acids that are continuously exposed to high concentration of oxygen (Ney et al.,1990).Despite their well-equipped antioxidant defense system, erythrocytes may be oxidatively injured, because of exposure to toxic chemicals like pyrethroids.

4.1.4. Effects of lambda cyhalothrin on haematological system

Lambda cyhalothrin (LCT) appears to be a third generation type II pyrethroid, displays significant toxicological changes in rabbit peripheral blood lymphocytes (Georgieva, 2006), but there is no information regarding the LCT-induced morphological changes in mammalian erythrocytes.

4.1.5. Role of taurine in haematological system

Taurine, 2-aminoethanesulfonic acid is reported to find clinical application against a variety of pollutants where cellular damage is a result of reactive oxygen species (Issabeagloo et al.,2011). Through its cytoprotective, osmoregulatory, and membrane stabilization properties, taurine shows the ability to mitigat chlorpyrifos and lead induced hematotoxicity in the rats (Akande et al, 2014). The current study is designed to examine the lambda cyhalothrin induced damages inflicted on the rat

blood cells and to get to know the possible ameliorative role of taurine in these situations.

4.2. Materials and methods

4.2.1. Chemicals

Lambda cyhalothrin(LCT) 5% emulsifiable concentrate (EC) was procured from RPC Agro Industries, Kolkata. Taurine was purchased from Sigma Aldrich Inc., USA. Hydrogen peroxide(H_2O_2), Red blood cell dilution fluid, White blood cell dilution fluid, Drabkin's diluents, Hydrochloric acid (HCl), Sulfo salicylic acid, Dithionitrobenzoic acid(DTNB), Tris-HCl, Pyrogallol, Thiobarbituric acid(TBA), *n*-Butanol-pyridine, Acetate buffer, Acetate buffer, Fructose, Ferric chloride($FeCl_3$), Glacial acetic acid,Sodium chloride(NaCl), Phosphate buffer(PBS),Disodium hydrogen phosphate(Na_2HPO_4),Potasium di hydrogen phosphate (KH_2PO_4),Sodium di hydrogen phosphate(NaH_2PO_4), Sodium hydroxide(NaOH), Ethylenediaminetetraacetic acid(EDTA), *n*-Butanol-pyridine, Pentobarbital sodium, reduced glutathione(GSH), 1-Chloro- 2,4-Dinitrochlorobenzene(CDNB),2-vinylpyridine,Sodium azide,Histopaque-1077,Glutaraldehyde and other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA; Himedia India, Ltd., Mumbai, India; and Merck India, Ltd., Mumbai, India for the experimentation.

4.2.2. Animal care

For the present study mature Wistar male albino rats (weighing 130-150 g) were taken and kept under controlled temperature(25 ± 2◦C) and light conditions (12h-

light–dark cycle) with free access to water and standard laboratory feed throughout the period of experimentation i.e. 14 consecutive days. The rats were acclimatized for a week before the beginning of the experiments. All the animal experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC), registered under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India. All animal treatment and surgical procedures were carried out in accordance with the relevant laws and guidelines of the CPCSEA.

4.2.3. Treatment protocol

Group	Dose
Gr-I: Control	No treatment
Gr II: Taurine control	50mg/kg body wt.
Gr III: LCT low dose	1/7 of LD_{50} value i.e., 10.83 mg/kg body wt.
Gr IV: Taurine + LCT low dose	50mg/kg body wt. + 10.83 mg/kg body wt
Gr V: LCT high dose	1/5 of LD_{50}value i.e.,15.17mg/kg body wt.
Gr VI: Taurine + LCT high dose	50mg/kg body wt. + 15.17mg/kg body wt.

Total animals were divided into six groups (n=6). Final doses of LCT of the study for the present treatments were selected by preliminary investigations followed by oral LD_{50}dose 75.85mg/kg body wt. (Sharma et al.,2010). Taurine was applied at the dose level of 50 mg/kg body wt.(Ozden et al.,2009; Cetiner et al.,2005).

4.2.4. Sample collection

After the last day of treatment, all the animals were sacrificed under light anesthesia using pentobarbital sodium and blood samples were collected from all the treated and control animals for the assessment of oxidative stress parameters, erythrocyte morphology and other haematological parameters.

4.2.5. Separation of erythrocytes

Using ethylene-diamine-tetra-acetic acid (EDTA) as an anticoagulant, 2 ml of blood was collected from the hepatic vein of each rat and was poured upon the same amount of histopaque-1077. Then it was centrifuged at 2000 rpm for 30 minutes to separate the erythrocytes. By removing plasma and leukocytes, only erythrocytes were suspended in phosphate buffer (0.1 M, pH 7.4) at 5% (v/v) concentration and were used for the assay of oxidative stress in rat erythrocytes.

4.2.6. Study on oxidative stress parameters in rat erythrocytes

4.2.6.1. Estimation of malondialdehyde (MDA)

MDA of erythrocytes suspension was determined according to modified method of Ohkawa et al. (Ohkawa et al.,1979) and the absorbance was taken at 535nm.

4.2.6.2. Estimation of reduced glutathione (GSH)

Reduced glutathione of erythrocytes was carried out according to the method of Griffith,(Griffith, 1981) and expressed as µg/mg Hb.

4.2.6.3. Estimation of oxidized glutathione (GSSG)

Oxidized glutathione was determined according to the method of Griffith,(Griffith, 1980). The absorbance was taken at 412 nm within 1min.

4.2.6.4. Superoxide dismutase (SOD) activity

SOD activity of erythrocytes was measured according to the method of Marklund and Marklund,(Marklund and Marklund, 1974). Briefly, in a spectrophotometric cuvette, 2 ml of Tris-HCl(50 mM), 100 µl of 2 mM pyrogallol in the presence of 1 mM EDTA and 10 µl of erythrocytes suspensions were added and the reading was taken at 420 nm for 3 min. The enzyme activity was estimated by measuring the percentage inhibition of the pyrogallol autoxidation by SOD.

4.2.6.5. Assay of catalase (CAT) activity

Catalase activity was measured by adding 1ml of 30 mM H_2O_2, 1.9 ml of 50 mM phosphate buffer and 0.1 ml of erythrocyte samples (in 0.05M Tris-HCl) in a glass cuvette. After mixing, six readings were noted at 240nm at 30 sec interval (Aebi ,1974).

4.2.6.6. Evaluation of glutathione-s-transferase (GST) activity

Glutathione-s-transferase activity in erythrocytes was estimated spectrophotometrically according to the method of Habig et al. (Habig et al., 1974). Reaction mixture contained 0.1 ml of erythrocytes suspension, 2.8ml of PBS, 0.1ml of GSH and 50µl of 60mM CDNB. All the contents were taken in a cuvette and reading was noted at 340nm. The values were expressed in µmol CDNB conjugate formed/min/ per milligram of Hb for erythrocytes.

4.2.6.7. Assay of glutathione peroxidase (GP_X) activity

Determination of GPx was carried out by the method of Rotruck et al.(Rotruck et al.,1973). Briefly, the reaction mixture contained 0.2ml of 0.4M Phosphate buffer

(pH-7), 0.1ml of 10mM sodium azide, 0.2ml of erythrocytes suspension in phosphate buffer(pH-7), 0.2ml of 4mM reduced glutathione, and 0.1ml of 2.5mM hydrogen peroxide(H_2O_2). The contents were incubated for 10min at 37^0C, and 0.4ml of 10% TCA was added to stop the reaction and centrifuged at 3200 rpm for 20min. Then 1 ml of 5,5'-dithiobisnitrobenzoic acid (DTNB) and 3 ml of di sodium hydrogen phosphate (Na_2HPO_4) were added to supernatant and the optical density was measured at 420 nm.

4.2.7. Scanning electron microscopic (SEM) study of rat erythrocytes

Scanning electron microscopic study (Goel et al., 2006) of rat erythrocytes were done by taking blood sample from each animal. Erythrocytes were separated using Histopaque-1077 and then 500µl of erythrocytes were immediately fixed in 2.5% glutaraldehyde made in 0.1 M phosphate buffer (pH 7.4). After 1 h of fixation, cells were centrifuged at 1000–1500 rpm and pellets were suspended in triple distilled water. After single washing, the final pellet was again suspended in triple distilled water. A drop of the sample was smeared on the metallic SEM stubs, loaded with a conductive silver tape on its top. The stubs were then coated with gold to a thickness of 100 Å using a sputter-ion coater, for 4–5 min and the specimens were finally ready to observe under scanning electron microscope.

4.2.8. Total erythrocyte count (Red Blood Cell count)

Total erythrocyte count (Wintrobe, 1967) was done by diluting blood in 1:200 dilutions with RBC dilution fluid and then total erythrocytes were counted in Neubaur haemocytometer chamber and were expressed as $\times 10^6$ mm^{-3}.

4.2.9. Estimation of haemoglobin percentage

The haemoglobin percentage was measured by cyanmethemoglobin method (Dacie and Lewis 1975).

4.2.10. Packed cell volume (PCV)

Packed cell volume (PCV) was measured by taking anticoagulated whole blood, which was centrifuged at 3000 rpm for 30 minutes in a Wintrobe`s tube. The erythrocytes were settled down at the bottom (Wintrobe, 1967).The PCV was determined by the height of erythrocyte column which is directly read from the graduation mark on the Wintrobe`s tube

4.2.11. Red cell indices

Red cell indices, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were measured from the total erythrocyte count, haemoglobin percentage and haematocrit value(Wintrobe, 1967).

I. Mean corpuscular volume (MCV): The average volume of erythrocyte is known as mean corpuscular volume and is expressed in ‘Femtolitres’.

$$\text{MCV} = \frac{\text{Haematocrit (\%)}}{\text{RBC count in million}} \times 100$$

II. Mean corpuscular haemoglobin (MCH): The average weight of haemoglobin content in erythrocyte is called mean corpuscular haemoglobin and is expressed in ‘Picograms’.

$$\text{MCH} = \frac{\text{Haemoglobin (g/dl)}}{\text{RBC count in million}} \times 10$$

III. Mean corpuscular haemoglobin concentration (MCHC): Expression of the average haemoglobin concentration per unit volume of packed red cell is defining as mean corpuscular haemoglobin concentration and is expressed in '%'.

$$\text{MCHC} = \frac{\text{MCH}}{\text{MCV}} \text{ or } \frac{\text{Hb (gm/dl)}}{\text{Hct (\%)}} \times 100$$

4.2.12. Total leukocyte count (TLC)

Total leukocyte count (Wintrobe, 1967) was done by diluting blood in 1:20 dilution with white blood corpusal (WBC) dilution fluid and then total leukocytes were counted in Neubaur haemocytometer chamber.

4.2.13. Differential leukocyte count (DLC)

Thin blood smear was made by anticoagulant-added whole blood in a clean glass slide and was stained with Leishman`s stain and then was observed under oil immersion objective of the microscope. An area of the blood smears slightly before than `tail end` was chosen where the morphology of the white cells is clearly visible. The percentage of granulocytes and agranulocytes were calculated (Wintrobe, 1967).

4.2.14. Statistical analysis

The data were analyzed to achieve mean values and standard errors for all treated and control samples. Statistical analyses of the collected data were completed by a one-way analysis of variance (ANOVA), followed multiple comparison two tail t-

testfor analysis between groups, using Origin 6.1 software. Results were presented as mean ± SEM. Difference was considered statistically significant when $p< 0.05$.

4.3. Results

4.3.1. Alterations in oxidative stress parameters

The results of the present study showed that MDA level was significantly increased ($p<0.001$) in erythrocytes of the LCT treated groups compared to the control where MDA level decreased significantly ($p<0.001$) in taurine + LCT-treated groups (fig-4.2.A).

The GSH levels in erythrocytes of high dose LCT-treated rat were altered significantly ($p<0.001$) compared to control (fig-4.2.B). Administration of taurine to the low and high dose LCT-treated animals resulted in restoration of erythrocyte GSH levels significantly ($p<0.05$). A significant increase ($p<0.001$) in GSSG level in high dose LCT treated rat erythrocytes was seen in figure-4.1.C. It was significantly ($p<0.001$) reduced after pretreatment of taurine.

In figure 4.2.D the lambda cyhalothrin caused a significant decrease ($p<0.01$) in the activity of SOD in treated group compared to the control. Pretreatment of taurine along with lambda cyhalothrin improved the parameters significantly ($p<0.05$). Activity of CAT was diminished significantly ($p<0.001$) in LCT treated group (Fig-4.2.E). Taurine supplementation along with lambda cyhalothrin caused increase CAT activity significantly ($p<0.001$).

Significantly decreased in the activity of GST and GP_X were also observed in the LCT-intoxicated rats (Fig-4.2.F, 4.2. G). Pretreatment of taurine was found to exhibit a protective effect on the GST and GP_X activity.

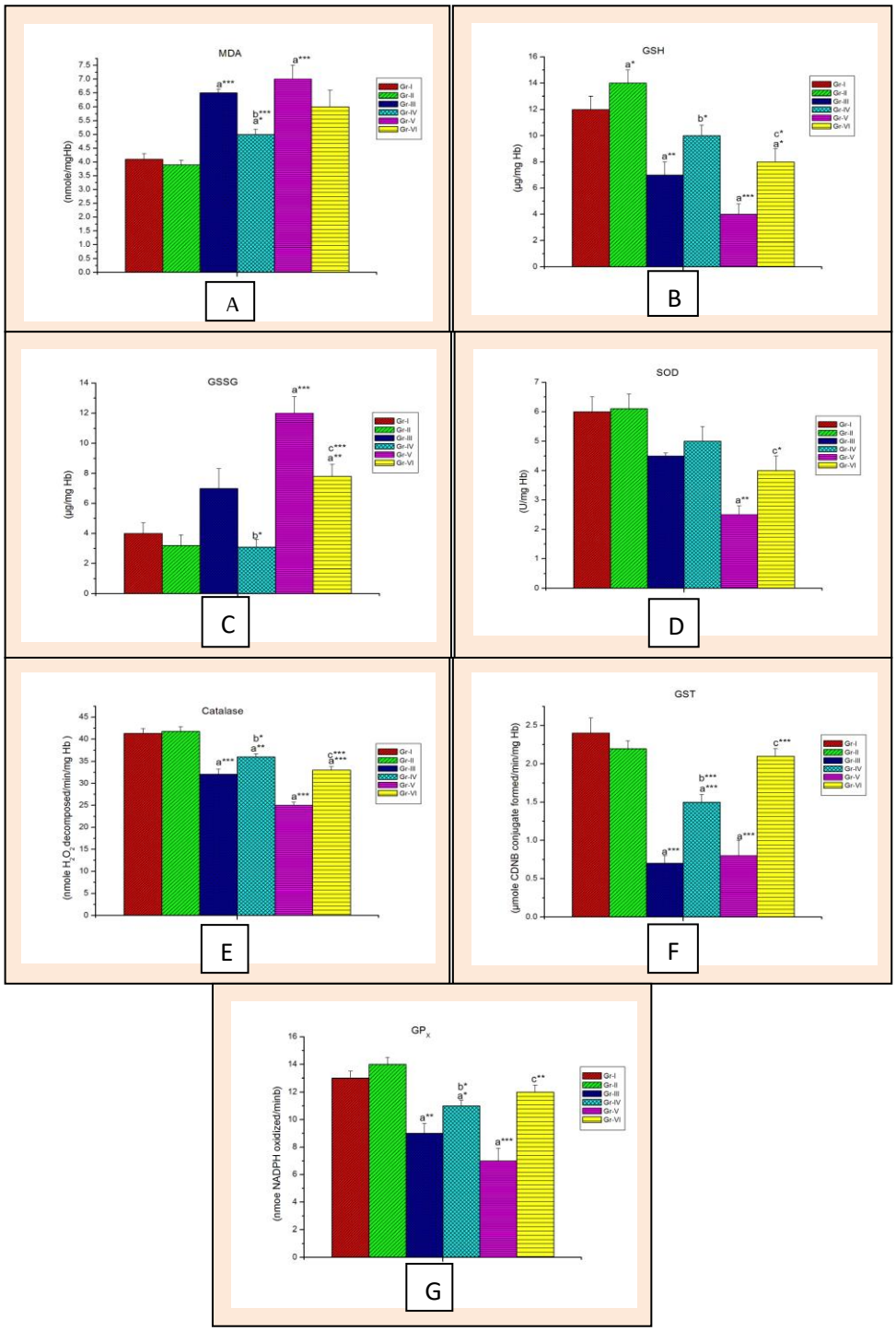


Figure-4.2. Effect of lambda cyhalothrin and taurine on lipid peroxidation (MDA) and antioxidant status in control and experimental group of rat erythrocytes. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group I versus all other groups; superscript b, Group III versus Group IV and superscript c, Group V versus Group VI (*indicates $p<0.05$,** indicates $p<0.01$, *** indicates $p<0.001$). A= Effect of lambda cyhalothrin and taurine on MDA, B= Effect of lambda cyhalothrin and taurine on GSH, C= Effect of lambda cyhalothrin and taurine on GSSG, D=Effect of lambda cyhalothrin and taurine on SOD, E=Effect of lambda cyhalothrin and taurine on CAT, F=Effect of lambda cyhalothrin and taurine on GST, G=Effect of lambda cyhalothrin and taurine on GPx.

4.3.2. Scanning electron microscopic observations of rat erythrocyte morphology.

Effects of LCT intoxication along with pretreatment of taurine on rat erythrocytes were observed under scanning electron microscope and the results are depicted in figure 4.3.

Normal control animals showed perfect discocytes (fig 4.3.Gr-I). No significant morphological abnormalities in blood cells were observed in the animals treated with taurine alone (fig 4.3. Gr-II).However, animals treated with LCT in low doses showed morphological alterations from discocytes (D) to stomatocytes (S) and leptocytes (fig 4.3. Gr-III).The red cells with stoma in middle part of the cells called stomatocytes along with folded bowl shaped. Few ovalocytes(O) were observed in high dose LCT treated animals (fig 4.3,Gr-V).Certain irregularly created and

contracted cells with numerous projections known as echinocytes(E) were also visible in the LCT treated high dose group animals (fig 4.3.Gr-V). Dacrocytes(T) i.e. tear drop like structure were also found in this LCT treated high dose group (fig - 4.3,Gr-V). Protective effects of taurine were evident after the pretreatment of taurine followed by LCT, where the drastic alterations in the shape of the blood cells were restored close to the normal appearance of the cells (fig 4.3, Gr-IV and Gr-VI).Despite all these protective effects of taurine, very low population of echinocytes were still present in control and taurine control group (fig-4.3, Gr-I and Gr-II).

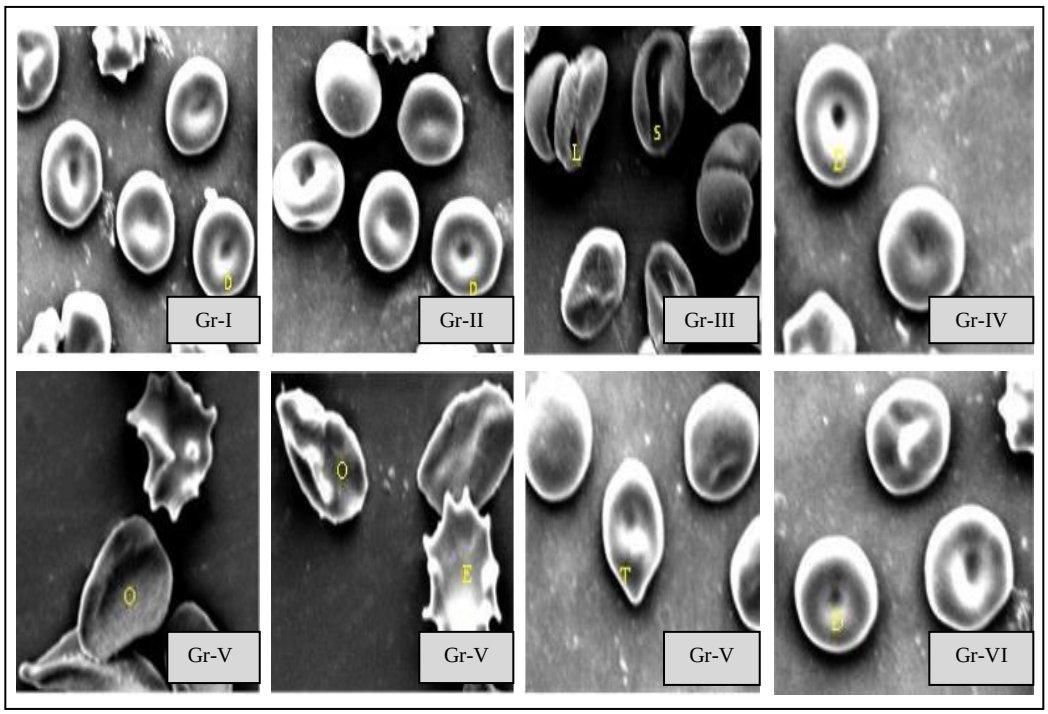


Figure-4.3.scanning electron photomicrograph of erythrocytes of control and treated rats. Bar scale = 10 µm. Discocytes (D), Leptocyte (L), stomatocyte (S). ovalocyte (O), echinocyte (E),dacrocyte or tear drop like structure (T). Bar scale = 10 µm.

4.3.3. Effects on haemogram

In LCT treated groups total erythrocyte count and haemoglobin (%) were decreased significantly ($p<0.001$) at dose-dependent manner (Table 4.1) which demonstrated the toxic effects of LCT. Pretreatment of taurine reduced the toxic effects of LCT and caused significant increase in total erythrocyte count and haemoglobin percentage. PCV, MCH and MCHC were decreased significantly ($p<0.001$) in LCT treated group compared to control group (Table 4.1.) whereas MCV value was increased significantly in case of LCT-intoxicated group (Table 4.1). Taurine restored these parameters towards more or less control level (Table 4.1).

Table 4.1. Effect of lambda cyhalothrin and taurine on total erythrocyte (RBC) count, Haemoglobin concentration (Hb conc.), PCV (Packed cell volume), Mean cell volume (MCV), Mean cell haemoglobin (MCH) and Mean corpuscular haemoglobin concentration (MCHC) in control and experimental group of rat.

Parameters	Group-I	Group-II	Group-III	Group-IV	Group-V	Group-VI
Total Erythrocyte Count ($\times 10^6$ / mm^3)	7.3±0.09	7.5±0.15	6.3±0.12 a***	7±0.1 a* b**	5.8±0.18 a***	6.05±0.14 a***
Hb percentage (gm/dl)	14.73±0.03	14.7±0.05	11.86±0.04 a***	13.4±0.07 a***b***	10.31±0.04 a***	11.5±0.15 a***c***
PCV (%)	44±0.14	44.8±0.15a**	43.68±0.07	44.48±0.22a***b**	41.05±0.31 a***	42±0.17 a*** c*
MCV (fl)	60.27±0.26	59.73±0.2	67.62±0.08 a***	61.43±0.21 a**b***	70.69±0.02 a***	68.86±0.23 a***c***
MCH (pg)	20.18±0.02	19.6±0.09 a***	18.82±0.03 a***	19.14±0.05a*** b***	17.78±0.06 a***	18.85±0.03 a***c***
MCHC (%)	33.48±0.05 a***	32.81±0.58 a***	27.8±0.58 a***	31.16±0.04 a***b***	25.14±0.57 a***	27.37±0.79 a***c***

Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (*indicates $p<0.05$,** indicates $p<0.01$and *** represents $p<0.001$).

4.3.4. Effects on leukogram

Leukocyte count and lymphocyte percentage (Table 4.2) were increased significantly ($p<0.001$) in LCT treated groups. The results showed that the treatment of taurine to the rats exhibited significant decrease in leukocyte count and lymphocyte percentage compared to rats receiving only LCT. Neutrophil count also decreased significantly ($p<0.001$) at dose-dependent manner in case of LCT treated rats. In LCT treated low and high dose groups, eosinophil and monocyte percentage were decreased significantly ($p<0.001$) also in a dose-dependent manner compared to that of control.

Table 4.2. Effect of lambda cyhalothrin and taurine on total leukocyte count, lymphocyte count, neutrophil count, eosinophil count, and monocyte count in control and experimental group of rat.

Parameters	Group-I	Group-II	Group-III	Group-IV	Group-V	Group-VI
Total leukocyte count ($\times 10^6/\mu l$)	6.6± 0.12	6.6±0.06	8.4±0.05 a***	7.9± 0.02a*** b***	9±0.23 a***	8.3±0.1 a***c**
Lymphocyte count (%)	56.13±0.13	56.05±0.13	60.15± 0.26 a***	58.0±0.16a***b***	65.83± 0.17a***	62.05±0.26a***c***
Neutrophil count (%)	36.9±0.57	37.15±0.83	34.0±0.54a*	35.7±0.28	29.97± 0.5a***	32.05±0.5a***
Eosinophil count (%)	2.5±0.06	2.6±0.09	2±0.1a**	2.4±0.12 b*	1.4±0.06a***	2±0.10a**c***
Monocyte count (%)	4.4±0.19	4.2±0.14	3.45±0.16a**	3.9±0.12 a*b*	2.8±0.10a***	3.0±0.22 a***

Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (*indicates $p<0.05$, ** indicates $p<0.01$and *** represents $p<0.001$).

4.4. Discussion

In the study lambda cyhalothrin intoxication created alterations in oxidative stress parameters in rat erythrocytes as well as produced a notable variation in the morphological appearance of the rat erythrocytes and remarkable changes in the haematological parameters. Protective effect of taurine was also shown in the results. The erythrocytes in normal physiological situations are resistant to oxidative damage due to their well-equipped biological and protective mechanisms by enhanced antioxidant enzymes, such as catalase(CAT), Glutathione peroxidase (GP_X), superoxide dismutase (SOD) and glutathione reductase(GR)(Sies,1997). It must also be mentioned that the erythrocytes may be susceptible to oxidative damage because of the presence of heme-iron, PUFA and oxygen, under oxidative stress and it may trigger the reactions that make oxidative changes in red blood cells (Clemens and Waller,1997). It has been reported that pyrethroids prompt oxidative stress, as displayed by elevation of lipid peroxidation products (Banerjee et al.,1999). LCT is a α-cyano moiety comprising type-II pyrethroid which instigates the oxidative stress. Nasuti et al.and Prasanthi et al. noticed that oxidative damage was done in erythrocytes because of lipophilicity of pyrethroids (Nasuti et al., 2003; Prasanthi et al., 2005). Elevated malondialdehyde (MDA) level in erythrocytes treated with LCT is an agreement with the findings of Nasuti and Prasanthi (Prasanthi et al., 2005).The pretreatment of taurine in conjunction with lambda cyhalothrin reduced the increased level of MDA towards its normal limit. The normalization of MDA level in erythrocytes following taurine pretreatment is very likely because its antioxidant

properties, as has been shown previously (Cabre et al., 1999). Reduced glutathione (GSH) is considered as a notable biomolecule that serves against chemically created oxidative stress. It acts as a free radical scavenger and neutralizes the radicals that are involved in biological damage (Nicotera and Orrenius,1986). Hence, the measurement of its activity is necessary to evaluate the oxidative stress and antioxidant status created by LCT. In the current study, GSH content reduced notably in the erythrocytes of LCT treated rat compared to the control. Noteworthy deterioration in the GSH level by LCT exposure is either because of increased utilization or reduced production of GSH. Taurine pretreatment may lead to an enhancement in GSH levels by directing cysteine into the GSH synthesis as cysteine is the precursor of GSH (Hagar, 2004). Elevated oxidized glutathione (GSSG) level in case of LCT-treated erythrocytes and reduced GSSG in taurine treated erythrocytes also substantiate the earlier findings. This study shows that the reduction in the activity of superoxide dismutase, a copper-zinc-containing enzyme and CAT, a heam-containing enzyme in lambda-cyhalothrin intoxicated erythrocytes can be elucidated by the increase of lipid peroxidation followed by increase in MDA content. LCT created stress is, perhaps, the reason for over production of ROS that increases singlet oxygen and peroxyl radicals, which reduce SOD and CAT by their effective use. Glutathione peroxidase, a selenium comprising tetrameric glycoprotein found in mammalian erythrocytes helps to stop lipid peroxidation of the cell membrane (Brigelius-Flohé,1999). Lambda cyhalothrin exposure causes a reduction in the activity of GPx, perhaps, because of the depleted GSH, as the activity of GPx relies upon the level of GSH. Increased use of GPx to detoxify the pesticide induced

free radicals, also substantiate the above result (Hayes and Pulford, 1995). Glutathione-s-transferase are detoxifying enzymes that catalyze the conjugation of a variety of electrophilic substrates to the thiol group of glutathione, releasing less toxic forms (Hayes and Pulford,1995) . T he notable decrease of GST activity in erythrocytes after exposure of lambda cyhalothrin may suggest enough detoxification of pesticide in rat erythrocytes. Taurine administration reversed all the abnormalities because of its stimulatory effect on endogenous antioxidants (Saad and Al-Rikabi,2002). This study tells us that the radical alterations in the red cell morphology of the animals exposed to LCT. The normal presence of the erythrocytes was changed into many different forms including echinocytes, stomatocytes, dacrocytes and few spherocytes also. Echinocyte form is thought to be a structurally pathological membrane defect, happening also during the smear preparation. LCT may disturb the structure of lipids situated in the erythrocyte membrane (Suwalsky et al.,2004) . This means that erythrocytes in individuals exposed to LCT poisoning shall not survive their whole life span of 120-130 days, but are supposed to be removed as echinocytes. This leads to low hemoglobin levels because of LCT toxicity (Shashi and Meenakshi, 2012). Stomatocyte has the stoma in the middle part, normally found in liver disease, also noticed in LCT treated animals. Because of the lack of related studies in this field, the acceptable elucidations for such findings are the abnormal erythropoiesis, or the defects on the erythrocyte membrane lipid bilayer. Perhaps, it is because of insufficient haemoglobin formation, decreased water permeability across erythrocyte membranes, increased erythrocyte aging, the

rate of oxygen release by erythrocytes, reduced thermo stability of erythrocytes, or augmented erythropoiesis to compensate anemia (Tkeshelashvili et al.,1989) .

Pretreatment of taurine followed by LCT enhanced the morphology of the red blood cells. The preventive effects of taurine are most likely because of its function as a direct antioxidant by scavenging reactive oxygen radicals, inhibition of lipid peroxidation and as an indirect antioxidant by averting changes in erythrocyte membrane permeability resulting from oxidative damage in many tissues including erythrocytes (Waters et al.,2001).

A notable change in haemogram, leukogram and red cell indices were noticed in rats exposure to lambda cyhalothrin suggests the physiological dysfunction of the haemopoietic system of rats. The decline in erythrocyte counts noticed with LCT treatment may be because of haemolysis as a result of type-II pyrethroid exposure which leads to haemorrhage and reduced erythropoiesis (Mandal et al., 1986).Substantial decline in erythrocyte count and haemoglobin percentage could perhaps be due to suppression of erythropoiesis and haem synthesis, and also due to destruction of erythrocyte in hemopoietic tissue (Fetoui et al., 2008). Lysis of erythrocyte is created by chemicals that cause harm to erythrocyte membrane and it leads to oxidative injury to haemoglobin, or may damage the anti-oxidative protective mechanism. Enhanced hemolysis generally causes decline in haemoglobin, erythrocyte count and are accompanied by high reticulocytes count, enhanced anisocytosis, increased red cell distribution width and volumes. Few authors have documented similar results with the treatment of cypermethrin in rats (Manna et al., 2004). Haemoglobin percentage and haematocrit values have direct

connection to erythrocyte count (Moustafa et al., 2012) due to the synergistic link among these blood parameters in all vertebrates. Reduction in haemoglobin percentage in the current study could be because of the reduced biosynthesis of haem in bone marrow enhanced rate of destruction of erythrocytes or declining rate of erythrocytes formation.

The packed cell volume(PCV) suggests oxygen carrying capacity of blood and the degree of stress on animal health. In high dose lambda cyhalothrin treated group PCV level declined. In agreement with the current result reduced erythrocyte count, haemoglobin percentage and PCV levels were also documented in rats treated with deltamethrin (Yekeen et al.,2007).

In our study mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were reduced in lambda cyhalothrin treated groups, which is, perhaps, suggestive of macrocytic and hypochromic anaemia (Barger,2003) .Because of lower erythrocyte count, haemoglobin percentage and PCV in LCT treated animals, MCH and MCHC value were diminished. In the current study, the rise in mean corpuscular volume (MCV) and decline in MCHC also suggests the probability of macrocytic and hypochromic anaemia (Barger,2003; Latimer et al.,2004) perhaps, because of the increased activity of bone marrow and deficiency of some hemopoietic factors. Increased MCV may also be noticed in regenerative anaemia because of haemolysis and haemorrhages.

The rise in leukocyte was also reported in lambda cyhalothrin intoxicated groups, possibly due to the activation of immune system of the body (Yousef et al.,2003).

This may lead to an increase in release of leukocytes from bone marrow storage pool into the blood. The principle function of leukocytes is to safeguard against foreign bodies, which is achieved by leukocytosis and antibody production**.** Pathological leukocytosis, may be formed because of the exposure of chemicals or acute haemorrhages and haemolysis. Leukocytosis may be increased because of the resistance of the animal for localization of the inflammatory response. Another probable reason of leukocytosis is, the severe haemorrhages in liver and lungs (Latimer et al.,2004). This rise is, perhaps, connected to a rise in lymphocyte percentage.

However, pretreatment with taurine has a strong protective effect against lambda cyhalothrin created toxicity in haematological parameters of rats. The mechanism underlying haemato-protection of taurine is, perhaps, connected to its anti-anemic qualities which effectively supports to hemopoiesis. A number of investigators noted that taurine safeguards several organs in the body against toxicity and oxidative stress because of exposure of heavy metals and other toxins as well as drugs (Manna et al., 2009; Parildar et al.,2008).

4.5. Conclusion

In conclusion, it may be said that the lambda cyhalothrin treatment creates toxicity by producing oxidative stress and structural changes in erythrocytes and by alteration of haematological parameters in rat. Collectively, these data suggest that taurine pretreatment can potentially be considered as an intervention in human subject

against haematological dysfunction with accidental exposure of lambda cyhalothrin and related pyrethroids.

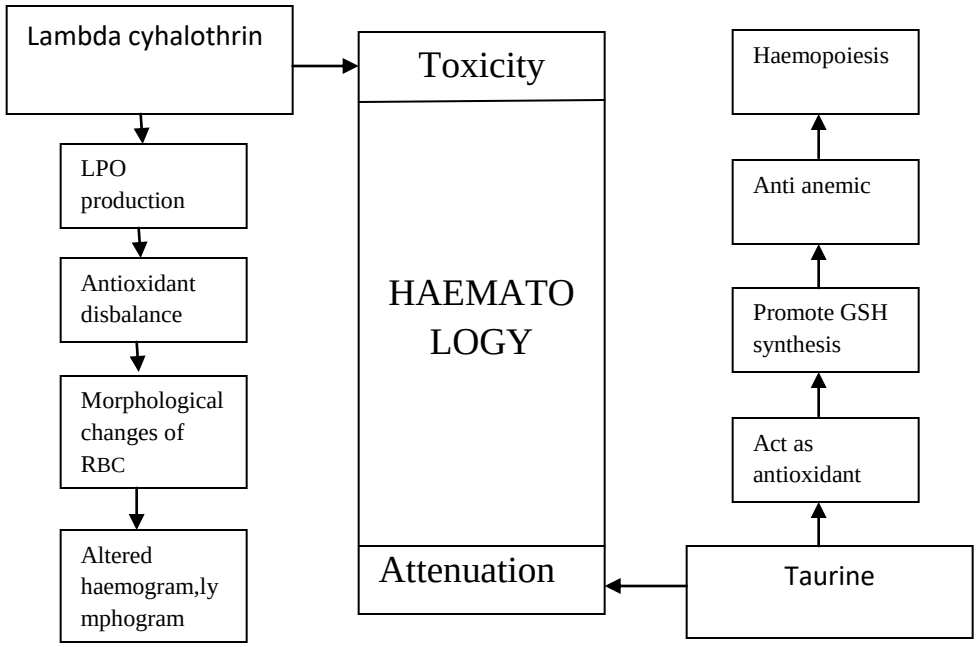


Figure4.4 shows the schematic diagram of LCT induced haematotoxicity

4.6. References

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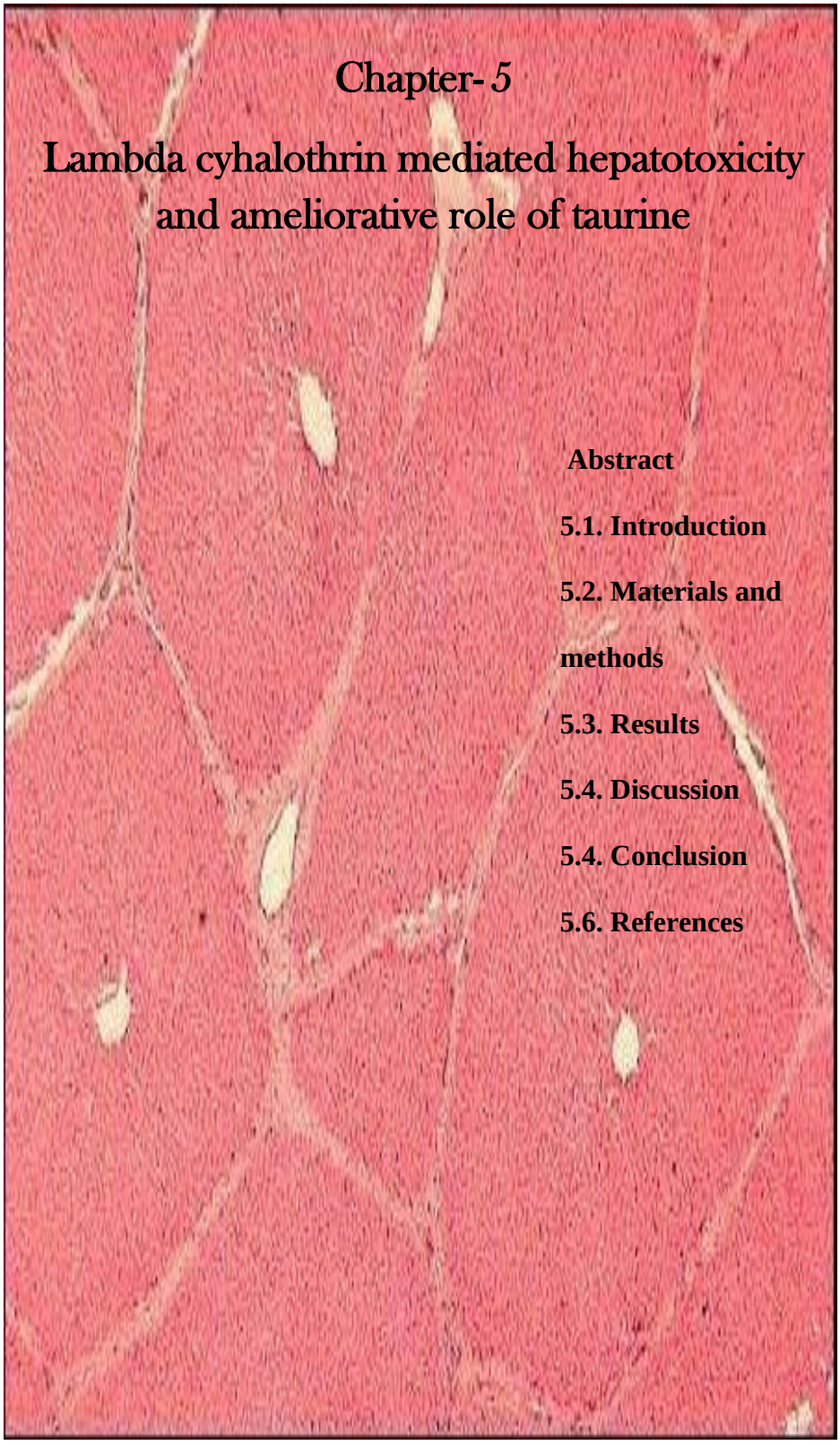


Chapter- 5

Lambda cyhalothrin mediated hepatotoxicity and ameliorative role of taurine

Abstract

Lambda cyhalothrin, a third generation type II pyrethroid, is used predominantly in agriculture production and animal husbandry. Liver is a target organ and primary site of detoxification and biotransformation and plays important role in metabolism to maintain energy level and structural stability of body. Taurine is a major intracellular free β-amino acid, can able to protect the body against toxicity. The aim of the present study was to investigate lambda cyhalothrin-induced hepatotoxicity and to search out the possible role of taurine for the attenuation of this toxicity. Male rats were randomly divided into six groups and lambda cyhalothrin was orally administered at two dose levels (10.83mg/kg body wt., 15.17mg/kg body wt.) alone or in combination with taurine pretreatment (50mg/kg body wt) for 14 consecutive days. Lambda cyhalothrin treatment generated excess reactive oxygen species which caused severe DNA damage and decreased mitochondrial membrane potential, ultimately induced apoptosis in hepatic cells. A significant increase in malondialdehyde and depletion of reduced glutathione level, superoxide dismutase, catalase, glutathione-s-transferase and glutathione peroxidase were observed in lambda cyhalothrin treated rat liver in a dose-dependent fashion. Significant changes in blood glucose level with a marked decline in glycogen content also indicated the hepatic dysfunction in lambda cyhalothrin treated rats. This was also confirmed by the altered activities of serum hepatic biomarker enzymes, lipid profiles and histoarchitecture of liver tissue in LCT intoxicated rats. Pretreatment of taurine mitigated the abnormalities. These findings pointed out the toxic effect of lambda cyhalothrin in rat liver and also revealed the protective action of taurine against this pyrethroid.

Rini Ghosh, Tuhina Das, Anurag Paramanik, Sujata Maiti Choudhury. Taurine improves lambda cyhalothrin induced biochemical alterations in Wistar rat liver. International Journal of Bioassays.2016; 5: 5059-065

5.1. Introduction

Liver is a major organ regulating homeostasis by accomplishing various functions within the body. It is the target for toxicity because of its responsibility in clearing and metabolizing chemicals through the process called detoxification (Larrey, 2003). In addition, liver works for diverse body functions including protein biosynthesis, salvaging different metabolic intermediates, playing roles in carbohydrate and lipid metabolism etc., therefore, any disturbance to liver is capable of changing the entire body physiology up to creating complications for subsistence of any individual. Liver damage caused by toxic chemicals has been acknowledged as a toxicological problem. Hepatotoxicity is a very common ailment resulting in serious impediments ranging from severe metabolic disorders to even mortality (Patel et al., 2008). Hepatotoxicity can arise in many ways, reflected as changes in the levels of serum cholesterol, serum levels of liver enzymes, or bilirubin (Malaguarnera et al.,2012). For the initial assessment, it is necessary to measure serum alanine and aspartate aminotransferase, alkaline phosphatase, bilirubin, direct and indirect. Xenobiotics induced hepatotoxicity is associated with the production of free radicals, which are not only toxic themselves, but also implicated in the pathophysiology of many diseases. Mechanism of hepatotoxicity can also be investigated through mitochondrial dysfunction and DNA damage (Fromenty and Pessayre,1995).

5.1.1. Pesticide induced hepatotoxicity

In spite of harmful effects of pesticides on environment and other living organisms, unfortunately the most efficient and perhaps the only useful way to fight against

pests is now using of chemical pesticides (Dehghani,2010). India is one of the principal users of agricultural pesticides to increase crop yield and in vector control program. Pesticide induced liver disorders can be life threatening. Many studies have implicated that the fundamental mechanism of toxicity induced by pesticides is oxidative damage (Celik et al., 2009; Kalender et al.,2010). Pesticides boost the creation of reactive oxygen species (ROS) during the reaction and react with biological molecules, which consecutively create oxidative stress, ultimately damaging membranes and other tissues (Heikal et al.,2012; Rai and Sharma.,2007).

5.1.2. Pyrethroid mediated hepatotoxicity

High doses of fenvalerate has been associated with decrease in body mass, increase in liver mass, and proliferation of the smooth endoplasmic reticulum in hepatic cells including induction of the activity of microsomal enzymes (El-Sewedy et al.,1982; WHO ,1990).Oral administration of fenvalerate produce degenerative changes in the liver, centrilobular necrosis, nuclear degeneration, fatty change, bile duct proliferation and round cell infiltration (Mani et al.,2004). Cypermethrin induced toxicity was manifested by elevated activity of enzymes along with those of other molecules in serum of cypermethrin intoxicated rats.

5.1.3. Effect of lambda cyhalothrin on hepatic system

Lambda cyhalothrin, a synthetic type II pyrethroid. Hepatic aspartate transaminase, alanine transaminase activity, and malondialdehde content along with the activities of antioxidant enzymes were reduced significantly in rat after lambda cyhalothrin

treatment for 4week (Madkour,2012). Zebra fish intoxicated with lambda cyhalothrin and Neemgold in different concentrations altered the total liver protein, total free amino acids, and nucleic acids (Ahmad et al ,2012).

5.1.4.Attenuating role of taurine in hepatic system

Taurine (2-aminoethane sulphonic acid) is a prime free intracellular non- protein sulphur amino acid (Atmaca, 2004), found in the liver at high concentrations. Several studies have revealed that taurine safeguards effects against chemically-induced hepatotoxicity (Heidari et al, 2013; Hartman et al, 2010; Liao et al, 2008). Furthermore, by trim down oxidative stress, increasing mitochondrial activity and modulating cytoplasmic and mitochondrial calcium homeostasis taurine has been found to stop toxin-mediated hepatic injuries (Asha and Devadasan, 2013). Moreover, taurine acts as an antioxidant in biological systems, scavenge ROS, attenuate lipid peroxidation and, as a consequence, stabilizes biological membranes (Wang et al., 2013; Higuchi et al., 2012).

Liver is the most important and the largest gland of the body and is the vital site of metabolism and detoxification that control various abnormalities as a result of exposure to the toxins of extrinsic as well as intrinsic forms. It is also the place of biotransformation by which a toxic composite has been switched in less harmful form to reduce toxicity (Paliwal et al.,2009), therefore, liver has been selected as experimental organ in the present study.

The goal of the current study is to highlight the effects of lambda cyhalothrin on hepatic oxidative stress, DNA fragmentation, changes in the activities of serum

hepatic biomarker enzymes, lipid profiles and histological alterations in rats as a model of mammals have been examined and to find out the putative ameliorative role of taurine for the attenuation of lambda cyhalothrin induced hepatotoxicity.

5.2. Materials and methods

5.2.1. Chemicals and reagents

Lambda cyhalothrin 5% emulsifiable concentrate (RPC Agro Industries, Kolkata), taurine (Sigma Aldrich Inc. USA). Hydrogen peroxide(H_2O_2), Hydrochloric acid (HCl), Sulfo salicylic acid, Dithionitrobenzoic acid (DTNB), Tris-HCl, Pyrogallol, Thiobarbituric acid(TBA), *n*-Butanol-pyridine, Acetate buffer, Sodium dodecyl sulfate, Sodium chloride(NaCl), Phosphate buffer (PBS), Disodium hydrogen phosphate($Na_2HPO_{4)}$, Potasium di hydrogen phosphate (KH_2PO_4),Sodium di hydrogen phosphate(NaH_2PO_4), 2-vinyl pyridine, Magnesium chloride($MgCl_2$), Sodium hydroxide(NaOH), Pentobarbital sodium, reduced glutathione(GSH), DL-alanine, α-Ketoglutaric acid, Glycerol, 1-Chloro- 2,4-Dinitrochlorobenzene(CDNB),2-vinylpyridine,Sodium azide, 2′, 7′-dichlodihydrofluorescein diacetate ($DCFH_2$-DA), Rhodamine-123 and other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA; Himedia India, Ltd., Mumbai, India; and Merck India, Ltd., Mumbai, India for the experimentation.

5.2.2. Animal mode

Wistar albino male rats (weighing 130-150 g) were chosen as an experimental animal in the present study on the basis of easy availability and handling under normal laboratory conditions, was maintained on the standard laboratory feed and water throughout the period of experimentation i.e. 14 consecutive days. Experimental protocol was approved by the Institutional Animal Ethical Committee, registered under CPCSEA. All animal treatment and surgical procedures were carried out according to the relevant laws and guidelines of the CPCSEA.

5.2.3. Treatment protocol

A commercial formulation of lambda cyhalothrin 5% emulsifiable concentrate (EC) was used for our experiments. It was in emulsion form and dilutions were done in distilled water to get the test concentrations. The test concentrations of lambda cyhalothrin were calculated from the percentage of the active ingredient of commercial formulation of lambda cyhalothrin.

The animals were divided into six groups of six rats each.

Group I: Distilled water control (no treatment).

Group II: Taurine control (50mg/kg body wt).

Group III: Lambda cyhalothrin low dose (10.83mg/kg body wt. i.e. $1/7^{th}$ of LD_{50} value).

Group IV: Taurine (50mg/kg body wt) + lambda cyhalothrin low dose (10.83mg/kg body wt.).

Group V: Lambda cyhalothrin high dose (15.17mg/kg body wt. i.e. 1/5 of LD_{50} value).

Group VI: Taurine (50mg/kg body wt) + lambda cyhalothrin high dose (15.17mg/kg body wt.)

Dose levels for the present treatments were finalized according to preliminary investigations following the LD_{50} of 75.85mg/kg body wt. Taurine was applied at the dose level of 50 mg/kg body wt. (Ozden et al., 2009; Cetiner et al., 2005). Lambda cyhalothrin, taurine and distilled water were administered once daily by oral gavage for 14 consecutive days. Animal's weight was taken daily and the dose was adjusted accordingly.

5.2.4. Determination of intracellular ROS generation using 2′,7′-dichlorodihydrofluorosceindiacetate in hepatic cell

Cells from the liver of experimental animals were isolated by the collagenase perfusion technique according to the method of Seglen with some modification (Seglen,1976). Isolated hepatic cells from the above stated different groups were incubated with 1 μg ml^{-1}2′,7′-dichlorodihydrofluorosceindiacetate (DCFH2-DA) at 37^0C for 30 min. Then the cells were washed with phosphate buffer saline (PBS, pH 7.4) / fresh culture media and centrifuged at 1200 rpm to remove the excess dye, DCFH2-DA. The cellular intensity were then analyzed in Hitachi F-7000 Fluorescence Spectrophotometer using excitation at 498 nm and 530 nm emission

and the image was taken by fluoroscence microscope (LEICADFC295, Germany)(Roy et al.,2008).

5.2.5. Determination of hepatic mitochondrial membrane potential (ΔΨm)

Schedule cells were incubated with 1.5 mM (0.5 µl) of Rhodamine 123 dye for 15 min at 37ºC in a humidified incubator. The cells were then washed three times with PBS. The cellular fluorescence intensity of Rhodamine 123 was measured using flow cytometer (BD FACSVerse) and Cell Quest software(M'Bemba-Meka et al.,2006).

5.2.6. DNA fragmentation study in hepatic cell by alkaline comet assay

DNA fragmentation was evaluated by alkaline comet assay with some modifications (Alcântara et al., 2011). Briefly, isolated hepatic cells were added with 0.7% agarose for carry out the comet assay. The glass slide was smeared with 1% agarose and covered with cell. After that slides were transfered in lysis buffer for electrophorized. Then the slides were stained with ethidium bromide and image was captured under fluorescence microscope (LEICA DFC295, Germany). The comet tail length was calculated as the distance between the end of heads and end of each tail. Tail moments were defined as the product of the percentage of DNA in each tail, and the distance between the mean of the head and tail distributions. Percentage of DNA (tail) was presented as: %DNA (tail) = TA × TAI × 100/ [(TA × TAI) + (HA × HAI)]; where TA= tail area, TAI = tail area intensity, HA= head area and HAI = head area intensity.

5.2.7. Estimation of lipid peroxidation and antioxidant status of liver

Liver tissue was homogenized (20mg/ml) and lipid peroxidation was determined in terms of malondialdehyde (MDA)(Ohkawa et al., 1979). The absorbance of supernatants was taken at 535nm in spectrophotometer (UV-245 Shimadzu, Japan). Reduced and oxidized glutathione of liver tissue homogenate(20mg/ml pbs) were estimated according to the method of Griffith((Griffith, 1980; (Griffith, 1981). Superoxide dismutase (SOD) of tissue homogenate (20mg/ml Tris HCl) was assessed according to the method of (Marklund and Marklund, 1974). Catalase activity of tissue homogenate (20mg/ml Tris HCl) was estimated by the decomposition of hydrogen peroxide according to the protocol of Aebi (Aebi, 1974). GPx activity of liver homogenate(20mg/ml pbs) was measured by the method described by Rotruck et al. (Rotruck et al., 1973).Glutathione-S-transferase (GST) activity of liver homogenate(20mg/ml pbs) was quantified spectrophotometrically (Habig et al., 1974). The activity of glutathione reductase of liver homogenate (20mg/ml pbs) was determined by the method of Williams and Arscott.(Williams and Arscott, 1971).Reading was take at 340nm and was expressed as nmole/NADPH consumed/min/mg protein. Detail methods were described in previous chapter 3,4.

5.2.8. Determination of blood glucose level

Blood glucose was estimated by Nelson method adapted from Somogyi's method**.** The blood was deproteinized by adding 9.5 ml of 5% zinc sulphate solution in 1 ml of blood sample. Then 9.5 ml of 4.5% barium hydroxide solution was included and the mixture was allowed to stand for 15 min for complete precipitation and was

filtered. For the preparation of sample, standard and blank, 0.5 ml of blood filtrate, 0.5 ml of 0.025mg/ml of glucose and 0.5 ml of distilled water were taken respectively in separate test tubes and 1ml of alkaline copper reagent were added in each test tubes and were boiled in a boiling water bath for 20min. Then those were allowed to cool in room temperature and 1 ml of arseno-molybdate reagent was mixed to each test tubes and volume was made upto 10 ml. Readings were taken at 540 nm in spectrophotometer (Nelson, 1944).

5.2.9. Estimation of liver glycogen

Tissue homogenate (100 mg/ml in hot 80% ethanol) was centrifuged at 8000 × g for 20 min. The residue was collected and dried in a hot water bath. To the collected residue, 5ml of distilled water and 6ml of 52% perchloric acid were mixed. The extraction process was done at 0^0C for 20min. Then the collected material was centrifuged at 8000×g for 15 min. 0.2 ml supernatant was transferred in a graduated test tube to make 1 ml volume by distilled water. Standards in graded concentrations were prepared by using working standard solution and all these volumes were made up to 1ml by distilled water. Then 4 ml of anthrone reagent was added to all test tubes and were heated in boiling water bath for 8 minutes. After cooling in a room temperature the optical density was noted at 630 nm. The amount of sample glycogen was measured from standard curve, prepared with standard glucose solution. Glycogen present in sample was expressed in µg of glucose/ mg of tissue (Sadasivam and Manickam et al.,1996).

5.2.10. Activity of serum glutamate-oxaloacetate transaminase (SGOT) and glutamate- pyruvate transaminase (SGPT)

Activity of serum glutamate-oxaloacetate transaminase (SGOT) and glutamate-pyruvate transaminase (SGPT) were assessed by the method of Goel (Goel,1988). For SGOT sample, 1 ml of buffer substrate (2.66gm aspartic acid, 60mg α-ketoglutaric acid and, 20.5ml of 1(N) NaOH and 100ml of volume was made by 0.1M phosphate buffer, pH7.4) and for SGPT sample, 1 ml of buffer substrate (1.78 gm DL-alanine, 30mg α-ketoglutaric acid, 20 ml of 0.1M phosphate buffer and 1.25ml of 0.4(N) NaOH) were taken and waited for 5min at 37^0C. Then 0.2ml of serum sample was mixed and incubated at 37^0C for 60 min. To prepare standard, 0.2 ml of working standard (200µM/100 ml) was taken in a test tube and 0.8ml of buffer substrate was added. For blank, 1.0 ml of buffer substrate was taken. In each of sample, standard and blank test tubes, 1ml of DNPH solution were added and waited for another 20 min. Then 10ml of 0.4(N) NaOH was mixed and waited for 10 minutes. Finally the readings were taken in spectrophotometer (UV-245 Shimadzu, Japan) at 520 nm

5.2.11. Estimation of serum alkaline phosphatase (ALP)

For sample preparation, 1.0ml of PNPP buffer (1mM PNPP in 0.02M Tris alkaline buffer, pH 7.5), 0.25ml of sample serum, 1.75ml redistilled water were taken in a centrifuge tube and mixed. To prepare standard, four standard solutions were prepared by mixing 5, 10, 20, 40 µg/ml of PNP (10mg% PNP) and 3.85, 3.80, 3.70,

3.50ml redistilled water respectively and 0.01 ml of 0.1M NaOH in each standard solution. All samples and standard solutions were incubated at 37^0C for 30 min. Then 0.1ml of NaOH and 0.9ml of redistilled water were added to each sample and standard solution and was centrifuged at 2000 rpm for 10min. The reading of each supernatant was taken in spectrophotometer at 420 nm. Amount of PNP liberated was measured in a spectrophotometer (UV-245 Shimadzu, Japan) at 420 nm against blank (Malamy and Horeker, 1966).

5.2.12. Estimation of serum lactate dehydrogenase

Lactate dehydrogenase, a cytoplasmic enzyme, was estimated by measuring the change in absorbance at 340 nm in solution containing NADH and pyruvate (Reeves and Fimognari,1966).

5.2.13. Measurement of tissue protein

Different dilutions (10µl to 50µl) of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water. The final volume in each of the test tubes is 6 ml. From these different dilutions, protein reagents (98:1:1) consisting of sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH), sodium potassium tartarate in distilled water, copper sulphate (Cu_2SO_4) in distilled water were added to different test tubes and 10 µl of homogenate(20mg/ml) and 500 µl of normal saline (0.9 gm%) were also added. The solutions were mixed well. Then 500µl of reagent Folin-Ciocalteau solution was added to each tube and incubated at 37^0C for 30 min. The standards were prepared similarly. The optical density was measured at 660 nm. The

absorbance was plotted against protein concentration to get a standard calibration curve (Lowry et al.,1951).

5.2.14. Serum bilirubin and lipid profile studies

Serum bilirubin was determined using commercial diagnostic reagent kit. Total cholesterol (TC), triglyceride (TG) and high density lipoprotein cholesterol (HDL) were estimated by using commercial diagnostic reagent kit (CREST BIOSYSTEMS). Very low density lipoprotein (VLDL) was calculated using following formula (TG/5) given by (Friedwalds et al.,1972) . LDL concentration (mg/dl) was estimated indirectly from the measured levels of TG, HDL, and TC using equation LDL= TC - (VLDL+HDL).

5.2.15. Histopathological study

For histological study, excised liver samples from the experimental animals of each group were fixed in Bouin solution. Samples were fixed for overnight and after dehydration were processed for paraffin embedding following the microtome technique. The sections were processed in alcohol-xylene series and were stained with haematoxylin and eosin and finally the sections were examined under light microscope for the evaluation of histopathological changes.

5.2.16. Statistical analysis

All the parameters were assayed in triplicate manner. The data was expressed as Mean±SEM. The differences between the means of each group were tested using a

one way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA). P<0.05 was considered to indicate a statistically significant difference.

5.3. Results

5.3.1. Determination of liver index

At the end of the experimental course, there was significant difference in liver index between treated and control rats. A significant reduction in liver index was recorded in rats treated with LCT compared to the control. The pre-administration of taurine with LCT shows an ameliorating effect(fig 5.1.).

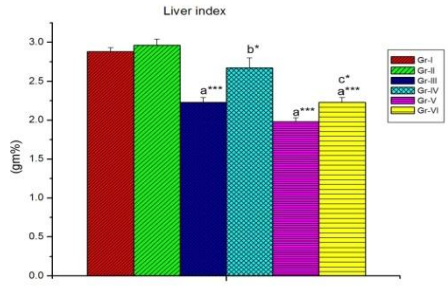


Figure 5.1 shows the liver index of control and treated rat. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (*indicates p<0.05, *** indicates p<0.001).

5.3.2. Measurement of intra cellular reactive oxygen species in hepatic cell

Hepatic cells exposed to lambda cyhalothrin (LCT) showed significant increased in DCF fluorescence intensity in term of ROS production in hepatic cells. The results also indicate a protective effect of taurine against LCT induced toxicity (Fig 5.2.B).The fluorescence images are correlated with the fluorescence intensity level (fig 5.2.A). 2,7- dichlorofluoresceindiacetate (DCFH-DA) was used to monitor oxidation in biological systems. After diffusing in the cell membrane, the fluorescent probe, DCFH-DA, is hydrolyzed by intracellular esterase to non-fluorescent dichlorofluorescein (DCFH). DCFH is then trapped inside the cells and oxidized to fluorescent DCF by peroxides in the presence of ROS (Le Bel et al.,1992).The trapped intracellular fluorescent dye DCF used to assess and detect intracellular ROS.

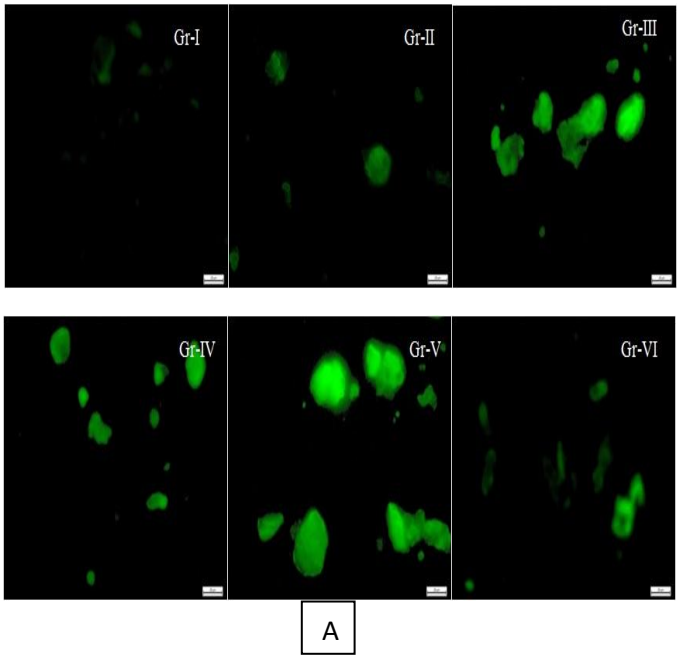


Figure 5.2.A represents the photomicrography of ROS formation in hepatic cell.5.2.B represents the DCF fluorescence intensity in term of ROS production in hepatic cells. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (*** indicates $p<0.001$).

5.3.3. Alteration of mitochondrial membrane potential (MMP) in hepatic cell

Analysis of mitochondrial membrane potential is based on Rhodamine 123 fluorescence intensity. In LCT intoxicated rat a depletion of MMP was noted compared to control rat (fig-5.3). Taurine treatment normalized the altered MMP.

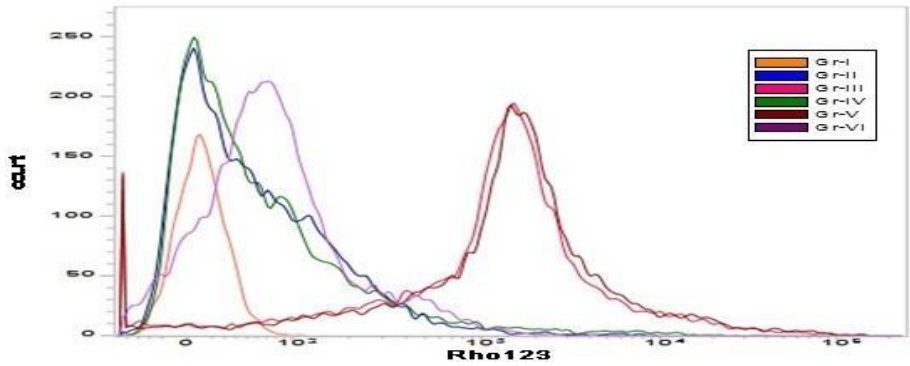
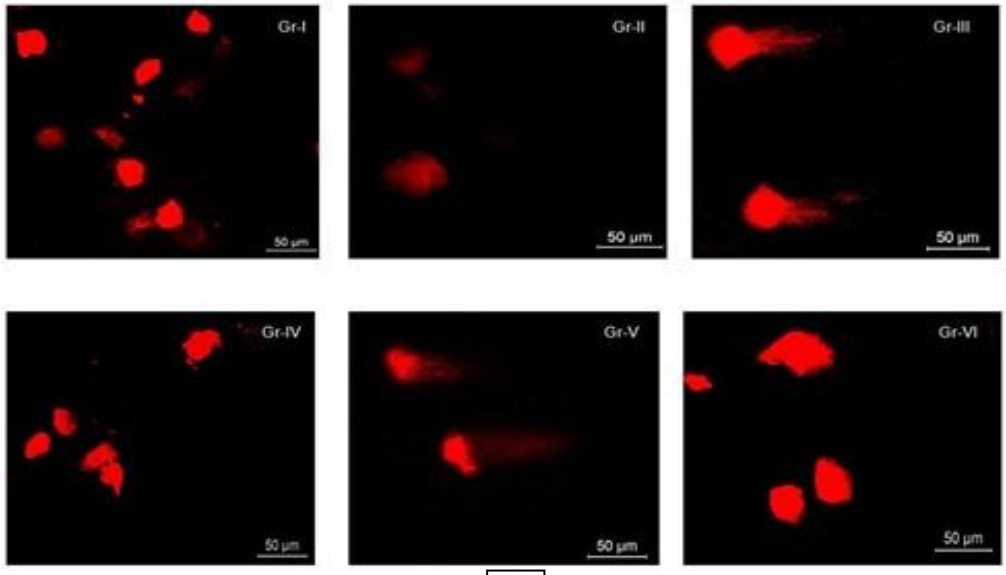


Figure-5.3 shows the mitochondrial membrane potential in hepatic cell, analyzed by flow cytometry using rhodamine-123 as fluorescent dye.

5.3.4. Detection of hepatic DNA damage using comet assay

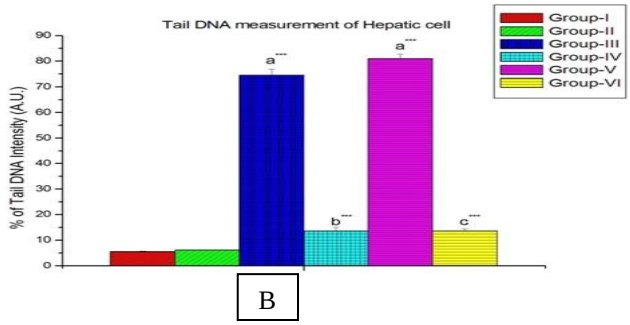
DNA damage was detected by the comet assay. Using a very small sample of cells, this test used to assess the genotoxic effect by evaluating tail length, tail intensity of

single DNA. As shown in the figure-5.4.A, no comet like appearance was found in control group (Gr-I). After LCT treatment (Gr-III, V), well-formed comet tail were clearly visualized. Pre treatment of taurine decreases the effect of LCT on hepatic cell (Gr-IV, VI).Percentage of tail DNA intensity was increased significantly in LCT intoxicated rat. Pre treatement of taurine reduced the damage caused by LCT exposure (fig-5.4.B).



A

Figure-5.4A represents the photomicrographic image of comet assay



B

Figure-5.4.B represents the graphical representation of tail DNA intensity measurement of hepatic cells. Results are expressed as Mean±SEM. Analysis is done

by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (*** indicates $p<0.001$).

5.3.5. Lipid peroxidation and antioxidant status in liver

The effect of LCT and taurine on malondialdehyde (MDA content) in treated and control rat is shown in fig-5.5.A. MDA level was significantly higher ($p< 0.01$) in LCT treated group compared to control in a dose dependent manner. Taurine restored it to a good extent.As depicted in fig-5.5.B, the reduced glutathione (GSH) level in LCT intoxicated groups were significantly ($p<0.001$) decreased compared to the control group. However, the GSH level was significantly improved in taurine pretreated group. Activity of oxidized glutathione (GSSG) level in LCT treated animals is shown in fig-5.5.C. GSSG level in LCT treated groups was increased significantly compared to control in a dose-dependent manner, where pretreatment of taurine restored the normal status.LCT exposure significantly ($p <0.001$) decreased the activities of superoxide dismutase (SOD), catalase (CAT) compared to control group. Moreover, the pretreatment of taurine caused a restoration in these enzymatic antioxidants activity. (fig-5.5.D, E).The activities of glutathione peroxidase ($GP_{X)}$ and glutathione-S-transferase (GST), glutathione reductase (GR) were significantly ($p< 0.001$) reduced after LCT treatment. Pre treatment of taurine significantly increased activities in respect to alone LCT-exposed groups (fig-5.5.F, G, H).

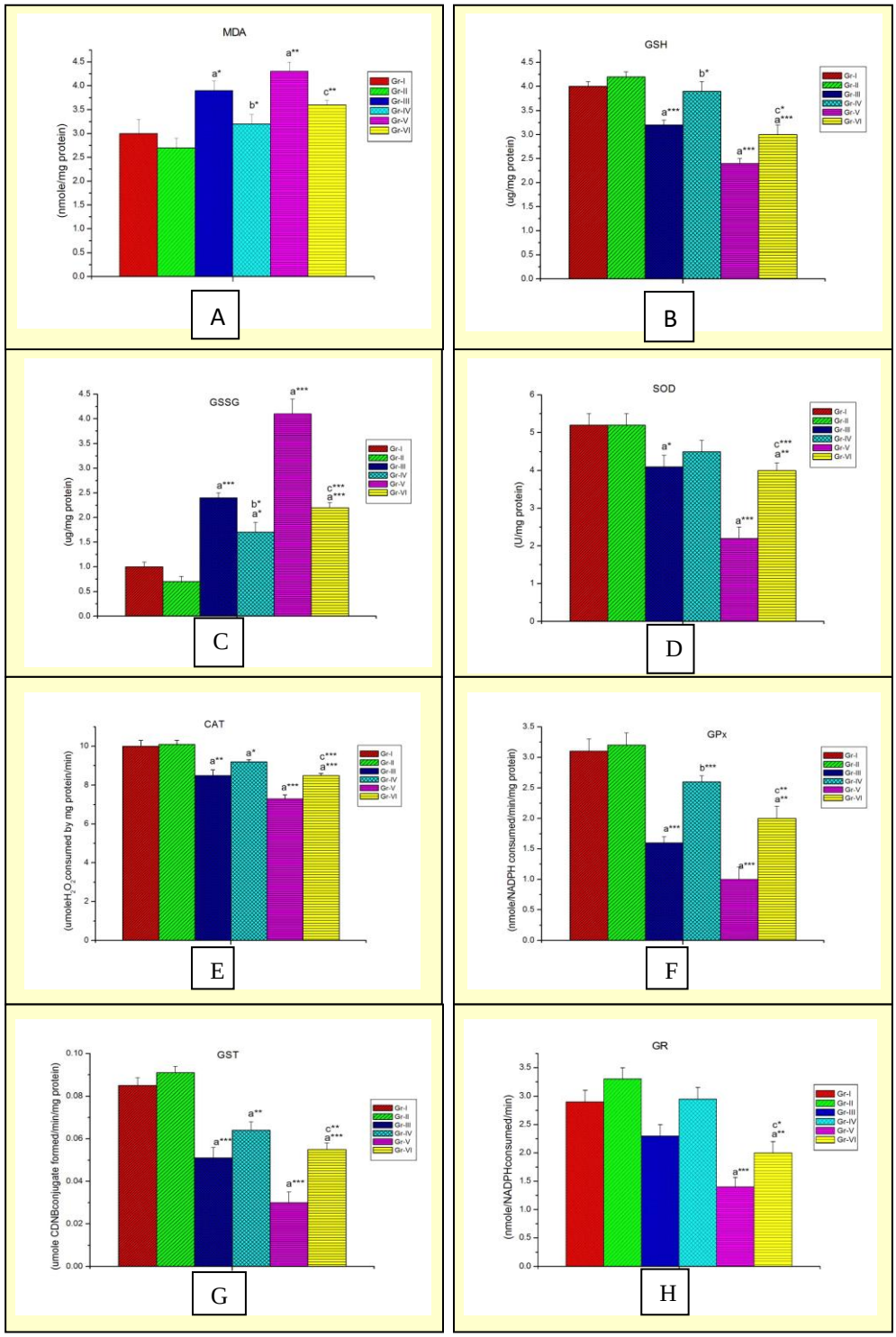

MDA
(nmole/mg protein)
Gr-I
Gr-II
Gr-III
Gr-IV
Gr-V
Gr-VI
A
GSH
(ug/mg protein)
B
GSSG
(ug/mg protein)
C
SOD
(U/mg protein)
D
CAT
(umoleH2O2consumed by mg protein/min)
E
GPx
(nmole/NADPH consumed/min/mg protein)
F
GST
(umole CDNBconjugate formed/min/mg protein)
G
GR
(nmole/NADPHconsumed/min)
H

Figure-5.5 shows the lipid peroxidation and antioxidant status alterations induced by LCT and the protective effect of taurine. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (*indicates $p<0.05$,** indicates$p<0.01$, *** indicates $p<0.001$).A=malondialdehyde (MDA level) ,B=reduced glutathione (GSH), C=oxidized glutathione (GSSG) , D=superoxide dismutase (SOD), E=catalase (CAT), F= glutathione peroxidase ($GP_{X)}$.G=glutathione-S-transferase (GST),H=glutathione reductase(GR).

5.3.6. Blood glucose and liver glycogen content

Biochemical analysis showed a significant increase ($p<0.001$) in blood glucose (fig-5.6A) with a marked reduction in serum glycogen (fig-5.6B) in LCT-exposed group. However, pre-treatment of taurine can able to prevent lambda cyhalothrin induced toxicity significantly.

5.3.7. Estimation of the activity of marker serum enzymes

As shown in fig-5.6.lambda cyhalothrin induced hepatotoxicity was confirmed by significant ($p<0.001$) elevated activities of serum glutamate-oxaloacetate transaminase (ALT/SGOT) (fig-5.6.C), serum glutamate-pyruvate transaminase (AST /SGPT) (fig-5.6.D) and serum alkaline phosphatase (ALP) (fig-5.6.E) and lactate dehydrogenase (LDH) (fig-5.6.F). No significant changes were observed after taurine treatment alone. On the other hand, pre treatment with taurine along with

lambda cyhalothrin had significantly reduced the above stated liver biomarker serum enzymes.

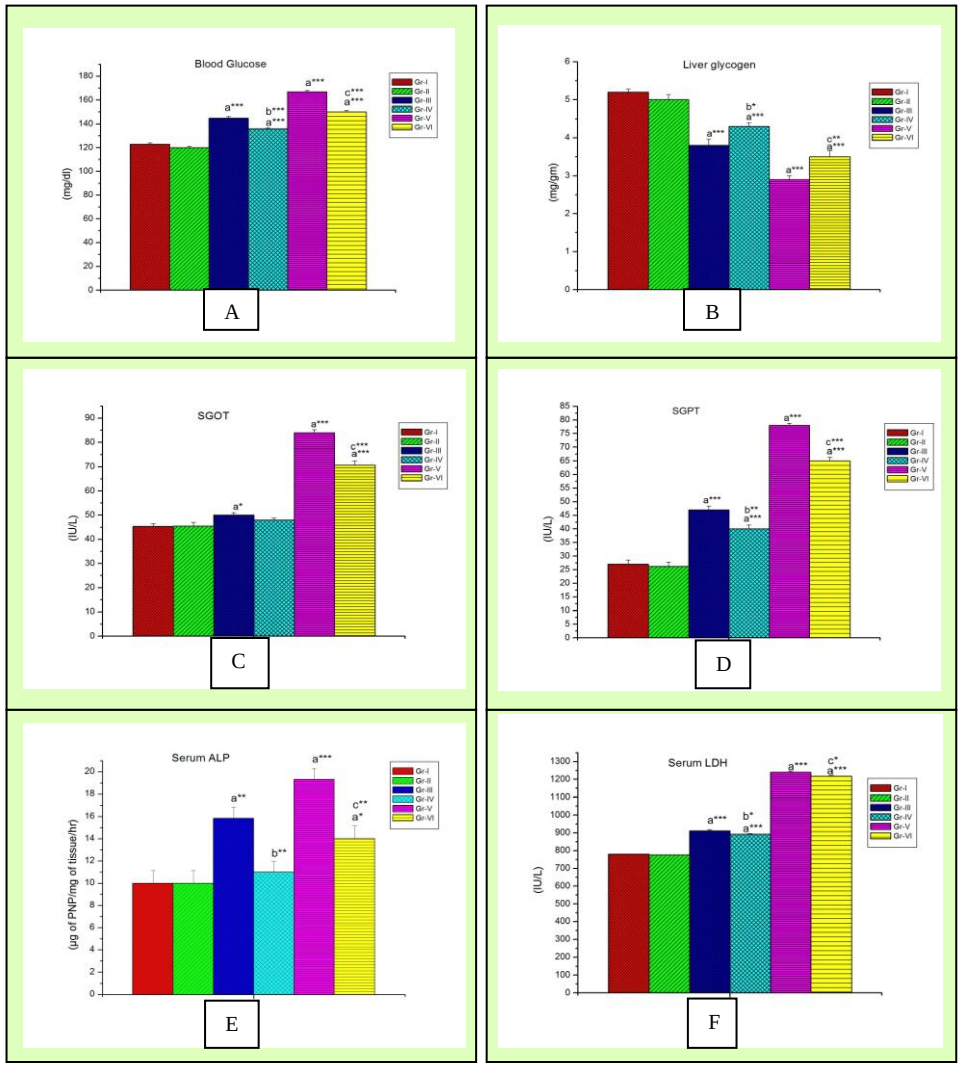


Figure-5.6 shows the biochemical changes after taurine pre treatment in LCT treated rats. Data represent Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI

(*indicates $p<0.05$,** indicates $p<0.01$, *** indicates $p<0.001$).A=Blood glucose. B=Liver glycogen. C=SGOT. D=SGPT. E=Serum ALP. F= Serum LDH.

5.3.8. Determination of lipid profile and serum bilirubin

Table 5.1. reflect the significant elevated level of serum total cholesterol (TC), serum triglyceride (TG) and serum low density lipoprotein (LDL), serum very low density lipoprotein (VLDL) along with reduced high density lipoprotein cholesterol (HDL) level in lambda cyhalothrin intoxicated rat. The significant elevation ($p<0.001$) in total serum bilirubin level in pyrethroid treated groups compared to control were observed in this experiment.

5.3.9. Effect on histopathology of liver tissue

The histopathological pictures of liver tissue are presented in fig-5.7. Histological profile of control animals showed normal hepatic cyto-architecture. LCT intoxicated rats showed loss of radial arrangement of hepatocytes, congestion of central vein, dilatation of sinusoids between hepatocytes, blood congestion in central vein and bi-nucleation of hepatocytes. However, the liver section of the animals pretreated with taurine showed more or less similar to control group i.enormal hepatic cords and absence of severe congestion in central vein.

Table 5.1.Effect of LCT and taurine on lipid profile and serum bilirubin in male rats.

Parameters	Group-I	Group-II	Group-III	Group-IV	Group-V	Group-VI
TC (mg/dl)	120±2.4	115±1.5	135±1.59 a***	121.33±1.62 b***	145±1.29a***	126 ±1.32 c***
TG (mg/dl)	83.66 ±1.5	80±1.06a**	124.5±1.33 a***	92±1.13 b***	135±1.52 a***	126±1.18 a***c***
HDL (mg/dl)	48±1.5	52.83 ±1.19 a*	34±1.18 a***	40±0.93 a**b***	25±1.09 a***	31±0.96 a*** c**
VLDL (mg/dl)	16.73 ±0.29	16.0± 0.21	24.9±0.27 a***	18.4±0.22 a** b***	27±0.30 a***	25.2±0.24 a***c***
LDL (mg/dl)	55.26 ±2.2	46.6± 1.8 a*	76.1±2.5 a***	62.93±1.47a* b**	93±1.5a ***	69.8±1.98a** c***
BL(mg/dl)	0.3±0.05	0.4±0.06	1±0.07 a***	0.5±0.02 a**b***	1.7±0.1 a***	1.2±0.1 a***c***

Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI

(*indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001).Total cholesterol(TC),Triglyceride (TG),High density lipoprotein cholesterol (HDL),Very low density lipoproteins (VLDL),Low density lipoproteins (LDL), Bilirubin level(BL).

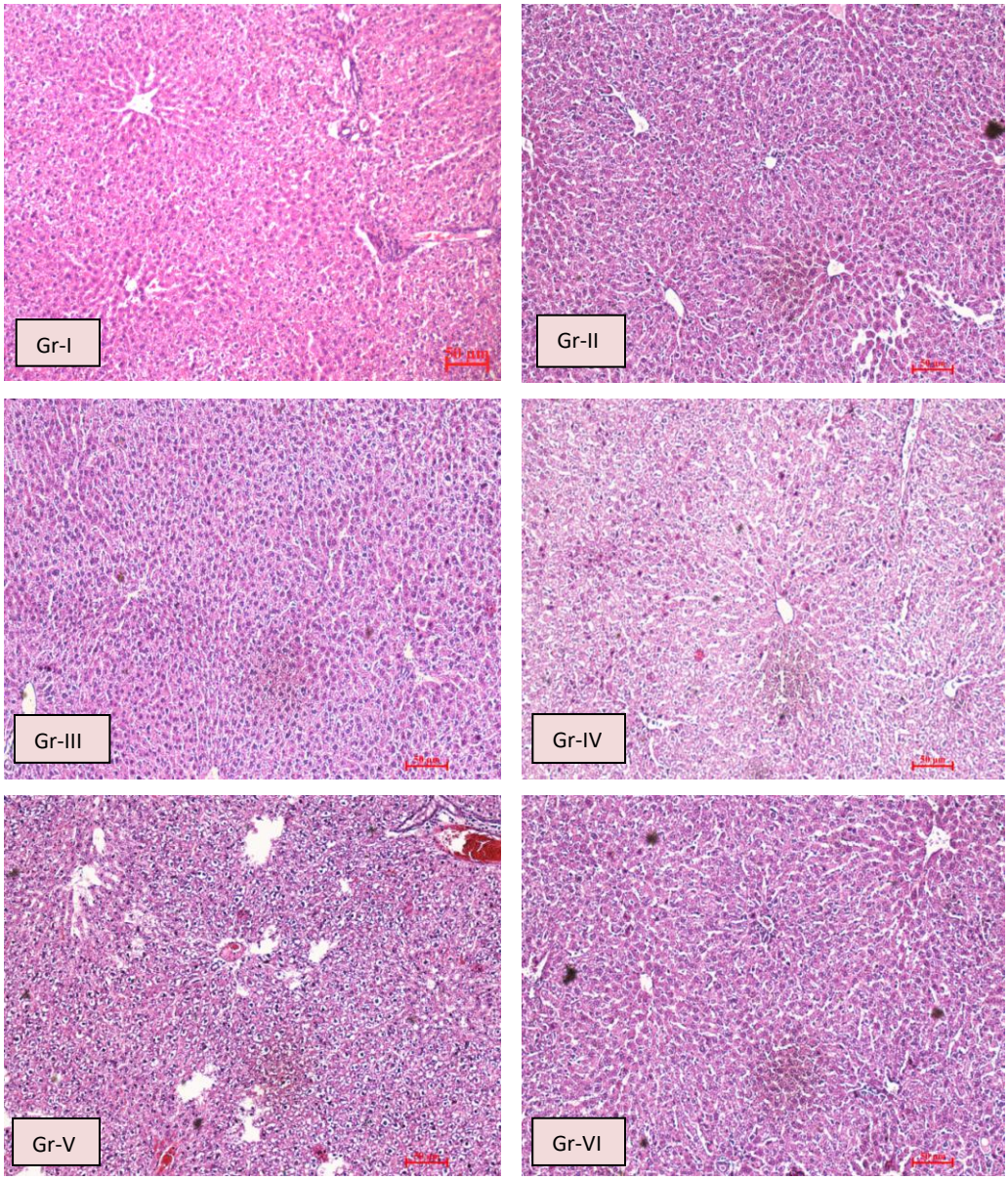


Figure-5.7. Histopathological examination of rat liver of control and experimental groups of rats. Specimens were stained with hematoxylin and eosin.

5.4. Discussion

Liver is one of the essential organs which play a crucial role in the mechanism of detoxification and abolition of toxic materials from the body. Constant exposure and intoxication to different types of exogenous toxins and pollutants including pesticides on a daily basis may produce hepatic abnormalities.

In toxicological studies, body, organ and relative organ weights are important criteria for evaluation of organ toxicity (Heikal et al., 2011) .In the present study, oral administration of LCT resulted in a significant reduction and elevation in body and liver weights of rats, respectively. The reduction in body weight may be due to the action of LCT that produced oxidative stress.

Oxidative stress takes place when the balance between antioxidants and ROS are disturbed either by diminution of antioxidants or elevation of ROS. In the present study, oxidative damage in hepatic cells was reflected through excess production of ROS in terms of increased DCF flurosence intensity in lambda cyhalothrin intoxicated rat. Excess production of ROS due to LCT exposure was confirmed by the production of fluorescent DCF simply correlate with the flurosence microscopic image. These kinds of results are also corelated with previous study where DNA fragmentation was provoked by cypermethrin in rat brain (Hussien et al., 2013). Hepatocyte damage mediated by oxidative stress is associated with the disruption of the mitochondrial membrane potential (MMP). The MMP connotes the mitochondrial energetic state in a living cell and is often utilized to measure the

activity of the mitochondrial respiratory chain, electron transport systems and the activation of the mitochondrial permeability transition (Ly et al., 2003). Reduced MMP in isolated hepatic cell may highlight towards the functional impairment of the hepatic system. Pretreatment with taurine decreased ROS production, thereby controlled DNA fragmentation and also improved the MMP in LCT intoxicated rat hepatic cells. This excess production of ROS may directly fragment the hepatic DNA which was noticed in the present study in terms of DNA damage detected by comet assay. Pretreatment with taurine, controlled excess ROS production by its antioxidant property and showed stimulatory effects on endogenous antioxidants (Saad and Rikabi, 2002) and facilitated to normalize rat hepatic status.

Oxidative stress was also verified through elevation in lipid peroxidation (LPO), reduction in antioxidant enzymes (SOD, CAT, GPx, GST and GR) and altered glutathione level. MDA has been used as a sensitive biomarker for oxidative damage. On the other hand, GSH takes part in the liver detoxification and regulating the thiosulphide status of the cell. It may be consumed for conjugation reaction, which mainly involves metabolism of xenobiotic agent. Many abnormal conditions noticeably decrease cellular GSH concentrations by non-enzymatic oxidation of GSH to glutathione disulfide (GSSG). The cause of this alteration of GSH to GSSG is oxidative stress (Grăvilă et al., 2010). The results of the present study indicated that the oxidative injury in liver of LCT treated rats was prominent and also evidenced by increased liver MDA, GSSG level and decreased liver GSH content. In the present result decrease of other antioxidant status in LCT treated rat establishes

the weakness to oxidative damage. These enzymes work mutually to abolish active oxygen species. Results were also in line with previous studies which have shown that deltamethrin induced hepatotoxicity increased MDA level, and decreased hepatic enzymatic and non-enzymatic antioxidant level. Taurine may accomplish inhibition of lipid peroxidation and also act as an indirect antioxidant by impeding changes in hepatic system resulting from oxidative damage (Timbrell et al., 1995; Hagar, 2004).

Hepatotoxicity could also be elucidated by the mess up of normal blood glucose and hepatic glycogen level. Increased fasting blood glucose levels in lambda cyhalothrin exposed rats were observed compared to the control group in the present study. The increased blood glucose level may be due to the interference of carbohydrate metabolism resulted from the alteration of the catecholamine levels (Goodman and Gilman, 1975) or phosphorylase activities or due to less peripheral glucose utilization. Observed reduced hepatic glycogen level in the present study may be a consequence of abruptly increased catabolism to meet higher energy demands caused by LCT intoxication.

In the assessment of liver damage by pyrethroids, the assay of hepatic biomarker serum enzyme levels such as alanine transaminase (ALT),aspartate transaminase (AST), and alkaline phosphatase(ALP) mostly used (El-Demerdash,2004; Stockham and Scott,2002). ALT is an enzyme that transfers -NH_2 group from the amino acid alanine to a keto acid acceptor. Aspartate transaminase plays a role in the

metabolism of the amino acid alanine. Hepatocytes contain a huge amount of aspartate (AST) and alanine transaminase (ALT) enzymes and these enzymes diffuse into plasma due to liver damage. ALT occurs in high amount in hepatocyte cytosol and is reflected as an indicator of liver cell damage (Kalender et al., 2010).Alkaline phosphatase (ALP) is used to diagnose liver injury and is reported that the level of ALP is increased due to liver diseases (Aturk et al., 2006).For the assessment of liver damage, the determination of the serum ALP is largely used (Bradberry et al., 2005). In the present investigation marked increase in liver ALT and AST under stress of pesticides has been observed. This elevation in the above said parameters have been well supported by different studies (Rao and Banerji ,1990; Sahni and Saxena, 2001). The increase in transaminase activity in the serum is indicative of cellular leakage and loss of functional integrity of hepatic cell membrane that release of the enzymes in sinusoidal spaces to intralobular vein (Rahman et al., 1996). The increased levels of enzymes were normalized to a good extent after 14 days pretreatment of taurine pointed out that it provided protection by stabilizing the structural integrity of the hepatocellular membrane against lambda cyhalothrin. Level of serum alkaline phosphatase is associated with the hepatocytes functioning. Bile canaliculi cells lining usually synthesize increased serum alkaline phosphatase in response to cholestasis and increased biliary pressure (Gaw et al.,1999) . Lambda cyhalothrin administration increased serum ALP level and it was brought to near normal level by taurine treatment. Hepatic damage was also reflected in LCT treated group through the altered serum lactate dehydrogenase, a sensitive intracellular enzyme and an

indicator of liver cell damage (Kim et al., 2001). Lactate dehydrogenase catalyzes the oxidation of L-lactate to pyruvate by hydrogen transferring with intervention of NAD+ as a hydrogen acceptor. Any change in LDH activity designates change in the lactate production from pyruvate under anaerobic conditions favouring the reoxidation of NADH. This permits glycolysis to carry on in the absence of oxygen by generating sufficient NAD. Elevation in the specific activity of LDH in experimental animals when compared to respective controls observed in the present investigation may be due to forward reaction of LDH, namely pyruvate to lactate which may be operative during LCT toxicity. It is also to meet energy demands when aerobic conditions are lowered due to diminished TCA cycle enzyme activities (Martin et al.,1983). Increased activity of LDH is a characteristic feature of a shift from aerobic to anaerobic metabolism leading to an elevated rate of pyruvate conversion into lactate, resulting in lactic acidosis (Chinoy et al.,1996). The LDH activity increases during conditions favoring anaerobic respiration to meet energy demands, when aerobic respiration is lowered (Murray et al., 1995). Increased LDH activity was supported by several authors (VasanthaSena, 2002; Manna et al., 2004).Pretreatment with taurine caused apparent normalization in the LDH level.

Bilirubin is a compound formed from the breakdown of hemoglobin. Bilirubin is one of the important biochemical indices used to assess hepatotoxicity as liver plays a key role in bilirubin metabolism. In our finding increased level of total bilirubin in LCT treated rats may be due to increased amounts of free haemoglobin resulted from increased destruction of red blood cells. The bilirubin level depends not only on the

amount of haemoglobin broken down, but also on the capability of the liver to excrete the increased amount of bilirubin present in it (Stephen et al.,1997) .So another possible mechanism is to blockage of biliary tract in LCT treated rat. Water insoluble bilirubin within the hepatocytes is conjugated with glucuronic acid and formed bilirubin diglucoronide by glucoronosyl transferase then excreted in the bile in water soluble form. May be LCT act on this step and lead to hyperbilirubinemia. In studies observing the role of taurine in improving hyperbilirubinemia, it may be assumed that taurine may improve excretion of bile, blood flow, and enhances the functions of hepatocytes (Miyata et al., 2006; Guertin et al., 1991).In LCT exposed rats, the rise in total serum cholesterol level could be due to obstruction in liver bile ducts causing decline or interruption of its secretion to the duodenum consequently producing cholestasis (Saxena and Sharma 1999). The disturbance in lipoprotein formation is one of the factors leading to accumulation of cholesterol in pesticide treated mice (Hassan et al., 1995) and it may be a reason for elevated cholesterol in LCT exposed rats. Administration with taurine may decrease the levels of total cholesterol. The hypocholesterolemic effect of taurine has been reported in mice (Kamata et al., 1996), rats (Sugiama et al., 1989; Sugiama et al., 1984), and humans (Zhang et al., 2004), but its mechanism is not well established. Taurine takes a part in conjugation reaction with bile acids in the liver. Taurine increases bile acid synthesis (Yamanaka et al., 1986) by simultaneous upsurge in the mRNA expression and activity of cholesterol 7α-hydroxylase, a rate-limiting enzyme in hepatic bile acid synthesis (Ebihara et al., 2006). The primary mechanisms accountable for the

hypocholesterolemic action of taurine may be due to the increased conversion of cholesterol into bile acids through the activation of cholesterol 7α-hydroxylase.

Triglycerides are free fatty acids esters of glycerol. Liver causes biosynthesis and assimilation of lipoproteins like LDL and VLDL through which triglycerides are secreted into circulation (Shen et al., 1998). Rise of these lipoproteins in current study causes increase of serum triglyceride. In the present study, pretreatment of taurine significantly alleviate lambda cyhalothrin induced rise in the liver triglyceride level. Decrease serum HDL is associated with elevated serum cholesterol level in LCT treated rat because HDL mainly plays an important role in cholesterol efflux from tissues (Shakoori et al., 1988).Pretreatment of taurine try to normalized the above said changes.

Some important histopathological findings were observed in rat liver after exposure to different doses of LCT. Those were the loss of radial arrangement in hepatocytes, congestion of central vein, dilated sinusoids between hepatocytes, blood congestion in central vein. Histological data from the current study suggest that the active ingredient of LCT may damage the normal architecture of liver through the generation of reactive oxygen species. The underlying mechanism may be attributed to type II pyrethroid LCT which has a α –cyano moiety, therefore its toxicity may be due to release of cyanohydrins, which are unstable under physiological conditions and further decompose to cyanides and aldehyde which in turn could act as a source of free radical. However, pretreatment with taurine exhibited relatively normal cyto-

architecture of liver as control rat which confirms the hepato protective effect of taurine.

5.5. Conclusion

From the present study it may be concluded that the lambda cyhalothrin exposure produced hepatotoxicity by the over production of ROS, alteration of antioxidant level, hepatocellular DNA fragmentation and decreased mitochondrial membrane potential, hepato-histological abnormality in rat liver. Collectively, these data suggested that taurine pretreatment played an ameliorative role in lambda cyhalothrin mediated hepatic dysfunction, hepato-histological abnormality and oxidative injury in the liver of Wistar rat. The mechanism underlying hepato-protection of taurine may be related to its function as a direct antioxidant by scavenging ROS and accomplishing inhibition of lipid peroxidation and also by the stimulatory effects on endogenous antioxidants.

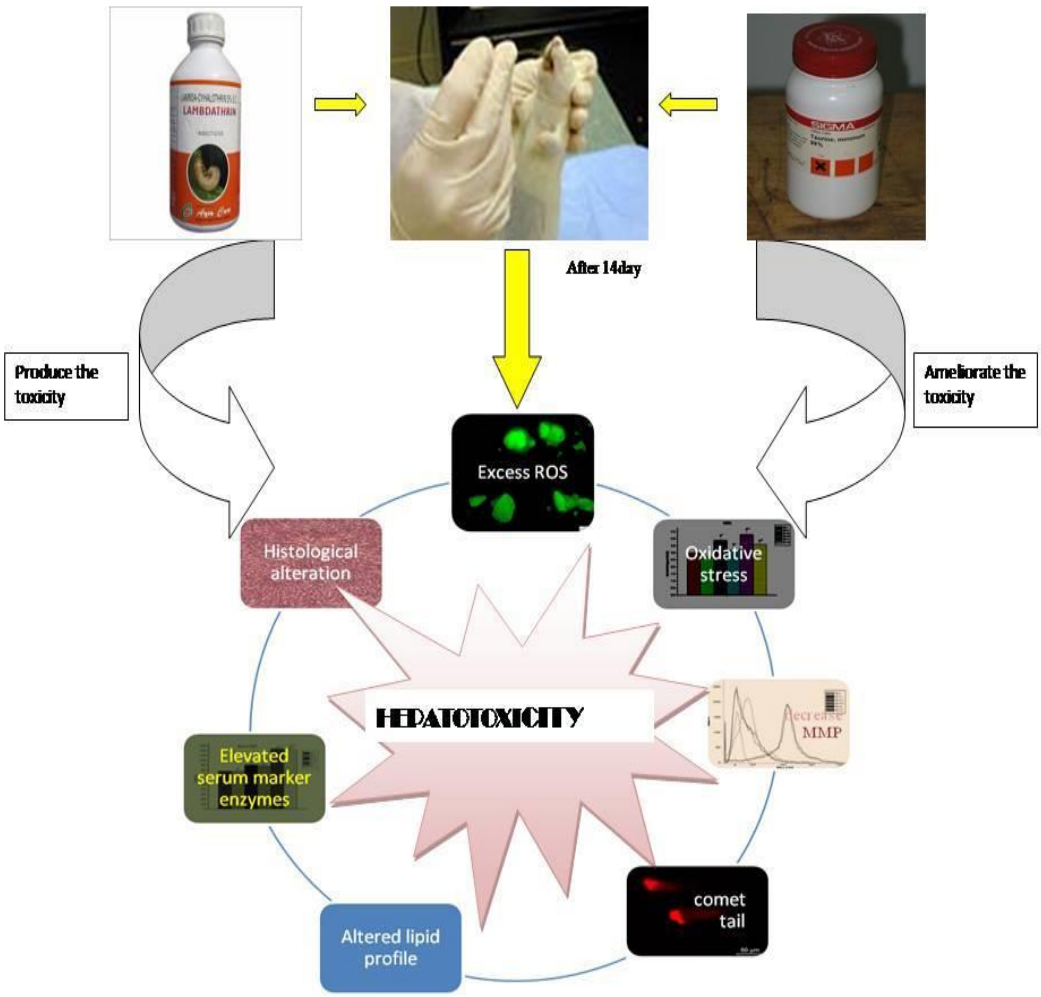


Figure-5.8 shows the proposed LCT induced hepatotoxicity and its amelioration by taurine

5.6. References

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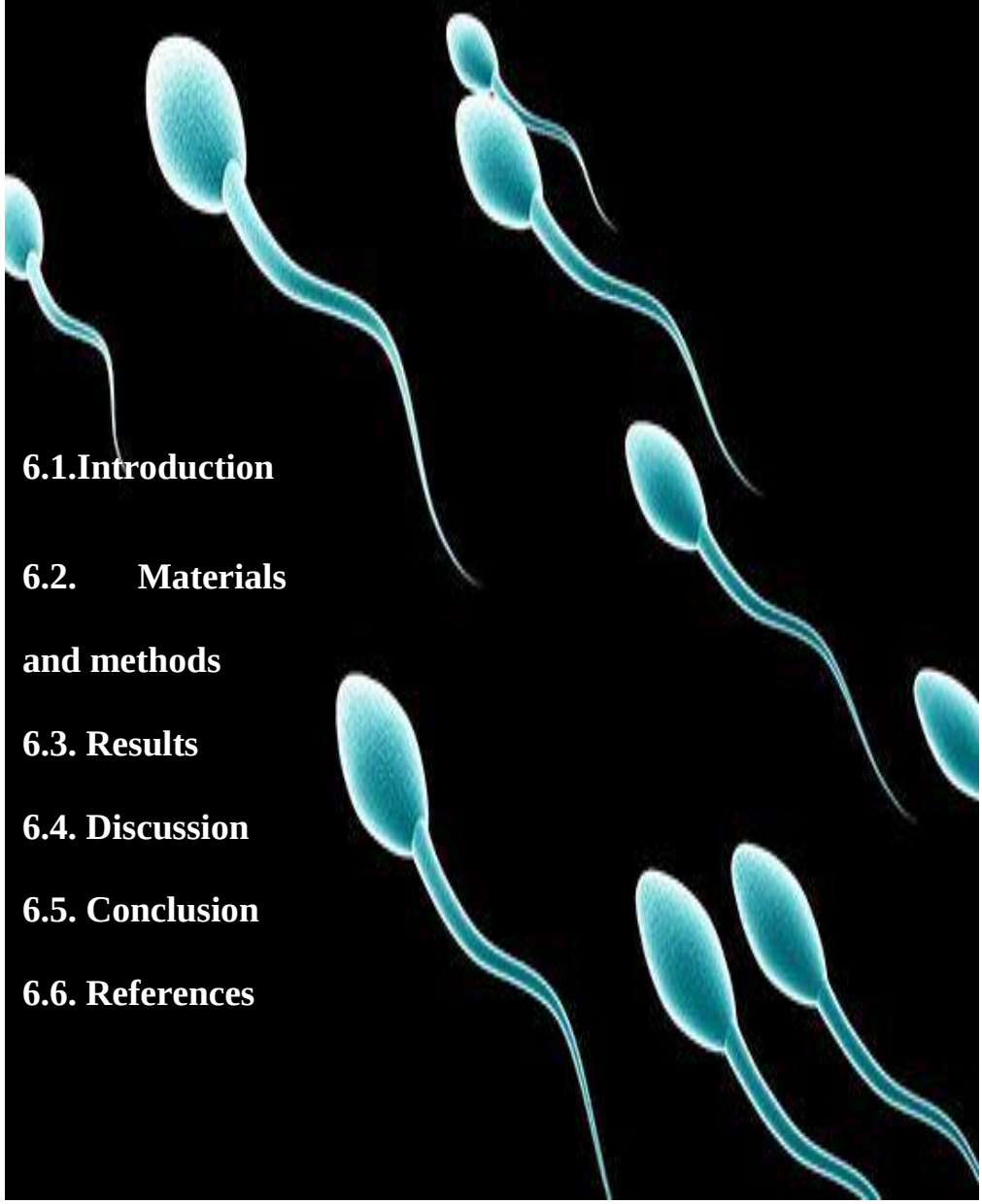
Chapter-6

Lambda cyhalothrin induced male reproductive system toxicity and its prevention by taurine



Abstract

Lambda cyhalothrin(LCT) is a type II pyrethroid insecticide and may cause serious environmental pollution and health problems. Taurine, 2-amino ethanesulphonic acid is an essential amino acid, plays important roles in numerous physiological functions. The present study was conducted to evaluate the adverse effects of lambda cyhalothrin on the reproductive system of male Wistar rats, and also to evaluate the protective role of taurine under these conditions**.** In the study sexually mature male rats were orally received lambda cyhalothrin at two different dose levels (10.83mg/kg body wt. i.e. $1/7^{th}$ of LD_{50} value and15.17mg/kg body wt. i.e. 1/5 of LD_{50} value) for 14 consecutive days along with pre-treatment of taurine(50mg/kg body wt.).A significant decrease in sperm count, viability, motility, seminal fructose concentration, hypo osmotic swelling, testicular reduced glutathione, testicular antioxidant activity and increase in testicular acid phosphatase, malondialdehyde and oxidized glutathione were observed in lambda cyhalothrin intoxicated rats. LCT treatment elevated ROS induced sperm DNA fragmentation observed by comet and TUNEL assay as well as reduced the sperm mitochondrial membrane potential. Spermatogenic and steroidogenic arrest or disorder also reflected by the alteration in the activity of testicular $\Delta^5$3β- and 17β-hydroxy steroid dehydrogenase with their suppressed protein expression level along with StAR and P450scc with the possible compensation of adrenal steroidogenesis. Decreased serum level of testosterone and pituitary gonadotrophins were also detected in LCT intoxicated rat. LCT induced toxicity was also confirmed by the histopathological studies. Taurine shows the protective effect against LCT induced reproductive toxicity in male rat by playing a role as an antioxidant and by stimulating spermatogenesis and steroidogenesis through its effect on hypothalamo-pituitary-gonadal axis. Therefore, the results reveal the toxic effect of lambda cyhalothrin on male reproductive system of rat and also point out towards the beneficial influences of taurine in this situation.

Rini Ghosh, Tuhina Das, Anurag Paramanik and Sujata Maiti Choudhury.Testicular oxidative stress and biochemical perturbations induced by lambda cyhalothrin and the protective effects of taurine in rats. World journal of pharmacy and harmaceutical sciences.2016; 5: 1486-1502.

6.1. Introduction

Due to escalation of farming in order to obtain higher yields, the use of synthetic pesticides has raised quickly in the last fifty years globally. However, over dependence on chemicals not only resulted in a high cost of production but also responsible for irreversible damage to the environment and long term health problems to humans and other living system.

6.1.1. Male reproductive physiology

The reproductive role of the male is to produce and deliver sperm to impregnate the female. To carry out these functions, a male has internal and external sexual organs. These structures include the testes, several tubules that carry sperm out of the testes, various glands, and the penis. In most mammalian species, including human, the male's external reproductive organs are the scrotum and penis. The internal reproductive organs consist of gonads that produce gametes (sperm cells) and hormones, accessory glands that secrete products essential to sperm movement, and ducts that carry out the sperm and glandular secretions (Campbell and Reece, 2005). Inside the testis is a network of fine-diameter tubes called seminiferous tubules. Seminiferous tubules lie within the testicular parenchyma and contain the Sertoli cells and the developing germinal cells (Campbell and Reece, 2005).

The Leydig cells are clustered within the interstitial compartment of the testes. The Leydig cells are the main source of androgen production in the male (Hooker, 1994). These cells are considered to be steroidogenic even in foetal development and

throughout life allowing for hormonal signaling throughout the body and to androgen dependent organs, especially the testes (Ewing and Zirkin, 1983).

The Sertoli cells lie adjacent to the seminiferous tubules and support the development and maturation of the germinal cells (spermatagonia). Sertoli cells, nourish, support, and protect developing germ cells, which undergo cell division by meiosis to form spermatozoa (Johnson et al., 2008). Sertoli cells provide immunological support by structurally supplying tight junctions (blood-testis barrier) and by phagocytizing cytoplasm and surplus spermatozoa material (Berndtson et al., 1987). In addition, Sertoli cells also provide essential protein complexes to support differentiation, growth, and maturation to the developing spermatagonia and spermatocytes, specifically androgen binding protein (ABP), transferrin, activin, and inhibin (Suresh et al., 2011). Testosterone is primarily important for the Sertoli cell development and maturation.

The production of testosterone is a complex cellular mechanism. Testosterone is necessary for normal testicle function, and in addition, essential for androgen dependent organs including the epididymis and accessory sex glands to produce fructose and citric acid, which are vital sources of energy and a vehicle for sperm. Cholesterol is the essential precursor to androgen synthesis and is available through metabolic acquisition in plasma or lipid droplets. Conversion of cholesterol to pregnenolone is the rate limiting step to androgen synthesis and is controlled by hydroxylation with catalytic properties from enzymatic side-chain cleavage complex in the mitochondria, specifically cytochrome P_{450} side chain cleavage (Hall, 1994; Kallen et al., 1998). Pregnenolone is further processed by enzymatic activity to

progestin steroid precursors through two different pathways. Cleavage of the two-carbon side-chain allows for irreversible production of weak androgens that can be further processed to stronger androgens including testosterone and the more biologically active dihydrotestosterone (DHT) by the 5α-reductase enzyme (Senger, 2004).

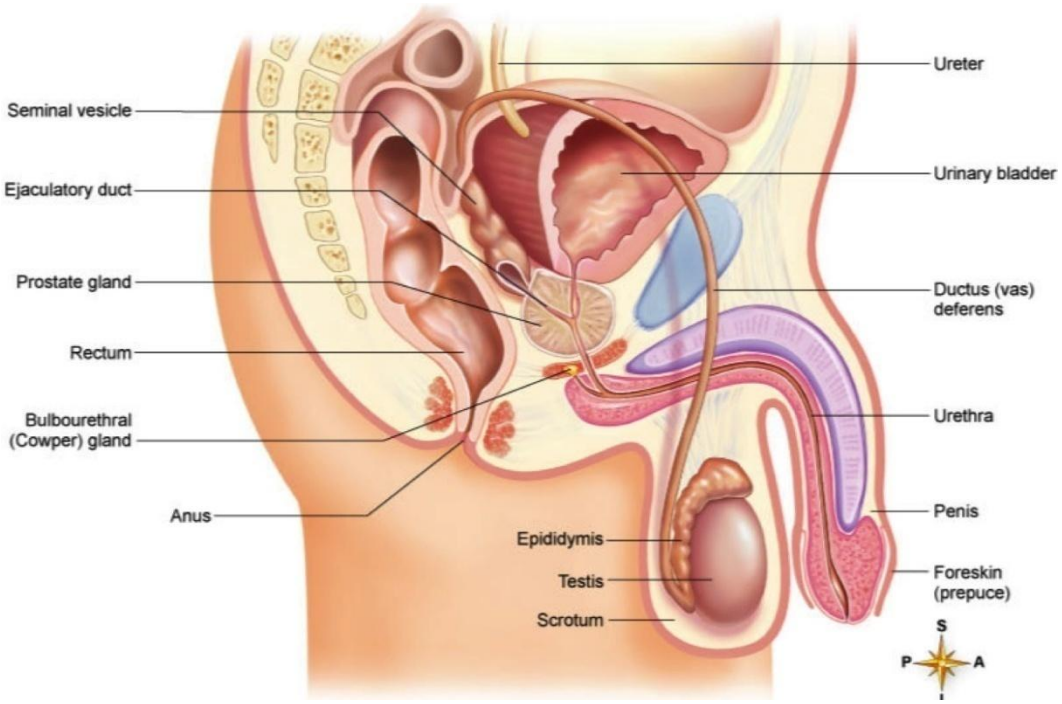


Figure 6.1: Organization of male reproductive organs.
Source: Mosby item and derived items (copyright) 2016, 2006,2002,1997,1992 by Mosby, Inc., an affiliate of Elsevier Inc.

6.1.2. Effects of pesticides on male reproductive system

Pesticides can influence the male reproductive system at one or at multiple sites including testes, the accessory sex glands, the central nervous system, and the neuroendocrine system (Moline et al., 2000). These may directly injure spermatozoa, by altering the endocrine function in any stage of hormonal regulation (hormone

synthesis, release, storage, transport, and clearance; receptor recognition and binding) and Sertoli cell or Leydig cell function.

To boost the production of reactive oxygen species (ROS) is one of main mechanism of pesticides induced toxicity. Connection between the production of excess reactive oxygen species (ROS) and male infertility was already established (Makker et al., 2009). Elevated ROS spoil the sperm and testicular functioning that is responsible for impaired spermatogenesis, suppression of steroidogenesis, and androgen synthesis through lipid peroxidation and DNA damage (Allen et al., 2004). Elevated ROS interfere in the completion of chromatin packing of sperm DNA during spermatogenesis when protamine replacement occurs in elongating spermatids (Smith and Haaf, 1998). Temporary nicks linked to the topoisomerases activity, facilitate histone-protamine replacement, but if these nicks are not fixed by the increased ROS, they will evolve into DNA fragmentation on mature sperm. Other internal causes of DNA fragmentation may occur due to post-testicular oxidative stress and apoptosis.

6.1.3. Effects of pyrethroid insecticides on male reproductive system

Recent researches indicate that pyrethroid induced reproductive toxicity is also mediated by two different mechanisms of direct influencing of cells and or by affecting the essential biochemical responses (Colborn, 1998; Mclachlan, 2001). Decreased semen quality and increased sperm DNA damage in relation to urinary metabolites of pyrethroid insecticides in human were already established (Meeker et al., 2008). Similarly various pathological effects of pyrethroids on reproductive performance of animals have been already reviewed. In different studies, the

histopathological effect of pyrethroids on testes and epididymis shows a correlation with altered sperm characteristics and reduction in sperm count, testosterone concentration and fertility.

6.1.4. Lambda cyhalothrin (LCT) as a reproductive toxicant

Ratnasooriya et al. conclude that LCT treatment to male rat impaired sextual compitance (Ratnasooriya et al., 2000). Poor fertility and cell damage in male rat causeed by LCT was also reported by Oshoke et al. (Oshoke et al.,2016). But there was a lack of information regarding the role of LCT on steroidogenesis and hormonal level.

6.1.5. Mitigating role of taurine in reproductive system

Taurine, 2-aminoethanesulfonic acid is a conditionally essential nutrient can be biosynthesized by male reproductive organs (Li et al., 2006). Taurine has been spotted in Leydig cells, vascular endothelial cells, and some other interstitial cells of testis and epithelial cells of efferent ducts in rats (Lobo et al., 2000). It may act as an antioxidant (Alzubaidi and Diwan, 2013), capacitating agent (Alzubaidi and Diwan, 2013), membrane-stabilized factor (Alzubaidi and Diwan,2013) and motility factor (Boatman et al.,1990) in sperm. Antioxidant property of taurine is also well-established in reproductive system and therefore, could be considered as a potent candidate to mitigate the male reproductive toxicity.

The aim of the present study is to find out the toxic effect of LCT in male reproductive system and also try to search out the attenuate role of taurine against LCT induced reproductive toxicity.

6.2. Materials and methods

6.2.1. Chemicals and reagents

Lambda cyhalothrin 5% emulsifiable concentrate (RPC Agro Industries, Kolkata), taurine (Sigma Aldrich Inc. USA). Hydrogen peroxide(H_2O_2), Hydrochloric acid (HCl), Sulfo salicylic acid, Dithionitrobenzoic acid (DTNB), Tris-HCl, Pyrogallol, Thiobarbituric acid(TBA), *n*-Butanol-pyridine, Acetate buffer, Acetate buffer, Fructose, Ferric chloride($FeCl_3$), Sodium dodecyl sulfate, Glacial acetic acid, Sodium chloride(NaCl), Phosphate buffer (PBS), Disodium hydrogen phosphate(Na_2HPO_4), Potasium di hydrogen phosphate (KH_2PO_4),Sodium di hydrogen phosphate(NaH_2PO_4), 2-vinyl pyridine, Magnesium chloride($MgCl_2$),Sodium hydroxide(NaOH), Zinc sulphate ($ZnSO_4$),Ferric chloride($FeCl_3$), Bis-polyacrylamide, Cholesterol, Ethylenediaminetetraacetic acid(EDTA), Pentobarbital sodium, reduced glutathione(GSH), 1-Chloro- 2,4-Dinitrochlorobenzene(CDNB),2-vinylpyridine,Sodium azide, Tetra sodium pyrophosphate (TNaPP), Nicotinamide adenine dinucleotide, Testosterone, Dehydroepiandrosterone (DHEA), Radioimmiuno precipitation assay buffer (RIPA) lysis buffer, 2′, 7′-dichlodihydrofluorescein diacetate ($DCFH_2$-DA), Rhodamine-123, Glucose-6-phosphate, Tris-buffered saline with Tween-20 (TBST), Nitro-blue tetrazolium, 5-bromo-4-chloro-3′-indolyphosphate, Polyvinylidene difluoride (PVDF), Methanol, antibodies (Cell Signaling Technology Beverly, MA, USA) and other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA; Himedia India, Ltd., Mumbai, India; and Merck India, Ltd., Mumbai, India for the experimentation.

6.2.2. Animal care

Healthy Wistar albino male rats (weighing 130-150 g) were chosen for this experiment. Standard laboratory feed and water were provided throughout the period of experimentation i.e. 14 consecutive days. Experimental protocol and surgical methods were approved by the Institutional Animal Ethical Committee, registered under CPCSEA.

6.2.3. Treatment protocol

The total animals were divided into six groups of six rats each and design as Group I: Distilled water control (no treatment), Group II: Taurine control (taurine at a dose level of 50mg/kg body wt.)(Ozden et al., 2009; Cetiner et al., 2005), Group III: Lambda cyhalothrin low dose (lambda cyhalothrin at a dose level of 10.83mg/ kg body wt.) (Sharma et al., 2010), Group IV: Taurine (50mg/kg body wt.)+lambda cyhalothrin low dose (10.83mg/kg body wt.), Group V: Lambda cyhalothrin high dose (lambda cyhalothrin at a dose level of 15.17mg/kg body wt.), and Group VI: Taurine(50mg/kg body wt.)+ lambda cyhalothrin high dose (15.17mg/kg body wt.). The route of application preferred for the study was daily oral gavage .At the end of the doses; the animals were fasted overnight, anesthetized using pentobarbital sodium and sacrificed by cervical dislocation on 15th day. Animal's weight was taken daily and the dose was adjusted accordingly.

6.2.4. Sample collection

After sacrificed, serum and one parts of the collected testis from control and treated rats were immediately stored at -80 ºC until analysis. The other part of the testis was

taken for histological analysis. Epididymis were collected and washed immediately for sperm collection.

6.2.5. Determination of testicular index

Testes of sacrificed male Wister albino rat were dissected from its body and all fats were removed from it. Then their weights were taken to calculate testicular index using the following formula:

$$\text{Testicular index} = \frac{\text{Testicular weight}}{\text{Body weight}} \times 100$$

6.2.6. Study on sperm parameters

6.2.6.1. Sperm count

Sperm suspension from the caudal epididymis was diluted (1:100) with phosphate buffer and delivered a drop of the dilution into the Neubauer haemocytometer chamber. The numbers of spermatozoa were counted under a light microscope and finally expressed as $\times 10^6 ml^{-1}$(WHO, 1999).

6.2.6.2. Assay of sperm viability

The eosin-nigrosin staining was considered to determine the sperm viability (WHO, 1999). One drop of sperm suspensions was added with two drops of 1% eosin. Three drops of 10% nigrosin were added and mixed well, after 30 seconds. A drop of mixture was placed on a clean glass slide to make a smear and allowed to air dry. The prepared slide was examined under the light microscope. Pink-stained dead spermatozoa and unstained live spermatozoa were counted and the viability of spermatozoa was expressed as the percent of viable spermatozoa .

6.2.6.3. Sperm motility

Sperm motility was evaluated and averaged by counting the motile and non-motile spermatozoa followed by counting of total sperm, under a light microscope and expressed as the percent motility (WHO, 1999).

6.2.6.4. Study of sperm morphology

The numbers of abnormal spermatozoa were examined under the microscope and expressed as a percentage of the total number of spermatozoa (WHO, 1999**).** At first two drop of eosin stain was added to the one drop of sperm suspension and kept for 5 min. at 37°C. After that a drop of mixture was placed on a clean slide and spread smoothly to make a thin film. The film was air dried and then observed under a microscope.

6.2.6.5. Hypo-osmotic swelling (HOS) test

Liquefied semen (0.1 ml) was added with 1ml of pre warmed swelling solution (0.735 gm sodium citrate dehydrate and 1.351 gm fructose in 100 ml distilled water) and was mixed gently with the pipette. Kept at 37°C for at least 30 minutes and examined the tail curling of sperm cells under microscope (Nikon Eclipse, LV100POL) **(**WHO, 1999**)**.

6.2.6.6. Estimation of sperm intracellular reactive oxygen species (ROS) generation

The intracellular ROS formation of sperm cells was investigated using the $DCFH_2DA$ as an indicator to detect the intracellular reactive oxygen species

produced according to Roy et al. (Roy et al., 2008) Oxidation results in the formation of fluorescent DCF inside the cells was apply to assess and detect intracellular ROS by flow cytometer (BD FACSVerse) and Cell Quest software.

6.2.6.7. Assay of sperm mitochondrial membrane potential (MMP)

The cationic fluorescent dye rhodamine-123 has been utilized for the estimation of mitochondrial membrane potential. Rhodamine-123 is a cell-permeable, cationic dye that can be readily sequestered by active mitochondria inproportion to the mitochondrial membrane potential. MMP is proportional to up taken rhodamine-123 which was measured in flow cytometer (BD FACSVerse) and Cell Quest software (M'Bemba-Meka et al., 2006).

6.2.6.8. Sperm DNA fragmentation study by alkaline comet assay

The alkaline comet assay was performed according to the method of Alcântara et al., (Alcântara et al., 2011). The comet tail length was calculated as the distance between the end of nuclei heads and end of each tail. %DNA (tail) = TA × TAI × 100/ [(TA × TAI) + (HA × HAI)]; where TA= tail area, TAI = tail area intensity, HA= head area and HAI = head area intensity.Detail method was describe in previous chapter 5.

6.2.6.9. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay.

DNA damage was performed by using the TUNEL assay using an *in situ* Cell Death Detection Kit, Fluorescein,Version:16(Cat No: 11684795910).

6.2.7. Assay of testicular lipid peroxidation, reduced and oxidized glutathione

Quantitative measurement of lipid peroxidation was performed in testicular tissue homogenate (20mg/ml in phosphate buffer) according to the method of Ohkawa et al. based on the formation of thiobarbituric acid reactive substances (TBARS) in term of malondialdehyde (MDA) formation (Ohkawa et al.,1979).

Testicular reduced glutathione (GSH) estimation was performed by the method of Griffith. The level of GSH was expressed as µg/mg protein (Griffith, 1981).

The oxidized glutathione (GSSG) level was measured using 2-vinylpyidine according to the method of Griffith. The level of GSSG was expressed as µg/mg protein (Griffith, 1980).

6.2.8. Determination of activities of testicular superoxide dismutase, catalase, glutathione s-transferase , glutathione peroxidase and glutathione reductase

The specific activity of testicular superoxide dismutase (SOD) was determined from its ability to inhibit the auto-oxidation of pyrogallol according to Marklund and Marklund,(Marklund and Marklund, 1974).

Catalase(CAT) is responsible for the conversion of H_2O_2 into water. So the testicular CAT activity was measured spectrophotometrically at 240 nm by calculating the rate of degradation of H_2O_2 according to the method of Aebi (Aebi, 1974).

Testicular glutathione-s-transferase (GST) activity was measured spectrophotometrically using 1-chloro-2,4-dinitrobenzene as electrophilic substrate that binds to GSH with the participation of the enzyme and forms a colored GSH-substrate complex, detected at 340 nm and the activity of GST in testis was expressed in terms of µmol/min/mg protein(Habig et al.,1974).

The activity of GPx was determined by the method of Rotruck et al. with slight modification (Rotruck et al.,1973).The absorbance of the reaction product was assayed at 412nm.

The activity of glutathione reductase was determined by the method of Williams & Arscott. Reading was taken at 340nm and was expressed as nmole/NADPH consumed/min/mg protein Williams and Arscott(Williams and Arscott, 1971).All the method were described in detail in previous chapter 4.

6.2.9. Assay of testicular glucose-6-phosphate dehydrogenase activity

Glucose-6-phosphate dehydrogenase activity was measured spectrophotometrically (Langdon, 1966). One unit of enzyme activity is expressed as that quantity which catalyses the reduction of 1 μM of NADP per minute by using glucose-6-phosphate as a substrate in this reaction the activity of this enzyme was recorded and absorbance was measured at 340 nm.

6.2.10. Measurement of seminal fructose concentration

Seminal fructose concentration was estimated by the method of Karvonen and Malm. (Karvonen and Malm, 1955). The reading was taken at 470 nm in spectrophotometer (UV-245 Shimadzu, Japan).Detail method was described in chapter3.

6.2.11. Assay of testicular acid phosphatase

The acid phosphatase activity was measured using *p*-nitrophenol phosphate as a substrate (Vanha-Perttula and Nikkanen, 1973). Amount of PNP liberate was measured spectrophotometrically at 420 nm.

6.2.12. Estimation of testicular and adrenal cholesterol

Testicular and adrenal cholesterol were measured according to Zlatkis et al. (Zlatkis et al., 1953).

6.2.13. Assay of testicular and adrenal of Δ^5, 3β-hydroxysteroid dehydrogenase (Δ^5, 3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD) activity

The activity of $\Delta^5$3β-HSD was assayed according to the methods described by Talalay. One unit of the enzyme activity was equivalent to a change in the absorbance of 0.001 U/min at 340 nm(Talalay, 1962).

For the measurement of 17β-HSD activity, the supernatant was added with 440µM of sodium pyrophosphate buffer, 960µl of bovine serum albumin, and 40µl of ethanol containing testosterone. The enzyme activity was measured after the addition of 100µl NAD to the tissue supernatant mixture in a spectrophotometer at 340 nm against a blank (without NAD). Result is expressed as one unit of enzyme activity at 340 nm (Jarabak et al., 1962).

6.2.14. Estimation of tissue protein

Different dilutions (10µl to 50µl) of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water. The final volume in each of the test tubes is 6 ml. From these different dilutions, protein reagents (98:1:1) consisting of sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH), sodium potassium tartarate in distilled water, copper sulphate (Cu_2SO_4) in distilled water were added to different test tubes and 10 µl of sample and 500 µl of normal saline (0.9 gm%) were also added. The solutions were mixed well. Then 500µl of reagent Folin-Ciocalteau

solution was added to each tube and incubated at 37^0C for 30 min. The standards were prepared similarly. The optical density was measured at 660 nm . The absorbance was plotted against protein concentration to get a standard calibration curve.

6.2.15. Western blot analysis of testicular steroidogenic pathway

Total testicular protein was isolated from each animal and Western blotting was performed. Briefly, tissues of testis were homogenized in 100 mg/ ml of ice-cold RIPA lysis buffer.

Total protein (50 µg protein) were separated on sodium dodecyl sulfate-polyacrylamide electrophoretic gel (SDS-PAGE) and transferred to PVDF membranes. The membranes were stained with Coomassie blue to visualize the amount of total protein transferred in each lane. To reduce nonspecific binding, membranes were preincubated for 2 h on a rocker at room temperature in a blocking buffer containing 5% non-fat dried milk, 5 M NaCl, 20 mM Tris-base, and 0.15% Tween-20, pH 8. Membranes were probed using a specific primary antibody against GAPDH (1: 1500 dilution), anti-3β-HSD (1:500 dilution), anti 17β-HSD (1:1500 dilution), anti-StAR (1:1000 dilution), anti- P450scc, diluted in 5% BSA in Tris-buffered saline with Tween-20 (TBST) overnight at 4°C. After being washed three times (5 min each) in TBST, membranes were incubated at room temperature for 1 h with ALP-conjugated suitable secondary antibodies (1:10 000) against the primary antibodies. Membranes were washed three times in TBST. Then proteins were visualized after staining with NBT-BCIP buffer. Then the picture was captured by Gel Doc (Bio-Rad). Densitometry of the appropriate sized bands was measured using

molecular imaging software (Image J 148-jdk 6 software)(Mandal et al., 2006 ; Nteeba et al., 2014).

6.2.16. Hormonal assays

For the quantitative determination of testosterone hormone ELISA Kit, Version: 5.1 - ALPCO (CATALOG NO: ABIN365714.) was used. Luteinizing hormone was assayed by ELISA Kit (CATALOG NO: ABIN365711.). Quantitative determination of follicle-stimulation hormone was done by ELISA Kit (CATALOG NO**:** ABIN365553**)** according to the manufacturer's instructions.

6.2.17. Histological study

For histological study, specimens were fixed in bouin solution, dehydrated in alcohols and embedded in paraffin. Tissue section from each animal was stained with hematoxylin and eosin stain and observed under light microscope for histopathological assessment.

6.2.18. Statistical analysis

The data were analyzed to obtain mean values and standard error for all treated and control samples. Statistical analyses of the collected data were done by a one way ANOVA, followed multiple comparison two tail t-test, using Origin 6.1 software. Difference was considered significant when $P < 0.05$.

6.3. Results

6.3.1. Testicular index, sperm parameters and morphology

Decrease in testicular index, sperm count, viability, motility, hypo-osmotic swelling and increase in sperm abnormality was observed in the LCT treated group compared to the control group (Table.6.1.). Pretreatment with taurine increased testicular index and also improved the quantity and quality of semen to a good extent.

Table 6.1. Effect of LCT and taurine on testicular index, sperm parameters and morphology in male rats.

Results are expressed as Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group I versus all

Group	Testicular index	Sperm count (million/ml)	Sperm viability (%)	Sperm motility (%)	Sperm morphological abnormalities (%)	Hypo osmotic swelling (%)
Gr-I	1.49±0.04	18±1.5	88±0.8	80±1.3	16±1.4	77.3±0.6
Gr-II	1.57±0.01	19±1	90±0.8	83±1.1	15±0.7	78.5±0.5
Gr-III	1.33±0.03 a*	13±0.8 a*	66±1.1a***	66±0.8 a***	23±0.9 a**	44.66±0.4a***
Gr-IV	1.36±0.02 a*	16.26±1 b*	74±1.3 a***b**	73±1.3 b***	18±1 b**	52.5±0.5a***b***
Gr-V	1.08±0.03 a***	9.16±0.65a***	42.8±0.9 a***	42±0.8 a***	28±1.3 a***	31.66±0.3a***
Gr-VI	1.28±0.04 a** c**	15±0.9 c***	59.1±1.1 a***c***	59±1.1 a***c***	24±1 a**c*	46.2±0.8a***c***

other groups; superscript b, Group III versus Group IV and superscript c, Group V versus Group VI. (*indicates p<0.05,** indicates p<0.01, *** indicates p<0.001).

6.3.3. Measurement of sperm intracellular reactive oxygen species (ROS) generation and mitochondrial membrane potential

To verify the status of the oxidative stress in control and LCT treated rat the production of fluorescent DCF was measured after H_2DCFDAstaining (fig 6.2.). Results demonstrated that intoxication of LCT increased ROS generation where pretreatment of taurine reduced the toxic effect of LCT.

Rhodamine-123 is widely used to make dynamic measurements of mitochondrial membrane potential. After exposure to LCT a marked decline in sperm mitochondrial membrane potential was observed. Protective effect of taurine was prominent in figure 6.3.

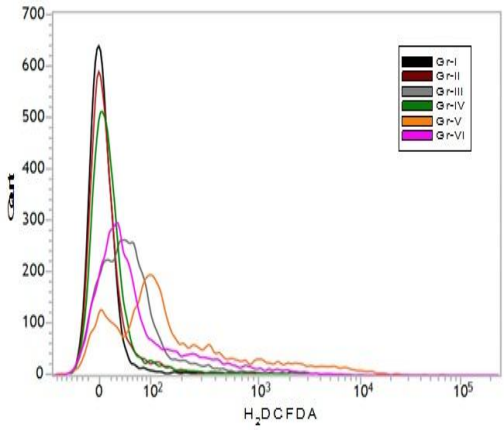


Figure 6.2

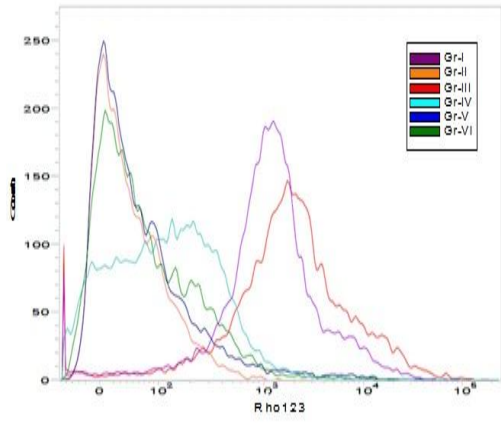


Figure 6.3

Figure 6.2.represents the flow cytometric analysis of excess ROS formation in epididymal spermatozoa stained with H_2DCFDA.

Figure 6.3 represents the flow cytometric analysis of mitochondrial membrane potential.

6.3.4. Sperm DNA damage

The genotoxic effect of LCT was confirmed by this comet assay. The significant increase ($p < 0.001$) in the percentage of tail DNA intensity in LCT treated group compared to control may be as a result of direct DNA strand breakage by LCT induced excess free radical generation. Effect was reduced after pretreatment of taurine (fig 6.4.A).The fluorescence images (fig-6.4.B) were highly correlated with the percentage of tail DNA intensity level.

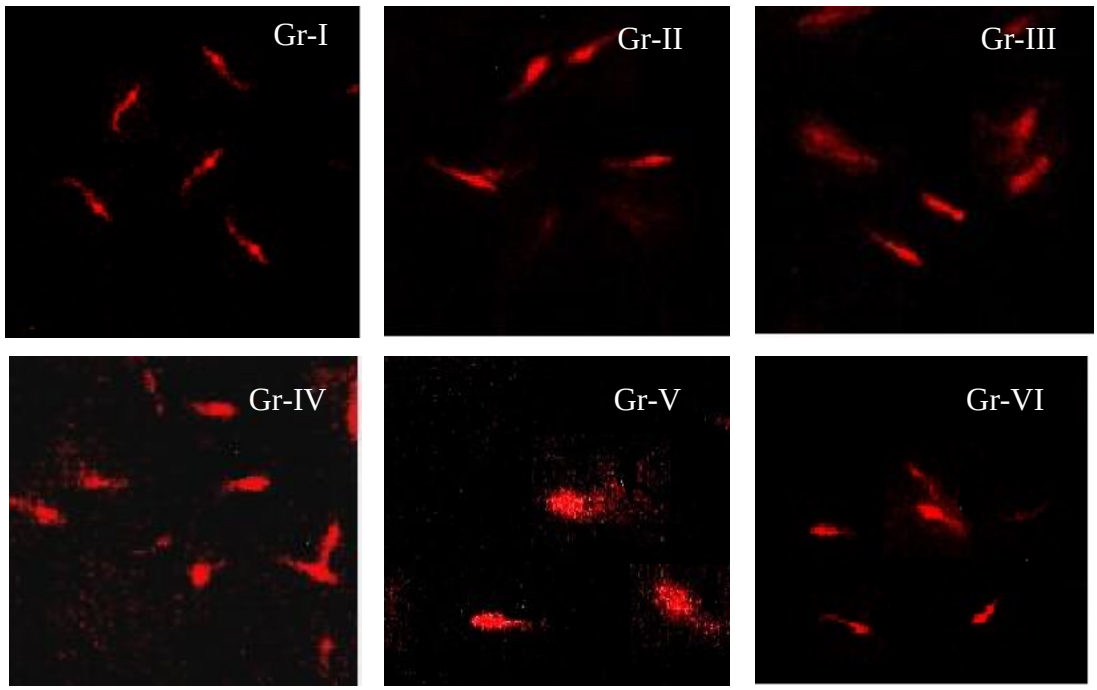


Figure-6.4.A represents the effect of LCT and taurine on DNA fragmentation study by Comet Assay.

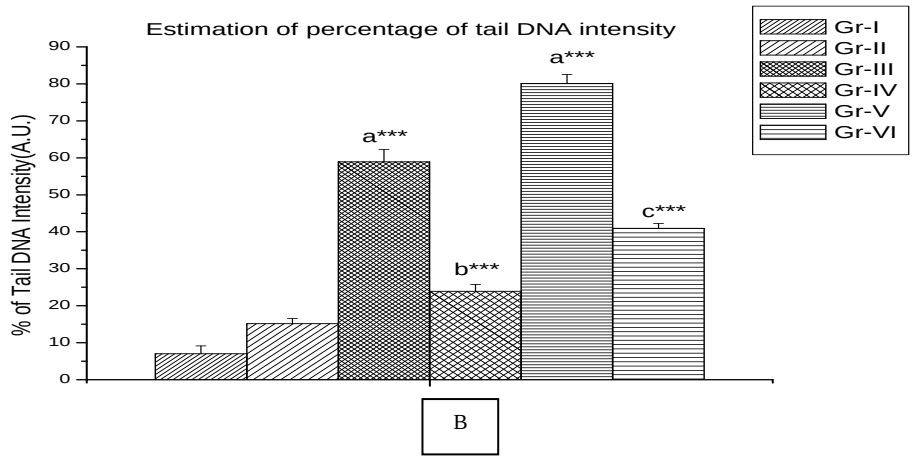


Figure-6.4.B shows the effect of LCT and taurine on percentage of tail DNA intensity in male albino rat.

6.3.5. Analysis of Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay

Terminal deoxynucleotidyltransferase mediated dUTP nick end labeling (TUNEL) assay using flow cytometry shown around 15%, and 25% TUNEL positive respectively in LCT intoxicated rat, indicates apoptotic cells with the presence of DNA fragments. Pretreatment of taurine shows the protective effect on LCT induced DNA damage by reducing the TUNEL positive cell (fig-6.5).

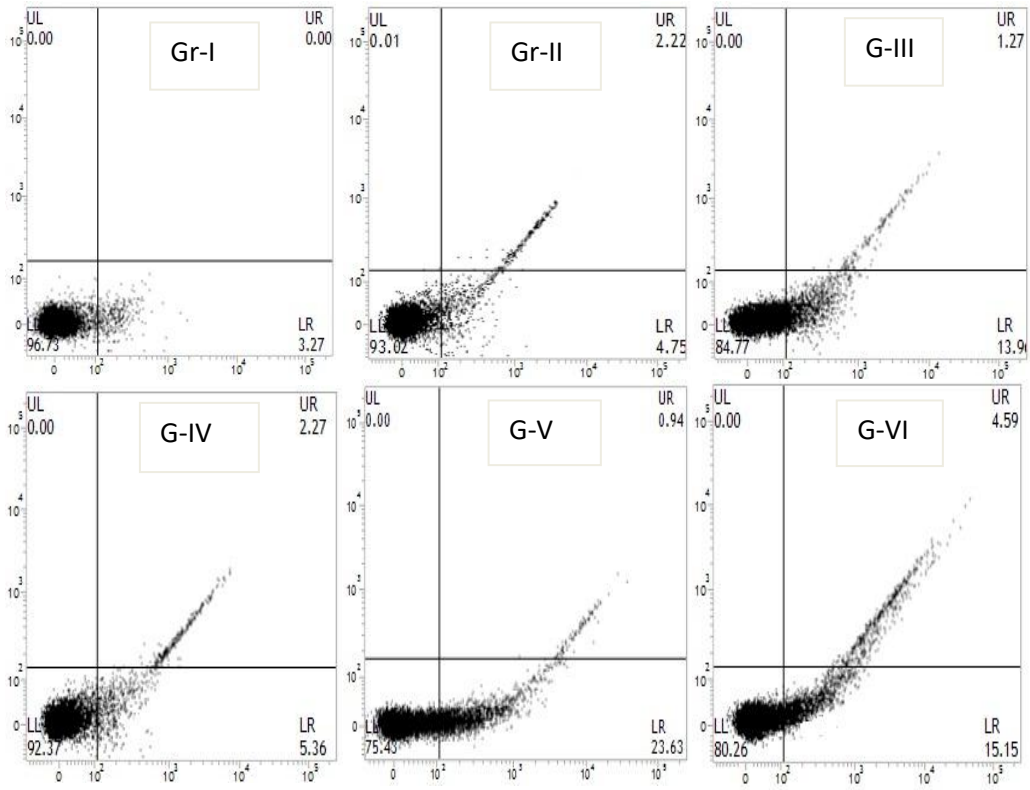


Figure-6.5.A represent the dot plot of DNA strand breaks, analyzed by flow cytometry in control and treated rats.

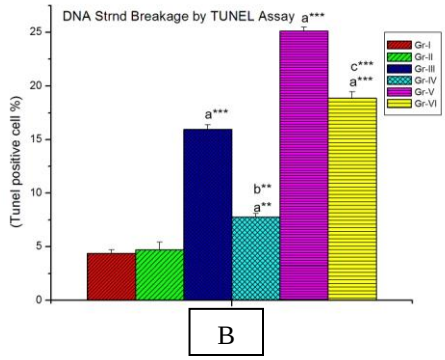


Figure-6.5.B: Analysis of TUNEL positive cell percentage. Data are presented as Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI.

Asterisks represents the different level of significance (**indicates $p<0.01$,*** indicates $p<0.001$).

6.3.6. Testicular lipid peroxidation and antioxidant status

Figure 6.6.depicts the levels of testicular malondialdehyde (MDA) (fig-6.6.A) and GSH content (fig-6.6.B) in control and treated rat. A significant increase ($p<0.001$) in MDA content and oxidized glutathione (GSSG) were observed in rat testes exposed to LCT. On the other hand, a significant decrease was observed in reduced glutathione (GSH) content. Results of antioxidant enzymes activities were also presented in figure 6.6. From the result it was well-established that LCT treatment showed significant inhibition of the activities of antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase ($GP_{X)}$. glutathione-S-transferase (GST), glutathione reductase]. The activity of the malondialdehyde and antioxidant enzymes were enhanced in taurine pretreated groups.

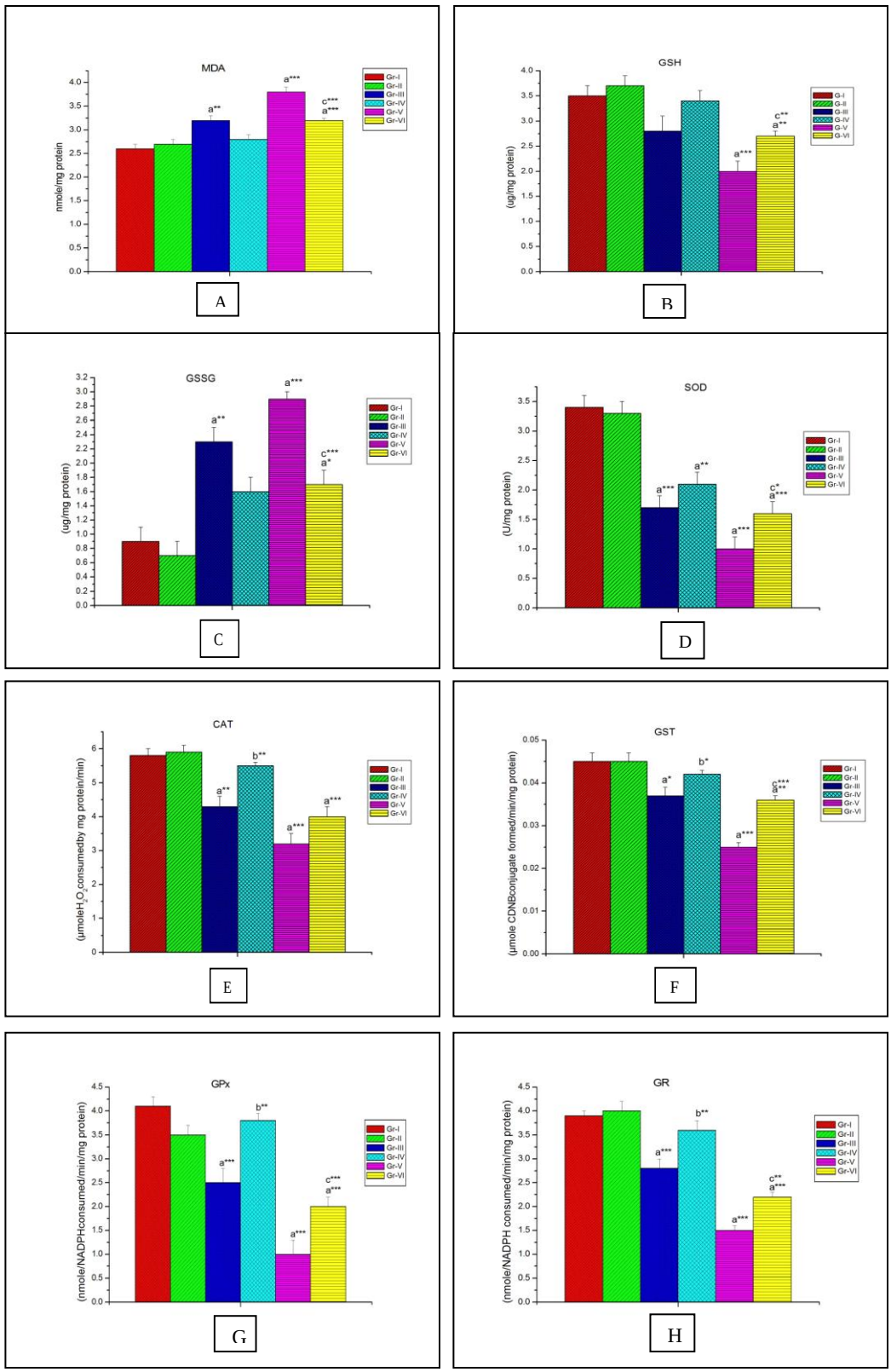

MDA
nmole/mg protein
Gr-I
Gr-II
Gr-III
Gr-IV
Gr-V
Gr-VI
A
GSH
(ug/mg protein)
G-I
G-II
G-III
G-IV
G-V
G-VI
B
GSSG
(ug/mg protein)
C
SOD
(U/mg protein)
D
CAT
(µmoleH2O2consumedby mg protein/min)
E
GST
(µmole CDNBconjugate formed/min/mg protein)
F
GPx
(nmole/NADPHconsumed/min/mg protein)
G
GR
(nmole/NADPH consumed/min/mg protein)
H

Figure-6.6: Graphical presentation of lipid peroxidation and antioxidant status in control and treated group of rat. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI. (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$). A=malondialdehyde (MDA level) level),B=reduced glutathione (GSH), C=oxidized glutathione (GSSG) , D=superoxide dismutase (SOD), E=catalase (CAT), F= glutathione peroxidase (GP_X).G=glutathione-S-transferase (GST),H=glutathione reductase(GR).

6.3.7. Effect of Glucose 6 phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase activity in LCT treated rat was found to be decreased compared to that of control. Pretreatment of taurine brought back the normal status significantly (fig-6.7).

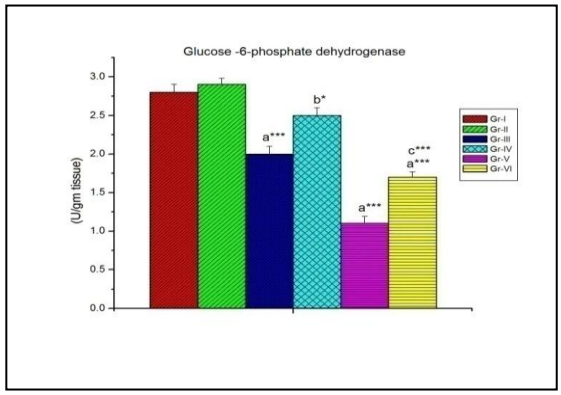


Figure-6.7 represents the glucose-6-phosphate dehydrogenase activity in control and treated group of rat. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c

Group V versus Group VI (* indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001).

6.3.2. Effect on seminal fructose concentration and acid phosphatase activity

Seminal fructose concentration was found to be decreased in response to LCT, compared to control (fig.6.8A).Taurine pretreatment in LCT treated rats rescued it from LCT induced toxicity.

Acid phosphatase activities were elevated in the testis after LCT treatment, in comparison to the controls (fig.6.8B). Pretreatment of taurine restored towards normal status.

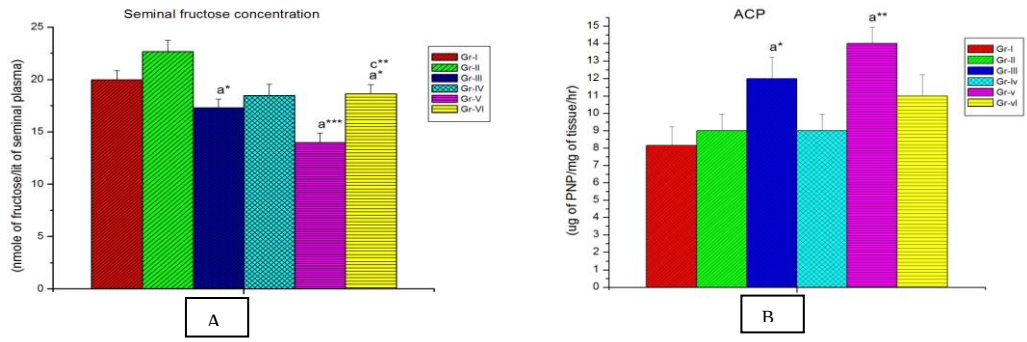


Figure-6.8 shows the seminal fructose concentration and acid phosphatase activity in control and LCT induced rat with protective effect of taurine.

Results are expressed as Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group I versus all other groups; superscript b, Group III versus Group IV and superscript c, Group V versus Group VI. (*indicates p<0.05,** indicates p<0.01, *** indicates p<0.001).A= seminal fructose concentration. B=acid phosphatase activity.

6.3.8. Effect on testicular cholesterol, and activities of testicular Δ^5,3β-hydroxysteroid dehydrogenase (Δ^5,3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD)

Testicular cholesterol was increased in LCT treated rat compared to control and taurine control group. The activities of testicular key androgenic enzymes like Δ^5,3β -HSD and 17 β -HSD along with their expressions were studied. The inhibitory responses of LCT were noted in Δ^5,3β -HSD and 17β -HSD activities. LCT exposure decreased the expressions of these testicular androgenic proteins, whereas pre treatment of taurine with LCT increased the activity and expressions of these testicular proteins to the control level(fig-6.9).

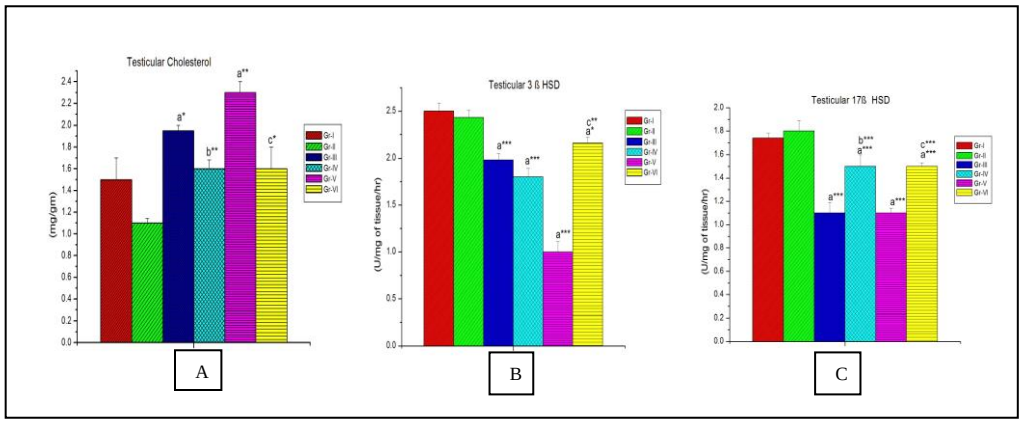
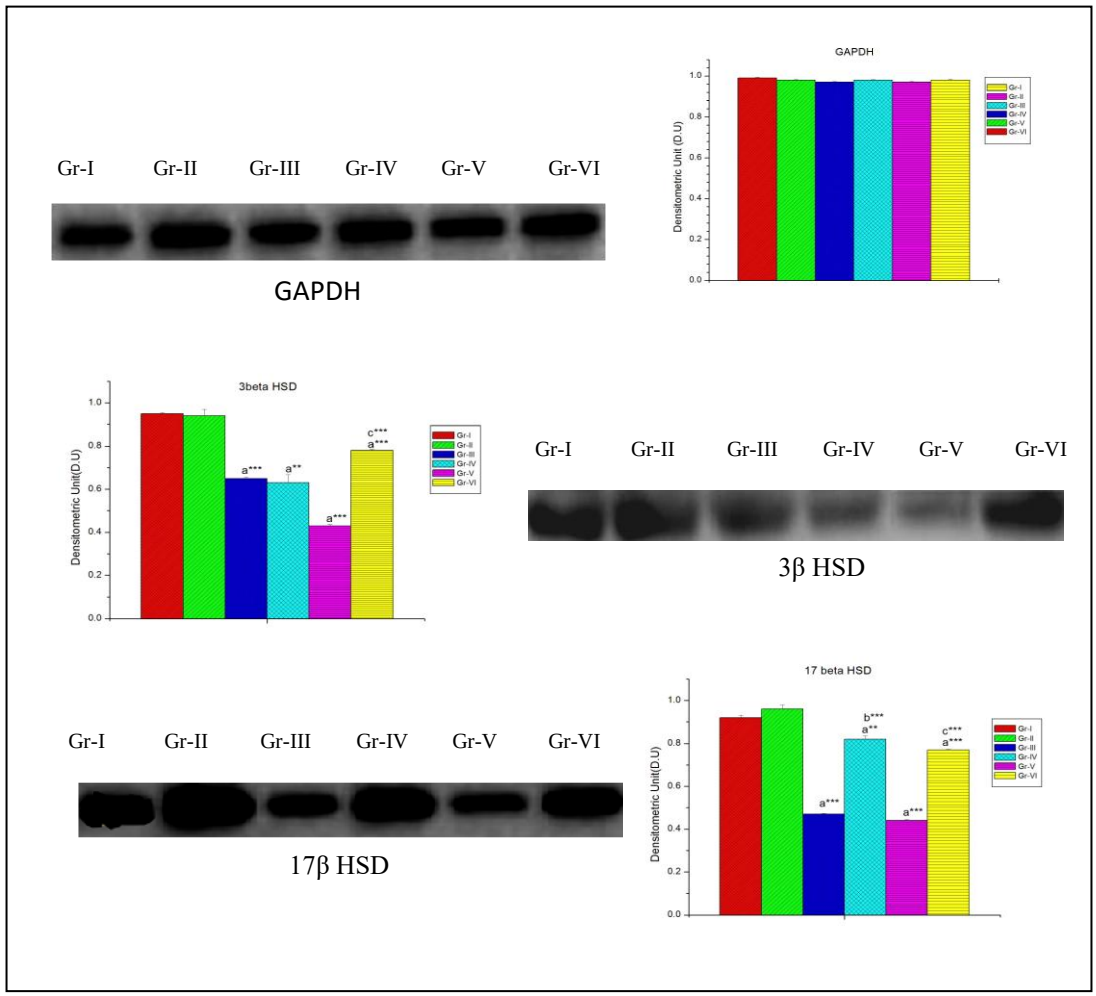


Figure-6.9 represents the testicular cholesterol (fig.A), and activities of testicular Δ^5,3β-hydroxysteroid dehydrogenase (Δ^5,3β-HSD) (fig.B), 17β-hydroxysteroid dehydrogenase (17β-HSD) (fig.C) in control and treated group of rat. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

6.3.9.Western blot analysis of testicular Δ^5, 3β-HSD, 17β–HSD, steroidogenic acute regulatory protein (StAR) and P450scc(cholesterol side chain cleavage enzyme)

Western blot analysis of Δ^5, 3β-HSD, 17β–HSD, StAR and P450scc revealed that the LCT exposure dramatically decreased these protein expressions compared to the control. However, taurine pretreatment with LCT significantly restored the above said parameters up to the control level (fig-6.10).



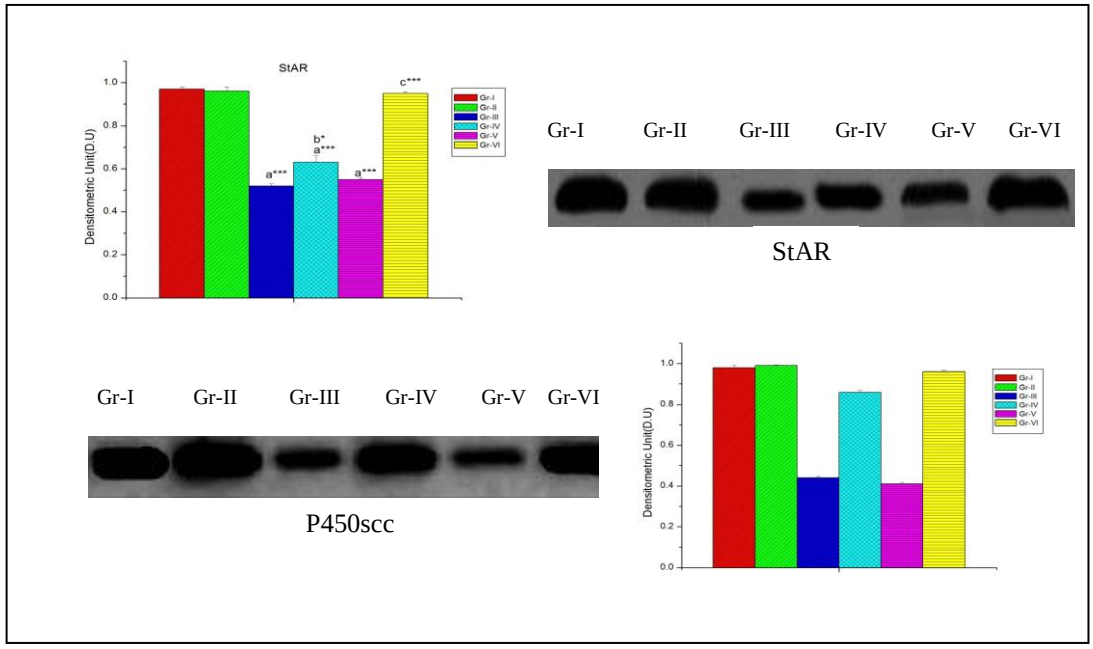


Figure 6.10 shows the expression of GAPDH, Δ^5,3β-hydroxysteroid dehydrogenase (Δ^5,3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), StAR, P450scc in control and treated rat. Gr- I=control, Gr-II=taurine control, Gr-III=LCT low dose, Gr-IV=taurine+LCT low dose, Gr-V= LCT high dose, Gr-VI=taurine+LCT high dose group.

6.3.10.Effect on adrenal cholesterol and the activities of Δ^5, 3β-HSD and 17β-HSD

The adrenal cholesterol was found to be decreased in LCT intoxicated rat. The activities of adrenal Δ^5, 3β-HSD and 17β-HSD were increased in LCT treated rat whereas pre treatment of taurine decreased the activity up to the control level(fig 6.10).

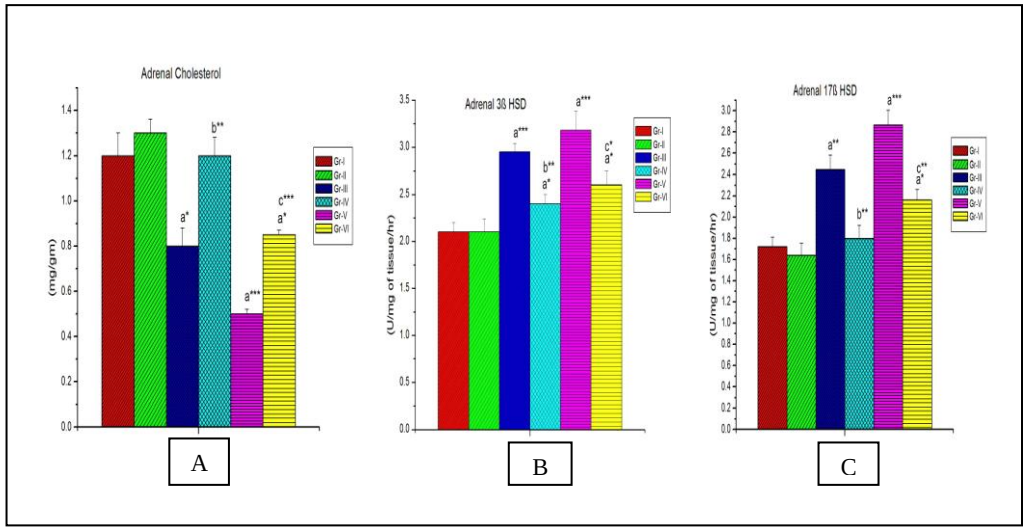


Figure-6.11 represents the adrenal cholesterol (fig.A), and activities of adrenal Δ^5,3β-hydroxysteroid dehydrogenase (Δ^5,3β-HSD) (fig.B), 17β-hydroxysteroid dehydrogenase (17β-HSD)(fig.C) in control and treated group of rat.Results are expressed as Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

6.3.11. Serum Levels of Testosterone, LH, and FSH

Table 6.2 shows the results of serum testosterone, LH and FSH obtained from control and LCT exposure group of rat. The serum levels of these hormone were reduced significantly in LCT treated group ($p<0.001$), while the levels of testosterone; FSH and LH were near normal after pretreatment of taurine with LCT.

Table 6.2. Effect of LCT and taurine on serum testosterone, LH and FSH level

	Testosterone(ng/ml)	LH (mIU/ml)	FSH(mIU/ml)
Gr-I	4.1±0.05	0.7±0.05	0.88±0.03
Gr-II	4.3±0.13	0.7±0.04	0.83±0.03
Gr-III	3.4±0.13a***	0.5±0.05a*	0.46±0.05a***
Gr-IV	4±0.13b*	0.58±0.01a*	0.68±0.02 a***b**
Gr-V	1.3±0.14 a***	0.38±0.01a***	0.26±0.02 a***
Gr-VI	3.2±0.18 a*** c***	0.49±0.01a**c**	0.53±0.02a*** c***

Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

6.3.12. Histopathological findings

The histological sections demonstrated apparently normal seminiferous tubules in control animals with sperm cell series at different stages of development and they were well stained. The lumens were filled with a good population of mature sperm cells.The testes of rats that received LCT at two dose levels indicates testicular atrophy. Seminiferous tubules with altered luminal diameter and poor staining intensity showed that the population of spermatozoa was markedly reduced compared with the control group. Similar degenerative changes in the seminiferous tubules and testicular atrophy have been reported in experimental animals with

various insecticides treatment. However treatment with taurine more or less normalized the above mentioned parameters to a good extent.

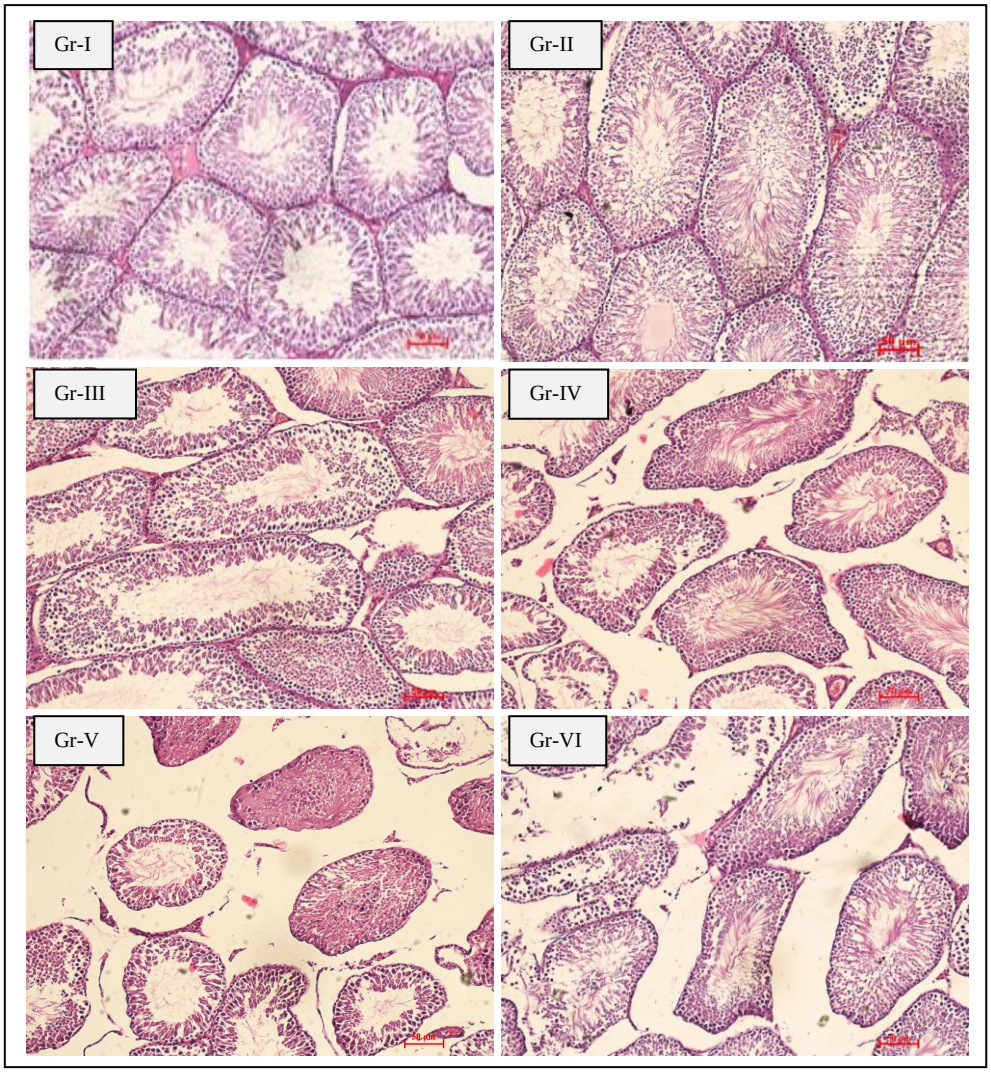


Figure-6.12.Histological pictures of testis of control and experimental groups of rats by hematoxylin and eosin staining

6.4. Discussion

The testicular index relies on both testicular weight and body weight. Reduction in the testicular weight on lambda cyhalothrin (LCT) exposure is perhaps due to decreased tubule size, reduced number of germ cells and enlarged spermatids

(Sanchez-Pena et al.,2004). The testicular index reduced by LCT intoxication successfully raised by the treatment of taurine. Perhaps, this is due to the preventive role of taurine on testicular damage. Sperm count is one of the most sensitive tests for spermatogenesis and it is highly correlated with fertility. The decrease in epididymal sperm count after LCT treatment may be due to the inhibition in spermatogenesis. The decrease in epididymal sperm count may directly depends upon the hormonal input from the hypothalamico-pituitary-testicular axis and its effect on functional testicular epithelium (Dua and Vaidya ,1996).

Decrease in sperm motility, live sperm, and increase in number of abnormal sperm may be due to direct effect of the LCT on matured and stored sperm in epididymis. Androgen insufficiency which in turn disturbed the testicular function may be an indication towards the adverse effect of LCT on the physical characteristics of sperm. This type of results also indicates that LCT might have crossed the blood testes barrier and have interfered with functioning of testicular epithelium since it is the germinal epithelium of the testes that produce the sperm cells (Dua and Vaidya,1996).Another possible mechanism for decrease in sperm motility, live sperm, and increase in number of abnormal sperm may be also due to increased reactive oxygen species (ROS) production by LCT as observed in this study. Pesticide induced ROS production is known to affect sperm motility, live spermadversely (El-Demerdash et al., 2004; Joshi et al., 2011; Elbetieha et al., 2001). This can be explained by another study where dicofol induced blockage of gonadotropin production and/or release by the pituitary reduced testosterone

production by leydig cells was responsible for the arrest of spermatogenesis (Vanage et al., 1997).

The hypo-osmotic (HOS) swelling test developed by (Jeyendran et al., 1984) predicts the membrane integrity by determining the ability of the sperm membrane to maintain equilibrium (Ahmadi and Soon-Chye,1992) between the sperm cell and its environment. Hypo-osmotic stress causes influx of the fluid to the sperm that produces sperm tail coiling or swelling like balloon. A higher percentage of swollen sperm indicates the presence of sperm with a functional and undamaged plasma membrane (Ramu and Jeyendran,2013). In our result lower percentage of swollen sperm in LCT treated rat indicates structural and functional impairment of sperm plasma membrane. Comparatively higher percentage of swollen sperm was noted in case of tauine pre-treated group that indicate a protective effect of taurine against LCT toxicity.

Depending on the physiological role of taurine in acting as antioxidant agent (Alzubaidi and Diwan, 2013) it protects against loss of motility and kept spermatozoa motile. In addition, taurine increased hypo-osmotic swelling as it acted as a membrane stabilizing factor (Alzubaidi and Diwan, 2013) by inhibiting sperm Na^{+},K^{+}-ATPase activity to protect the sperm plasma membrane from the free radicals and oxidation specially when taurine is the major amino acid of sperm cell and seminal fluid (Agarwal et al., 2003; Yang et al., 2010).

Reactive oxygen species (ROS) are essential intermediates in oxidative metabolism. Nevertheless, when oxidative stress occurs, ROS are generated in excess and consequently may damage cells by oxidizing lipids, disrupting DNA and proteins.

Mammalian sperm cells contain a high amount of polyunsaturated fatty acid that undergoes oxidation to affect important sperm parameters (Aitken et al., 1989). The intracellular amounts of ROS were measured by a fluorometric assay with 2, 7-dichlorofluoresce indiacetate (DCFH-DA). Huge production of ROS in LCT induced group of animal was confirmed by the fluorescence microscopic image that detected the presence of elevated fluorescent DCF in LCT- treated rat.

Mitochondria are considered one of the main endogenous sources of reactive oxygen species (ROS) production. Moreover, the elevated level of ROS can cause oxidation of the mitochondrial pore and, thereby, disrupting the mitochondrial membrane potential (MMP), an indicator of functional impairment on the reproductive system (Gravance et al.,2001). Decrease in MMP was seen in LCT-treated rats. A positive correlation between poor sperm mitochondrial function and diminished motility and reduced fertility was already reported (Marchetti et al., 2002; Piasecka and Kawiak, 2003).Taurine pretreatment alleviated it to a good extent.

The exact mechanisms of DNA damage was not completely understood but several studies point out the possibility that oxidative and mechanical events could be responsible for DNA fragmentation and base modification (Perez-Cerezales et al., 2010).Comet assay is overriding other assays due to its sensitivity in detecting DNA damage, robustness, viability of application to any eukaryotic cell and is economical (de Andrade et al.,2004;Ateeq et al.,2005). Under standard conditions, the comet assay detects the amount of cells with DNA single strand breaks. In our study the raised length of migration observed in the treated rat could be reflecting DNA single strand breaks. The mechanism of action of synthetic pyrethroid on DNA is still

obscure. The effect is assumed to be unique chemical interactions causing strand breaks and the suppression of DNA repair systems and related processes; however, with the present knowledge, it is difficult to comment on the exact mechanism.

DNA fragmentation is one of the important feature can also be detected using the terminal deoxynucleotidyltransferase–mediated dUTPnickend labeling assay, known as the TUNEL assay. It was proposed by several authors that DNA strand breaks was the main evidence for apoptosis in human spermatozoa (Gandini et al.,2000;Barroso et al.,2000). However, it is important to note that DNA fragmentation in mature sperm can have other origins besides apoptosis, as it can occur during or after DNA packaging in spermiogenesis or as a result of oxidative stress (Barroso et al., 2000). In the current study the TUNEL positive cell detected by this method found to be higher in LCT treated rat compare to control. This damage is also compatible with the previous findings obtained by using the comet assay.

Several study indicates that pesticide-induced toxicity was associated with lipid peroxidation (Mansour and Mossa, 2009; Mossa et al., 2013).Lipid peroxidation is believed to be one of the main markers of ROS-mediated damage. In our experiment malondialdehyde (MDA) level in LCT treatment was found to be significantly higher than that of control. Excessive production of reactive oxygen species (ROS) thought to be associated with the inhibition of endogenous antioxidant defense system that are involved in neutralization of toxic effects of these free radicals by donating electrons to these toxic species. In this study, glutathione (GSH) which is a major cellular antioxidant that directly terminates ROS(Visioli and Hagen, 2011) has been found to be reduced from normal level. Superoxide dismutase that popular as a chain

breaking antioxidant, plays a vital role in protection against deleterious effects of lipid peroxidation (Sullivan et al., 2000) while catalase together with glutathione peroxidase reduces the H_2O_2 into H_2O and oxygen to prevent oxidative stress and in maintaining cell homeostasis.ROS has also been known to decrease the detoxification system produced by glutathione s-transferase (GST) (Yamamoto and Yamashita, 1999). The significant decrease of testicular GST activity in LCT intoxicated rats may indicates insufficient detoxification process(Hayes et al.,2005). Taurine supplementation may recover the normal status by directing cysteine, the precursor of GSH into the GSH synthesis pathways. The significant changes in the activity of G-6-PD indicate that LCT affects metabolic pathways in the testis. Glucose-6 phosphate dehydrogenase is a key enzyme for maintenance of redox potential in cells (Farhud and Yazdanpanah, 2008). Glucose-6-phosphate dehydrogenase leads to NADPH production through pentose phosphate pathway. It also acts as a cofactor for other anti-oxidant enzymes like glutathione reductase (Farhud and Yazdanpanah, 2008).Reduced testicular glucose-6-phosphate dehydrogenase activity indicates towards low production of NADPH, important as a central reductant and regulator of redox potential (Tian et al., 1999).

Our results revealed that pretreatment of taurine with LCT to treated animals reverse the level of enzymatic and non enzymatic antioxidant parameters.

Seminal vesicular secretion is important for sperm motility and stability of sperm chromatin and fructose is its principal secretory product which delivers nutrients for the semen. Any significant change in the fructose content of the seminal vesicle indicates the abnormal functioning of seminal vesicle (Gonzales and Villena, 2001).

The seminal plasma fructose induces the glycolytic metabolism of spermatozoa and pyrethroid treatment reported to reduce seminal vesicular fructose content that hampered the secretory ability of the seminal vesicle (Gupta et al., 2002) which was seen in this study.

Testicular acid phosphatase activity was an important marker to assess the toxicity. Dilatation of blood capillaries in between seminiferous tubules is the result of acid phosphatase enzyme activity. The increase in acid phospahatase enzyme activity could be explained on the bases of enhancement of cell membrane permeability with disturbance in the transphosphorylation process as a result of cellular degeneration (Linder et al.1988).

Testicular inhibitory action of LCT was observed by the elevation of testicular cholesterol level resulted from the inhibition of testicular androgenesis (Morris and Chaikoff, 1959) due to non-utilization testicular cholesterol that produce testosterone. A significant decrease in the activities of steroidogenic key enzymes (Δ^5, 3β-HSD and 17β-HSD) was highlighted in LCT treated groups indicates the inhibition in testicular androgenesis (Murono and Payne, 1979).These type of result may also confirmed by the decreased proteins expressions of these key testicular enzymes (Δ5, 3β-HSD and 17β-HSD).StAR is expressed in steroidogenic tissues (Stocco and Clark,1996) and acts exclusively on the outer mitochondrial membrane, regulate the cholesterol transport (West et al.,2001). Our results shows that the testicular protein expression of StAR was significantly decreased in LCT exposed rat indicating the low transport of cholesterol from outer to inner mitochondria. Once cholesterol is transferred to the inner mitochondrial membrane by StAR, it is

converted to pregnenolone, which is catalyzed by P450scc present in the inner mitochondrial membrane (Jefcoate et al., 1992; Hanukoglu,1992). P450scc is part of the cholesterol side chain cleavage enzyme system .The function of P450scc is to convert cholesterol to pregnenolone which is a rate-limiting step in steroidogenesis. The decrease of P450scc protein expression level can lead to the decrease of progesterone, which ultimately lead to less gonadal steroidogenesis. Decreased adrenal cholesterol level along with elevated activitiesof adrenal key enzymes (Δ^5, 3β-HSD and 17β-HSD) in LCT treated rat compared to control also indicates the compensatory steroidogenesis due to LCT exposure.The co-administration of taurine in LCT treated rats has resulted a significant protection in testicular steroidogenesis, StAR, and key androgenic enzymes like Δ^5, 3β-HSD and 17 β -HSD expressions.

Testosterone (T), the main steroid sex-hormone in male albino rats, is secreted by Leydig cells of the testes under the control of complex neuroendocrine interactions. Testosterone is needed for the continued production of different generations of germ cells in the seminiferous tubules. Because testosterone plays a crucial role in malereproductive system, disruption of its productionmay impair male reproductive health. Reduced testosterone level in LCT treated group may lead to the separation of germ cells from the epithelium of the seminiferous tubules (Zitzmann, 2008). In the current study, significant decrease in testosterone level, may be also as a result of direct action of LCT on Leydig cells, which are the main site of testicular androgen biosynthesis.

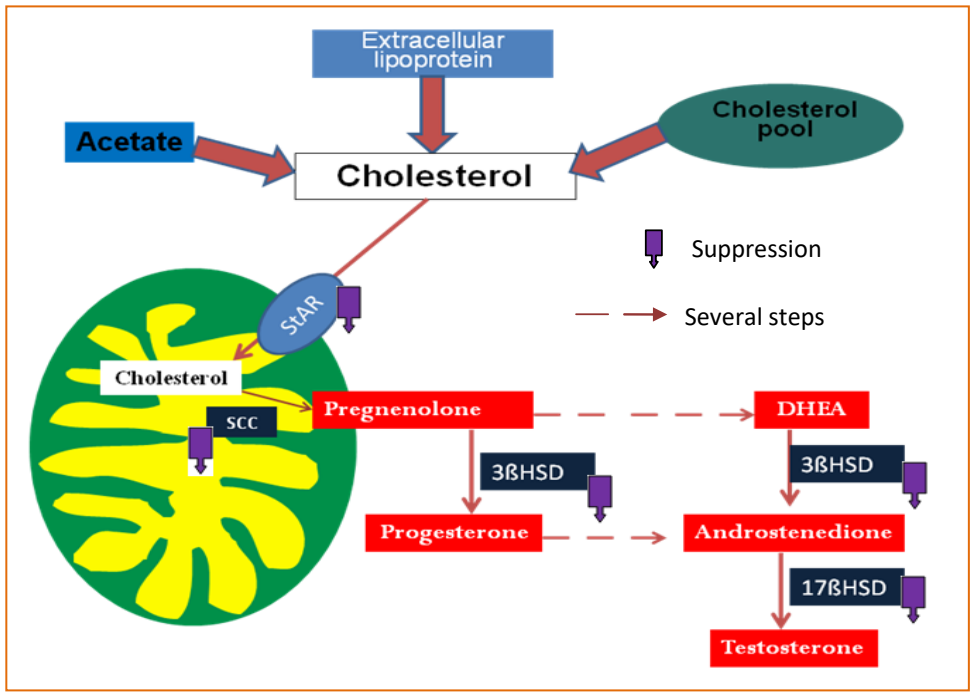


Figure 6.13: Graphical representation of proposed mechanism of LCT induced testicular steroidogenic toxicity.

Decreased LH in LCT treated rat may be the another reason of low level of testosterone as testosterone and other androgen production from the Leydig cells are controlled by thegonadotropins from the anterior pituitary, specifically luteinizing hormone (LH)that is synthesized and released from the anterior pituitary by the stimulation ofgonadotropin-releasing hormone (GnRH) from the hypothalamus (Schanbacher, 1982).

According to the Singh and Pandey, the changes in the activities of steroidogenic enzymes Δ^5, 3β-HSD and 17β-HSD and their protein expression lead to inhibition of testicular androgen biosynthesis(Singh and Pandey,1990).

Taurine through the action on hypothalamo-pituitary gonadal axis, may stimulate LH and FSH secretion and can regulate the testosterone production from testes by interacting with membrane receptors on the Leydig cells and stimulates them for the conversion of cholesterol to testosterone (Xiao et al.,1997; Yang et al.,2007).

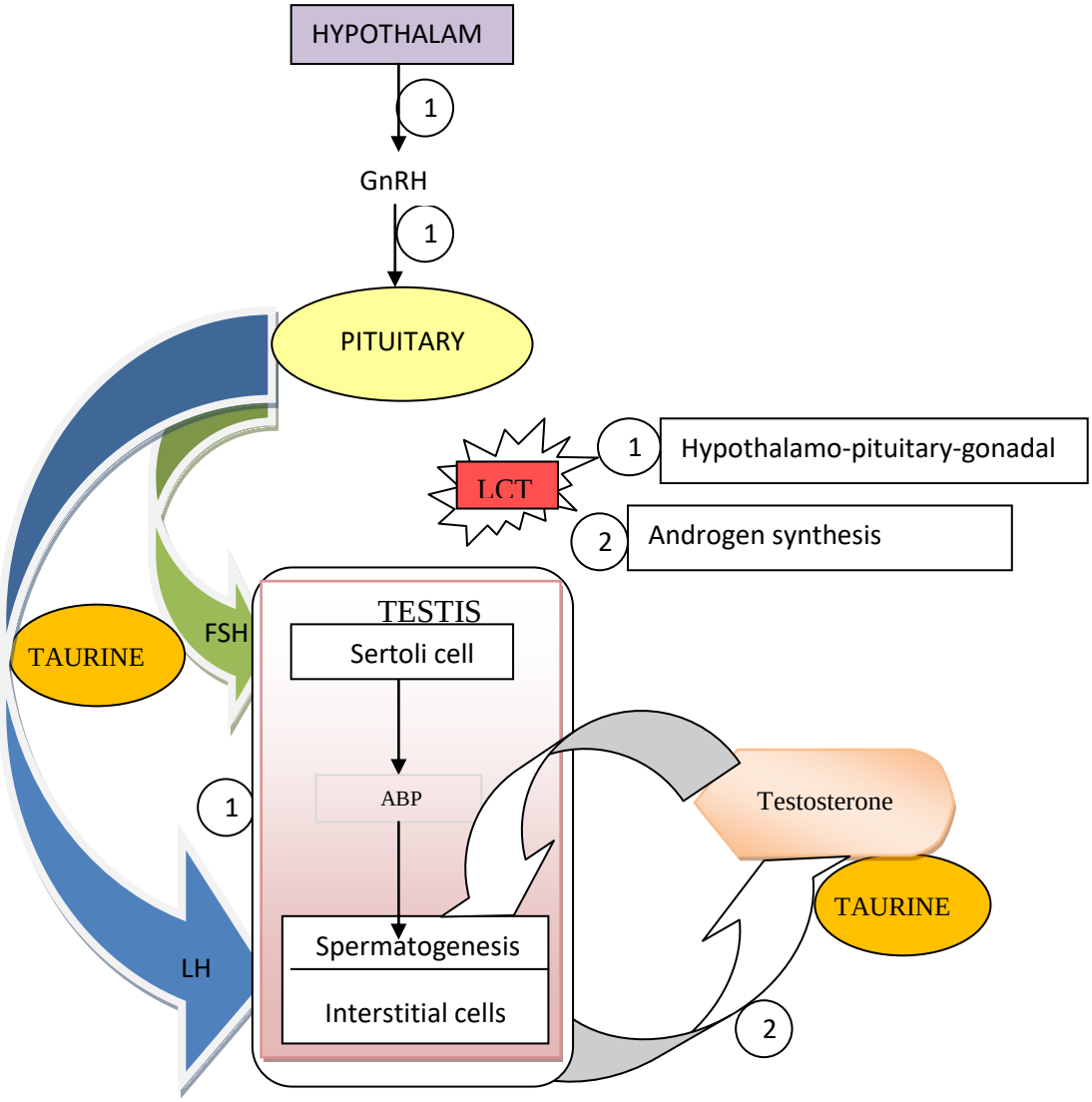


Figure 6.14: Proposed mechanism of taurine mediated alleviation on LCT induced hypothalamo-pituetary-gonadal axis deregulation

In the current study, animals treated with LCT exhibited the loss of intact general architecture and the consistency of seminiferous tubules with poor staining intensity compared with the control. The luminal diameter of the seminiferous tubules was also changed and the population of spermatozoa was markedly reduced in LCT treated group. Similar result was reported by Kumar and Nagar (Kumar and Nagar, 2014). These results may be due to LCT induced oxidative stress responsible for structural compromise in the testis.

6.5. Conclusion

From the present study, it may be concluded that LCT impaired testicular structure and functions through free radicals generation, interfered in the spermatogenesis, testicular steroidogenesis by altering key steroidogenic enzymes, StAR protein expression and testicular antioxidant status. LCT inhibited the androgen production acting primarily at the level of hypothalamo-pituitary-gonadal axis to inhibit the release of gonadotropins. Simultaneously, taurine caused improvement in the spermatogenesis, testicular androgenesis and attenuated the oxidative stress by improving in the antioxidant statusin LCT treated animals. The findings suggest the possible effectiveness of taurine as a therapeutic adjunct in LCT induced male reproductive system toxicity.

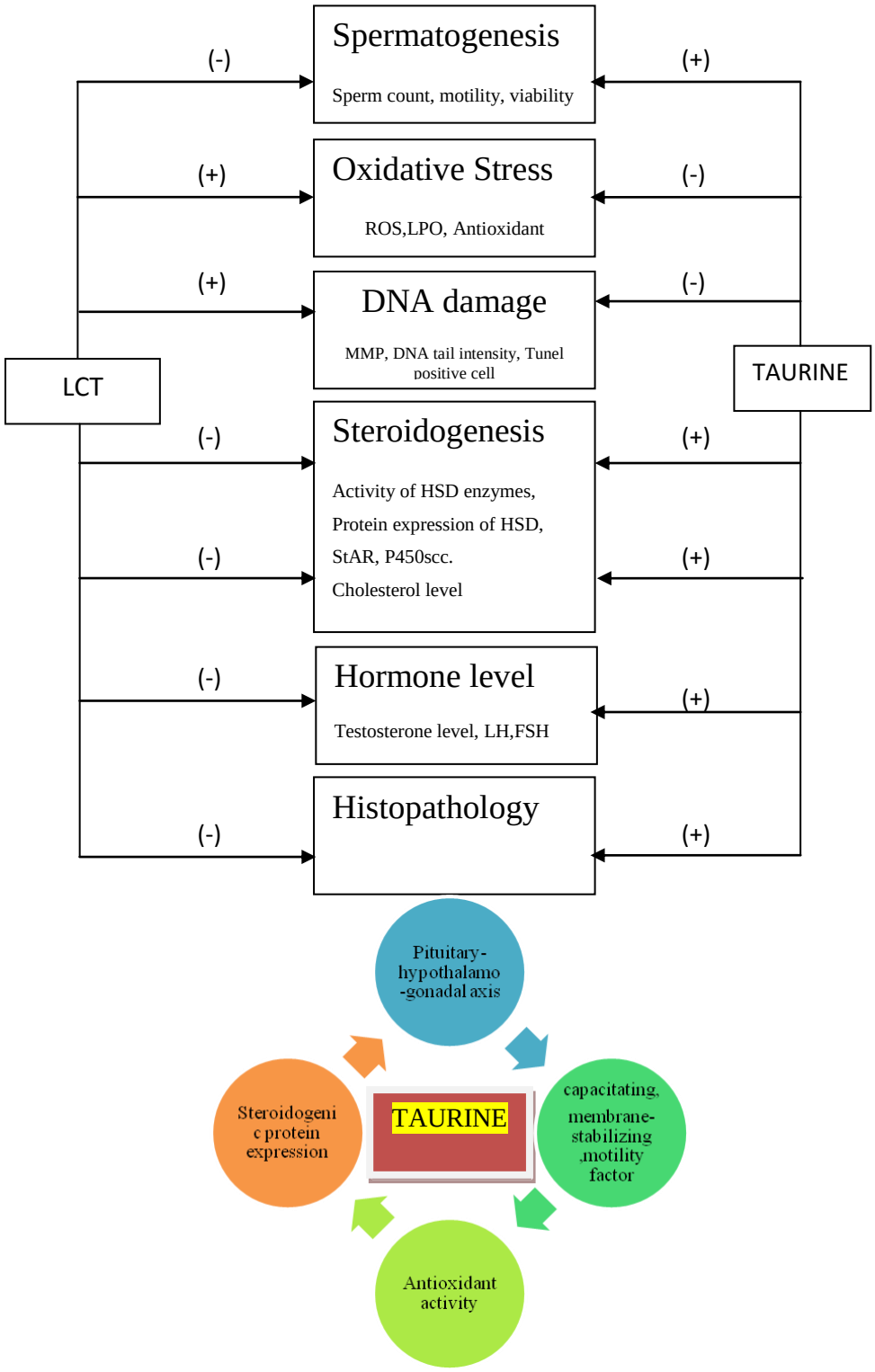


Figure6.15: Schematic diagram of LCT induced reproductive toxicity and its attenuation by taurine

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Chapter-7
Lambda cyhalothrin induced reproductive toxicity in mature female rat and taurine attenuates the toxicity

Abstract

The extensive use of chemical compound namely pesticide leads to serious environmental pollution and health hazards. In the present study sexually mature female rats were received lambda cyhalothrin(LCT) at two different doses (6.3 mg/ kg body wt. and 11.33mg/ kg body wt. respectively) once daily by oral gavage for 14 consecutive days along with pre-treatment of taurine(50mg/kg body wt.). In addition, increase in malondialdehyde level, accompanied by a reduction in reduced glutathione and antioxidant enzymes (superoxide dismutase, catalase, glutathione-s-transferase, glutathione peroxidase, glutathione reductase)activity in LCT-intoxicated rat ovary exhibited the toxic potential of LCT in female rat. Increase in ovarian cholesterol was found in LCT treated group compared to control rat. Diminished activity of ovarian $\Delta^5$3β- and 17β-hydroxysteroid dehydrogenase(HSD) with an increased adrenal $\Delta^5$3β- and 17β-HSD activity in LCT-treated rat were observed as a result of LCT exposure. Suppressed protein expression of 17β-HSD and steroidogenic acute regulatory protein (StAR) also reflected in LCT intoxicated groups. Hormonal imbalance was prominent by significantly reduction of the estradiol, progesterone and gonadotropic hormone level in LCT treated rat. Histopathological alterationsof the LCT treated ovaries displayed various degenerative changes. However pre-treatment of taurine potentially mitigates the toxic effect of LCT. In conclusion, exposure of female rats to LCT produces reproductive toxicity and thereby exhibits a threat to both environment and human health.

Rini Ghosh, Sujata Maiti Choudhury . Alteration in estrous cycle, lipid peroxidation and antioxidant status in female rat after exposure to lambda cyhalothrin and its attenuation by taurine.International Journal of Bioassays.2015; 4 (11): 4526-4532.

7.1. Introduction

Reproductive toxicity is a much more serious problem than any disease. Although researchs have been conducted to associate occupational exposure to pesticides with fertility problems in men (Larsen et al., 1998), studies related to female reproductive toxicity are scarce. Reason may be the rate of exposure to pesticides is higher in case of men, because men usually apply pesticides whereas women get exposed through re-entry activities only. Another reason may be that the assessment of fertility in women is more complicated than fertility in men. Pesticides which are not directly used by humans but indirectly they enter in human body and cause reproductive toxicity through several different mechanisms like direct damage to the structure of cells, interference with biochemical processes necessary for normal cell function, and biotransformation resulting in toxic metabolites. Reproductive effects in women induced by pesticide exposure are decreased fertility, spontaneous abortions, premature birth, low birth weight, developmental abnormalities, and ovarian disorders (Abell et al.,2000; Idrovo et al.,2005).By interfering with the female hormonal function pesticide cause negative effects on the reproductive system. Pesticides that may disrupt the hormonal function are often called endocrine disrupting chemicals, involved in mimicking or blocking the actions of the steroid hormone by directly binding to the hormone receptors either as agonists or antagonists (Waters et al., 2001).

7.1.1. Pyrethriod induced toxicity

Few studies have reported ovarian histological alterations in pyrethriod treated animals. Pyrethroids mediated endocrine disruption , alteration in calcium homeostasis in ovary (He et al., 2006) and atrophy in endometrial glands of pyrethroid exposed non-pregnant animals have been documented. Pyrethroids also lead to teratogenecity or reproductive toxicity .Dose dependent increased in foetal mortality (Biernacki et al., 1995) and significant decreased in number of foetuses vs. number of corpus luteam (embryonic resorption) along with delayed ossification of bones (Biernacki et al., 1995) were recorded in cypermethrin exposed female rabbits during gestation. Reduction in the implantation sites, corpora lutea and recovered foetuses from uterine horns and induction incidence of pre- and post-implantation losses and early mortality rate in pyrethroid intoxicated animals have been reported (Shukla and Taneja, 2002). In female rats serum progesterone level has been reduced after long time exposure to pyrethroid (Sangha et al., 2013). Not just one but several of the mechanisms are involved in the pathophysiological pathways explaining the role of pesticide exposure in fertility problems and other reproductive effects.

7.1.2.Lambda cyhalothrin mediated female reproductive toxicity in mammalian

Synthetic pyrethroid lambda cyhalothrin(LCT), has been found to accumulate in biological membranes due to its lipophilic nature and leading to oxidative damage (Michelangeli et al.,1990). Low doses of the LCT for long time produced maximum level of lipid peroxidation and reduction in activity of antioxidant enzymes on 14-21 days of pregnancy (Tukhtaev et al.,2012).However, information on the status of steroidogenesis and histoarchitecture of ovary is publicly unavailable.

7.1.3. Amiliorative effect of taurine

Taurine is an essential amino acid and is present at high concentrations in many tissues and play significant role on various physiological functions as antioxidant (Alzubaidi and Diwan, 2013), and membrane stabilizer by reducing reactive oxygen species.

The present study was carried out to investigate the effect of LCT on reproductive system of female rat and also to find out the exact role of taurine in this situation.

7.2. Materials and methods

7.2.1. Chemicals and reagents

Lambda cyhalothrin 5% emulsifiable concentrate (RPC Agro Industries, Kolkata), taurine (Sigma Aldrich Inc. USA). Hydrogen peroxide(H_2O_2), Hydrochloric acid (HCl), Sulfo salicylic acid, Dithionitrobenzoic acid (DTNB), Tris-HCl, Pyrogallol, Thiobarbituric acid(TBA), *n*-Butanol-pyridine, Acetate buffer, Ferric chloride($FeCl_3$), Sodium dodecyl sulfate, Glacial acetic acid, Sodium chloride(NaCl), Phosphate buffer (PBS), Disodium hydrogen phosphate(Na_2HPO_4), Potasium di hydrogen phosphate (KH_2PO_4),Sodium di hydrogen phosphate(NaH_2PO_4), 2-vinyl pyridine, Magnesium chloride($MgCl_2$),Sodium hydroxide(NaOH), Ferric chloride($FeCl_3$), Bis-polyacrylamide, Cholesterol, Ethylenediaminetetraacetic acid(EDTA), Pentobarbital sodium, reduced glutathione(GSH), 1-Chloro- 2,4-Dinitrochlorobenzene(CDNB),2-vinylpyridine,Sodium azide, Tetra sodium pyrophosphate (TNaPP), Nicotinamide adenine dinucleotide, Testosterone, Dehydroepiandrosterone (DHEA), Radioimmiuno precipitation assay buffer (RIPA)

lysis buffer, Tris-buffered saline with Tween-20 (TBST), Nitro-blue tetrazolium, 5-bromo-4-chloro-3′-indolyphosphate, Polyvinylidene difluoride (PVDF), Methanol, antibodies (Cell Signaling Technology Beverly, MA, USA) and other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA; Himedia India, Ltd., Mumbai, India; and Merck India, Ltd., Mumbai, India for the experimentation.

. 7.2.2. Animal care and treatment

Mature 36 Wistar female albino rats weighing 130-150g were acclimatized for one week before the treatments temperature of 25°±2°C with 12 hrs light-dark cycle. The animals were given the standard laboratory feed and water adequately throughout the period of experimentation. Experimental protocol was approved by the Institutional Animal Ethical Committee, registered under CPCSEA. All animal treatment and surgical procedures were carried out according to the relevant laws and guidelines of the CPCSEA.

7.2.3. Treatment protocol

Rats were randomly divided into six groups, each containing six animals.

Group I: Distilled water control

Group II: Taurine control (50mg/kg body wt)

Group III: Lambda-cyhalothrin low dose (1/9 of LD_{50} value i.e., 6.3 mg/kg body wt.)

Group IV: Taurine (50mg/kg body wt) + lambda-cyhalothrin low dose (6.3 mg/kg body wt.)

Group V: Lambda-cyhalothrin high dose (1/5 of LD_{50}value i.e.,11.33mg/kg body wt.)

Group VI: Taurine (50mg/kg body wt) + lambda-cyhalothrin high dose (11.33mg/kg body wt.).

Lambda cyhalothrin 5% emulsifiable concentrate (EC), a commercial formulation was used for this experiment following the oral LD_{50} 56.69 mg/kg body wt. respectively in female rats (Sharma et al., 2010). Taurine was applied at the dose level of 50 mg/kg body wt. which was effectively used to ameliorate the toxicity induced by various xenobiotics (Ozden et al., 2009; Cetiner et al.,2005).

7.2.4. Sample collection

After sacrificed, serum and one parts of the collected ovary from control and treated rats were immediately stored at -80 ºC until analysis. The other part of the ovary was taken for histological analysis.

7.2.5.Estimation of ovarian index

Ovarian index was measured by using the following formula:

$$\text{Ovarian index} = \frac{\text{Ovarian weight (g)}}{\text{Body weight (g)}} \times 100$$

7.2.6.Estimation of oxidative stress parameters

7.2.6.1.Ovarian malondialdehyde (MDA): The malondialdehyde of ovarian homogenate(20mg/ml) was assayed by Ohkawa et al., (Ohkawa et al., 1979). Detailed method was described in the previous chapter.

7.2.6.2.Ovarian reduced glutathione (GSH): The reduced glutathione of tissue homogenate was measured by the method of Griffith. Reading was taken at 412-420nm (Griffith, 1981). Detailed method was described in the previous chapter.

7.2.6.3. Ovarian oxidized glutathione (GSSG): Ovarian oxidized glutathione was executed by the method of Griffith (1980) and the reading was taken at 412 nm within 1min (Griffith, 1980). Detailed method was described in the previous chapter.

7.2.6.4.Ovarian superoxide dismutase (SOD): SOD of ovarian homogenate was carried out according to the method of Marklund and Marklund (Marklund and Marklund, 1974)and the reading was noted in the spectrophotometer at 420 nm for 3 min. Detailed method was described in the previous chapter.

7.2.6.5.Ovarian catalase (CAT): According to the method of Aebi, the ovarian catalase was estimated (Aebi, 1974). Detailed method was described in the previous chapter.

7.2.6.6.Ovarian glutathione-s-transferase (GST): Ovarian GST was assayed by the method of (Habig et al., 1974). Detailed method was described in the previous chapter.

7.2.6.7.Ovarian glutathione peroxidase (GPx): Ovarian GPx was measured according to the method of (Rotruck et al., 1973). Detailed method was described in the previous chapter.

7.2.6.8.Ovarian glutathione reductase (GR): Ovarian GR was estimated according to Williams and Arscott, (Williams and Arscott,1971) and the reading was taken at 340nm. Detailed method was described in the previous chapter.

7.2.7. Estimation of ovarian and adrenal cholesterol level

Ovarian and adrenal cholesterol were measured according to the method of (Zlatkis et al., 1953). Detailed method was described in the previous chapter.

7.2.8. Biochemical assay of tissue (ovary and adrenal) $\Delta^5$3β-HSD activity and 17β-HSD

Activity of ovary and adrenal $\Delta^5$3β-HSD was measured according to the method of **(**Talalay, 1962). Detailed method was described in the previous chapter.

Activity of ovarian 17β-HSD was measured according to (Jarabak et al., 1962).Result is expressed as one unit of enzyme activity is equivalent to a change in absorbency of 0.001/min at 340 nm (Jarabak et al., 1962). Detailed method was described in the previous chapter.

7.2.9.Estimation of tissue protein

Tissue protein was estimated as previously described method(Lowry et al.,1951)

7.2.10. Western blot analysis of ovarian 17β-HSD and steroidogenic acute regulatory protein (StAR)

Ovaries were crushed in a homogenizer and allow to cool at -80ºC.Tissue fragments were transferred in to RIPA lysis buffer (Archanco et al.,2003). After 1hr sample were centrifuged at 12000rpm at 4ºC for 15min.Supernatent was recovered and the protein content was measured by Lowry method. Western blot analysis was performed as describe previously (Mandal et al., 2006 ; Nteeba et al., 2014). Then proteins were visualized after staining with NBT-BCIP buffer. Then the picture was captured by Gel Doc (Bio-Rad). Densitometry of the appropriate sized bands was

measured using molecular imaging software (Image J 148-jdk 6 software). Detailed method was described in the previous chapter.

7.2.11. Estimation of gonadotropin hormones (Lueteinizing hormone and follicle-stimulating hormone)

For the quantitative determination of Luetenizing hormone ELISA Kit CATALOG NO:ABIN365711.)was used.

Quantitative determination of follicle-stimulating hormone was done by ELISA Kit (CATALOG NO**:** ABIN365553**)** according to the manufacturer's instructions.

7.2.12. Determination of progesterone and estradiol hormones

The progesterone levels in serum of female rats were analyzed by using ELISA Kit CATALOG NO: LS-F10045. For the quantitative determination of estradiol level commercial ELISA kit CATALOG NO: ABIN365135 was used.

7.2.13. Histological examination

For histological examination under light microscope, specimens were fixed in bouins than dehydrated in alcohols and embedded in paraffin. Five micron tissue sections from each group of rat were stained with hematoxyl in and eosin stain (H&E) for general histopathological examination.

7.3. Results

7.3.1. Effect on ovarian index

The ovarian index of LCT exposed rats was decreased significantly ($p<0.001$) in a dose dependent manner compared to the rats of the control group (Table-1). Taurine increased the ovarian index of LCT induced rats significantly.

Table 7.1.Effect of LCT and taurine on ovarian index

Group	Ovarian index
Group-I (control)	0.040±0.0002
Group-II (Taurine control)	0.038±0.0004
Group-III (LCT, 6.3mg/kg body wt.)	0.029±0.0003a***
Group-IV (Taurine+LCT, 6.3mg /kg body wt.)	0.032±0.0006a***b*
Group-V (LCT, 11.33mg/kg body wt.)	0.023±0.00031a***
Group-VI (Taurine+LCT, 11.33mg /kg body wt.)	0.027±0.001a***c***

Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (*indicates $p<0.05$,** indicates $p<0.01$, *** indicates $p<0.001$).

7.3.2. Impact on oxidative stress

The effect of LCT and taurine on ovarian malondialdehyde (MDA) in female rat was shown in figure 7.1A. In LCT treated group, MDA content increased significantly ($p<0.001$) compared to the control group in dose dependent manner where treatment of taurine decreased the LCT toxicity and restored the normal status of the ovary to a great extent.

Figure 7.1B shows the effect of taurine on ovarian GSH level in lambda cyhalothrin exposed female albino rat. From this figure it is observed that GSH were decreased

significantly ($p<0.001$) in LCT treated groups compared to the control which were alleviated by taurine. The effect of taurine on ovarian GSSG level in lambda cyhalothrin induced female albino rat is shown in figure 7.1C. GSSG level was significantly higher ($p<0.001$) in rats of LCT treated high dose group. Taurine restored it to a good extent. As presented in figure 7.1D, the activity of SOD in the LCT treated groups were significantly decreased in compare to the control group. However, pretreatment with taurine resulted in a significant increase in the activity of SOD in LCT treated group animals respectively. As depicted in figure 7.1E, the activities of CAT in the LCT treated low and high dose groups were significantly ($p<0.001$) decreased compared to the control group. However, the activity of CAT was significantly increased by taurine pretreatment in low and high dose group animals. Activity of GST on LCT treated low and high dose animals is shown in figure 7.1F. GST level was increased significantly in case of LCT treated high dose group compared to control, where pretreatment of taurine restored the normal status.As shown in figure 7.1G, the activity of glutathione peroxidase and glutathione reductase (figure 7.1H) in the LCT treated group was significantly decreased in ovary. However, pretreatment with taurine resulted in a significant increase in the activity of glutathione peroxidase

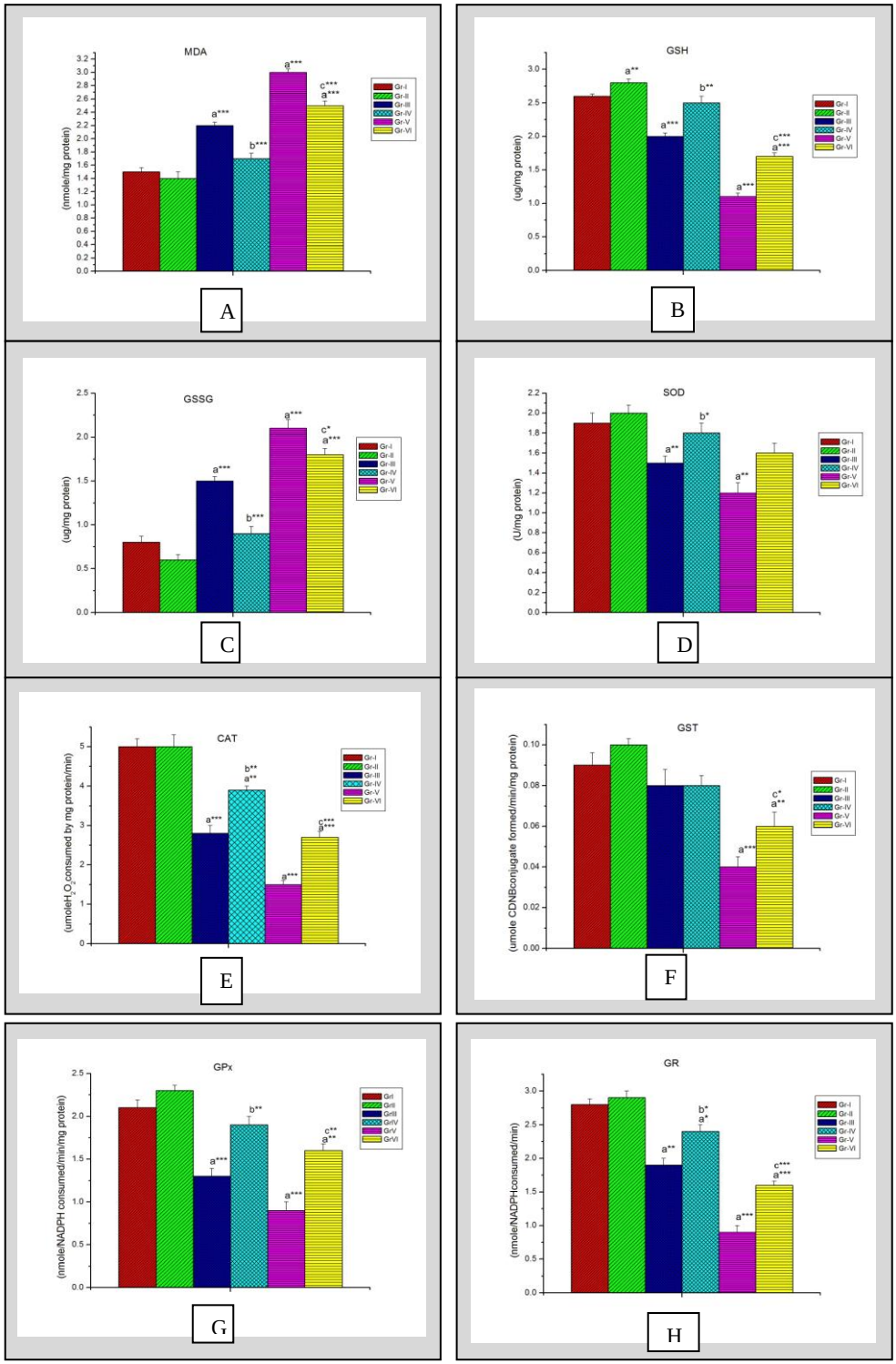


Figure-7.1 represents the MDA content and antioxidant status in control and treated group of rat. Results are expressed as Mean±SEM. Analysis is done by one way

ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).A=MDA content, B=GSHlevel, C=GSSG level, D=SOD.E=CAT, F=GST, G=GPx, H=GR.

7.3.3. Tissues cholesterol and steroidogenic key enzymes activity

The toxic effect of LCT on steroidogenesis was assessed by measuring ovarian and adrenal cholesterol level in control and treated rat. Ovarian cholesterol levels was significantly increased ($p<0.001$) by the treatment of LCT (Table.7.2) where adrenal cholesterol level was significantly decreased ($p<0.001$) from the basal level. Pretreatment of taurine significantly alleviated the effect. Activity of ovarian Δ^5, 3β-HSD and 17 β-HSD were significantly decreased ($p<0.001$) by the treatment of LCT (Table.7.2) where activity of adrenal Δ^5,3β-HSD and 17β-HSD were shown to be induced significantly ($p<0.001$) from the basal level. Pretreatment of taurine significantly alleviated the toxic effect.

Table 7.2.Effect of LCT and taurine on ovarian and adrenal cholesterol and activities of ovarian and adrenal Δ^5,3β-HSD and 17β-HSD

Parameters	Group-I	Group-II	Group-III	Group-IV	Group-V	Group-VI
Ovarian cholesterol(mg/gm)	1.5±0.1	1.3±0.06	2±0.08 a***	1.8±0.06 a*	3.1±0.1 a***	2.2±0.08 a***c***
Ovarian Δ^5,3β-HSD(U/ mg of tissue/hr)	2.1±0.08	2.15±0.1	1.5±0.08 a***	1.9±0.09 b**	0.8±0.09 a***	1.4±0.1 a***c**
Ovarian 17β-HSD(U/mg of tissue/hr)	1.55 ±0.07	1.6±0.1	1.05±0.08 a*	1.4±0.1	0.8±0.07 a***	1.2±0.07 a*c*
Adrenal cholesterol(mg/gm)	1.6 ±0.07	1.6±0.04	0.9±0.08 a***	1.3±0.02 a**b***	0.4±0.02 a***	0.7±0.04a***c***
Adrenal Δ^5,3β-HSD(U/ mg of tissue/hr)	1.9±0.15	1.8±0.15	2.3±0.1	2.05±0.08	2.8±0.1 a***	2.35±0.09 a**c*
Adrenal 17β-HSD(U/mg of tissue/hr)	1.4±0.1	1.3±0.08	1.9±0.09 a**	1.6±0.1	2.19±0.08 a***	1.8±0.09 a*c*

Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups,

superscript b Group III versus Group IV and superscript c Group V versus Group VI (*indicates p<0.05,** indicates p<0.01, *** indicates p<0.001).

7.3.4. Protein expression of 17β-HSD and steroidogenic acute regulatory protein (StAR)

Protein expression observed by western blot analysis revealed significant decrease following LCT exposure with respect to the control and taurine co administered group.

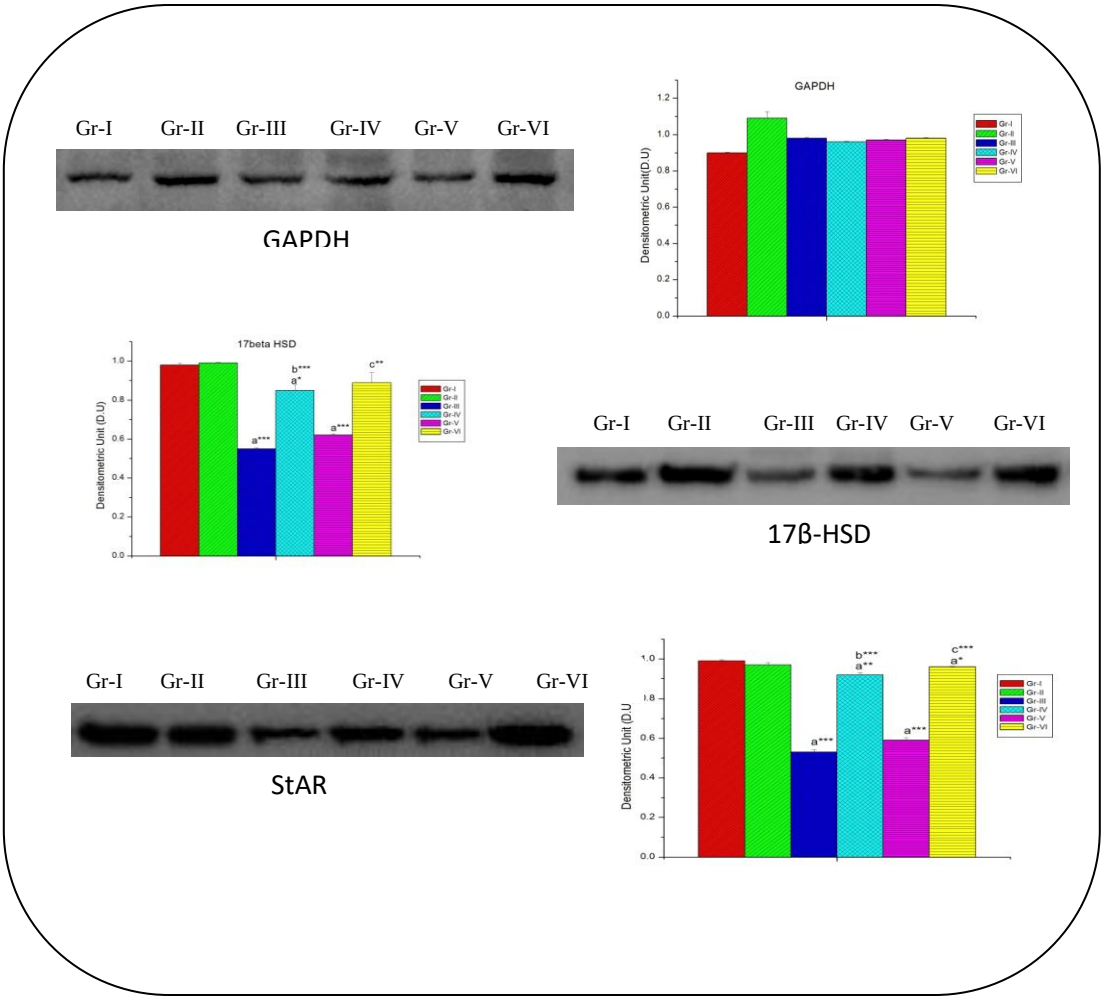


Figure 7.2. Shows the expression levels of ovarian GAPDH, 17β-HSD, StAR in control and treatedrats. Gr-I=control,Gr-II=taurine control, Gr-III=LCT Low

dose,Gr-IV=taurine+LCT low dose,Gr-V= LCT Highdose,Gr-VI=taurine+LCT high dose group.

7.3.5.Hormonal changes

7.3.5.1.Luteinizing hormone (LH): The serum LH levels of LCT treated groups were significantly ($p<0.001$) lower compared with the control. A significant protection was found in taurine pre treated group.

7.3.5.2. Follicle stimulating hormone (FSH): FSH level of LCT treated rat were also significantly ($p<0.001$) different from the control. Level was near normal to control in taurine pre treated group.

7.3.5.3. Levels of steroid hormones

7.3.5.3.1. Progesterone: There was a significant decreased ($p<0.001$)in the level of progesterone in response to LCT for 14 days of experiment compared to the control group(Table 7.3).

7.3.5.3.2. Estrogen

After LCT treatment the levels of estrogen (also known as oestrogen) found to be decrease when compared to the control group .In the LCT treated rats that received taurine also, these parameters was similar to the control levels (Table 7.3).However, pre treatement of taurine effectively mitigated the toxicity.

Table7.3. Effect of LCT and taurine on serum gonadotropins and progesterone, estrogen level

Group	Progesterone(ng/ml)	LH (mIU/ml)	FSH(mIU/ml)	Estradiol(ng/ml)
Gr-I	19.2±0.6	0.66±0.03	0.9±0.02	26.83±1
Gr-II	19.17±0.8	0.63±0.04	0.9±0.03	27±1.4
Gr-III	16.4±0.96 a*	0.5±0.03a**	0.5±0.02a***	22.83±0.09a*
Gr-IV	18±0.57	0.66±0.03b**	0.7±0.01a*** b***	24.33±1.3
Gr-V	12.2±0.86 a***	0.33±0.02a***	0.4±0.01 a***	18.5±0.8a***
Gr-VI	15±0.8 a**c*	0.46±0.03a** c**	0.7±0.03a*** c***	23.83±1.24c**

Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (*indicates $p<0.05$,** indicates$p<0.01$, *** indicates $p<0.001$).

7.3.6.Histological observation of ovary

Histological examination of the ovary showed a significant alteration in the architecture of follicles after LCT treatment in comparison with the control rats (fig7.3.). A significant protection was found after taurine supplementation.

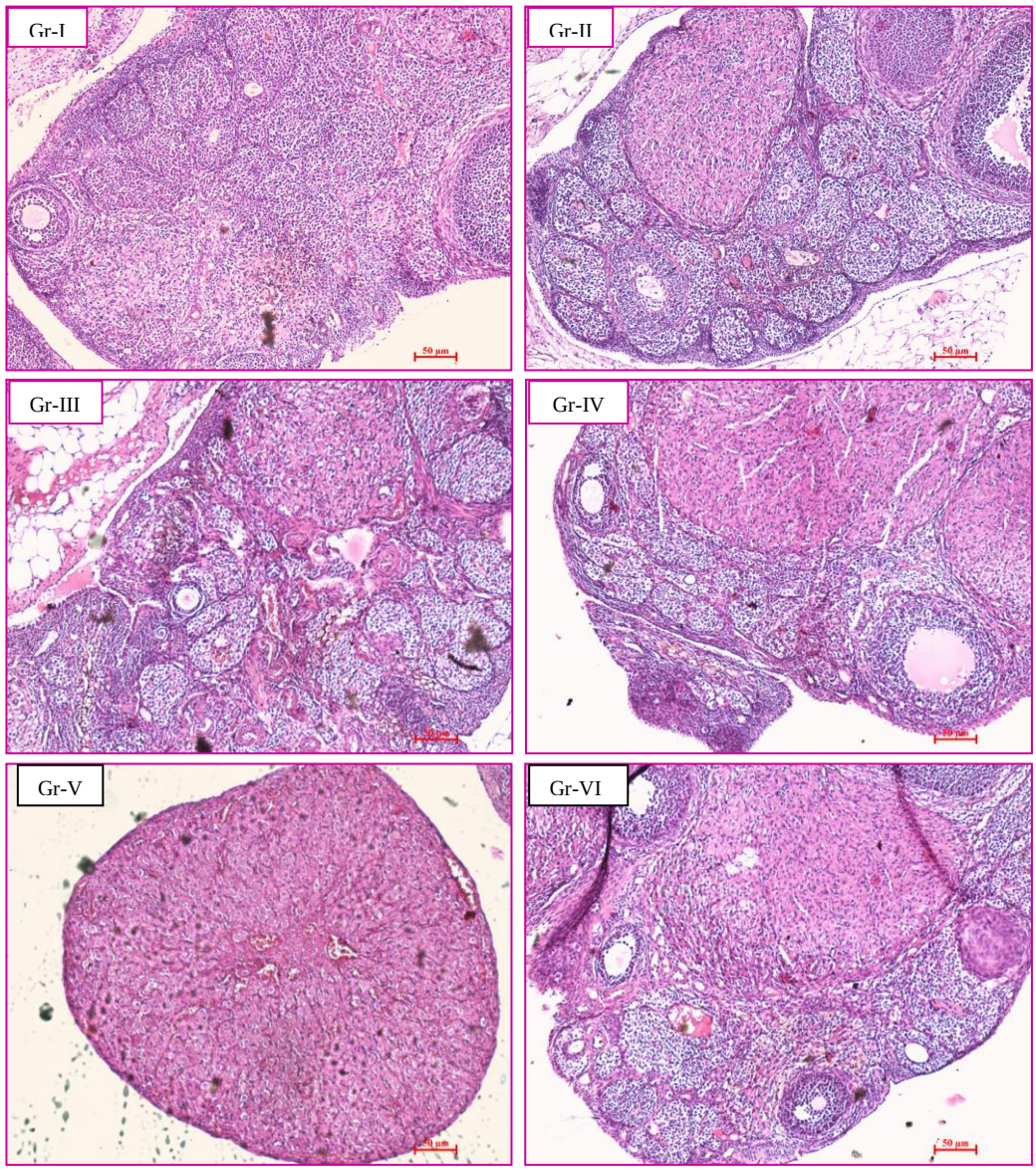
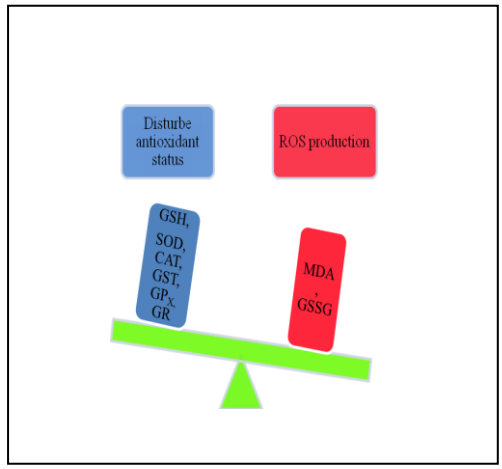


Figure-7.3. Histological pictures of ovary of control and experimental groups of rats after hematoxylin and eosin staining

7.4. Discussion

The ovary is the most important sex organs in the female reproductive system. Normal ovarian biochemical parameters, hormonal level, and histomorphology of the ovary are indicative of the normal sexual processes in the female rat.

In this study decrease in ovarian index in case of LCT treated rats compared to control may be due to reduced number of ovarian germ cells. Other possibility was insufficient female gonadotropins level that decrease in body and organ weights by inducing the atrophy of somatic tissues and ovarian tissue. In addition to this, one of the most common explanations of tissue damage is oxidative stress which may take place in LCT exposed rats and altered antioxidant status. Different studies showed that LCT significantly increases the level of malondialdehyde, the end product of lipid peroxidation in the liver and kidneys of rats, whereas the activity of antioxidant enzymes decreased (Fetoui et al.,2009; Fetoui et al.,2010).Ansari et al. established that the intoxication of LCT in different periods of postnatal ontogenesis causes oxidative stress, manifested by a significant increase in MDA level with suppressed activity of antioxidant enzymes (SOD, CAT) in brain tissue(Ansari et al.,2012). The ability of LCT to induce a pronounced oxidative stress was also confirmed in in-vitro study (El-Demerdash, 2007; Abdallah et al., 2011) but there was no clear information regarding the toxic effect of LCT on female rats. In the study it is found that LCT administration to rats



resulted in a marked increase in the lipid peroxidation as indicated by the increase in the level of malondialdehyde (MDA), that may be due to LCT induced increased excess ROS level. Antioxidant enzymes are believed to be a primary defense that prevents biological molecules from oxidative damage. In the detoxification of the reactive toxic metabolites, GSH, the most important biological molecules, play an important role. So, in the present study reduction in GSH levels which is utilized for the detoxification of reactive toxic substances may be an indication of LCT induced oxidative stress. An increased level of GSSG correlates the GSH level. According to the results, the activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase in LCT treated rats ovaries were significantly decreased. There was no significant change found in GST level in LCT treated low dose group but marked alteration has been observed in LCT treated group. These results suggested that LCT has the capability to generate excess free radical induced oxidative damage evidenced by alterations in various antioxidant enzymes. Similar result was found in Salama et al.(Salama et al.,2005). Pretreatment of taurine diminished lipid peroxidation either by scavenging or quenching oxygen-derived free radicals, hydrogen peroxide or hypochlorous acid directly, or by binding free metal ion species like Fe^{2+} or Cu^{2+} by its sulfonic acid group. On the other hand, since cysteine is a precursor of taurine and GSH, taurine supplementation may cause enhancement in GSH levels by directing cysteine into the GSH synthesis pathway(Hagar,2004).Therefore, improved GSH levels after taurine treatment may play an additional role in decreasing oxidative stress. The stimulatory effects of

taurine on endogenous antioxidants were also established by other studies (Erdem et al.,2000; Saad and Al-Rikabi,2002).

The low steroidogenesis was also evident by the decreased activity of steroidogenic enzymes (Δ^5-3β-HSD, 17β-HSD) and reduced protein expression of 17β-HSD as they are the key regulatory enzymes in ovarian steroidogenesis (Hinshelwood et al., 1994). A reduction of adrenal cholesterol and an increased activity of steroidogenic enzymes in adrenal may be due to the compensated steroidogenesis in adrenal gland (Maiti Choudhury et al., 2011).To preserve female reproduction and maintain fertility hormonal balance is important. Female reproductive function can be compromised by exposure to toxic chemicals at a variety of sites, including the hypothalamus, pituitary gland, ovary and reproductive tract. Crisp et al. reported that pesticides are endocrine disruptors where they mimic, enhance (agonists), or inhibit (antagonists) the action of endogenous hormones (Crisp et al., 1998).Disruption can occur in all stages (Bretveld et al.,2006) of hormonal regulation, hormone synthesis, hormone release and storage, hormone transport and clearance, hormone receptor recognition and binding (Bretveld et al., 2006). These can be disturbed by estrogen/progesterone imbalance. Disruption of any of these sites can ultimately manifest as a disruption of ovarian function, resulting in infertility (Schreinemachers, 2003). Ovary is responsible for oogenesis and estrogen secretion. FSH and LH of the anterior pituitary influence this process, as well as, also regulate to production of progesterone and estrogen by the sex organs (Murray et al.,2000).In the present study inhibitory effect of LCT on the secretion of pituitary gonadotrophins (FSH and LH) was clearly observed. The possibility of low serum levels of gonadrotrophins in this

experiment may be due to the elevated level of plasma glucocorticoids in LCT treated rats though this parameter was not investigated in this study. The level of estrogen (also known as oestrogen) and progesterone are controlled by pituitary gonadotropins and hypothalamus-releasing gonadotrophin releasing hormone (Lerner, 1969). A decrease in the serum levels of estradiol in LCT treated rats may occur due to the inhibition of ovarian steroidogenic protein and enzyme activity which is evident from protein expression level of ovarian of StAR in LCT exposed rat indicating the low transport of cholesterol from outer to inner mitochondria and 17β-HSD, catalyzes the final steps in estrogen biosynthesis. Previous studies have shown that LCT is an estrogen mimicking agent as identified in MCF-7 cell study (Meirong et al., 2008). Furthermore, Nejaty et al., reported that estrogen significantly inhibited basal progesterone production which was the important cause of LCT induced low level of progesterone(Nejaty et al., 2001).Taurine showed ameliorating effect on the reproductive hormones concentration and that may attributed to the role of taurine to stimulate secretion of LH and FSH through its effect on hypothalamo-pituitary gonadal axis.The results came in agreement with the studies of Xiao et al and Yang et al.(Xiao et al.,1997; Yang et al.,2007).

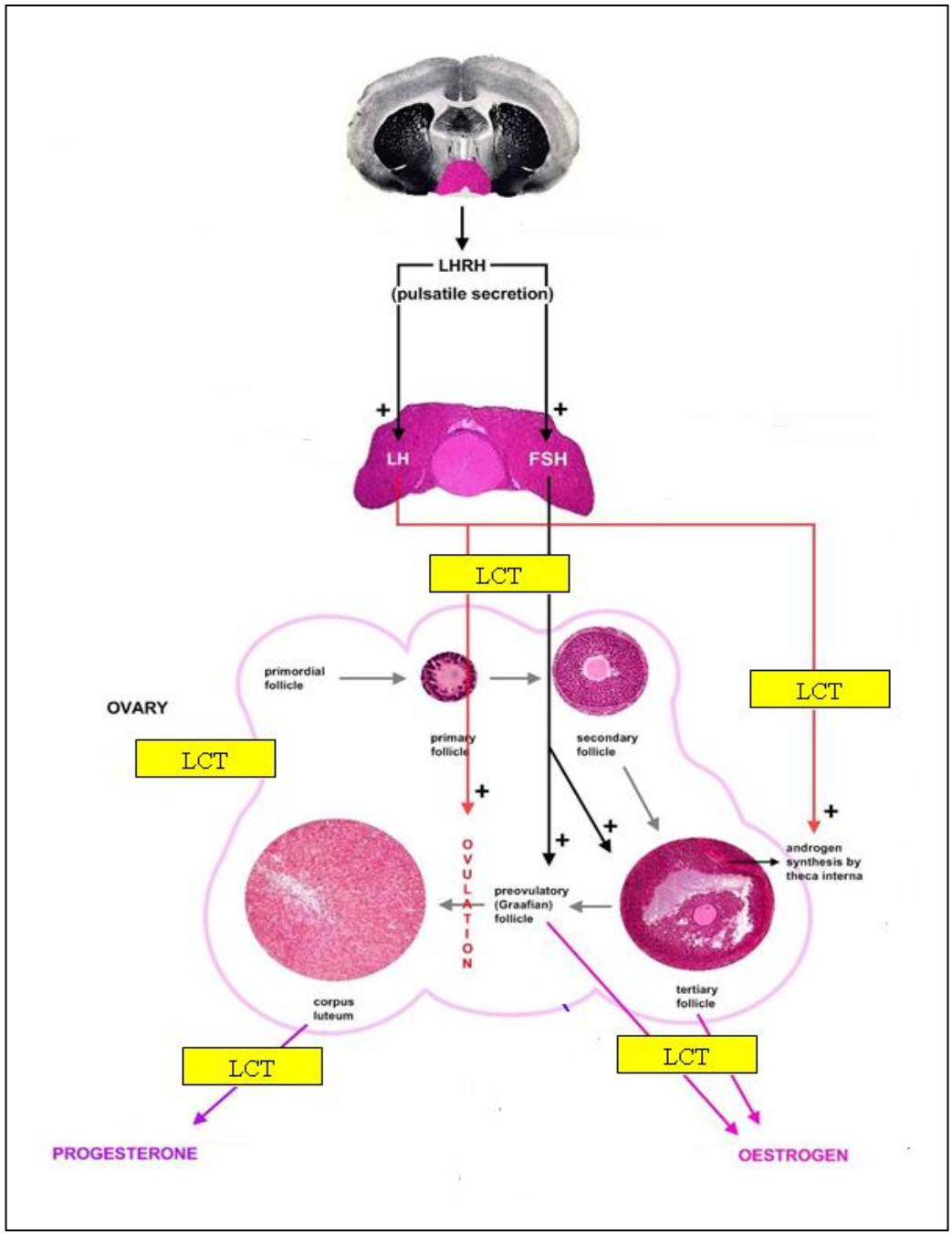


Figure7.4.Graphical representation of LCT induced reproductive toxicity.

Histopathological study in pyrethroid treated group suggest that LCT may promote the disorders of follicular maturation, structural degenerations of ovary presumably by lowering the level of gonadotropin and estradiol and by promoting the oxidative

stress induced damage of the tissues of ovary. Pretreatment of taurine restored the normal architecture of ovary to a great extent.

7.5. Conclusion

LCT exposure altered the female reproductive function by producing oxidative damage, disturbing the steroidogenic pathway and affecting the female hypothalmo-pituitary-gonadal axis. Histological study also established the female gonadal toxicity. Taurine pretreatment with LCT restored these parameters towards control level indicating the protective role of taurine on ovarian anomalies after LCT treatment.

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Chapter-8

Summary and conclusion

Registered pesticides are considered to be an essential pest management tool whose benefits overweight health and environmental risks. Some regulatory agencies also believed this fact. But history of toxicological research has shown that no pesticide, particularly insecticides, is harmless. Even the common trend of replacing organophosphates with putatively safer insecticide pyrethroids may not be a benign alternative. However, certain pyrethroids are reported to exhibit a potential for neurotoxicity, hepatotoxicity, reproductive toxicity, endocrine disruption and other systemic toxicity.

Lambda cyhalothrin (LCT) is a potent synthetic type II α-cyano-pyrethroid used worldwide to control a wide range of insects and ectoparasites in agriculture production, forestry, animal husbandry and public health applications. But, the widespread use of these substances has led to serious health problems.

The present study was designed to investigate the toxic effect of lambda cyhalothin in haematological, hepatic and reproductive system of Wistar rat, and the protective effect of taurine (a sulphur containing β-amino acid) in this situation.

Firstly, the dose dependent systemic toxicity of LCT was carried out at different dose levels in male and female Wistar rats. The results showed that oral administration of LCT for 14 consecutive days exhibited its haematological, hepatic and gonadal toxicity at 10.83 mg/kg body wt (1/7th LD50 dose) in male and ovarian toxicity at 6.29 mg/kg body wt. (1/9th LD50 dose) in female rat and also on above dose levels.

Lambda cyhalothrin induced haematological, hepatic, male and female reproductive toxicity were investigated in rat and alleviation of these toxicities by taurine was also studied. Lambda cyhalothrin (10.83 and 15.17mg/kg body wt. for male and 6.29 mg/kg body wt. and 11.33mg/ kg body wt. for female) was orally administered alone or combined with pretreated taurine (50mg/kg body wt.) for 14 consecutive days.

In haematological study, a significant decrease in erythrocyte count, haemoglobin percentage, haematocrit and red cell indices were observed and a significant increase in white blood cells and lymphocyte count were also detected. The results also showed an increase in malondialdehyde, oxidized glutathione level and depletion of reduced glutathione level, the activities of superoxide dismutase, catalase, glutathione-s-transferase and glutathione peroxidase in rat erythrocytes. A marked alteration in the morphology of lambda cyhalothrin intoxicated rat erythrocytes was seen by scanning electron microscopic study. However, pretreatment with taurine significantly restored the above said parameters by reducing oxidative stress.

Production of excess intracellular reactive oxygen species (ROS) in hepatic cells of LCT exposed rat triggered severe DNA damage and decreased mitochondrial membrane potential, ultimately induced apoptosis in hepatic cells. A significant increase in oxidative stress by the elevation of lipid peroxidation and depletion of reduced glutathione level and antioxidant enzymes were noticed in lambda cyhalothrin treated rat liver in a dose-dependent fashion. Significant changes in blood glucose level with a marked decline in glycogen content also indicated the hepatic dysfunction in lambda cyhalothrin treated rats. Hepatic damage was also

confirmed by the altered activities of serum hepatic biomarker enzymes, lipid profiles and liver histoarchitecture in LCT intoxicated rats. Pretreatment of taurine alleviated the LCT induced hepato-toxicity as an antioxidant to a good extent.

Male reproductive alteration was evaluated by the assessment of important sperm parameters, testicular biochemical parameters, enzyme activity and protein expression of steroidogenic pathway and endocrine status in testes. A significant decrease in sperm count, viability, motility, hypo-osmotic swelling, seminal fructose concentration, testicular reduced glutathione, testicular antioxidant activity and increase in testicular acid phosphatase, malondialdehyde and oxidized glutathione were observed in lambda cyhalothrin intoxicated rats. Subsequently, LCT treatment reduced the mitochondrial membrane potential in sperm cell. ROS induced DNA damage was reflected in comet assay and was supported by the TUNEL assay. Result also reflected the spermatogenic and steroidogenic arrest or disorder by altering the activity and protein expression level of testicular $\Delta^5$3β- and 17β-hydroxysteroid dehydrogenase(HSD) along with the downregulation of steroidogenic acute regulatory protein (StAR) and P450scc. Decreased serum level of androgen and pituitary gonadotrophins were also reported in LCT intoxicated rat. LCT induced toxicity was also confirmed by the testicular histopathology. Pretreatment of taurine reduced the harmful effects of lambda cyhalothrin by altering the hypothalamo-pituitary testicular axis towards normal levels and by ameliorating oxidative stress in LCT exposed animals.

Female reproductive toxicity induced by LCT was reflected through the oxidative damage in ovary, disruption in the ovarian biomarkers and key enzyme activities and protein expression of steroidogenic pathway and alterations in the serum hormonal levels of hypothalamo-pituitary ovarian axis in female rat. Histological analysis correlates the above said alterations. The pretreatment of taurine restored these parameters towards the control level indicating the preventive role of taurine on LCT imposed ovarian toxicity.

There are substantial scientific findings regarding the LCT induced haematological, hepatic, male and female reproductive toxicity and the pharmacological significance of taurine in attenuating system specific toxicity in this study. These considerable findings have utmost clinical significance and stimulate further detailed investigations in these areas.

Paper Publication

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ORIGINAL RESEARCH ARTICLE

INTERNATIONAL JOURNAL OF BIOASSAYS
ISSN: 2278-778X
CODEN: IJBNHY
OPEN ACCESS

Alteration in estrous cycle, lipid peroxidation and antioxidant status in female rat after exposure to lambda cyhalothrin and its attenuation by taurine

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Received for publication: August 26, 2015; **Accepted**: September 21, 2015

Abstract: Lambda cyhalothrin, a broad spectrum pyrethroid, is being extensively used against a wide range of pests in agricultural practices. The present study was highlighted to investigate the effects of the lambda cyhalothrin on estrous cycle and oxidative stress in rat ovary, also to find out the protective role of taurine against lambda cyhalothrin induced ovarian toxicity. Mature 36 female Wistar rats were divided in six groups and they were received oral administration of lambda cyhalothrin at two dose levels (8.33 and 16.66 mg/kg body wt) for consecutive14 days. Taurine (50mg/kg body wt) was treated before lambda cyhalothrin administration in the combined taurine-lambda cyhalothrin treated groups. Lambda cyhalothrin caused significant alterations in estrous cycle with an increase in diestrous index compared to control rats. Lambda cyhalothrin treatment also produced oxidative stress in ovary by significant increase in malondialdehyde level, accompanied by a reduction in reduced glutathione and antioxidant enzymes (superoxide dismutase, catalase, glutathione-s-transferase, glutathione peroxidase, glutathione reductase). The present study revealed that the pretreatment of taurine was able to ameliorate lambda cyhalothrin induced ovarian oxidative stress.

Key Words: Lambda cyhalothrin; Taurine; Estrous cycle; Oxidative stress; Antioxidant enzymes.

INTRODUCTION

The undesired effects of pesticide have been recognized as a serious public health concern during the past decades. Insecticide exposure have known to cause problems and outbreak of diseases among animals and human[1].Chronic neurological syndromes, malignant tumors, immunosuppressive and teratogenic effects in experimental animals are often caused by prolonged exposure to insecticides[2].Ovary, which has a key role in female reproduction, is one of the affected organs in mammals [3,4].

Disease vectors such as cockroaches, mosquitoes, ticks and flies are controlled by pyrethroids, structural derivatives of naturally occurring pyrethrins and have greater potency over organochlorines, organophosphates and carbamates. They affect the nerve fiber of the disease vectors by binding to a protein that regulates the voltage-gated sodium channel.

During the past few years, quantification of free radical generation and antioxidant defense status has become an important aspect in assessing the toxicity of any chemical. One of the important molecular mechanisms involved in pesticide-induced toxicity assumed to be the lipid per-oxidation[5-12]. In normal physiological condition, reactive oxygen species (ROS) and antioxidants remain more or less in balanced level. When this balance is disturbed towards an increase in ROS, oxidative stress occurs. ROS affect multiple physiological processes from oocyte maturation to fertilization; produce alterations in metabolic reactions, embryo development and pregnancy.

Lambda cyhalothrin(LCT), a third generation type-II synthetic pyrethroid, is widely used to control insect pest in agriculture, public health, homes and gardens.Due to its rapid metabolism and excretion it may create problems in non-target species if applied indiscriminately. LCT has been found to accumulate in biological membranes leading to oxidative damage due to its lipophilic nature[13]. The toxicity of LCT to mammals and its ability to induce oxidative stress *in vivo* and *in vitro* have been established from various reports[14-18]. However, information on the status of lipid peroxidation and antioxidant defence system of this pesticide in ovary is publically unavailable.

Taurine, 2-aminoethanesulphonic acid is an essential amino acid and is present at high concentrations in many tissues. It plays important roles in various physiological functions including bile acids conjugation, calcium level modulation and maintenance of osmolarity, antioxidation and membrane stabilization [19, 20]. Its beneficial effects in various physiological and pathological conditions by reducing reactive oxygen species[21-23]and preventing DNA damage[24, 25] were also reported.

Female reproductive function specially the oxidative status of female reproductive system can be altered by the exposure of toxic chemicals like pyrethroids. The present study was carried out to assess the effect of oral exposure of LCT on estrous cycle as well as on ovarian oxidative status of female Wistar rat and to find out whether there is any the protective role of taurine against the LCT induced effects.

MATERIALS AND METHODS

Chemicals and Reagents:

Lambda cyhalothrin 5%Emulsifiable Concentrate (EC) was procured from RPC Agro Industries, Kolkata. Taurine and 1, 2dichloro-4-nitrobenzene (CDNB) was purchased from Sigma-Aldrich. Thiobarbituric acid, 5, 5' Dithiobis-2-nitrobenzoic acid (DTNB), ethylene di tetra acetic acid (EDTA), oxidized and reduced forms of glutathione and hydrogen peroxide were all purchased from Sigma Chemical, USA. All other chemicals used were of the finest analytical grade.

Animal care and treatment:

Mature 36 Wistar female albino rats weighing 130-150g were acclimatized for 1 week before the treatments at

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temperature of 25°±2°C with 12 hrs light-dark cycle. The animals were given the standard laboratory feed and water adequately throughout the period of experimentation. Rats were randomly divided into six groups, of six animals each. The experimental protocol was approved by the Institutional Animal Ethics Committee. The experiments were designed as:

1. Group I: Control (2 ml distilled water /kg body wt.)
2. Group II: Taurine control (50mg/kg body wt)
3. Group III: Lambda cyhalothrin low dose (8.33mg/body wt.)
4. Group IV: Taurine (50mg/kg body wt) +lambda cyhalothrin low dose (8.33mg/body wt.)
5. Group V: Lambda cyhalothrin high dose (16.66mg/body wt.)
6. Group VI: Taurine (50mg/kg body wt) + lambda cyhalothrin high dose (16.66mg/body wt.)

After one hour of the treatment of taurine (50mg/kg body wt), lambda cyhalothrin was administered at two dose levels (8.33mg, and 16.66mg /kg body wt.)[26] for consecutive 14 days. All animals were observed at least once daily to notice whether there was any behavioral change or signs of intoxications. Food and water consumption were monitored daily. Animal's weight was taken daily and the dose was adjusted accordingly.

Sample collection

Body weight of each rat was taken during the treatment period and before sacrifice. All rats were sacrificed by rapid decapitation after 24 hrs of the last dose. The ovaries were removed immediately and adhering fats were cleaned. Then weights of the ovaries were recorded and stored properly for the determination of oxidative stress biomarkers.

Estimation of Ovarian index

Ovarian index was measured by using the following formula:

$$\text{Ovarian index} = \frac{\text{Ovarian weight (g)}}{\text{Body weight (g)}} \times 100$$

Study of Estrous cycle[27]:

Vaginal smear histology is used to understand the estrus cycle as an index of ovarian functions. A detailed comparative estrous cycle study was designed in female rats exposed to two above mentioned doses of LCT and also pretreatment of taurine.

Collection of Vaginal smear:

The vaginal smear was collected daily in the morning (11.00-11.30a.m) through a Pasteur pipette filled with normal saline (0.9%NaCl) by inserting the tip into the rat's vagina. Vaginal lavage was taken on glass slides. A different glass slide was used for each rat for the specific dose. One drop was collected with a clean tip from each rat.

Staining and microscopic observations[28]:

The slides containing the vaginal smear were stained with hematoxylin and eosin. All the stained slides were examined under light microscope. The phases of estrous cycle were confirmed depending on the presence of different characteristic cells. The following formula was used for measuring the diestrous index:

$$\text{Diestrous index} = \frac{\text{No. of days with clear diestrous smear}}{\text{Total duration of treatment}} \times 100$$

Estimation of oxidative stress parameters:

Ovarian malondialdehyde (MDA)[29]: The reaction mixture consisted of 0.5ml of ovarian homogenate,1 ml of TBA(0.8%)-TCA (20%) mixture. Then the mixture was boiled for 45 min at 95°C. After cooling at room temperature it was centrifuged at 4000 rpm for 10 min and the reading was taken at 535nm.

Ovarian reduced glutathione (GSH)[30]: The assay mixture contained 200µl of ovarian homogenate and 100 µl of sulfosalicylic acid. The mixture was centrifuged for 10 min at 3000 rpm. Then 1.8 ml of DTNB was added with the supernatant and was shaken well. Reading was taken at 412-420nm.

Ovarian oxidized glutathione (GSSG)[31]: 100µl of ovarian homogenate was mixed with 2µl of 2-vinyl pyridine and was incubated for 1hr at 37°C. Then 250µl of sulfosalicylic acid (4gm %) was added with it and was kept in room temperature for 30min. It is centrifuged at 2000 rpm for 10 min. Then 200 µl of supernatant was added with 2 ml of DTNB (4mg %) and the reading was taken at 412 nm within 1min.

Ovarian superoxide dismutase (SOD)[32]: At first 2 ml of 50 mM Tris Hcl, 20µl of 10 mM pyrogallol and 20 µl of ovarian homogenates were poured in a spectrophotometric cuvette and the absorbance was noted in the spectrophotometer at 420 nm for 3 min. The enzyme activity was estimated by measuring the percentage inhibition of the pyrogallol autoxidation by SOD.

Ovarian catalase (CAT)[33]: The reaction mixture consisted of 0.5ml of H_2O_2, 2.5ml of double distilled water and 40µl of ovarian homogenate prepared in 0.05M trisHCl and was taken in a cuvette. After mixing, six readings were noted at 240nm in 30sec interval.

Ovarian glutathione-s-transferase (GST)[34]: Ovarian homogenate (0.1 ml), 2.8ml of PBS,0.1ml of GSH and 50µl of 60mM CDNB were taken in a cuvette and reading was noted at 340nm.

Ovarian glutathione peroxidase (GPx)[35]: Ovarian tissue homogenate was added with 2.5 mM H_2O_2, 0.4 M sodium phosphate buffer, 10 mM sodium azide and reduced glutathione and volume made upto 2 ml with distilled water and was incubated for 5min at 37°C. Then 10% TCA was mixed with reaction mixture. After that the mixture was centrifuged and the supernatant was mixed with 1 ml of DTNB and 3 ml of Na_2HPO_4. Reading was taken at 412nm.

Ovarian glutathione reductase (GR)[36] : 2ml of GSSG, 20µl of 12 mM $NADPHNa_4$ and 2.68ml of PBS were added with 100µl of ovarian homogenate and the reading was taken at 340nm.

RESULTS

Effects on ovarian index

The ovarian index of LCT exposed rats was decreased significantly ($p<0.001$) in a dose dependent manner compared to the rats of the control group (Table-1). Taurine increased the ovarian index of LCT induced rats significantly.

Effects on estrous cycle

Result showed a significant decrease ($p<0.001$) in frequency of estrous cycle. In case of treated animals, most of the rats exhibit abnormal estrous cycle. The duration of proestrous, estrous and metestrous of LCT treated rats of two dose groups were reduced significantly in a dose dependent manner compared to the rats of the control group (Table-2). In high dose LCT treated group, diestrous phase came earlier and persisted for a long time compared to the low dose LCT treated group. A significant increase was observed in the duration of diestrous phase and diestrous index of treated rats in a dose dependent manner (Table-2). Taurine decreased the duration of diestrous phase and diestrous index of LCT exposed rats significantly ($p<0.001$).

Effects on oxidative stress

The effect of taurine on ovarian malon-di-aldehyde (MDA) in lambda cyhalothrin exposed female albino rat is shown in Figure 1. In LCT treated group, MDA content increased significantly ($p<0.001$) compared to the control group in dose dependent manner where treatment of taurine decreased the LCT toxicity and restored the normal status of the ovary to a great extent.

Figure 2 shows the effect of taurine on ovarian GSH in lambda cyhalothrin exposed female albino rat. From this figure it is observed that GSH were decreased significantly ($p<0.001$) in LCT treated groups compared to the control which were alleviated by taurine.

The effect of taurine on ovarian GSSG level in lambda cyhalothrin induced female albino rat is shown in Figure 3. GSSG level was significantly higher ($p<0.001$) in rats of LCT treated high dose group. Taurine restored it to a good extent.

As presented in Figure 4, the activity of SOD in the LCT treated low and high dose were significantly decreased ($p<0.05$) and ($p<0.001$) compared to the control group. However, pretreatment with taurine resulted in a significant increase in the activity of SOD in low dose ($p<0.05$) and in high dose ($p<0.001$) group animals respectively.

As depicted in Figure 5, the activities of CAT in the LCT treated low and high dose groups were significantly ($p<0.001$) decreased compared to the control group. However, the activity of CAT was significantly increased by taurine pretreatment in low ($p<0.01$) and ($p<0.05$) high dose group animals.

Activity of GST on LCT treated low and high dose animals is shown in Figure 6. GST level was increased significantly at dose dependent manner in case of LCT treated group compared to control, where pretreatment of taurine restored the normal status.

As shown in figure 7, the activity of glutathione peroxidase and glutathione reductase in the LCT treated group was significantly decreased in ovary. However, pretreatment with taurine resulted in a significant increase in the activity of glutathione peroxidase and reductase.

Table 1: Effect of taurine on ovarian index in lambda cyhalothrin exposed female albino rat.

Group	Ovarian Index
Group-I (control)	0.030±0.0003
Group-II (Taurine control)	0.029±0.0004
Group-III (LCT, 8.33mg/body wt.)	0.027±0.0003a***
Group-IV (Taurine+LCT, 8.33mg /body wt.)	0.0286±0.00055a***b*
Group-V (LCT, 16.66mg/body wt.)	0.024±0.00031a***
Group-VI (Taurine+LCT, 16.66mg /body wt.)	0.028±0.001a***c***

Results are expressed as Mean±SEM (N=6). Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Probability values with different superscripts (a, b, c) differ from each other significantly. Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates $p<0.05$, *** indicates $p<0.001$).

Table 2: Effect of taurine on estrous cycle in lambda cyhalothrin exposed female albino rat.

Group	No of estrus cycle	Proestrous	Estrous	Metestrous	Diestrous	Diestrous Index (DI)
Group-I	5.7±0.2	5.3±0.2	7.5.2±0.3	4.5±0.3	12±0.4	84.02±5.4
Group-II	5.3±0.2	5.2±0.4	4.2±0.3a***	4.3±0.2	11.7±0.3	82.66±2.3
Group-III	2.5±0.4a***	2.8±0.4 a***	6±0.4a*b*	3.5±0.4	18±0.6a***	128.57±4.1a***
Group-IV	4±0.25a***b*	4.2±0.4a**b*	3.3±0.3a***	4±0.4	15.5±0.2a***b**	120.24±5.6a***
Group-V	1.3±0.2a***	1.33±0.3a***	3.3±0.3a***	1.2±0.2a***	23±0.4a***	164.28±5.8a***
Group-VI	2.8±0.16a***	2.7±0.3a***c*	4.8±0.4a***c*	3.3±0.2a*c***	18.16±0.5a***c***	135.7±3.7a***c**

Results are expressed as Mean±SEM (N=6). Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Probability values with different superscripts (a, b, c) differ from each other significantly Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisks represents the different level of significance (*indicates $p<0.05$, **indicates $p<0.01$,*** indicates $p<0.001$).

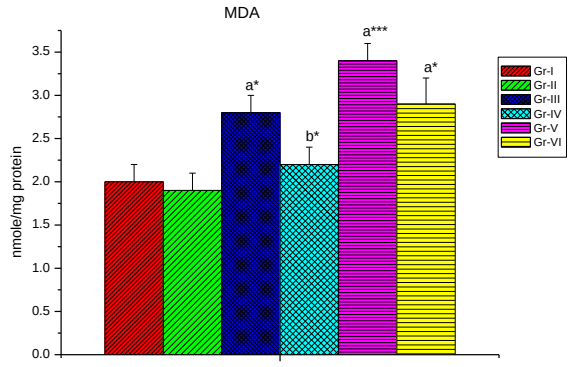

Figure 1: Effect of taurine on ovarian Malon-di-aldehyde (MDA) in lambda cyhalothrin exposed female albino rat.Results are expressed as Mean±SEM (N=6). Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Probability values with different superscripts (a,b) differ from each other significantly.Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV. Asterisks represents the different level of significance (* indicates p<0.05, *** indicates p<0.001).

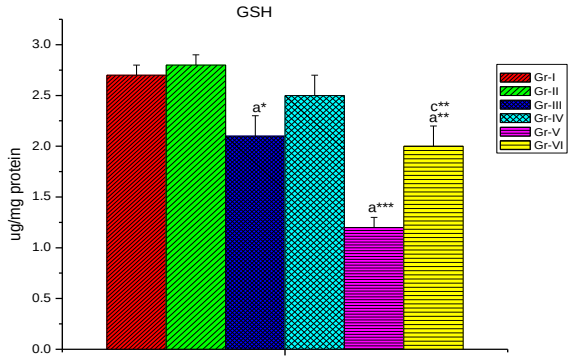

Figure 2: Effect of taurine on ovarian Reduced glutathione (GSH) in lambda cyhalothrin exposed female albino rat.Results are expressed as Mean±SEM (N=6). Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Probability values with different superscripts (a, c) differ from each other significantly.Superscript a, Group-I versus all other groups; Superscript c Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05,**indicates p<0.01,*** indicates p<0.001).

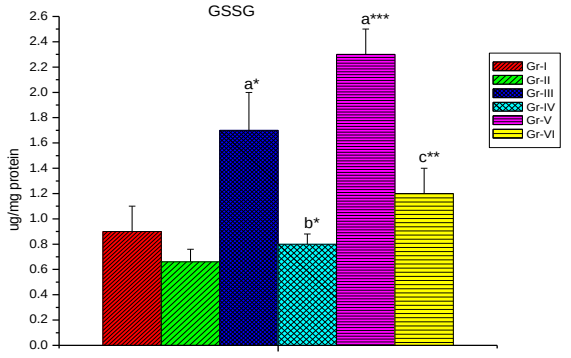

Figure 3: Effect of taurine on ovarian Oxidized glutathione (GSSG) in lambda cyhalothrin exposed female albino rat.Results are expressed as Mean±SEM (N=6). Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Probability values with different superscripts (a, b, c) differ from each other significantly.Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05,**indicates p<0.01,*** indicates p<0.001).

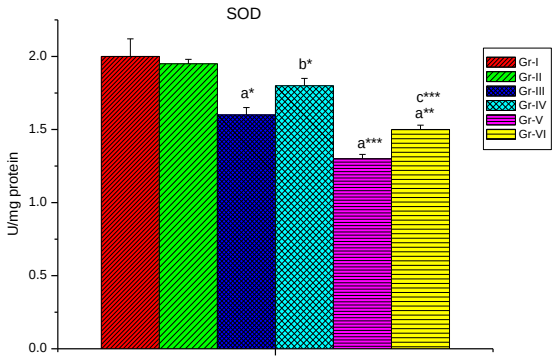

Figure 4: Effect of taurine on ovarian Super oxide dismutase (SOD) in lambda cyhalothrin exposed female albino rat. Results are expressed as Mean±SEM (N=6). Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Probability values with different superscripts (a, b, c) differ from each other significantly. Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisks represents the different level of significance (*indicates p<0.05,**indicates p<0.01,*** indicates p<0.001).

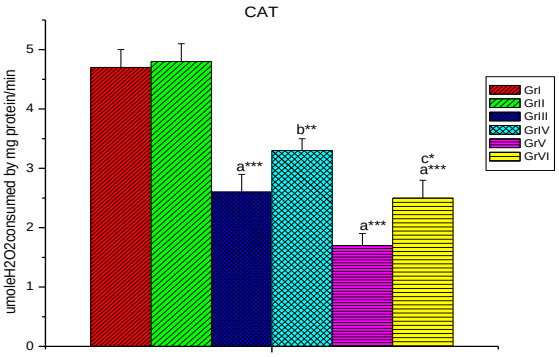

Figure 5: Effect of taurine on ovarian Catalase (CAT) in lambda cyhalothrin exposed female albino rat. Results are expressed as Mean±SEM (N=6). Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Probability values with different superscripts (a, b, c) differ from each other significantly.Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05,**indicates p<0.01,*** indicates p<0.001).

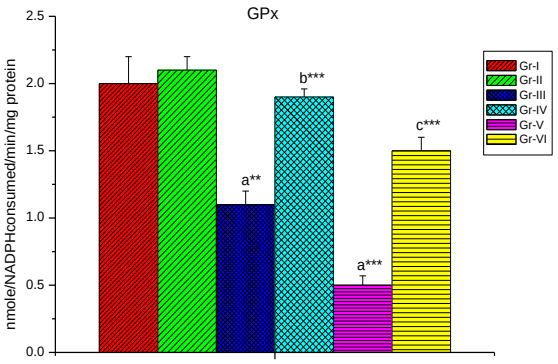

Figure 6: Effect of taurine on ovarian Glutathione peroxidase in lambda cyhalothrin exposed female albino rat. Results are expressed as Mean±SEM (N=6). Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Probability values with different superscripts (a, b, c) differ from each other significantly Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisks represents the different level of significance (**indicates p<0.01,*** indicates p<0.001).

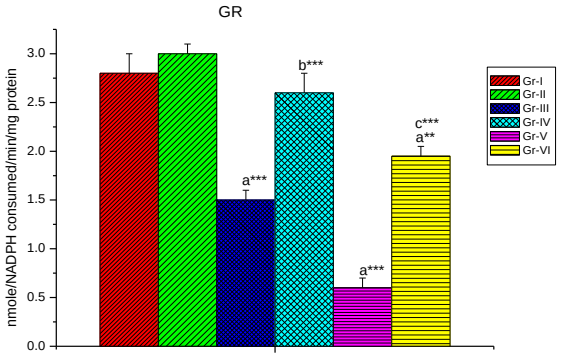

Figure 7: Effect of taurine on ovarian Glutathione reductase in lambda cyhalothrin exposed female albino rat. Results are expressed as Mean±SEM (N=6). Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Probability values with different superscripts (a, b, c) differ from each other significantly Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisks represents the different level of significance (**indicates p<0.01,*** indicates p<0.001).

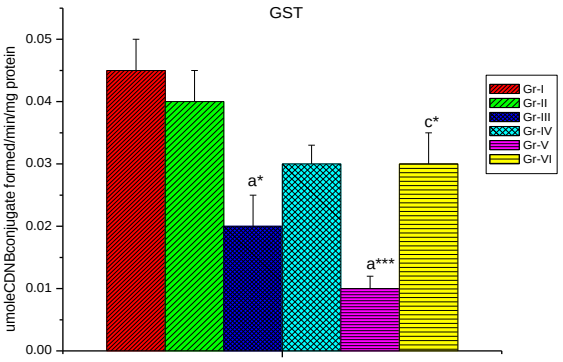

Figure 8: Effect of taurine on ovarian Glutathione-S-transferase (GST) in lambda cyhalothrin exposed female albino rat. Results are expressed as Mean±SEM (N=6). Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Probability values with different superscripts (a, c) differ from each other significantly.Superscript a, Group-I versus all other groups; Superscript c Group-V versus Group-VI. Asterisks represents the different level of significance (*indicates p<0.05,*** indicates p<0.001).

DISCUSSION

The present study was planned to evaluate the adverse effects of LCT on female rats and its attenuation by taurine. In this study we have examined the ovarian index and estrous cycle as the basic benchmarks for the ovarian toxicity. In this study, a decrease in ovarian index in LCT treated rats may be due to decreased number of ovarian germ cells. Other possible reason for LCT induced loss of body wt. and ovarian weights expressed as ovarian index may be due to endocrine disruption, inhibition of steroidogenic enzyme activity. Inadequate female gonadotropins are also responsible for the reduction in body and ovarian weights by inducing the atrophy of somatic and ovarian tissues. In addition to this, one of the most common reasons of tissues damage is oxidative stress which was seen in LCT exposed rats in this study.

In our study we have observed that the rats in control group shows regular estrous cycle but reduction in the estrous cycle was noticed in the LCT exposed rats. The duration of proestrus, estrous and metestrous also decreased with a consequent increase in the duration of diestrous in each estrous cycle in a dose dependent manner compared to control rats. Moreover, the diestrous index has been increased significantly in LCT treated rats. All the above findings were in agreement with previous observations[37].

LCT induced toxic manifestations may also be associated with induction of oxidative stress through the formation of free radicals and alteration in antioxidant systems. It was reported that LCT significantly increased the level of MDA in the liver and kidneys of rats, whereas the activity of antioxidant enzymes (SOD, CAT) was decreased[16,17]. Treatment with ascorbic acid, an antioxidant vitamin, caused a significant reduction of toxic effects of this pesticide. The administration of LCT in different periods of postnatal ontogenesis was also reported to enhance oxidative stress by a significant increase in MDA level and suppressed activity of antioxidant enzymes (SOD, CAT) in brain tissue[38]. The ability of LCT to induce a pronounced oxidative stress was also demonstrated in a vitro study[14, 18]. There is no clear information regarding the toxic effect of LCT on female rats. In our study we found that administration of LCT to rats resulted in a marked dose-dependent increase in the lipid peroxidation as indicated by the increase in the level of malondialdehyde (MDA), that may be due to LCT induced increased ROS level.

GSH, one of the most important biological molecules, play a key role in the detoxification of the reactive toxic metabolites. Decline in GSH levels in ovary after LCT treatment may be an indication of oxidative

stress, whereas GSH is utilized for the detoxification of reactive toxic substances. An increased level of GSSG also reflects the oxidative stress of ovary. Normal cellular functioning depends on a balance between ROS production and antioxidant defense mechanisms present in the cell. Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage.

According to the results, the activities of SOD, CAT, glutathione peroxidase and glutathione reductase in ovaries of LCT treated rats were significantly decreased. There was no significant changes found in GST level in LCT treated low dose group but marked alteration has been found in LCT treated high dose group. These results suggested that LCT has the capability to induce free radicals and oxidative damage as evidenced by alterations in various antioxidant enzymes [39]. Reduction of antioxidant enzymes levels may be due to the direct effect on the enzymes against LCT-induced ROS generation.

Taurine administration reversed all these abnormalities of above mentioned ovarian parameters to a good extent. It diminished lipid peroxidation either by scavenging or quenching oxygen-derived free radicals, hydrogen peroxide or hypochlorous acid directly, or by binding free metal ion species like Fe^{2+} or Cu^{2+} by its sulfonic acid group. By decreasing carbonyl group production, taurine suggested to decrease enhanced oxidative damage[40, 41]. On the other hand, since cysteine is a precursor of taurine and GSH, taurine supplementation may cause enhancement in GSH levels by directing cysteine into the GSH synthesis pathway[41,42]. Therefore, increased GSH levels after taurine treatment may play an additional role in decreasing oxidative stress. The stimulatory effects of taurine on endogenous antioxidants were also established by other studies [43,44].

CONCLUSION

Considering the above results we may conclude that LCT has the potency to induce toxicity as well as oxidative stress in ovary; thereby alter normal estrous cycle and oxidative stress parameters. Administration of taurine attenuates the toxicity of LCT in ovary.

ACKNOWLEDGMENT

The authors are thankful to authority of Vidyasagar University, Midnapore, India for providing all the facilities to execute this study.

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CITE THIS ARTICLE AS:
Rini Ghosh and Sujata Maiti Choudhury. Alteration in estrous cycle, lipid peroxidation and antioxidant status in female rat after exposure to lambda cyhalothrin and its attenuation by taurine. *International Journal of Bioassays* 4.11 (2015): 4526-4532.

Source of support: Vidyasagar University, Midnapore, India
Conflict of interest: None Declared

TOXICOLOGY AND FORENSIC MEDICINE

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Open Journal

http://dx.doi.org/10.17140/TFMOJ-1-107

Research

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Volume 1 : Issue 2
Article Ref. #: 1000TFMOJ1107

Article History
Received: September 10th, 2016
Accepted: October 5th, 2016
Published: October 6th, 2016

Citation
Ghosh R, Das T, Paramanik A, Maiti Choudhury S. Lambda cyhalothrin elicited dose response toxicity on haematological, hepatic, gonadal and lipid metabolic biomarkers in rat and possible modulatory role of taurine. *Toxicol Forensic Med Open J*. 2016; 1(2): 42-51. doi: 10.17140/TFMOJ-1-107

Lambda Cyhalothrin Elicited Dose Response Toxicity on Haematological, Hepatic, Gonadal and Lipid Metabolic Biomarkers in Rat and Possible Modulatory Role of Taurine

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ABSTRACT

Extensive application of pesticides is usually accompanied with serious problems of pollution and health hazards. Lambda-cyhalothrin (LCT), a type II synthetic pyrethroid, is widely used in agriculture, home pest control and protection of foodstuff. This study designed to evaluate the dose dependent haematological, hepatic and gonadal toxicity of LCT at different dose levels in Wistar rat. Investigations were also done to find out the toxic effect of lambda cyhalothrin on lipid metabolism in female rat and its amelioration by taurine. Rats were exposed to different doses of lambda cyhalothrin over a period of 14 consecutive days. Exposure to LCT produced ataxia, agitation, rolling and also tremors which were considered as the signs of toxicity. Significant decrease in erythrocyte count, haemoglobin percentage, seminal fructose concentration, hepatic and testicular reduced glutathione (GSH) content was observed. Increase in leukocyte count, serum aspartic and alanine transaminase, hepatic and testicular malondialdehyde (MDA), testicular and ovarian cholesterol after LCT treatment were seen in male rats at the dose level of 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50}). Elevated ovarian cholesterol and MDA and reduced 3β hydroxy steroid dehydrogenase (HSD) and GSH level were also observed in lambda cyhalothrin exposed female rat at the dose level of 6.29 mg/kg body wt. ($1/9^{th}$ LD_{50}). LCT caused increase in serum triglyceride, cholesterol, low density lipoproteins (LDL), very low density lipoproteins (VLDL) and bilirubin and decrease in serum high density lipoproteins (HDL) in female rat. Taurine pretreatment ameliorated LCT induced altered lipid metabolic biomarkers in female rat.

KEYWORDS: Lambda cyhalothrin; Taurine; Wistar rat; Hepatic and gonadal toxicity; Lipid metabolism.

ABBREVIATIONS: LCT: Lambda-cyhalothrin; GSH: Glutathione; MDA: Malondialdehyde; HSD: Hydroxy Steroid Dehydrogenase; LDL: Low Density Lipoproteins; VLDL: Very Low Density Lipoproteins; HDL: High Density Lipoproteins; DNA: Deoxyribonucleic acid; EC: Emulsifiable Concentrate; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals.

INTRODUCTION

The interaction of xenobiotics with the biological system is a multifaceted phenomenon, which comprises interplay between the environment, the host and the chemical substance. Chemical contagion as a result of pesticide introduction has been assumed as one of the factors for the deterioration of natural fauna. Indiscriminate application of pesticides is usually accompanied with serious problems of pollution and health hazards.[1]

http://dx.doi.org/10.17140/TFMOJ-1-107

Pyrethroids, derivatives of natural pyrethrins have been well known for their high effectiveness against insects and can be easily biodegraded than other types of pesticides.[2] In spite of claims of low mammalian toxicity of pyrethroid, several investigations reported the toxicological evidence of pyrethroid among various species of animals.[3] Pyrethroids are easily absorbed through gastrointestinal and respiratory tract due to their lipophilic nature and also make them easier to be stored in the lipid rich internal tissues like body fat, skin, liver, kidney, central and peripheral nervous systems. Hemato-biochemical studies are important for the analysis of the functional status of animals to suspected toxic agents. It may act as a strong evidence against toxicity of contaminated pyrethroid insecticides. Recent reports have clarified that exposure to pyrethroid leads to a significant modifications in hematological findings.[4] On the other hand the liver is the first organ encountered when toxicants enter into the body. The liver was found to accumulate a huge pyrethroid residues as it is the primary site for pyrethroid metabolism. A large number of man-made chemicals such as pesticides stated to produce liver damage.[5] Several investigations also reported that pesticides adversely affect the testicular functions in experimental animals[6-9] as well as they are potent endocrine disrupters.[10,11] Pesticides are responsible for oxidative stress that causes free radicals generation, leading to deoxyribonucleic acid (DNA) fragmentation.[12,13]

Lambda-cyhalothrin (LCT), a type II pyrethroid pesticide, is used worldwide to control pests in a variety of agricultural crops. LCT is chemically alpha-cyano-3phenoxybenzy3-(2-chloro-3,3,3 trifluoropropenyl)-2,2,dimethylcyclo- propane carboxylate. It revealed that lambda-cyhalothrin is moderately toxic for mammals[12,13] and highly toxic for fish, aquatic invertebrates and bees. LCT at low concentrations can cause death in these species.[14,15] The degree of concentration and nature of solvent are important for the toxicity of lambda cyhalothrin.[16]

Taurine (2-aminoethane sulphonic acid), a free intracellular sulfonated beta amino acid, is present in many animal tissues especially muscle, brain, liver, heart, etc.[17] From the metabolism of methionine and cysteine, taurine is derived and is much more concentrated in pro-inflammatory cells such as polymorphonuclear phagocytes and in the retina.[18] Taurine takes part in bile acid conjugation, detoxification, osmoregulation and modulation of cellular calcium level.[19-21] Taurine acts as major antioxidant in most living organism which attributes its ability to stabilize biomembranes[17] and to scavenge reactive oxygen species.[22]

For any pesticide, the exposure concentration is important in the alteration of its toxicity. So in our present study, LCT were administered at different concentrations to find out the exact toxic dose levels of LCT which can produce hematological, hepatic and gonadal toxicity in male and female Wistar rat. At the same time, investigation was also carried out to evaluate the protective role of taurine against LCT induced alterations in lipid metabolism of female rat.

MATERIALS AND METHODS

Chemicals and Reagents

Lambda cyhalothrin 5% emulsifiable concentrate (EC) was procured from RPC Agro Industries, Kolkata. Taurine was purchased Sigma Aldrich Inc., USA. All other chemicals used were of analytical grade and were purchased from Merck India Ltd, Himedia India Ltd, etc.

Animals and Care

For the present study mature Wistar male and female albino rats (weighing 130-150 g) were taken and the animals were housed in polypropylene cages at an ambient temperature of 25 °C ±2 °C with 12 hrs light-dark cycle. The rats were acclimatized for one week prior to different treatments. The standard laboratory feed and water were supplied throughout the period of experimentation. The present study was approved by the Institutional Animal Ethical Committee (IAEC), registered under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India and performed in accordance to the relevant laws and guidelines of the CPCSEA.

Experimental Design

A commercial formulation of lambda cyhalothrin 5% EC 'Karate' was used for the study. Dilution of lambda cyhalothrin was done in distilled water to acquire the test concentrations. The test concentrations of lambda cyhalothrin were measured from the percentage of the active ingredient present in above mentioned commercial formulation of lambda cyhalothrin.

Healthy mature Wistar rats (n=6, for control and each dose group) of either sex selected by random sampling, were used for the study. The rats were kept fasting for overnight providing only sufficient water, after which LCT were administered orally for 14 consecutive days at the dose level of 6.89 mg/kg body wt. (i.e. 1/11th LD_{50} dose) for male and 5.15 mg/kg body wt. (i.e. 1/11th LD_{50} dose) for female. Sharma et al[23] reported that oral LD_{50} dose of LCT for mature male and female rats were 75.85 and 56.69 mg/kg body wt. respectively. The procedure was repeated for the dose levels of 7.58(1/10th LD_{50}), 8.42(1/9th LD_{50}), 10.83(1/7th LD_{50}), 15.17(1/5th LD_{50}), 18.96(1/4th LD_{50}), 25.28(1/3rd LD_{50}) mg/kg body wt.[24] for male rats. For female rats, lambda-cyhalothrin were administered orally at the dose levels of 5.66(1/10th LD_{50}), 6.29(1/9th LD_{50}), 8.09(1/7th LD_{50}), 11.33(1/5th LD_{50}), 14.17(1/4th LD_{50}), 18.89(1/3rd LD_{50}) mg/kg body weight. Dose solutions were freshly prepared immediately before usage.

In another separate set of experiment, female rats were divided into the following six groups each containing six to as-

sess LCT induced toxicity to lipid metabolism and to find out the protective effect of taurine against this toxicity. Taurine was administered at the dose level of 50 mg/kg body wt., which was effectively used to protect the toxicity induced by various xenobiotics.[25,26]

1. Group A: Control (no treatment).
2. Group B: Taurine control (50 mg/kg body wt.).
3. Group C: LCT low dose (1/9 of LD_{50} value i.e., 6.3 mg/ kg body wt.).
4. Group D: Taurine (50 mg/kg body wt.)+lambda-cyhalothrin low dose (6.3 mg/kg body wt.).
5. Group E: Lambda-cyhalothrin high dose (1/5 of LD_{50} value i.e., 11.33 mg/kg body wt.).
6. Group F: Taurine (50 mg/kg body wt.)+lambda-cyhalothrin high dose (11.33 mg/kg body wt.).

At the end of the doses, the animals were fasted overnight. On 15th day, the rats were anesthetized with pentobarbital sodium (35 mg/kg) and sacrificed by cervical dislocation. Samples were collected and stored at -80 ºC until analysis.

Study on Body Weight

Body weight of individual overnight fasting male and female animals was taken regularly before the administration of LCT. After 24 hrs of the treatment of the last dose, all animals were weighed and then sacrificed.

Measurement of Haematological Parameters in Male Rat

Erythrocyte count: Erythrocyte count was done by the dilution of blood with red blood cell (RBC) dilution fluid (1:200) and the total erythrocyte counts[27] were expressed as $\times 10^6/mm^3$.

Estimation of haemoglobin percentage: The haemoglobin percentage was measured by cyanmethemoglobin method.[28] Using Drabkin's diluent as a blank, the optical density was measured at 540 nm.

Total leukocyte count: Blood was diluted (1:20) with white blood cell (WBC) dilution fluid and total leukocyte were counted using Neubaur haemocytometer.[27]

Study of Hepatic Biomarkers in Male Rat

Assay of serum glutamate-oxaloacetate transaminase (SGOT) and serum glutamate-pyruvate transaminase (SGPT): For SGOT sample, 1 ml of buffer substrate (2.66 gm aspartic acid, 60 mg α-ketoglutaric acid and, 20.5 ml of 1(N) NaOH and 100 ml of volume was made by 0.1 M phosphate buffer, pH-7.4) and for SGPT sample, 1 ml of buffer substrate (1.78 gm DL-alanine, 30 mg α-ketoglutaric acid, 20 ml of 0.1M phosphate buffer and 1.25 ml of 0.4(N) NaOH) were taken and waited for 5 min at 37 ºC. Then 0.2 ml of serum sample was mixed and incubated at 37 ºC for 60 min. To prepare standard, 0.2 ml of working standard (200 µM/100 ml) was taken in a test tube and 0.8 ml of buffer substrate was added. For blank, 1.0 ml of buffer substrate was taken. In each of sample, standard and blank test tubes, 1 ml of 2,4-dinitrophenylhydrazine hydrochloride (DNPH) solution were added and waited for another 20 min. Then 10 ml of 0.4(N) NaOH was mixed and waited for 10 minutes. Readings were taken at 520 nm in spectrophotometer[29] (UV-245 Shimadzu, Japan).

Assay of hepatic malondialdehyde (MDA): Malondialdehyde (MDA) was determined by the mixing of 1 ml of sample with 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of acetate buffer (20%, pH-3.5) and 1.5 ml of aqueous solution of thiobarbituric acid (0.8%) and the mixtures were boiled for 60 min at 95 ºC. After heating when the red pigment was produced, that was extracted with 5 ml of n-butanol-pyridine (15:1) and centrifuged at 5000 rpm for 10 min at room temperature. The optical density of supernatants was measured at 535 nm.[30]

Estimation of hepatic reduced glutathione (GSH) content: 100 µl of sulfosalicylic acid was mixed with 200 µl of sample. Then the mixture was allowed for centrifugation at 3000 rpm for 10 min. With the supernatant, 1.8 ml of DTNB was included and shaken well.[31] Final reading was noted at 412 nm.

Study of Male Reproductive Parameters

Measurement of seminal fructose concentration: In a centrifuge tube, 1 ml of diluted seminal plasma (five times dilution was done by mixing 0.1 ml of seminal plasma with 4.9 ml of distilled water) was added with 0.3 ml of 1.8 gm% $ZnSO_4$ and 2 ml of 0.1 M NaOH. After 15 min, the mixture was centrifuged at 2000 g to obtain the supernatant. Then seminal fructose concentration was measured by taking 0.5 ml of supernatant as sample, 0.5 ml of 0.14 mM and 0.28 mM fructose solutions as two standards and 0.5 ml of distilled water as blank. Then, 0.5 ml of indole reagent and 5 ml of concentrated HCl were added to each test tube. The test tubes were then incubated at 50 ºC for 20 min and were cooled in ice water and then in room temperature.[32] The reading was taken at 470 nm in spectrophotometer (UV-245 Shimadzu, Japan).

Estimation of testicular cholesterol: Testicular tissue was homogenized with 0.5% $FeCl_3$ solution at a conc. of 20 mg/ml. Supernatant was collected after centrifugation of the homogenized tissue at 2000 rpm for 10 min. Then 0.1 ml of supernatant was added with 6 ml of glacial acetic acid to prepare sample. Simultaneously 5.9 ml of glacial acetic acid was added with 0.1 ml of working standard and 0.1 ml of distilled water to prepare standard. Blank was prepared by mixing 6 ml of glacial acetic acid and 0.1 ml of distilled water. Then 4 ml of colour reagents were added to each, mixed vigorously and stand for 20 minutes for spectrophotometric reading at 570 nm against blank.[33]

Assay of testicular malondialdehyde (MDA) and reduced glutathione (GSH) content: Testicular malondialdehyde (MDA) and

reduced glutathione (GSH) content were measured by the respective above mentioned methods.[30,31]

Study of Female Reproductive Parameters

Estimation of ovarian Δ⁵, 3β-hydroxysteroid dehydrogenase (Δ⁵, 3β-HSD) activity: Homogenizing media was prepared by 20 ml of glycerol, 0.01 M EDTA in 0.05 M phosphate buffer in 100 ml with redistilled water. Tissue homogenate (20 mg/ml homogenizing media) was centrifuged at 10,000 rpm for 30 min at 4 ºC in a cold centrifuge. One ml of supernatant was mixed with 1 ml of sodium pyrophosphate buffer and 40 µl of dehydroepiandrosterone (DHEA). After addition of 0.1 ml of NAD, the activity of Δ^5,3β-HSD was measured at 340 nm against a blank (without NAD).[34]

Assay of ovarian cholesterol, malondialdehyde (MDA) and reduced glutathione (GSH) content: Ovarian cholesterol, malondialdehyde (MDA) and reduced glutathione (GSH) content were measured by the respective above mentioned methods.[33,30,31]

Study of the protective effect of taurine on serum bilirubin and lipid profile in female rat in selected dose levels

Serum bilirubin was determined by the method of Jendrassik and Grof using commercial diagnostic reagent kit.[35] Serum cholesterol (CHO), triglyceride (TG) and high density lipoprotein cholesterol (HDL) were estimated by the commercial diagnostic reagent kit.[36] VLDL was calculated using the formula (TG/5).[37] LDL concentration (mg/dL) was estimated indirectly from the concentrations of CHO, TG and HDL using the equation[37] LDL=CHO-(VLDL+HDL).

Statistical Analysis

The data was expressed as Mean±SEM. The differences between the means of each group were tested using a one way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA, USA). $p<0.05$ was considered to indicate a statistically significant difference.

RESULTS

General Observations

After each treatment of lambda cyhalothrin, the animals were kept under observation at least once in 30 min interval. Special attention was given up to 4 hrs from the time of treatment. Rats those were exposed to different doses of lambda cyhalothrin over a period of 14 days produce ataxia, agitation, rolling and also tremors which were considered as the signs of toxicity up to eleventh day of treatment after which the symptoms were reduced.

Changes in Body Weight, Food and Water Intake

Significant changes in final body weight were found at the dose level of 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50}) in male rats and at the dose level of 5.15 mg/kg body wt. ($1/11^{th}$ LD_{50}) in female rat (Figure 1). No differences in food and water consumption were seen in last consecutive four days of treatment in the experimental schedule but there was little non-significant alterations observed in food consumption at the exposure dose levels of $1/4^{th}$ and $1/3^{rd}$ LD_{50}.

Effect on Haematological Parameters in Male Rat

To find out the dose dependent effect of LCT, some clinically significant biochemical parameters from each system were studied. From the study it was seen that total erythrocyte count was decreased significantly ($p<0.05$) from the dose level of 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50}) to 25.28 mg/kg body wt. ($1/3^{rd}$ LD_{50}) in male rat (Table 1). No significant changes were found below 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50} dose). Maximum toxic effect

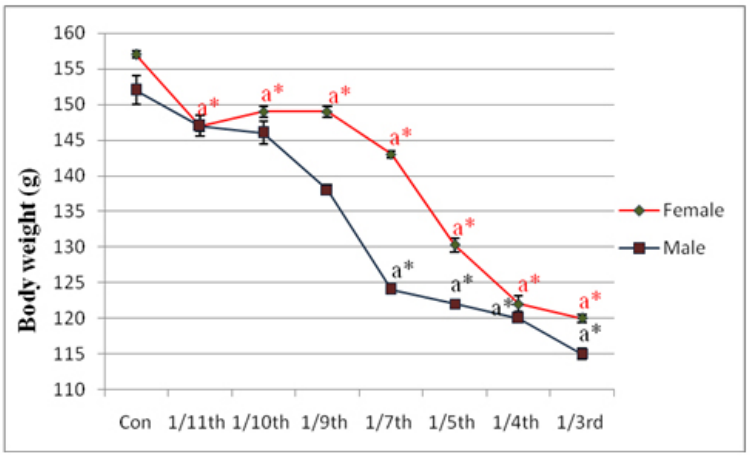

Figure 1: Effect of lambda cyhalothrin on body weight of male and female rat. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA. Superscript a, control group *versus* all other groups (*indicates $p<0.001$). (For male and female the LD_{50} values are different which are mentioned in materials and methods section).

Group	Erythrocyte count ($\times 10^6$ / mm^3)	Hb percentage (gm/dL)	Leukocyte count ($\times 10^6$/µl)
Control	7±0.3	14±0.5	6±0.17
1/11th LD_{50} dose (6.89 mg /kg body wt.)	7.2±0.2	14±1.1	6±0.11
1/10th LD_{50} dose (7.58 mg /kg body wt.)	7±0.1	13.3±1.4	6.03±0.14
1/9th LD_{50} dose (8.42 mg /kg body wt.)	7±0.06	13.6±0.6	6.03±0.15
1/7th LD_{50} dose (10.83 mg /kg body wt.)	6±0.2a*	10±0.6a**	8±0.11a***
1/5th LD_{50} dose (15.17 mg /kg body wt.)	5.9±0.1a*	8±0.6a**	9.2±0.12a***
1/4th LD_{50} dose (18.96 mg /kg body wt.)	5.9±0.2a*	8±0.6a**	9.1±0.17a***
1/3rd LD_{50} dose (25.28 mg /kg body wt.)	5.9±0.1a*	8±1.1a**	9.2±0.12a***

Table 1: The effect of lambda cyhalothrin on haematological parameters in male rat. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA. Superscript a, Control group *versus* all other groups (*indicates p<0.05,**indicates p<0.01, ***indicates p<0.001).

of LCT was exhibited at the 15.17 mg/kg body wt. (1/5th LD_{50} dose) without any mortality. Interestingly, the rate of mortality was increased from the dose level of 18.96 mg/kg body wt. (1/4th LD_{50}). Similar results were found in case of haemoglobin percentage (p<0.01). Table 1 displays the significant (p<0.001) increase in leukocyte count in lambda cyhalothrin treated male rats from the dose level of 1/7th LD_{50} to 1/3rd LD_{50}.

Effect on Hepatic Biomarkers in Male Rat

Activities of serum glutamate-oxaloacetate transaminase (SGOT) and serum glutamate-pyruvate transaminase (SGPT): The effects of LCT on SGOT, SGPT are shown in Figures 2A, and 2B respectively. The activity of these two important hepatic transaminase enzymes were increased significantly (p<0.001) with increase in the concentration of LCT as compared to the control rats. No significant alterations were noted below 10.83 mg/kg body wt. (1/7th LD_{50} dose) in male rat.

Hepatic lipid peroxidation and glutathione content: Hepatic malon-di-aldehyde (MDA) and reduced glutathione content in the control and experimental groups of male rats are shown in Figures 2C and 2D. MDA was found to be significantly increased (p<0.01) whereas reduced glutathione content was decreased significantly (p<0.001) in a dose-dependent manner from the dose level of 10.83 mg/kg body wt. (1/7th LD_{50} dose) onwards.

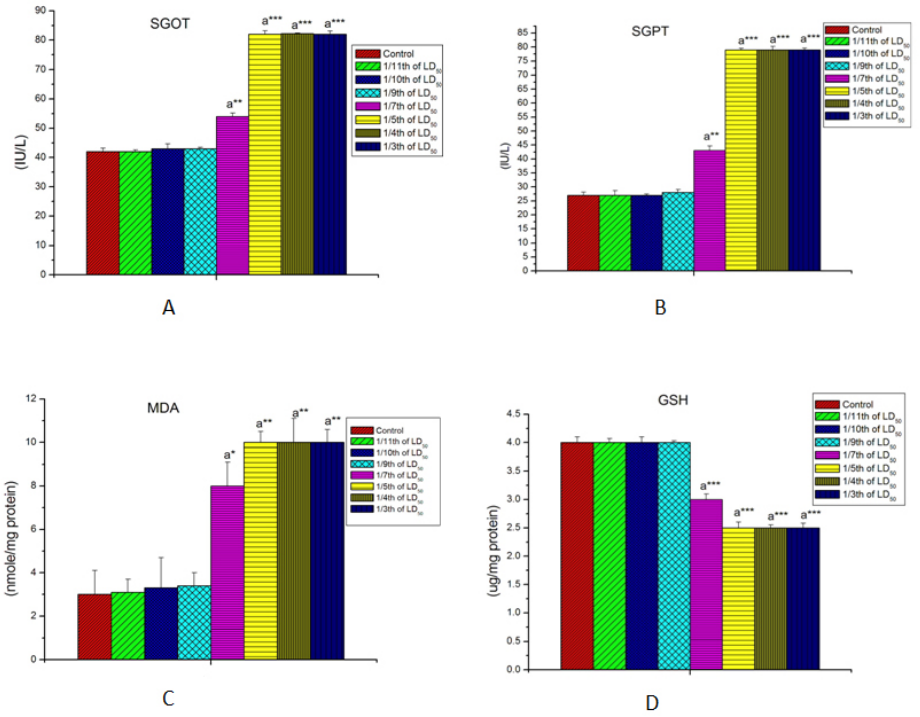

Figure 2: Effect of lambda cyhalothrin on some hepatic biomarkers in male rat.
A= Effect on SGOT. B= Effect on SGPT. C=Effect on liver MDA. D=Effect on liver GSH. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA. Superscript a, Control group *versus* all other groups (*indicates p<0.05, **indicates p<0.01, ***indicates p<0.001).

http://dx.doi.org/10.17140/TFMOJ-1-107

Effect on Male Reproductive System

Effect of LCT on seminal fructose concentration and testicular cholesterol: As shown in Table 2, the LCT induced reproductive toxicity by reducing seminal fructose concentration ($p<0.001$) and elevating testicular cholesterol level ($p<0.05$).

Impact of LCT on testicular oxidative stress and antioxidant status: Table 2, also shows the effect of LCT on MDA and GSH. Testicular MDA was found to be increased significantly ($p<0.01$) in response to LCT treatment whereas a significant decrease ($p<0.001$) in testicular GSH level was also noted. Significant changes were detected from the dose level of 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50} dose) onwards.

Effect on Female Reproductive Function

Ovarian steroidogenic key enzyme activities and cholesterol content: LCT induced ovarian toxicity was exhibited by significant diminution in ovarian steroidogenic enzyme activities and elevation in ovarian cholesterol content at the dose level of 6.29 mg/kg body wt. ($1/9^{th}$ LD_{50} dose) and were continued towards increased dose levels of LCT (Figures 3A and 3B).

Effect of LCT in malondialdehyde (MDA) and reduced glutathione (GSH) level: LCT intoxicated rats shows a marked dose-dependent increase ($p<0.001$) in the lipid peroxidation, specified in term of MDA (Figure 1). Decline in GSH levels in LCT treated rat may also an indication of oxidative stress as GSH is used

Group	Seminal fructose concentration (nmole of fructose/lit of seminal plasma)	Testicular Cholesterol (mg/gm)	Testicular MDA (nmole/mg protein)	Testicular GSH (µg/mg protein)
Control	20±0.3	1.4±0.1	2.5±0.2	3.5±0.03
$1/11^{th}LD_{50}$	20±0.6	1.4±0.2	2.6±0.2	3.4±0.12
$1/10^{th}$ LD_{50}	20±0.5	1.4±0.05	2.6±0.1	3.4±0.03
$1/9^{th}$ LD_{50}	19.5±0.2	1.4±0.1	2.6±0.3	3.4±0.09
$1/7^{th}$ LD_{50}	18±0.1a**	1.9±0.1a*	4±0.2a*	2.9±0.12a**
$1/5^{th}$ LD_{50}	15±0.3a***	2.4±0.3a*	5±0.3a**	2.36±0.22a***
$1/4^{th}$ LD_{50}	15±0.2a***	2.4±0.1a*	5±0.2a**	2.36±0.25a***
$1/3^{rd}$ D_{50}	15±0.4a***	2.4±0.05a*	5±0.5a**	2.36±0.24a***

Table 2: Shows the effect of lambda cyhalothrin on some male reproductive parameters.
Results are expressed as Mean±SEM. Analysis is done by one way ANOVA. Superscript a, control group *versus* all other groups (*indicates $p<0.05$, **indicates $p<0.01$, ***indicates $p<0.001$).

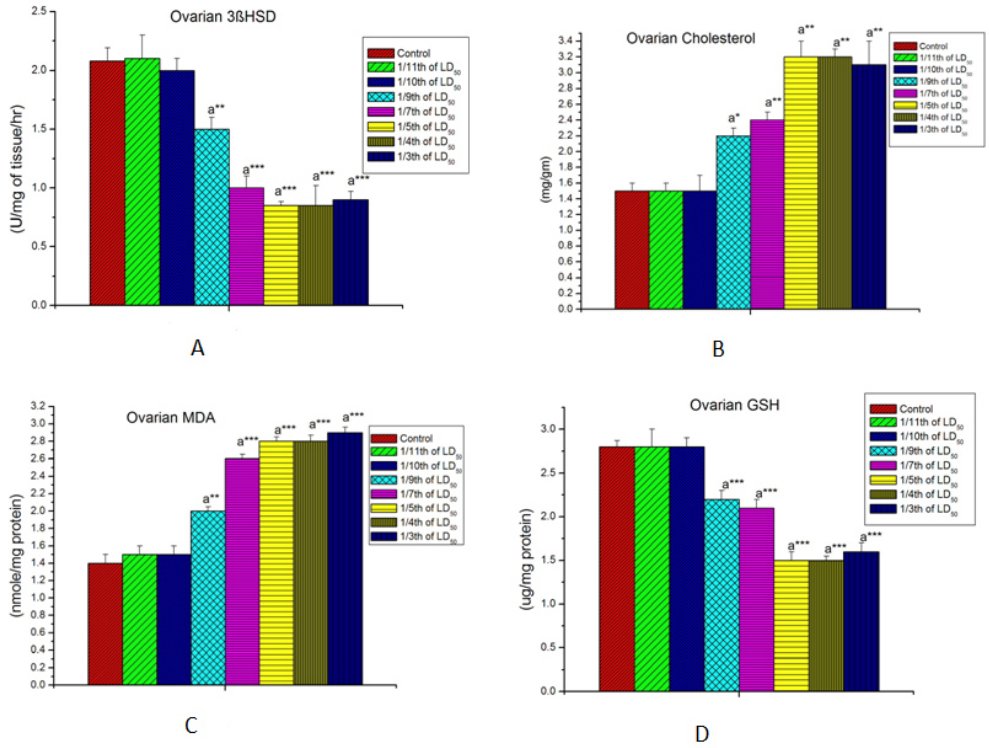

Figure 3: Effect of lambda cyhalothrin on female reproductive biomarkers.
A=Effect on ovarian 3β HSD. B= Effect on ovarian cholesterol. C= Effect on ovarian MDA. D=Effect on ovarian GSH. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA. Superscript a, control group *versus* all other groups (*indicates $p<0.05$, **indicates $p<0.01$, ***indicates $p<0.001$).

http://dx.doi.org/10.17140/TFMOJ-1-107

Parameters	Group-A	Group-B	Group-C	Group-D	Group-E	Group-F
Serum total Cholesterol (mg/dL)	120±0.7	112±1.5a***	138±0.8a***	127±1.3a***b***	149±1.5a***	137±1.2a***c***
Serum triglyceride (TG) (mg/dL)	98±1.0	92±1.0a**	110±1.0 a***	98±0.5b*	121±1.0a***	104±1.5a*c***
High density lipoprotein (HDL) (mg/dL)	45±0.7	51±1.0a***	38±0.2 a***	47±1.2b***	29±1.0a***	37±0.5 a***c***
Very low density lipoprotein (VLDL) (mg/dL)	19.6±0.2	18.4±0.2	21.6±0.1a***	20±0.3b*	24.2±0.2a***	20.8±0.4a**c***
Low density lipoproteins (LDL) (mg/dL)	55.3±1.8	42.6±2.7a**	78.4±0.8a***	60±0.2a*b***	95.8±0.7a***	79.2±1.4a**c***
Serum bilirubin (mg/dL)	0.3±0.05	0.4±0.06	1±0.07a***	0.5±0.02a**b***	1.7±0.1a***	1.2±0.1a***c**

Table 3: Effect of LCT and taurine on some biomarkers of lipid metabolism in female rats.
Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a, Group A (control group) *versus* all other groups, superscript b, Group C *versus* Group D and superscript c, Group E *versus* Group F (*indicates $p<0.05$, **indicates $p<0.01$, ***indicates $p<0.001$).

for the detoxification of reactive toxic substances resulted from LCT exposure at the dose level of 6.29 mg/kg body wt. ($1/9^{th}$ LD_{50} dose) (Figures 3C and 3D).

Selection of Experimental Dose of Lambda Cyhalothrin

From these above findings for male rats, 10.83($1/7^{th}$ LD_{50} dose) and 15.17($1/5^{th}$ LD_{50} dose) mg/kg body wt. and for female rats, 6.29 mg/kg body wt. ($1/9^{th}$ LD_{50} dose) and 11.33($1/5^{th}$ LD_{50} dose) mg/kg body wt. were selected as effective doses for our further studies. At the $1/7^{th}$ LD_{50} dose, significant alterations were seen in different systemic biochemical parameters of male rat. In female rat first significant toxic response was found at the dose level of 6.29 mg/kg body wt. ($1/9^{th}$ LD_{50} dose). So in case of male and female rat, $1/7^{th}$ LD_{50} dose and $1/9^{th}$ LD_{50} dose were considered as effective low dose. Better toxic response was found at $1/5^{th}$ LD_{50} dose for both male and female rat after which animal mortality was increased. So this dose was considered as effective high dose for future studies.

Effect on Lipid Profiles in Female Rat

The data from Table 3 reflected marked alterations in the levels of serum total cholesterol, lipoproteins and bilirubin. The total cholesterol concentration was found to be significantly increased ($p<0.001$) in the LCT treated group compared to control. Serum triglyceride, VLDL-cholesterol and LDL-cholesterol in the LCT treated rats were also found to be elevated when compared to normal rats where as HDL was markedly ($p<0.001$) reduced in LCT treated female rats. Our results also showed a significant increase of serum bilirubin level in LCT intoxicated rats. Pre treatment with taurine has caused the significant alleviation in serum total cholesterol, LDL cholesterol and HDL cholesterol in LCT treated rats. Pretreatment with taurine also improved the altered serum triglyceride and bilirubin level (Table 3).

DISCUSSION

The present study was carried out to assess the toxic effects of LCT on the different system of male, female Wistar rats, and also to search out the alleviating role of taurine under this toxic condition. In toxicological studies, body weight is a basic benchmark for evaluation of organ toxicity. In the present study, oral administration of LCT brought about a significant reduction in body weight of both male female rats.

A significant change in erythrocyte counts, haemoglobin percentage and leukocyte were detected in lambda cyhalothrin exposed rats and these pointed out the physiological disruption in the rat haemopoietic system. Haemolysis of blood cells[38] may cause the decrease in erythrocyte counts in LCT intoxicated rats which in turn tends to be responsible for the reduction in haemoglobin percentage. In the present study, decreased biosynthesis of haem in bone marrow may also lead to the significant reduction in haemoglobin percentage. Increased leukocyte in lambda cyhalothrin treated group may arise due to the immediate activation of the immune system of the body[39] against lambda cyhalothrin.

SGOT, SGPT are two important hepatic enzyme biomarkers of hepatotoxicity. In the present study a significant increase in SGOT, SGPT level after LCT treatment at different concentration point out towards active utilization of amino acids in energy-yielding metabolic processes like gluconeogenesis. Pyrethroids induced oxidative stress by the elevation of lipid peroxidation products.[40,41] Elevated MDA level in LCT intoxicated rat liver was in an agreement with the above statement. This type of result also suggested that LCT produces hepatic injury and pathogenesis through the generation of free radicals and by the alteration of antioxidant system. Decrease in cellular GSH concentrations may be through low production or non-enzymatic oxidation of GSH to glutathione disulfide (GSSG) due to oxidative stress in LCT treated rat liver at different dose levels.

Here the results also reflect the male reproductive dysfunction after LCT exposure at different dose levels. The reduction in fructose content in seminal fluid collected from LCT intoxicated rats were drawn attention towards the secretory ability of seminal vesicles and the nutritive potential for the semen. Previous studies[42-44] reported that ROS were involved in the toxicity of various pesticides. ROS inhibits steroidogenesis by disrupting cholesterol transport to mitochondria.[45] LCT elevated testicular cholesterol at the dose level of 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50} dose) and also on above dose levels. An increase in MDA, the mostly used biomarker of lipid peroxidation, indicates serious

cellular damage, inhibition of several enzymes and cellular functions.[46] GSH, one of the most potent biological molecules, play a key role in the detoxification of the reactive toxic metabolites. A considerable decline in GSH levels in liver LCT treated rat may be due to its utilization to challenge the common oxidative stress.

According to the results, increase in ovarian cholesterol and significant decrease in ovarian steroidogenic enzyme activity pointed out towards the LCT induced ovarian toxicity through diminishing steroidogenesis. The maintenance of high redox potential is a prerequisite for assuring the reproductive system functions in a healthy organism.[47,48] Increased ovarian MDA and decreased ovarian GSH level in LCT intoxicated rats compared to control may be an indication of oxidative stress due to LCT exposure at the dose level of 6.29 mg/kg body wt. ($1/9^{th}$ LD_{50} dose) and also on above dose levels.

The elevation in serum total cholesterol level in female rats was observed in the LCT treated groups indicating the hypercholesterolemic action of LCT due to blockage of liver bile ducts causing reduction or cessation of its secretion to the duodenum.[49] Pretreatment of taurine by its hypocholesterolemic action caused reduction in serum total cholesterol. Elevated serum triglyceride, VLDL-cholesterol and LDL-cholesterol with reduction of HDL in LCT treated rats may suggest that LCT has strong toxic potential to alter normal body physiology. These may be attributed to the increased fat catabolism in response to LCT.[50] Our results also showed a significant increase of serum bilirubin level in LCT intoxicated rats. The increased level of total bilirubin in treated female rats is an indicator of hyper-bilirubinemia, a useful indicator of hepatocellular dysfunction.[51,52] Pretreatment with taurine improved the altered lipid profile and bilirubin level in female rats. Taurine play a role in conjugation reaction with bile acids in the liver. Consequently, bile acid synthesis is increased[53] by simultaneous rise in the messenger RNA (mRNA) expression and activity of cholesterol 7α-hydroxylase, a rate-limiting enzyme in hepatic bile acid synthesis.[54] The primary mechanisms responsible for the hypocholesterolemic action of taurine may be due to the increased conversion of cholesterol into bile acids through the stimulation of cholesterol 7α-hydroxylase enzyme activity.

CONCLUSION

The present study showed that LCT exposure produced hematological, hepatic and gonadal toxicity at 10.83 mg/kg body wt. ($1/7^{th}$ LD_{50} dose) in male and at 6.29 mg/kg body wt. ($1/9^{th}$ LD_{50} dose) in female rat and also on above dose levels. Taurine has the potential to mitigate LCT induced altered lipid metabolic biomarkers in female rat.

ACKNOWLEDGMENT

The authors are thankful to the authority of Vidyasagar University, Midnapore, India for providing all the facilities to execute this study.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Open Access
CODEN: IJBNHY
ISSN: 2278-778X
Original Research Article
International Journal of Bioassays

Taurine improves lambda cyhalothrin induced biochemical alterations in Wistar rat liver

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Received: September 23, 2016; **Received:** September 29, 2016; **Accepted**: October 11, 2016
Available online: 1st November 2016

Abstract: Taurine is a major intracellular free β-amino acid, which can protect the body against toxicity. Lambda-cyhalothrin, a third-generation type II pyrethroid. is used predominantly in agriculture production and animal husbandry. The aim of the present study was to investigate lambda cyhalothrin-induced biochemical changes in rat liver and to search out the possible role of taurine for the attenuation of hepatotoxic biomarkers. Male rats were randomly divided into six groups and lambda cyhalothrin was orally administered at two dose levels (10.83mg/body wt., 15.17mg/body wt.) alone and in combination with taurine pretreatment (50mg/kg body wt) for 14 consecutive days. A significant change in blood glucose level with a marked decline in glycogen content were indicated the hepatic dysfunction in lambda cyhalothrin treated rats. This was also confirmed by the altered activities of serum hepatic biomarker enzymes and lipid profiles in LCT intoxicated rats. Pre-treatment of taurine mitigated the abnormalities. These findings pointed out the toxic effect of lambda cyhalothrin in rat liver and also revealed the protective action of taurine against this pyrethroid.

Key words: Taurine; Lambda cyhalothrin; Blood glucose; Serum hepatic biomarker enzymes.

Introduction

Taurine (2-aminoethane sulphonic acid) is a major free intracellular non- protein sulphur amino acid [1]found in milimolar concentrations in many animal tissues[1]. Taurine is present in the liver at high concentrations [2]. Several studies have reported that taurine has a defensive effect against chemically-induced hepatotoxicity[3-6]. Moreover, by trim down oxidative stress, enhancing mitochondrial activity and modulating cytoplasmic and mitochondrial calcium homeostasis taurine has been found to prevent toxin-mediated hepatic injuries[7]. Furthermore, taurine has been accounted to function as an antioxidant in biological systems, scavenge ROS, attenuate lipid peroxidation and as a consequence, stabilizes biological membranes[8,9].

Environmental pollution from pesticides is a vital topic that attracts broad spread public concern. In spite of harmful effects of pesticides on environment and other living organisms, currently the most efficient and perhaps the only useful way to fight against pests is chemical pesticides [10,11]. India is one of the principal users of agricultural pesticides to increase crop yield and in vector control program. The use of pesticides above the safe level may create serious threat to nontarget organisms in the environment.

A significant amount of pesticides and their metabolites become mixed in ponds; rivers and run-off from pastures. They are potentially toxic to animals[12]. Humans, as agricultural workers, or via food consumption are potentially exposed to these pesticides either directly or indirectly. In addition to careless contact at the time of spraying, entering sprayed farms, consuming foods infected by pesticides, drinking pesticide contaminating water, can cause diseases and abnormalities. Synthetic pesticides have always been under concern because of their effects on human-used agricultural products and their threat and risk to human beings' health[13]. Synthetic pyrethroids are the latest major class of broad-spectrum organic insecticides used in agricultural, veterinary and household applications. Due to their low mammalian toxicity, rapid breakdown in soil and remaining steady in sunlight, synthetic pyrethroids turn into a friendly group of insecticides but their volume of production point out that in future the misapplication and accidental contact may be very high.

Lambda-cyhalothrin (LCT), a synthetic type II pyrethroid is broadly used in agriculture, public health, house and in gardening for pest management[14]. Besides, lambda-cyhalothrin acts on farm animals to prevent and manage

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ectoparasites and in public health agendas. Residues of lambda-cyhalothrin have been accounted in vegetables and fruits, milk and blood of dairy cows and also in cattle meat [15,16]. The objectives of the present study are to highlight the effects of taurine on biochemical parameters of liver in male Wistar rats co-exposed to lambda cyhalothrin.

Materials and Methods

Chemicals and reagents

Lambda cyhalothrin 5% emulsifiable concentrate (EC) was procured from RPC Agro Industries, Kolkata. Taurine was purchased Sigma Aldrich Inc. USA. All other chemicals used were of analytical grade and were purchased from Merck India Ltd, Himedia India Ltd., etc.

Animal mode

Wistar albino male rats (weighing 130-150 g) were maintained on the standard laboratory feed and water throughout the period of experimentation i.e. 14 consecutive days. Experimental protocol was approved by the Institutional Animal Ethical Committee, registered under CPCSEA. All animal treatment and surgical procedures were carried out according to the relevant laws and guidelines of the CPCSEA.

Treatment protocol

A commercial formulation of lambda cyhalothrin 5% emulsifiable concentrate (EC) was used for our experiments. The animals were divided into six groups of six rats each. Group I: Distilled water control (no treatment). Group II: Taurine control (50mg/kg body wt). Group III: Lambda cyhalothrin low dose (10.83mg/body wt. i.e. 1/7th of LD_{50} value). Group IV: Taurine (50mg/kg body wt) +lambda cyhalothrin low dose (10.83mg/body wt.). Group V: Lambda cyhalothrin high dose (15.17mg/body wt. i.e. 1/5 of LD_{50} value). Group VI: Taurine (50mg/kg body wt) + lambda cyhalothrin high dose (15.17mg/body wt.)

We followed the dose 75.85-mg/kg body weight as the oral LD_{50} of lambda cyhalothrin in male rats[17] and it was also verified in our laboratory. Dose levels for the present treatments were finalized according to our preliminary investigations. In our experiments taurine was applied at the dose level of 50 mg/kg body wt. This dose was effectively used to ameliorate the toxicity induced by various xenobiotics [18,19]. Taurine was pre-treated before 1 hr of LCT exposure to build antioxidant pool or advanced protection in animal body before LCT exposure. Lambda-cyhalothrin, taurine and distilled water were administered once daily by oral gavage for 14 consecutive days. Animal's weight was taken daily and the dose was adjusted accordingly.

Determination of blood glucose level

Blood glucose was estimated by Nelson method adapted from Somogyi's method (Nelson, 1944)[20]. The blood was deproteinized by adding 9.5 ml of 5% zinc sulphate solution in 1 ml of blood sample. Then 9.5 ml of 4.5% barium hydroxide solution was included and the mixture was allowed to stand for 15 min for complete precipitation and was filtered. For the preparation of sample, standard and blank, 0.5 ml of blood filtrate, 0.5 ml of 0.025mg/ml of glucose and 0.5 ml of distilled water were taken respectively in separate test tubes and 1ml of alkaline copper reagent were added in each test tubes and were boiled in a boiling water bath for 20min. Then those were allowed to cool in room temperature and 1 ml of arseno-molybdate reagent was mixed to each test tubes and volume was made upto 10 ml. Readings were taken at 540 nm in spectrophotometer.

Estimation of liver glycogen

Tissue homogenate (100 mg/ml in hot 80% ethanol) was centrifuged at 8000 × g for 20 min. The residue was collected and dried in a hot water bath. To the collected residue, 5ml of distilled water and 6ml of 52% perchloric acid were mixed. The extraction process was done at 0^0C for 20min. Then the collected material was centrifuged at 8000×g for 15 min. 0.2 ml of supernatant was transferred in a graduated test tube to make 1 ml volume by distilled water. Standards in graded concentrations were prepared by using working standard solution and all these volumes were made up to 1ml by distilled water. Then 4 ml of anthrone reagent was added to all test tubes and were heated in boiling water bath for 8 minutes. After cooling in a room temperature, the reading was noted at 630 nm. The amount of sample glycogen was measured from standard curve, prepared with standard glucose solution. Glycogen present in sample was expressed in µg of glucose/ mg of tissue [21].

Activity of serum Glutamate-oxaloacetate transaminase (SGOT) and Glutamate-pyruvate transaminase (SGPT)

To prepare sample for SGOT, 1 ml of buffer substrate (prepared by taking 2.66gm aspartic acid, 60mg α-ketoglutaric acid and, 20.5ml of 1(N) NaOH and volume was made upto 100ml by 0.1M phosphate buffer, pH7.4) and for the preparation of sample for SGPT, 1 ml of buffer substrate (containing 1.78 gm DL-alanine, 30mg α-ketoglutaric acid, 20 ml of 0.1M phosphate buffer and 1.25ml of 0.4(N) NaOH) were taken and allowed to wait for 5min at 37^0C. Then 0.2ml of sample serum was added and incubated for 60 min at 37^0C. To prepare standard, 0.2 ml of working standard (200µM/100 ml) was receive in a test tube and 0.8ml of buffer substrate was added. For blank, 1.0 ml of buffer substrate was taken. In each of sample, standard and blank test tubes, 1ml

of DNPH solution were mixed and allowed to wait for another 20 min. Then 10ml of 0.4(N) NaOH was mixed and allowed to stand for 10 minutes. Finally, the readings were taken in spectrophotometer (UV-245 Shimadzu, Japan) at 520 nm [22].

Estimation of serum alkaline phosphatase (ALP)

For sample preparation, 1.0ml of PNPP buffer (1mM PNPP in 0.02M Tris alkaline buffer, pH 7.5), 0.25ml of sample serum, 1.75ml of redistilled water were taken in a centrifuge tube and mixed. To prepare standard, four standard solutions were prepared by mixing 5, 10, 20, 40 µg/ml of PNP (10mg% PNP) and 3.85, 3.80, 3.70, 3.50ml redistilled water respectively and 0.01 ml of 0.1M NaOH in each standard solution. All samples and standard solutions were incubated at 37^{0}C for 30 min. Then 0.1ml of NaOH and 0.9ml of redistilled water were added to each sample and standard solution and was centrifuged at 2000 rpm for 10min. The reading of each supernatant was taken in spectrophotometer at 420 nm. Amount of PNP liberated was measured in a spectrophotometer (UV-245 Shimadzu, Japan) at 420 nm against blank [23].

Estimation of serum lactate dehydrogenase

Lactate dehydrogenase, a cytoplasmic enzyme, was estimated by measuring the change in absorbance at 340 nm in solution containing NADH and pyruvate [24].

Serum bilirubin and Lipid Profile Studies

Serum bilirubin was determined[25] using commercial diagnostic reagent kit. Total cholesterol (TC), triglyceride (TG) and high density lipoprotein cholesterol (HDL) were estimated [26] by using commercial diagnostic reagent kit. Very low density lipoprotein (VLDL) was calculated using following formula (TG/5) given by [27]. LDL concentration (mg/dl) was estimated indirectly from the measured levels of TG, HDL, and TC using equation LDL = TC - (VLDL+HDL).

Statistical analysis

All the parameters were assayed in triplicate manner. The data was expressed as Mean±SEM, the differences between the means of each group were tested using a one-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA). P<0.05 was considered to indicate a statistically significant difference.

Results

Blood glucose and liver glycogen content

Biochemical analysis showed a significant increase ($p < 0.001$) in blood glucose (fig 1) with a marked reduction in serum glycogen (fig 2) in LCT-exposed group. However, pre-treatment of taurine can able to prevent lambda cyhalothrin induced toxicity significantly.

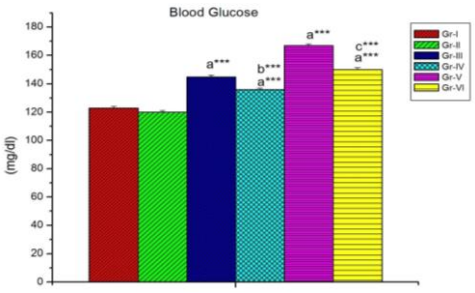

Fig-1 shows the effects of LCT and taurine on blood glucose. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (*** indicates p<0.001).

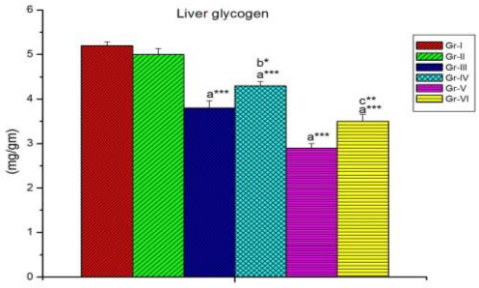

Fig-2 shows the effects of LCT and taurine on liver glycogen level. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (* indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001)

Estimation of serum enzymes

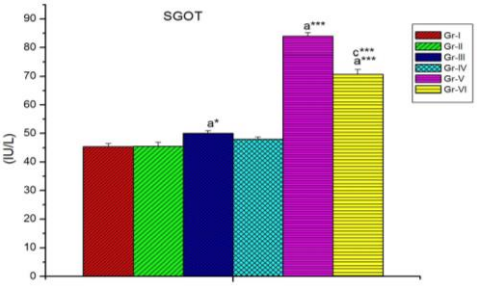

Fig-3 shows the effects of LCT and taurine on serum Glutamate-oxaloacetate transaminase (SGOT). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (* indicates p<0.05, *** indicates p<0.001).

As shown in fig-3,4 lambda cyhalothrin induced toxicity was confirmed by significant($p<0.001$) elevated activities of serum glutamate-oxalacetate transaminase (SGOT), serum glutamate-pyruvate transaminase (SGPT) and serum alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in fig 5,6. No significant changes were observed after taurine treatment alone. On the other hand, pre-treatment with taurine along with lambda cyhalothrin had significantly reduced the above stated serum enzymes.

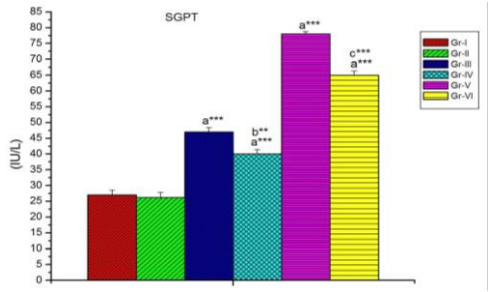

Fig-4 shows the effects of LCT and taurine on serum Glutamate-pyruvate transaminase (SGPT). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (** indicates $p<0.01$, *** indicates $p<0.001$).

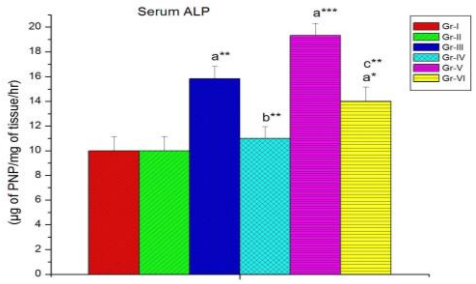

Fig-5 shows the effects of LCT and taurine on serum alkaline phosphatase activity. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

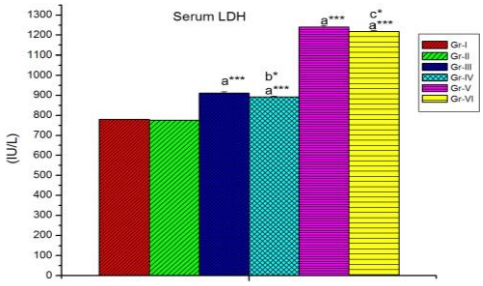

Fig-6 shows the effects of LCT and taurine on serum lactate dehydrogenase activity. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups; superscript b Group III versus Group IV and superscript c Group V versus Group VI (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$)

Determination of lipid profile and serum bilirubin

Table 1 reflect the significant elevated level of serum total cholesterol (TC), serum triglyceride (TG), serum low density lipoprotein (LDL), serum very low density lipoprotein (VLDL) along with reduced high density lipoprotein cholesterol (HDL) level in lambda cyhalothrin intoxicated rat (table 1). The significant elevation ($p<0.001$) in total serum bilirubin level in pyrethroid treated groups compared to control were observed in this experiment (table 1).

Table 1: Effect of LCT and taurine on lipid profile and serum bilirubin in male rats.

Parameters	Group-I	Group-II	Group-III	Group-IV	Group-V	Group-VI
Total Cholesterol (mg/dl)	120±2.4	115±1.5	135±1.59a***	121.33±1.62b***	145±1.29a***	126 ±1.32c***
Triglyceride (TG) (mg/dl)	83.66±1.5	80±1.06a**	124.5±1.33a***	92±1.13b***	135±1.52 ***	126±1.18a***c***
High density lipoprotein cholesterol (HDL) (mg/dl)	48±1.5	52.83±1.19 a*	34±1.18 a***	40±0.93a**b***	25±1.09 a***	31±0.96 a***c**
Very low density lipoproteins (VLDL) (mg/dl)	16.73±0.29	16.0±0.21	24.9±0.27a***	18.4±0.22a**b***	27±0.30a***	25.2±0.24a***c***
Low density lipoproteins (LDL) (mg/dl)	55.26±2.2	46.6±1.8a*	76.1±2.5a***	62.93±1.47a*b**	93±1.5a***	69.8±1.98a**c***
Bilirubin level (mg/dl)	0.3±0.05	0.4±0.06	1±0.07a***	0.5±0.02a**b***	1.7±0.1a***	1.2±0.1a***c***

Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a Group I versus all other groups, superscript b Group III versus Group IV and superscript c Group V versus Group VI (*indicates $p<0.05$, ** indicatesp<0.01, *** indicates $p<0.001$).

Discussion

Biochemical parameters are the sensitive indices and important tools for toxicological studies. In the present study, increased fasting blood glucose levels in lambda cyhalothrin-exposed rats were observed compared to the control group in the present study. The increased blood glucose level may be due to the interference of carbohydrate metabolism resulted from the alteration of the catecholamine levels [28] or phosphorylase activities or due to less peripheral glucose utilization.

Observed reduced hepatic glycogen level in the present study may be a consequence of abruptly increased catabolism to meet higher energy demands caused by LCT intoxication.

Raised levels of serum transaminase enzymes are indicators of cellular leakage and loss of functional integrity of hepatic cell membrane. Hepatocellular necrosis leads to high level of serum aspartate transminase, alanine transaminase. Elevated level of alanine transminase in the serum is a prominent index of liver damage. The increased levels of enzymes were normalized to a good extent after 14 days pre-treatment of taurine pointed out that it provided protection by stabilizing the structural integrity of the hepatocellular membrane against lambda cyhalothrin. Level of serum alkaline phosphatase is associated to the hepatocytes functioning. Bile canaliculi cells lining usually synthesize increased serum alkaline phosphatase in response to cholestasis and increased biliary pressure [29]. Lambda cyhalothrin administration increased serum ALP level and it was brought to near normal level by taurine treatment. Hepatic damage was also reflected in LCT treated group through the altered serum lactate dehydrogenase, a sensitive intracellular enzyme and an indicator of liver cell damage[30]. Pretreatment with taurine caused apparent normalization in the ALP and LDH level.

In the present study, increased total bilirubin level in LCT exposed rats may be due to increased haemoglobin percentage resulted from increased destruction of red blood cells. LCT may also block the biliary tract in treated rat which is another possible mechanism of LCT toxicity. In studies observing the role of taurine in improving hyperbilirubinemia, it may be assumed that taurine may improve excretion of bile, blood flow, and enhances the functions of hepatocytes [31,32].

In LCT exposed rats, the rise in total serum cholesterol level could be due to obstruction in liver bile ducts causing decline or interruption of its secretion to the duodenum consequently producing cholestasis[33]. The disturbance in lipoprotein formation is one of the factors leading to accumulation of cholesterol in pesticide treated mice[34] and it may be a reason for elevated cholesterol in LCT exposed rats. Administration with taurine may decrease the levels of total cholesterol. The hypocholesterolemic effect of taurine has been reported in mice [35], rats [36,37], and humans[38], but its mechanism is not well established. Taurine takes a part in conjugation reaction with bile acids in the liver. Taurine increases bile acid synthesis[39] by simultaneous upsurge in the mRNA expression and activity of cholesterol 7α-hydroxylase, a rate-limiting enzyme in hepatic bile acid synthesis[40]. The primary mechanisms accountable for the hypocholesterolemic action of taurine may be due to the increased conversion of cholesterol into bile acids through the activation of cholesterol 7α-hydroxylase.

Triglycerides are free fatty acids esters of glycerol. Liver causes biosynthesis and assimilation of lipoproteins like LDL and VLDL through which triglycerides are secreted into circulation [41]. Rise of these lipoproteins causes increase of serum triglyceride. In the present study, pre-treatment of taurine significantly alleviates lambda cyhalothrin induced rise in the liver triglyceride level. Decrease serum HDL is associated with elevated serum cholesterol level in LCT treated rat because HDL mainly plays an important role in cholesterol efflux from tissues [42]. Pretreatment of taurine tries to normalize the above said changes.

Conclusion

It may be concluded from the present study that the lambda cyhalothrin exposure produced biochemical alterations in treated rats liver. Collectively, our data also suggested that taurine pretreatment played an ameliorative role in lambda cyhalothrin mediated biochemical alterations in liver. The mechanism underlying protection of taurine may be related to its function as a direct antioxidant by scavenging ROS and also by the stimulatory effects on hepatic bile acid synthesis.

Acknowledgments

The authors are thankful to Vidyasagar University, Midnapore, India for providing the facilities to execute these studies.

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Cite this article as:

Rini Ghosh, Tuhina Das, Anurag Paramanik, Sujata Maiti Choudhury. Taurine improves lambda cyhalothrin induced biochemical alterations in wistar rat. *International Journal of Bioassays* 5.11 (2016): 5059-5065.

DOI: http://dx.doi.org/10.21746/ijbio.2016.11.0013

Source of support: Vidyasagar University, Midnapore, India.

Conflict of interest: None Declared

WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 6.041
Volume 5, Issue 11, 1486-1502 Research Article ISSN 2278 – 4357

TESTICULAR OXIDATIVE STRESS AND BIOCHEMICAL PERTURBATIONS INDUCED BY LAMBDA CYHALOTHRIN AND THE PROTECTIVE EFFECTS OF TAURINE IN RATS

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Article Received on
20 Sept. 2016,

Revised on 10 October 2016,
Accepted on 30 October 2016

DOI: 10.20959/wjpps201611-8058

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ABSTRACT

Lambda-cyhalothrin is a type II pyrethroid insecticide and may cause serious environmental pollution and health problems. Taurine, 2-amino ethanesulphonic acid is an essential amino acid, plays important roles in numerous physiological functions. The present study was conducted to evaluate the adverse effects of lambda cyhalothrin on the reproductive system of male Wistar rats and also to evaluate the protective role of taurine under these conditions**.** In the study sexually mature male rats were orally received lambda cyhalothrin at two different dose levels (10.83mg/body wt. i.e. 1/7th of LD_{50} value and 15.17mg/body wt. i.e. 1/5 of LD_{50} value) for 14 consecutive days along with pre-treatment of taurine (50mg/kg body wt.). A significant decrease in sperm motility, seminal fructose concentration, reduced glutathione and increase in testicular acid phosphatase, glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase activity, malondialdehyde and oxidized glutathione were observed in lambda cyhalothrin intoxicated rats. However, pretreatment with taurine significantly restored the above said parameters close to the normal level. The results disclose the toxic effect of lambda cyhalothrin on male reproductive system of rat and also point towards the beneficial influences of taurine in reducing the harmful effects of lambda cyhalothrin in this situation.

KEYWORDS: Lambda-cyhalothrin; Taurine; Testicular; Oxidative stress; Biochemical parameters.

INTRODUCTION

Although in terms of prosperity, green revolution has brought a windfall to the farmers in the field of agriculture, it is now showing its side-effects in the form of large-scale environmental degradation with chemical pesticides leading to the contamination of water, food and air. There has been indiscriminate use of pesticides in agriculture as well as in household and this is one of the major health issues in both developing and developed countries.[1] It disrupts the male reproduction in case of both humans and wildlife. Hence, testicular toxicity is of serious concern because a large number of pesticide is affecting badly the testicular functions in experimental animals.[2,3] The biggest group of broad-spectrum organic insecticides is synthetic pyrethroids which is used in agricultural, veterinary and household applications.[4] Lambda-cyhalothrin (LCT) is a type II pyrethroid, used mainly to control insect pest in agriculture, public health, homes and gardens. LCT is found in vegetables and fruits[5], milk and blood of dairy cows[6] as well as in cattle meat.[7] Placental transfer of LCT has been detected in goats.[8] It has also been noticed that LCT brings significant genotoxic and cytotoxic effects on human lymphocytes cultured *in vitro*[9], a dose dependent chromosomal aberrations in mice[10] and also changes in rabbit peripheral blood lymphocytes.[11] It has already been seen thatLCT causes hepatotoxicity.

Taurine (TA), a sulphur comprising β-amino acid is present in most animal tissues. It is required for the normal functioning of different organs.[12] Biosynthesis and dietary intake from meat and mostly sea food[13] are the sources of TA in the body. Furthermore, TA prevents toxin-mediated hepatic injuries by minimizing oxidative stress, strengthening mitochondrial function and regulating cytoplasmic and mitochondrial calcium homeostasis.[14] Apart from it, TA also brings nephroprotective effects, perhaps because of its antioxidant and membrane stabilization effects.[15,16]

It is our hypothesis in the current study that LCT administration in the male Wistar rats may bring bad effects on sperm motility which includestesticular biochemical and oxidative stress parameters. Moreover, we also hypothesized that TA may decrease the harmful effects of LCT on testicularbiochemical parameters in the rats based on its bioprotective effects on testis. The current study is significant as pyrethroid is priority chemical for risk assessment because of their multiple organ toxicity and there is a strong possibility of the co-exposure of humans and other living organisms in the ecosystem to LCT. So, the current study has been done to assess the testicular toxicity in terms of biochemical alterations in testis and oxidative

stress caused by LCT in male rat and the consequences of taurine on biochemical parameters in male Wistar rats to LCT.

MATERIALS AND METHODS

Chemicals and reagents

Lambda cyhalothrin 5% emulsifiable concentrate (EC) was procured from RPC Agro Industries, Kolkata. Taurine was purchased Sigma Aldrich Inc. USA. All other chemicals used were of analytical grade and were obtained from Merck India Ltd, Himedia India Ltd, etc.

Animal mode

Healthy Wistar albino male rats (*Rattusnorvegicus*) (weighing 130-150 g) were chosen for this experiment on the basis of easy availability and handling under normal laboratory conditions. The animals were fed on a standard dietand water*ad libitum* and kept in a temperature controlled environment (20–22◦C) with an alternating cycle of 12 hlight and dark.The experiments reported here were approved by the Institutional Animal Ethical Committee, registered under CPCSEA. All animal treatment and surgical procedures were carried out according to the relevant laws and guidelines of the CPCSEA.

Treatment protocol

Lambda cyhalothrin 5% emulsifiable concentrate (EC), a commercial formulation was used for this experiment. It was in emulsion form and dilutions were prepared in distilled water to obtain the test concentrations which were calculated from the percentage of the active ingredient of commercial formulation of lambda cyhalothrin following the oral LD_{50} 75.85-mg/kg body weight in male rats.[17] During the experimental duration, body weights were daily recorded and the doses of the tested compounds were prepared accordingly.

After acclimatization period for one week under laboratory conditions, rats were randomly divided into six groups each containing six rats. Group I: Distilled water control (no treatment), Rats in Group II (Taurine control) were received taurine at a dose of 50mg/kg body wt, based on previous work.[18,19] Group III:lambda cyhalothrinat a dose of 10.83mg/body wt. i.e. $1/7^{th}$ of LD_{50} valuefollowing oral LD_{50} 75.85-mg/kg body weight.[17] Group IV: the same dose of taurine as in group II, 60 min before the administration of lambda cyhalothrin low dose (10.83mg/body wt.). Group V: Lambda cyhalothrin high dose (15.17mg/body wt. i.e. 1/5 of LD_{50} value) and Group VI: same dose of Taurine (50mg/kg

body wt) as in group II before the administration of lambda cyhalothrin high dose (15.17mg/body wt.). The route of application chosen for the study was daily oral gavage for 14 consecutive days. At the end of the treatment schedule, the animals were fasted overnight, anesthetized using pentobarbital sodium (35mg/kg) and sacrificed by cervical dislocation on 15th day.

Sample collection

After sacrifice, testis from control and treated rats were collected and immediately stored at -80ºC until analysis. Epididymis were collected and washed immediately for sperm collection.

Determination of Testicular index

Testes of sacrificed male Wister albino rat were dissected from its body and all fats were removed fromit. Then their weights were taken to calculate testicular index using the following formula:

$$\text{Testicular index} = \frac{\text{Testicular weight}}{\text{Body weight}} \times 100$$

Sperm motility

Sperm motility was evaluated by the counting method of the motile and non -motile spermatozoa under microscope and expressed as the percent motility.[20]

Measurement of seminal fructose concentration

In a centrifuge tube, 1ml of diluted seminal plasma (five time dilution was done by mixing 0.1ml of seminal plasma with 4.9ml of distilled water) was mixed with 0.3ml of 1.8gm% $ZnSO_4$ and 2ml of 0.1M NaOH. To get the supernatant, the above mixture was centrifuged at 2000g after 15min. Then seminal fructoseconcentration was estimated by taking 0.5ml of supernatant as sample, 0.5ml of 0.14mM and 0.28mM fructose solutions as two standards and 0.5 ml of distilled water as blank and added 0.5 ml of indole reagent, 5 ml of concentrated HCl to each test tube. The test tubes were incubated at 50^0C for 20 min and were cooled in ice water and then in room temperature.[21] The reading was noted at 470 nm in spectrophotometer (UV-245 Shimadzu, Japan).

Assay of acid phosphatase

The acid phosphatase activity was measured in an acetate buffer at pH 4.5 using *p*-nitrophenol phosphate as a substrate.[22] Amount of PNP liberated was measured spectrophotometrically at 420 nm.

Activity oftesticularglutamate-oxalacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT)

To prepare sample for GOT, 1 ml of buffer substrate (prepared by taking 2.66gm aspartic acid, 60mg α-ketoglutaric acid and 20.5ml 1N NaOH and volume was made upto 100ml by 0.1M phosphate buffer,pH7.4) and for the preparation of sample for GPT, 1 ml of buffer substrate (containing 1.78 gm DL-alanine, 30mg α-ketoglutaric acid, 20 ml 0.1M phosphate buffer and 1.25ml of 0.4NNaOH) was taken separately and allowed to wait for 5min at 37^0C. Then 0.2ml tissue homogenate (20mg testis tissue/ml phosphate buffer) was added to eachsample and incubated for 60 min at 37^0C. To prepare standard, 0.2 ml of working standard (200µM/100 ml) was received in a test tube and 0.8ml of buffer substrate was added. For blank, 1.0 ml of buffer substrate was taken. In each of sample, standard and blank test tubes, 1ml of DNPH solution were mixed and allowed to wait for another 20 min. Then 10ml of 0.4NNaOH was mixed and allowed to stand for 10 minutes. Readings were measured in spectrophotometer (UV-245 Shimadzu, Japan) at 520 nm.[23]

Estimation of lipid peroxidation

Quantitative measurement of lipid peroxidation was performed in testicular tissue homogenate (20mg/ml in phosphate buffer) according to the method of Ohkawa et al.[24] based on the formation of thiobarbituric acid reactive substances (TBARS) in term of malondialdehyde(MDA) formation. The absorbance of supernatants was taken at 535nm in spectrophotometer (UV-245 Shimadzu, Japan).

Determination of reduced glutathione and oxidized glutathione

Testicular reduced glutathione (GSH) estimation was performed by the method of Griffith.[25] The GSH was expressed as µg/mg protein. The reaction mixture contained 200µl of tissue homogenate (20mg/ml in phosphate buffer), 100 µl of sulfosalicylic acidand centrifuged for 10 min at 3000 rpm. After addition of 1.8 ml of DTNB with the supernatant, reading was taken at 420nm.

The oxidized glutathione (GSSG) was measured according to the method of Griffith[26] using 2µl of 2-vinyl pyridine, 250µl of sulfosalicylic acid (4 gm %) and 2 ml of DTNB (4mg %). The reading was taken at 412 nm. The GSSG was expressed as µg/mg protein.

Assay of of glutathione-S-transferase

Glutathione-S-transferase (GST) activity was quantified spectrophotometrically.[27] Briefly in a cuvette, 0.1 ml of tissue homogenate(20mg/ml in phosphate buffer), 2.8ml of 20mg/ml in phosphate buffer, 0.1ml of GSH and 50µl of 60mM CDNB were taken, mixed and reading was noted at 340nm. The values were expressed in µmol CDNB conjugate formed/min/ per milligram protein.

Statistical analysis

All the parameters were assayed in triplicate manner. The data was expressed as Mean±SEM. The differences between the means of each group were tested using a one-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA). $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Effect on testicular index

The testicular index was decreased significantly ($p<0.001$) in a dose dependent manner in LCT treated group which was elevated significantly ($p<0.01$) by the pre-treatment of taurine(fig.1).

Sperm motility

Significant decrease ($p<0.001$) in sperm motility was observed in the LCT treated group compared to the control group (fig.2). Pre-treatment with taurine significantly ($p<0.001$) increased the sperm motility to a good extent.

Effect on seminal fructose concentration

Seminal fructose concentration was found to be decrease significantly ($p<0.001$) ina dose dependent manner in LCT treated group, compared to control (fig.3). Pre-supplementation of taurinerescuesit from LCT induced toxicity.

Assay of acid phosphatase activity

Acid phosphatase activity was significantly elevated($p<0.01$) in the testis after LCT treatment, in comparison to the controls (fig.4). Pretreatment of taurine reduced it but not significantly.

Estimation of testicular glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) activity

Testicularglutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) activity in LCT treated rat testis were found to be increased compared to that of control. Taurine restored these parameters towards more or less control level (fig.5, 6).

Lipid peroxidation and glutathione content

A significant increase ($p<0.001$) in MDA concentrations was observed in rat testes exposed to LCT pyrethroid in a dose dependent manner (fig.7).

On the other hand, a significant decrease ($p<0.001$) in GSH content (fig.8) with asignificant dose dependent increase in GSSG level in LCT treated rat testes was seen (fig.9).

Protective effects of taurine were evident after the pretreatment of taurine followed by LCT, where the alterations in the lipid peroxidation and glutathione content were restored close to the normal level.

Glutathione-S-transferase activity

From the result it is well-established that LCT treatment showed significant inhibition of antioxidant enzyme glutathione-S-transferase (GST) activities. The activity of the GST was better in taurine pre-treated group (fig.10).

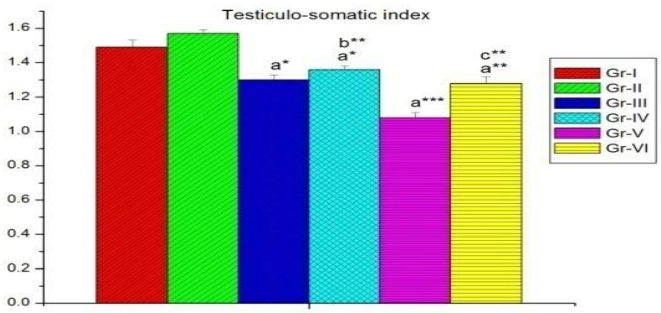

Figure1 shows the effect of taurine on testiculo-somatic index in LCT induced male rat. Results are expressed as Mean±SEM. Analysis is done by one way ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b, Group-III versus Group-IV; Superscript c, Group-V versus Group-VI. Asterisks represent the different level of significance (* indicates $p<0.05$, ** indicates $p<0.01$, * indicates $p<0.001$).**

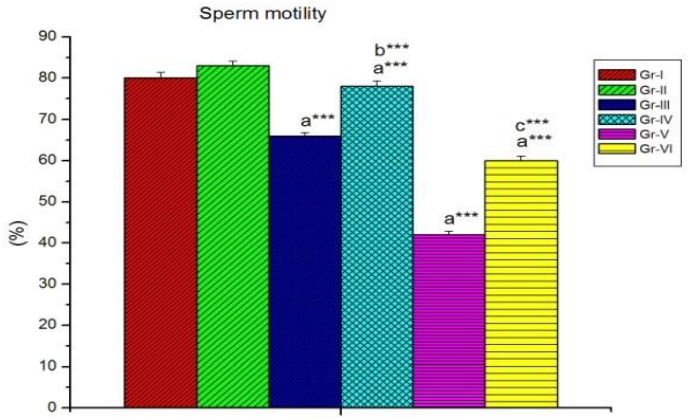

Figure 2. The effect of taurine on sperm motility in LCT induced male rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b, Group-III versus Group-IV; Superscript c, Group-V versus Group-VI. Asterisks represent the different level of significance (* indicates p<0.001).**

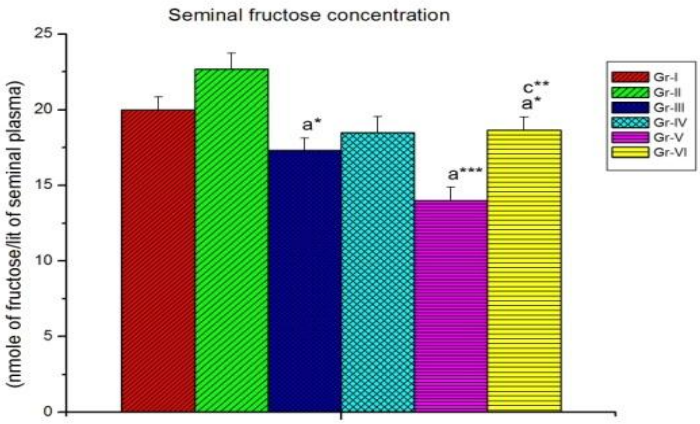

Figure 3 shows the effect of taurine on seminal fructose concentration in LCT induced male rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b, Group-III versus Group-IV; Superscript c, Group-V versus Group-VI. Asterisks represent the different level of significance (* indicates p<0.05, ** indicates p<0.01, * indicates p<0.001).**

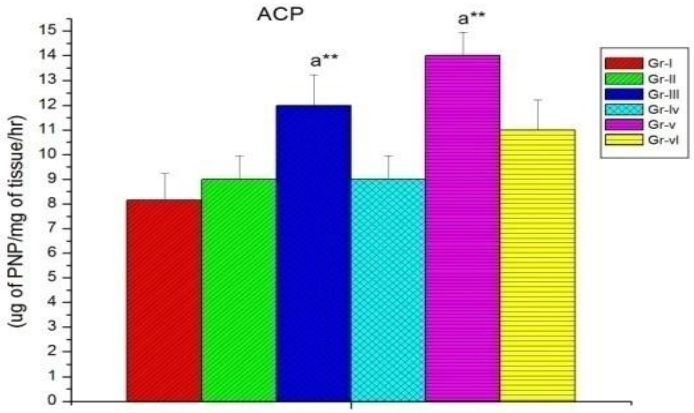

Figure 4. The effect of taurine on acid phosphatase activity in LCT induced male rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b, Group-III versus Group-IV; Superscript c, Group-V versus Group-VI. Asterisks represent the different level of significance (indicates p<0.01).**

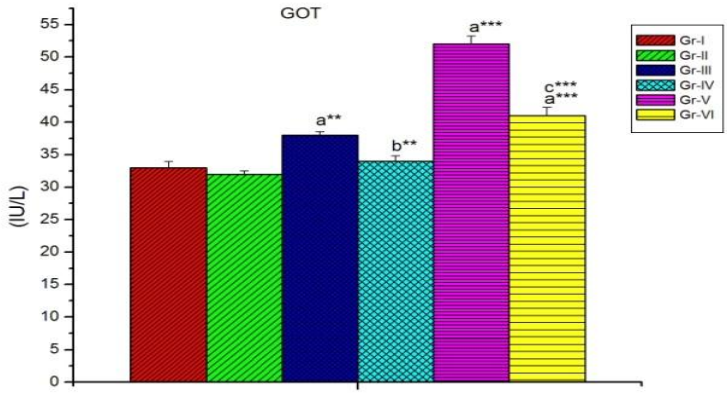

Figure 5 shows the effect of taurine on testicular glutamate-oxaloacetate transaminase (GOT) in LCT induced male rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b, Group-III versus Group-IV; Superscript c, Group-V versus Group-VI. Asterisks represent the different level ofsignificance (indicates p<0.01, *** indicates p<0.001).**

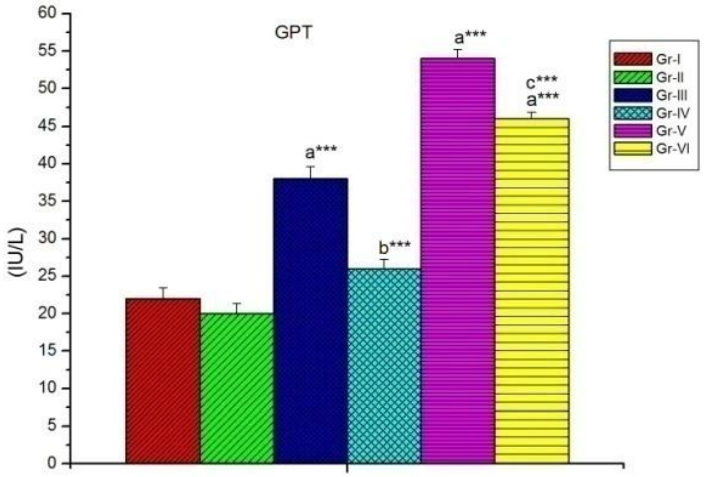

Figure 6. The effect of taurine on testicular glutamate-pyruvate transaminase (GPT) in LCT induced male rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b, Group-III versus Group-IV; Superscript c, Group-V versus Group-VI. Asterisks represents the different level ofsignificance (* indicates p<0.001).**

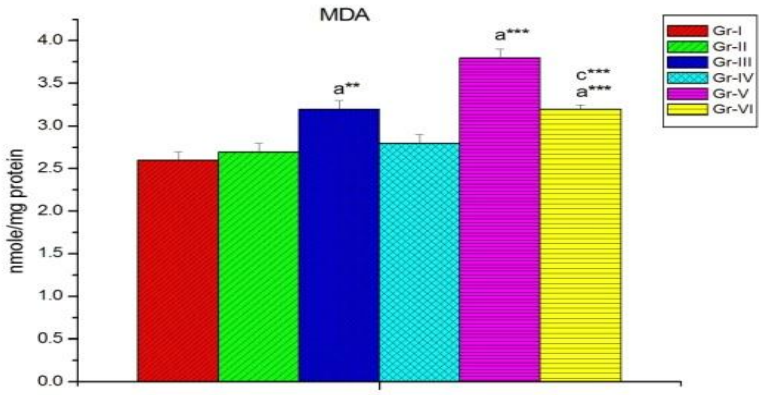

Figure 7 shows the effect of taurine on testicular malondialdehyde content in LCT induced male rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b, Group-III versus Group-IV; Superscript c, Group-V versus Group-VI. Asterisks represent the different level of significance (indicates p<0.01, *** indicates p<0.001).**

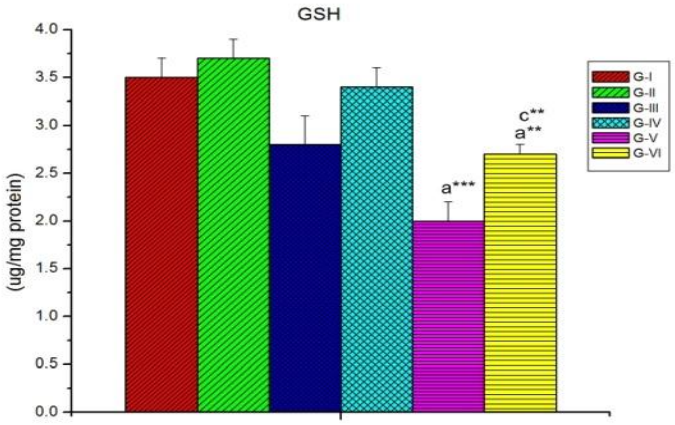

Figure 8. The effect of taurine on testicular reduced glutathione level in LCT induced male rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b, Group-III versus Group-IV; Superscript c, Group-V versus Group-VI. Asterisks represents the different level ofsignificance (indicates p<0.01, *** indicates p<0.001).**

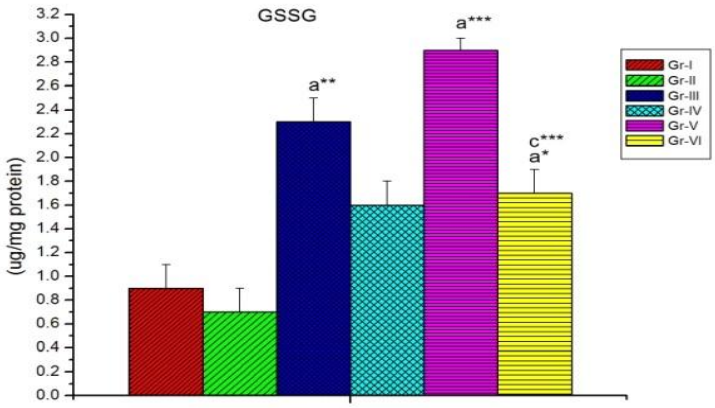

Figure 9 shows the effect of taurine on testicular oxidized glutathione in LCT induced male rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b, Group-III versus Group-IV; Superscript c, Group-V versus Group-VI. Asterisks represent the different level of significance (* indicates p<0.05, ** indicates p<0.01,* indicates p<0.001).**

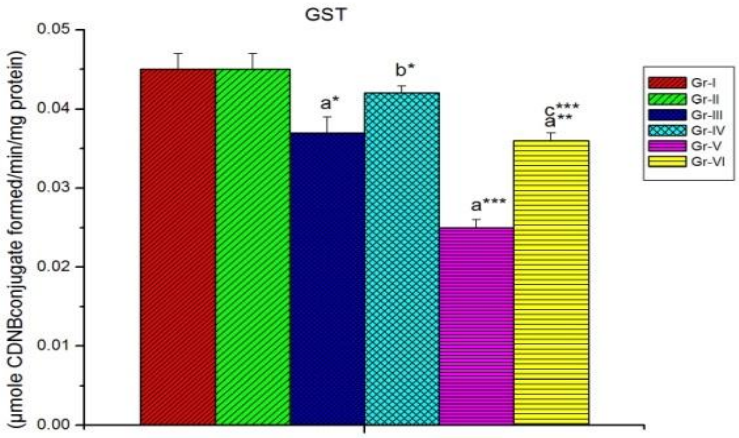

Figure 10 shows the effect of taurine on testicular glutathione –S-transferase activity in LCT induced male rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b, Group-III versus Group-IV; Superscript c, Group-V versus Group-VI. Asterisks represent the different level of significance (* indicates p<0.05, ** indicates p<0.01, * indicates p<0.001).**

DISCUSSION

The current study has been done to evaluate the harmful effects of lambda cyhalothrin (LCT) on the reproductive system of male Wistar rats and to assess the controlling role of taurine under the toxic situation. The testicular index relies on both testicular weight and body weight. Reduction in the testicular weight on LCT exposure is perhaps due to decreased tubule size, reduced number of germ cells and enlarged spermatids.[28] The testicular index reduced by LCT intoxication successfully raised by the treatment of taurine. Perhaps, this is due to the preventive role of taurine on testicular damage. Decline in sperm motility after oral administration of LCT was either by androgen deprivation effect of the pesticides or by low spermatogenesis. Another possibility was enhanced reactive oxygen species ROS production by pyrethroid exposure. Similar results were also reported earlier.[29,30,31] Taurine pretreatment significantly improves the sperm parameters, indicated the important role of taurine to improve the semen quality either by its effect on stimulation of testosterone secretion[32] or by maintaining the testicular homeostasis.

The useful biochemical indicators estimated in this experiment reveals the significant alteration in the seminal fructose content, the ‘marker’ for the functioning of seminal

vesicle[33], testicular acid phosphates, testicular glutamate pyruvate transaminase(GPT), glutamate oxaloacetate transaminase (GPT) activity, testicular malondialdehyde level and glutathione-S-transferase (GST) in LCT intoxicated rat compared to control. Fructose is the primary secretory product of seminal vesicle that offers nutrients for the semen also important for sperm motility and stability of sperm chromatin. Reducedfructose content indicated that the secretary ability of the seminal vesicle was hampered by the pyrethroid treatment[34], which adversely affect the nutritive potentials for the semen in turn affect sperm motility. In this experiment seminal fructose concentration was found to be significantly lower in the LCT intoxicated groups compared to the rats in the control group, with the exception of taurine treated groups. This type of result clearly indicates LCT induced testicular damage. Testicular acid phosphatase activity was an important marker to assess the male reproductive toxicity. Ittakes a role in the cell metabolic process, autolysis and also in the differentiation including many related processes. Dilatation of blood capillaries in between seminiferous tubules is the result of acid phosphatase enzyme activity. The increase in acid phospahatase enzyme activity could be explained on the basis of enhancement of cell membrane permeability with an interruption in the transphosphorylation process as a result of cellular degeneration.[35] Increased testicular GOT and GPT levels indicates towards the LCT induced testicular injury.[36] Taurine pretreatment significantly improves the sperm parameters, fructose content and normalize the GOT, GPT levels. Testicular acid phosphatase activity was improved but not significantly.

During pyrethroid metabolism, excess reactive oxygen species (ROS) wereproduced which caused oxidative stress in pyrethroid induced animals.[37] Lipid peroxidation is believed to be one of the main markers of ROS-mediated damage. In our experiment malondialdehyde (MDA) level in LCT treated group was found to be significantly higher than that of control. Excessive production of reactive oxygen species (ROS) is considered to be also associated with the inhibition of endogenous antioxidant defense system that are also responsible to neutralize the toxic effects of these free radicals by giving electrons to these toxic species. Keeping in mind that GSTs are detoxifying enzymes which effectively catalyzes the conjugation of a variety of electrophilic substrates to the thiol group of GSH to create less toxic forms.[38] The significant reduction of GST activity shows insufficient detoxification process in LCT intoxicated male rats. Yamamoto and Yamashita concluded that detoxification system created by GST may be reduced by the presence or action of ROS.[39] Co-administration of taurine notably improved these abnormalities to a great extent. It

decreased lipid peroxidation either by scavenging or reducing reactive oxygen species. In various studies, the invigorating effects of taurine on endogenous antioxidants were already confirmed. On the whole, the results not only have given strong support for the toxic effect of LCT, but have also indicated the preventive role of taurine in this condition.

CONCLUSION

From the above discussion it can be inferred that lambda cyhalothrin creates testicular toxicity, lipid peroxidation, antioxidant insufficiency in male rats and co-supplementation of taurine has therapeutic effects on lambda cyhalothrin-induced male reproductive toxicity.

CONFLICT OF INTEREST

Authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENT

The authors are thankful to Vidyasagar University, Midnapore for providing laboratory facilities to execute these studies.

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