Toxicity risk assessment of Pyriproxyfen on testicular tissue of adult Sprague Dawley rats.

By

Iqra bibi

DEPARTMENT OF ZOOLOGY
FACULTY OF BIOLOGICAL SCIENCES QUAID-I-AZAM UNIVERSITY ISLAMABAD, PAKISTAN

2023

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY.

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"In the Name of ALLAH, the most Beneficent, the most Merciful"

Toxicity risk assessment of Pyriproxyfen on testicular tissue of adult Sprague Dawley rats.

DEDICATION:

Dedicated to My Parents and Brothers who have always Supported me throughout my life. A special dedication to my father.

Toxicity risk assessment of Pyriproxyfen on testicular tissue of adult Sprague Dawley rats.

DECLARATION

I hereby certify that the work given in the following thesis is entirely original work and the material contained in this thesis is my original work. This work has never been presented for any other degree.

Iqra Bibi

Toxicity risk assessment of Pyriproxyfen on testicular tissue of adult Sprague Dawley rats.

CERTIFICATE

This dissertation "Toxicity risk assessment of Pyriproxyfen on testicular tissue of adult Sprague Dawley rats" submitted by **Ms. Iqra Bibi** is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Reproductive Physiology.

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*In the name of **Almighty Allah,** who is the most Beneficent, the most Gracious, most Altruistic and the most Merciful. I owe my gratitude to Allah who created us as a Muslim and blessed us with the knowledge to differentiate between right and wrong. I glory his name for giving me the strength to accomplish my every desire. He blessed us with the **Holy Prophet, Hazrat Muhammad** (SAW) for whom the whole universe is created and who enabled us to worship only one God. He (SAW) brought us out of darkness and enlightened the way of heaven.*

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LIST OF ABBREVIATIONS

EDCs	Endocrine disrupting chemicals
EPA	Environmental Protection Agency
EFSA	European Food Safety Authority
BMI	Body Mass Index
PCBs	Polychlorinated Biphenyls
WHO	World Health Organization
PIPs	Plant Incorporated Protectants.
IGR	Insect Growth Regulators
JH	Juvenile Hormones
PPF	Pyriproxyfen
mg/l	Milligram Per Liter
GUP	General Use Pesticide
Jha	Juvenile Hormone Agonists
JMPR	Joint FAO Meeting on Pesticide Residue
ROS	Reactive oxygen specie
POD	Guaiacol Peroxidase
Ml	Milliliter
CAT	Catalase
SOD	Super Oxide Dismutase
DEPPD	N,N-Diethyl-p-phenylenediamine sulphate salt
$FeSO_4$	Ferrous Sulphate
H_2O_2	Hydrogen Peroxide
PBS	Phosphate Buffer Saline
NADH	Sodium Hydroxide
Rpm	Revolution Per Minute
NO	Nitric Oxide
ANOVA	One Way Analysis of Variance

Toxicity risk assessment of Pyriproxyfen on testicular tissue of adult Sprague Dawley rats.

TA	Tunica Albuginea
SFT	Seminiferous Tubules
DCTA	Degenerative Changes in Tunica Albuginea
CSP	Clumping of Spermatozoa
MSP	Mature Spermatozoa
Ps	Primary Spermatozoa
Ss	Secondary Spermatozoa

Abstract:

Pyriproxyfen, (2-(1-methyl-2-(4-phenoxyphenoxy) ethoxy), is an aromatic, non-terpenoid, pyridine-based juvenile hormone that disrupts the equilibrium of hormones in the insects, strongly suppresses the embryogenesis of insects. It has been used on many crops for control of pests in many countries. The current study was intended to estimate the toxicity risk assessment of pyriproxyfen on testicular tissue of adult Sprague Dawley rats. To perform the current study, twenty adult male Sprague Dawley rats were separated into four groups: Control group was treated with 0.9% normal saline. Group 1 were given 62mg/kg of pyriproxyfen. Group 2 were provided with 124mg/kg of pyriproxyfen, and Group 3 were administrated with 186mg/kg of pyriproxyfen for 28 consecutive days. On the next day, all the animals were weighed and decapitated. Blood sample was drawn, that was followed by plasma extraction and kept at -20ºC for the analysis of biochemical parameters. For the analysis of histopathological changes, the right and left testis were fixed in 10% formalin. The results indicated that, pyriproxyfen administration indued decrease in the BMI and body weight at day 1, 14 and 28^{th} at high and low doses, while showed non-remarkable decrease($p<0.001$) in the weight of testis and accessory reproductive organs($p<0.05$) only at high dose. PPF showed increase in the weight of body organs as kidneys, liver, pancreas, brain, and heart and the blood glucose level showed highly remarkable decrease($p<0.001$) both at high and low doses. The antioxidant results showed highly notable increase($p<0.001$) in the level of SOD, ROS, and CAT at high and low doses of pyriproxyfen while POD showed non-remarkable increase($p=0.206$). histopathological changes in the testis of rats by the PPF are seen by decline in the number of sperms, increased immature spermatozoa and degenerative changes in tunica albuginea. The study concluded that pyriproxyfen decreases body weight, BMI, testis and accessory reproductive organ weights and blood glucose level, increases the weight of body organs such as heart, liver, pancreas, kidneys, and brain, increases level of SOD, POD, CAT and ROS, increases oxidative stress, and brings histopathological abnormalities in the testis at high dose while at low doses no significant changes were observed. Because of this finding, it is suggested that the usage of pyriproxyfen should be done with the proper cautions. These findings can be extended fairly to the human model as well, and this toxin can further be used to examine its impact on the reproductive system.

Introduction

Endocrine disrupting chemicals are described as "exogenous chemical or the combination of chemicals that interfere with any component of hormone activity" (Zoeller *et al.,* 2012; Gore *et al.,* 2015). According to US Environmental protection Agency (EPA), EDCs are termed as exogenous substances that hinders the synthesis, metabolism, secretion as well as endogenous hormones transport and elimination, that results in endocrine changes and disruption of homeostasis (Mnif *et al.,* 2011; Schug *et al.,* 2011; Coster et al., 2012; Nohynek *et al.,* 2013; Kabir *et al.,* 2015; Kiyama *et al.,* 2015). According to the criteria recommended by the European Commission's: EDCs shows three actions: Endocrine activity, pathologic or the detrimental endocrine-mediated activity and the Cause-effect relationship between the exposure of the substance and endocrine activity (EFSA. 2013; Salma *et al.,* 2016).

History:

The endocrine disruptive activity was first reported in 1920s, when the fertility issues were raised in the swine herds that fed on the mouldy grain (McNutt *et al.,* 1928). One of the most important early evidence of endocrine disruptive activity was occurred in Wisconsin, USA, in 1991 at the World Wildlife Fund (WWF) wingspread conference, where Colborn first given the name 'endocrine disrupter' which was followed up fifteen years later (Colborn *et al.,*1992; Hotchkiss *et al.,*2008). In 1960s and 1970s, one of the greater environmental movements was in the endocrine disruption filed all over the United States. *Rachel Carson* in his famous book *silent spring* about the harmful effects of endocrine disruptors. There were many milestone events occurred in the past from 1950s to 2000s in the field of endocrine disruptions as shown in figure 1.

The European Food Safety (EFSA) claims that most endocrine disruptive chemicals are synthetic chemicals, which disrupt the endocrine systems ability to bind to the receptors of hormones and results in the regulations of gene expression so epigenetic modifications occurred such as methylations and acetylation of the DNA molecule, and histone alterations (Baldi et al., 2008; Gore et al., 2015; Zama et al., 2010). EDCs can be divided into four categories based on their origin: Industrial (dichlorodiphenyltrichloroethane (DDT), PCBs, and alkylphenols), Agricultural (Herbicides, phytoestrogens, insecticides, fungicides, and pesticides), Residential (Phenols and biphenyls) and Pharmaceutical (propyl and butyl parabens) (Coster et al., 2012; Kabir et al.,2015; Monneret et al., 2017).

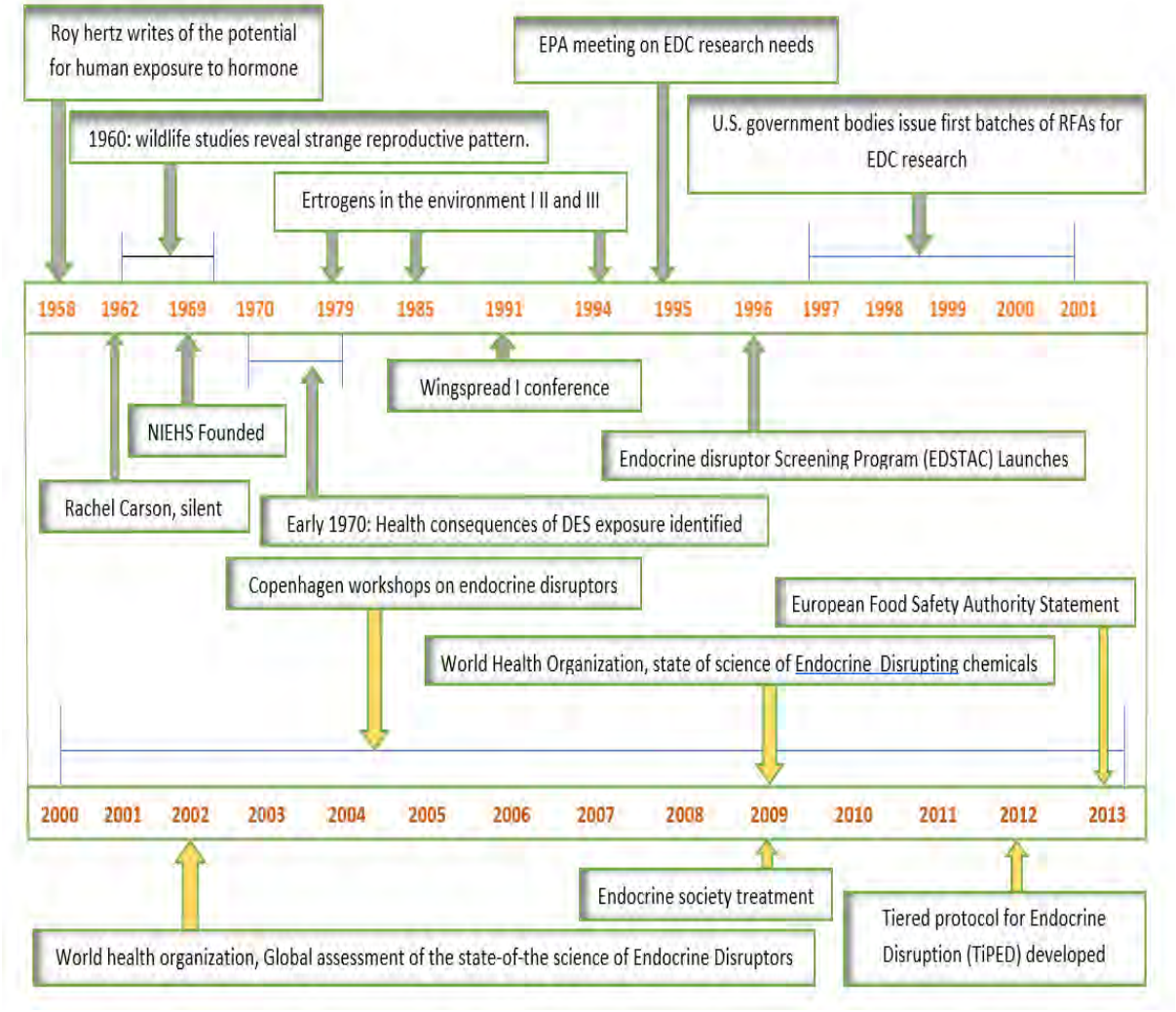


Fig 1. Significant developments in the EDC field.

EDCs may include heavy metals for example cobalt, lead, iron and nickel etc (Lubrano *et al.,* 2013). EDCs showed Different human body contamination pathways. The most typical exposure route includes absorption of food, inhalation, and direct exposure. Newborns expose to EDCs by the breastfeeding and interactions with baby products (Balaguer *et al.,* 2017; Gore *et al.,*2015; Heindel *et al.,*2015; Kabir *et al.,*2015). EDCs accumulate in adipose tissue because they have extended half-life and strong lipophilic nature (Sargis *et al.,* 2015; Heindel *et al.,* 2015). These two characteristics explain why EDCs can concentrate in any animal's adipose tissue (Barouki *et al.,* 2017).

Mechanism of endocrine disruption:

EDCs mostly act by the natural hormone's interference, because of their high capacity to bind with the receptors of estrogens and androgens (Tabb *et al.,* 2006). For example, EDCs can have agonist actions it means that it can bind to the various receptors of hormones and in turns leads to the activation of that receptor (CAR, PXR, and ER), before the stimulation of natural hormone actions. So, the receptors are blocked, and their functions is inhibited by this antagonist effect. EDCs lower the natural hormone concentrations and disrupt hormone production, transport, metabolism, and elimination.

The endocrine system is affected by the EDCs which has impact on the neurological, developmental and reproductive processes. The negative effects of EDCs are increased when they are associated with other EDCs (Nohynek *et al.,* 2013). The hypothalamic pituitary thyroid axis (HPT axis), hypothalamic pituitary gonadal axis (HPG axis), and hypothalamic pituitary adrenal axis (HPA axis) are regulated by EDCs (Zoeller *et al.,* 2012). However, EDCs mainly effect the HPG axis (Akhtar *et al.,* 1996; Cocco *et al.,* 2002; Leghait *et al.,* 2009; Sugiyama *et al.,* 2005).

After the discovery of DDT, in the year 1939, various pesticides have been discovered and used frequently with little or no limitations. By enlarging area under cultivation, mechanizing the process, cultivating hybrid plants with greater yield, and using techniques of pest management, the green revolution dramatically increased agricultural output in the year 1960s (Briggs *et al.,* 2009). So, this battle extensively needed the usage of pesticides. Mostly pesticides have positive impact on human health. In fact, pesticides used in pest management control in agriculture, and help in the control of plant diseases. They also guarantee higher food output, a reliable supply of food, and other advantages (Copper *et al.,* 2007).

Pesticides:

World Health Organization (WHO, 2020) claims, pesticides as chemicals that are applied to control pests for example, insects, fungus, animals, and unwanted plants. So, they play very important role in preventing the spread of illness. Insecticides are commonly used against insects, and their significance in public health has increased considering the number of people worldwide who suffer from infectious diseases brought by insects.

Numerous synthetic pesticides as well as insecticides are not bio-deteriorated easily and persist in water and soil for many years (Ahmad *et al.,* 2021; Namratha *et al.,* 2021; Singh and Singh, 2019). When these chemicals are accidently exposed to marine, aquatic, and terrestrial environment they negatively impact these habitats and drastically reduces the lifespan of many exposed creatures (Baralic *et al.,* 2020; Merdana *et al.,* 2021; Tahir *et al.,* 2021).

There are many ways to be exposed to pesticides, including agricultural drift, domestic pesticide use, and consuming pesticide residues. Studies on the biological surveillance show that, pesticide exposure to human is widespread. Food consumption is not the only way for the general population to be exposed. The population that lives near the location where the pesticides are used, produced, and discharged, are at greater risk to the pesticides exposure because of increased rate of interaction to the environment through breathing, or contact with soil and water (Mantovani *et al.,*2008; Peakall *et al.,* 2000; Whyatt *et al.,*2005).

Around two million pesticides are used annually, and over 800 chemicals that are active are sold in a variety composition worldwide (PAN-UK. Pesticide Action Network UK. 2009). More than 3 million human pesticide poisonings occur each year, resulting in roughly 250,000 mortalities globally, according to WHO reports (Yang et *al.,* 2007).

Pesticides contain numerous substances that have been recognized as endocrine disruptors (Andersen *et al.,* 2000; Kojima *et al.,* 2004; Lemaire *et al.,* 2006; Vinggaard *et al.,* 2000). Among them, insecticides are of 46%, herbicides are of 21%, fungicides are of 31%; some of them were discontinued from the environment but are still in the environment today.

World Health Organization (WHO) evaluate that every year, pesticides exposure results in 220,000 deaths (WHO: Our Planet, Our Health. Report of the WHO Commission, 1992). In some circumstances, it has been suggested that there may a connection between pesticides exposure and conditions like allergies, brain deformations, cancers and problems related to the reproductive system.

Classification of pesticides:

Pesticides are categorized based on their action mechanism, target site, and composition (Gangola *et al.,* 2015). Based on their chemical composition, and a description of the active ingredients, insecticides can be classified. Pesticides included four major types on basis of their chemical composition: organochlorine, organic phosphorus, carbamate, pyrethrin, and

pyrethroid. Biopesticide is another class of pesticide, this additional category of pesticides is subdivided into 3 major groups. Microbial pesticides, bio-chemical pesticides and plant incorporated protectants (PIPs). Biopesticides often referred to as biorational pesticides, originates from biotic sources such as viral sources, bacterial sources, fungus, etc. or from the bio-chemical origin like pheromones or insect growth regulators (IGRs).

Insect growth regulators:

Insect growth regulators (IGRs) are chemicals that impact and cause changes in the growth and reproduction of insects. This term is also known as "insect hormone mimics" since it mimics the actions of both juvenile hormone and insect hormone. *Schneiderman* invented the term "IGRs" in 1972. Insect growth regulators or mimics, can be divided into several categories for example chitin synthesis inhibitors, juvenile hormone analogues or mimics, molting hormone analogues, etc. Chromafenozide, lefenuron, kinoprene, methoxyfenozide, and pyriproxyfen are few of the most prominent IGRs (Tunaz *et al.,* 2004).

Juvenile hormone (JH):

Insect's metamorphosis and molting is regulated by the help of two hormones, one is the steroid hormone such as ecdysone and other is an acyclic terpenoid referred to as juvenile hormone (JH). The function of ecdysone is to initiate and regulate molting and of JH is to mediate molt's characteristics. There is no differentiation when JH is present. Ecdysone starts the process of altering the expression of certain genes required for the insect's metamorphosis when it is absent (Zhou *et al.,*2002). JH has five structural homologous types, each of which has one chiral center or more asymmetric center. Among these five types, JH-I and JH-III have been developed.

Pyriproxyfen (PPF):

Pyriproxyfen, 2-(1-methyl-2-(4-phenoxyphenoxy) ethoxy), was first formed in 1990 at the Sumitomo Chemical Company. PPF is an aromatic, non-terpenoid, pyridine- based powerful inhibitor of insect embryogenesis and adult emergence (Dhadialla et al., 1998).

Structure:

A pyridyl oxyethylene-replaced portion of the aliphatic chain makes up the phenyl carbonyl derivative known as pyriproxyfen as shown in figure 2. PPF is the mimic of potent JH that disrupts the balance of hormones in the insects, strongly suppresses the metamorphosis of the insects. So, the characteristics of adult insects for example, formation of egg and wings and maturation of reproductive organs and genitalia are disrupted. As a result, insects keep in the immature form (Harburguer *et al.,* 2009; Harwood *et al.,* 2016).

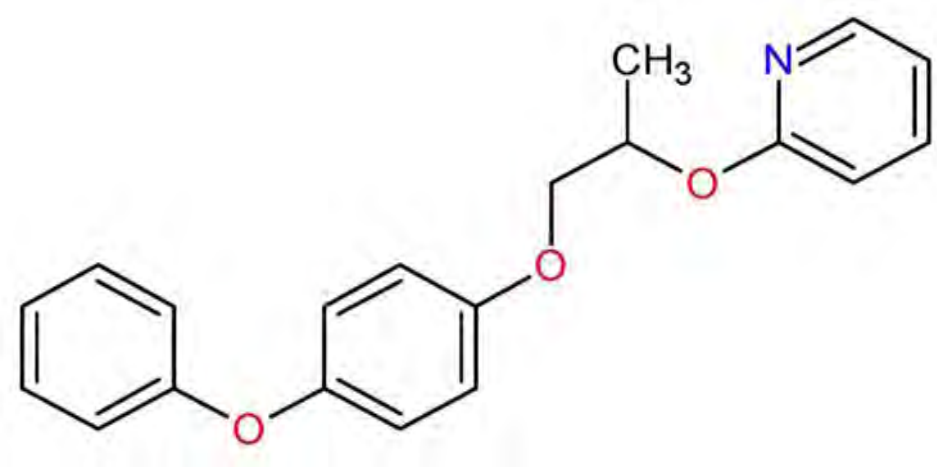


Fig 2: Two-dimensional structure of pyriproxyfen.

Properties:

PPF molecular formula is 321.5 gram per mole (g/mol) and its chemical formulae is $C_{20}H_{29}NO_3$. It has a 5.3Å diameter and length is about 18.4 that is fully extended. According to WHO guidelines and pesticide assessment for public health, PPF has melting point (M.P) of about 45-47 degree centigrade and vapor pressure (V.P) of about 1×10^{-7}. It has a PH of 6 and 0.367 mg/l water solubility (Sullivan *et al.,* 2000).

Pyriproxyfen is hydrophobic that predisposed to biomass and sediments, as indicated by its poor solubility in water and because of higher water partition coefficient. Additionally, due to its low vapor pressure, PPF is non-volatile and showed that there has been little to no dispersion into the environment. PPF is hardly hydrolyzed in water, however, the most important pathway of transformation is aquatic photolysis. PPF has half-life of 11.5 days (Sullivan *et al.,* 2000).

Pyriproxyfen has been used on many crops such as vegetables, apple, oranges, mango, pineapples, and coffee, for the control of pests in many countries (Sullivan *et al.,* 2008). Pyriproxyfen is manufactured as a part of mixed pesticide items, for example in carpet sprays, shampoos, and bug sprays etc. Pyriproxyfen belongs to the Environmental Protection Agency toxicity class III that is essentially non-toxic. As pyriproxyfen is General Use Pesticide (GUP), so the word CAUTION must be label on the items that contain PPF. in California, 106 items that contain PPF are presently registered. In 2005, California treated PPF of 9,946 pounds with cotton, almonds, oranges receiving 77% of the total. Less than 100 pounds each were used for public health and structural pest control (Sullivan *et al.,* 2008).

Arthropods, such as insects, weeds, and annual grasses, are frequently eradicated in with the help of drug pyriproxyfen. For vector control and public health initiatives, the WHO advice using PPF largely in drinking water (Tomlin *et al.,*2009; World Health Organization, 2006). PPF has ability to control insects that cause transformation of diseases, for example those insects that spread yellow fever, filariasis, dengue, and chikungunya demonstrated in the numerous studies. These insects include *Anopheles gambiae and arabiensis* (Jaffer *et al.,* 2015; Lwetoijera *et al.,* 2014) *culex quinque fasciatus.* (Jambulingam *et al.,* 2008). As a result, Pyriproxyfen has been employed globally in the public health initiatives in replacement of pesticides like pyrethroids that was causing resistance to insects (Lima *et al.,* 2011). There is different effect of PPF on insect including morphogenetic abnormalities and lower fecundity in adults as well as inhibit egg hatching and larval development (Maoz *et al.,* 2017; Ohba *et al.,* 2013).

Thermodynamic characteristics:

Pyriproxyfen is yellow waxy solid, density higher than water. Due to its low volatility, pyriproxyfen will not evaporate significantly into the atmosphere. As PPF is less volatile, so mammalian toxicity is minimum. Because of its concentration in the liquid formulations, pyriproxyfen in the vapor phase is expected to provide only a slight risk of exposure and moderately hazardous when inhaled. The U.S Environmental Protection Agency (EPA) has assessed the PPF possible reaching the surface water and sediments by the process of spraying because PPF is applied in agriculture on crops. PYR may be toxic to animals because its

solubility is very low, which make it simple to accumulate in the fatty tissues as long-term storage. According to WHO, daily recommended dose of PYR is 100mg/kg of body weight (Truong *et al.,* 2016).

Metabolism:

In environment such as water, plants, soil, insects, and animals, more than ten metabolites have been found. In soil -sludge system, the metabolites that are produced by the pyriproxyfen are metabolite A, metabolite B, metabolite C and metabolite D, E, F, G, H, I. In different organism different metabolites are found such as, in the larva of *Musca domestica* microsomes the major metabolites are metabolite A, metabolite E, metabolite F and metabolite I (Zhang *et al.,*1998). while in mammals like rats and mice, the pyriproxyfen breakdown into metabolite A, metabolite B, metabolite G, metabolite I (Yoshino *et al.,* 1996). The metabolites that have been found in different organisms as shown in figure 3.

Metabolites.	Name	Abbreviation
	Pyriproxyfen (4-phenoxyphenyl (RS)-2-(2pyridyloxy) propyl ether)	**PYR**
A	4-(4-hydroxyphenoxy) phenyl-(RS)-2-(2pyridyloxy) propyl ether	**4'-OH-PYR**
B	4-hydroxyphenyl (RS)-2(2-pyridyloxy) propyl ether	**DPH-PYR**
C	4-phenoxyphenol	**POP**
D	4,4'-oxydiphenol	**4'-OH-POP**
F	(RS)-2-hydroxypropyl4phenoxyphenylether4-(4-hydroxyphenoxy) phenyl (RS)-2hydroxypropylether	**POPA**
G	(RS)-2-(2-pyridyloxy) propanol	**4'-OH-POPA**
H	(RS)-2-(2-pyridyloxy) propionic acid	**PYPAC**
I	4-phenoxyphenyl (RS)-2(2-(5hydroxypyridyloxy)) propyl ether	**5"-OH-PYR**

Fig 3: Different metabolites of pyriproxyfen and their abbreviations:

Mechanism of action:

Pyriproxyfen efficiently competes for the juvenile hormone receptor because of their structural similarity with the juvenile hormone of insects. So PPF behave as juvenile hormone agonists (Jha) (Sullivan and Goh, 2008). Under normal situations, when larva gets ready to pupate, juvenile hormone production stops and the expressions of genes that are required for metamorphosis starts shifting. Pyriproxyfen is categorized as EDC (Sullivan *et al.,* 2008), because when JH or its analogues are present, homeostasis is disturbed, which directly affects the development of insects (Dhadialla *et al.,* 1998).

Toxicity:

The PPF potential to interrupt the endocrine system has been extensively reported in variety of the organisms such as invertebrates (Kakaley *et al.,* 2017; Tanaka *et al.,* 2018). PPF has variety of effects on the reproductive system, decrease fertility, bring changes in the storage lipids, the sex of infants shifting to male and variety of effects on ecology in the Daphnia (Ginjupalli *et al.,* 2015).

There are limited data available on the pyriproxyfen toxicity to the vertebrates like fish and fish ELS. There has been reports of *Xiphophorus maculatus* swimming abnormalities and embryo abnormalities at higher concentration of PPF (Caixeta *et al.,* 2016; Truong *et al.,* 2016). Data is also available on the ecotoxicological effects of fish larvae and adults (Brito *et al.,* 2017; Kreutz *et al.,* 2008; Pimpo *et al.,* 2013). The embryo of zebrafish that exposed to the pyriproxyfen at lower to the medium micro-molar concentrations at 120hpf showed neurotoxic effects (Truong *et al.,* 2016). Additionally, the microcephaly and other neurodevelopmental affects was not caused by the pyriproxyfen (Dzieciolowska *et al.,*2017), but PPF has been shown to interfere with the signaling of thyroid hormones (THs), that is important hormone for the amphibian growth (Denver *et al.,*2009; Spirhanzlova *et al.,*2018; Wegner *et al.,* 2016).

On the toxicity hazards of PYR, several studies lead to different conclusions. According to the Joint FAO Meeting on Pesticide Residues (JMPR), PYR was not considered to be a genotoxic or carcinogenic chemical (Invast *et al.,* 2008). PYR had no obvious effects on the stem cells number in the emerging central nervous system of zebrafish embryo's or on the abnormalities in the brain (Dzieciolowska *et al.,* 2017).

Oxidative stress caused by pyriproxyfen:

During the process of development, embryos are highly venerable to the toxics that are produced by the environment, such as pesticides (Yang *et al.,*2009). When the organism is under the stressors of environment, then showed oxidative stress. In the embryos of zebra fish, PPF at high doses, caused oxidative stress, so the level of antioxidants such as reactive oxygen species (ROS), increased in reaction to the stress caused by the PPF. the level of other antioxidants like peroxidases (POD) increased while the level of GSH declined. The histopathological determination in the zebra fish that were exposed to the pyriproxyfen showed weakening of the muscles of heart, hyperemia, as well as peri-cardia edema at high dose administration (Maharajan *et al.,* 2018).

Reproductive toxicity:

When the different concentrations of pyriproxyfen was applied on the rat pups. The results showed neuro- developmental abnormalities such as arhinencephaly and had less brain size (Evans *et al.,* 2016). When insecticides are persistently used, they lead to the disturbance of endocrine balance so harm the male reproductive system (Bergstrom *et al.,* 1996; Moller *et al.,* 2001). In mice, pyriproxyfen impairs the process of spermatogenesis and development of testis (Shahid *et al.,* 2019). So many studies on PPF identified its toxicity on different organisms but no research done on the evaluation of body mass index, level of antioxidants and histopathological abnormalities on rats, therefore the aim of this research is to determine the toxic risk assessment of pyriproxyfen on the reproductive system of adult male Sprague Dawley rats.

Aim and objectives:

This experiment has aim to investigate the toxic effect of pyriproxyfen on reproductive system of adult male rats. The objectives of this study are to:

- Determine the BMI changes caused by pyriproxyfen.
- Evaluate the body weight changes caused by pyriproxyfen.
- Calculate the Blood Glucose level changes caused by pyriproxyfen.
- Determine the oxidative stress, induce by pyriproxyfen in testes of rats.
- Evaluate histopathological changes induced by pyriproxyfen.

Materials and methods:

Current study was performed in the Lab of Reproductive Physiology, Zoology Department, Quaid-e-Azam University, Islamabad, Pakistan. Animals handling and all procedures were assessed and permitted by the ethical committee of the department which is research specific in animal handling. The recommendations for the appropriate maintenance and usage of research lab animals were considered while performing all the processes in the study.

Animals:

Twenty adult male Sprague Dawley rats (*Rattus norvegicus)* having average weight of 150±10g were taken from the primate faculty of Department of Animal Sciences, Quaid-e-Azam University. They were randomly kept into four groups and housed in separate stain stainless steel cages. These animals were kept during the experimentation, in well oxygenated room with temperature of 20-26˚C and twelve hours dark and light cycle for 28 days. Food chaw and tap water was fed to the animals ad libitum throughout experiment.

Pyriproxyfen:

Pyriproxyfen used in the experiment purchased from ANQA AGRO Multan, having PPF (10.8% EC) has an active ingredient.

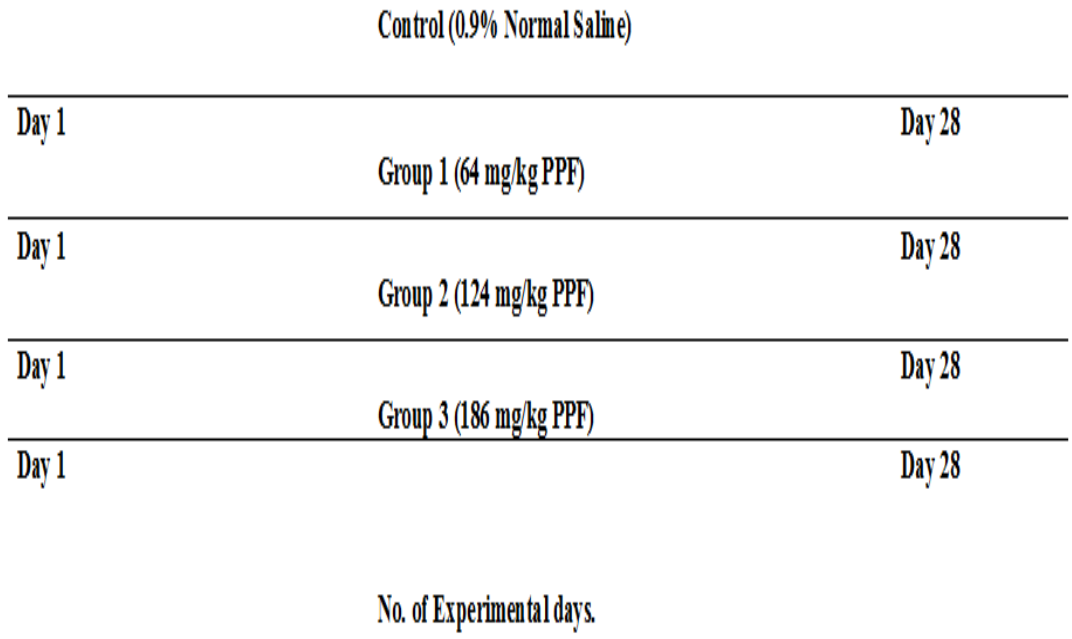
Preparation of Pyriproxyfen stock solution:

3ml PPF was mixed in 27ml of distal water to make final doses, and same process was repeated for 28 days to make fresh doses.

Experimental design

The current experiment aimed to estimate the reproductive toxicity of pyriproxyfen on male rats. The animals (n=5) were divided into four groups for the purposes of the study. For

around 28 consecutive days, all the doses were administered orally between 9 and 10 am, as shown in the below:



Control group:

The 'control group' was treated with 186mg/kg of 0.9% normal saline.

Group I:

Animals received 62mg/kg of pyriproxyfen.

Group II:

Rats were provided with 124mg/kg of pyriproxyfen.

Group III:

Animals were administered with 186mg/kg of pyriproxyfen.

Blood and Tissue collection:

The experiment was carried out over a period of 28 days, animals were weighed and decapitated on day 29. Following decapitation heparinized syringes were used to directly collect trunk blood, which was then stored in heparinized tubes. For 15 min, centrifuged blood

samples at a speed of 3000 rpm. Separated plasma was kept at -20ºC until analyzed. Following blood collection, reproductive organs including the testis and epididymis, were separated, and weighed after accessory fatty tissues were removed. Half of the reproductive organs were fixed in 10% PBS formalin solution having PH 7.4, for about 48 hours for the purpose of histology (fixation step), while the other half were stored at -80ºC for later used in oxidant/antioxidant study.

Determination of body weight:

Rats were weighed on day 1st, 14th and 28th of the experiment using top loading Sartorius Digital Balance (Germany).

Evaluation of Body mass index (BMI):

For the assessment of body mass index, the length of body (from nose to anus) was determined for all the rats on 28th day of the experiment. The calculation of BMI is as:

$$\text{Body mass index} = \text{Body weight in grams}/ (\text{Length in meter})^2 \ (m^2)$$

The normal level of BMI in male rats is (0.45 ± 0.02- 0.68 ± 0.05). (Novelli *et al.,* 2007).

Blood glucose level determination:

The blood glucose level was measured on the 1st, 14th and 28th days of the experiment using a EasyGluco Auto-coding (INFOPIA Co., Ltd. Korea) Glucometer. The measurement of glucose level was measured empty stomach. On the 1st day, blood was drawn from the tip of the tail by using sterile pricking needle and placed on the glucometer strips. The glucometer displayed a reading which was then recorded. The same experiment was carried out on all rats on days 14 and 28 and the readings were recorded. The normal blood glucose level in rats is 50-135mg/dl.

Determination of reproductive organ weight:

On the day 28, reproductive organs were separated, including the testis and epididymis, and then removed all the fatty tissues and weighed using top Sartorius Digital Balance (Germany). Similarly, accessory reproductive organs (seminal vesicles and prostate) were separated and weighted.

Body organs weight determination:

Body organs (brain, kidneys, heart, liver, and pancreas, were separated and then weighed on the 29th day of the experiment.

Biochemical analysis:

To the estimation of biochemical parameters, blood plasma was taken and kept at 20ºC. The frozen samples were thawed and utilized for the determination of antioxidant status of all the treatment groups and control groups.

Superoxide Dismutase activity (SOD):

SOD activity was evaluated by the procedure of (Kakkar *et al.,* 1984).

Reagents:

Sample 0.3ml

Phenazine methosulphate 0.1ml

Sodium pyrophosphate buffer 1.2ml

NADH 0.2ml

Glacial acetic acid 1ml

Procedure:

For the determination of SOD level, 0.3ml of sample was mixed with 0.1ml of 186µM phenazine methosulphate, 1.2ml of sodium pyrophosphate buffer having pH of 7.0 was added, then 0.2 ml of sodium hydroxide was added. At last, after one minute, the reaction was stopped by the addition of 1 ml of glacial acetic acid, the reading was observed at 560 nm. The findings were expressed as units per milligrams of protein.

Guaiacol peroxidase Activity (POD):

The activity of POD was determined using a technique proposed by (Chance and Maehly, 1955).

Reagents:

Homogenate 0.1ml

Phosphate buffer 2.5ml

H_2O_2 0.3ml

Guaiacol 0.1ml

Procedure:

For the estimation of POD activity, the reaction was maintained by adding 2.5ml of 50mM PB having pH 5.0, then 0.3ml of 40 mM H_2O_2, and 0.1ml of 20 mM guaiacol into 0.1ml of homogenate. Changes in the absorbance at 470 nm were observed after 1 minute. POD activity was recorded as 0.01-unit change in absorbance over a minute.

Estimation of Reactive Oxygen Species Assay:

The protocol of Nagasaka *et al.,* 2007 was used for the evaluation of ROS in testicular tissue.

Reagents:

Solution mixture 20µl

Buffer 1.2 ml

Homogenate 20µl

Procedure:

The pH of 0.1M of sodium acetate buffer was maintained at 4.8 by adding 4.1 gram of sodium acetate to 500 ml of distilled water. A second solution was prepared by adding 50 mg of $FeSO_4$ to 10ml of sodium acetate buffer. Both solutions were mixed 1:25 and incubated at room temperature in the dark for 20 minutes. Then 20µl of solution mixture, 1.2 ml of buffer and 20µl of homogenate were taken in the cuvette and absorbance was measured at 505 nanometer using Smart Spec TM plus spectrophotometer. 3 readings were obtained every 15 seconds for each sample.

Catalase (CAT) Activity:

With minor alterations, (Chance and Meahly, 1955) method was used to determine catalase activities.

Reagents:

Plasma 0.1ml

Phosphate buffer 2.5ml

H_2O_2 0.4ml.

Procedure:

In a cuvette, 0.1ml of plasma, 2.5 milliliter of 50mM PB having pH of 5.0 and H_2O_2 0.4 milliliter of 5.9 mM were mixed to measure the CAT level in the testicular tissue. After

one minute, there was a change in the absorbance of the solution at 240nm. CAT activity was noted as a 0.01unit variation in absorbance in one min.

Tissue histology:

PPF antifertility effect on the testis and epididymis were measured using histology of testicular and epididymal tissues. After the collection of tissues of testis and epididymis, the next procedures were carried out.

Fixation:

Tissue was placed in Sera----------for 4-6 hours.

Tissue was immersed in 10% of formalin----------for about 24 to 48 hours.

Dehydration:

At ambient temperature, tissues were dehydrated with ascending levels of alcohol after fixation as given,

For 60 minutes----------in 70% Ethanol.

For 60 minutes----------in 80% Ethanol

For 1 hour----------in 90% Ethanol

For 1 hour----------in 95% Ethanol

For 1.5 hours----------in 100% Ethanol I.

For 1.5 hours ----------in 100% Ethanol II.

For 1.5 hours ----------in 100% Ethanol III.

Embedding:

subsequent steps were carried out for embedding purpose. Placed the tissues in xylene and fixed in paraffin in the order.

For 60 minutes----------in xylene I.

For 60 minutes----------in xylene II.

For 2 hours----------in paraffin I at 58˚C.

Following that, the tissues were transported in molten wax within a boat. The wax was allowed to solidify after the removal of bubble. Trimmed and set on wooden blocks, tissue-filled paraffin wax blocks were sectioned using a knife or scalpel.

Albumen Slides preparation:

Preparation of Albumin:

Following procedure was carried out for the preparation of albumin:

- Two egg whites were added to 1200mL of deionized water.
- After that, stir for 5 minutes with a magnetic stirrer.
- Next, 4ml of ammonium hydroxide was added.
- For 5 minutes, stirred again.
- Then passing through a low-grade filter (coffee filter).
- In the end, albumin was kept in a glass bottle with a screw cap at 40 C and in the dark.

Coating Slides:

For slide coating following procedure was taken.

- Individually laid out slides were coated at a low setting on-slide warmer.
- Using a small clean paint brush, a thin layer of albumin was painted on each slide. The procedure is carried out at least three times.
- The slides were allowed to dry overnight on the heating plate at a low setting.
- Slide coated albumin were kept in their original packaging at room temperature until required.

Microtomy:

After embedding the tissues in paraffin, they were mounted on wooden blocks with melted wax. Seven µm thin sections were cut with a microtome (in which wooden blocks were placed). Tissue-filled long ribbons were stretched before being fixed in clean albumenized glass slides that had been previously prepared. These slides were stored on a Fischer slide warmer set to 60 degrees Celsius. After that, glass slides were placed in an incubator for a night to finish stretching**.**

Staining:

Slides were stained in the following grades:

- For 60-180 seconds----------in xylene I.
- For 180 seconds---------- in xylene II.
- For 60-180 seconds ----------100% alcohol.
- For 180 seconds----------90% alcohol.
- For 180 seconds ----------70% alcohol.
- For 180 seconds ----------washed with water.
- For 8 minutes----------haematoxylin.
- For 2 minutes----------washed with water.
- For 1 minute----------Acidified alcohol.
- For 120 seconds----------Washed with water.
- For 2 minutes ----------Bluing water.
- For 2 minutes----------Washed with water.
- 10 dips----------90% Alcohol.
- For 120 seconds ----------Eosin
- For 120 seconds ----------washed with water.
- For 5 minutes----------in 90% Alcohol.
- For 5 minutes----------in 100% Alcohol I
- For 5 minutes----------in 100% Alcohol II.

- For 5 minutes----------in 100% Alcohol III.
- For 5 minutes----------in Xylene I.
- For 5 minutes----------in Xylene II.

Then, on each slide, add 3 small drops of DPX were put to per mount, before per mount were dried placed cover slip gently over slides.

Light Microscopic Study:

Tissue sections (7µ thick) were observed under a light microscope at 40 magnifications (Nikon, 187842, Japan). All experimental groups, slides were examined. The testicular luminal and tubular diameters, epithelial height, and epididymal diameter (tubular and lumen) and epithelial height were calculated for Histomorphometry analysis using image J2x software, package programme.

Microphotography:

Microphotography was carried out using a Leica LB microscope (Germany) equipped with a Canon digital camera (Japan).

Statistical Analysis:

One-way analysis of variance (ANOVA) was carried out using SPSS software, and then post-hoc Tukey's test was performed to compare various groups. Mean ± SEM were used to present all the findings. The significance level was set to $p<0.05$.

Results:

Body weight:

There was non-remarkable decrease in body weight recorded among all the groups when compared with the control at day 1. No significant decrease(p=0.142) was noted at day 1, At day 14 the change (p=0.084) was noted and at day 28 (p=0.407) was observed. There was no significant decrease in body weight was observed in body weight at day 1, 14 and 28, when comparison was made among groups at high and low doses of the pyriproxyfen.

Table 1: Mean ± SEM body weight(g) among adult male rats in control and treated groups.

Parameters		Experimental groups			
Body weight(g)	**Days**	**Group 1**	**Group 2**	**Group 3**	**P-value statistics**
	1	155.49 ± 1.89	146.48 ± 1.05	143.48 ± 1.24	138.87 ± 1.26	0.142
	14	146.88 ± 0.88	141.87 ± 1.61	136.40 ± 1.26	130.59 ± 1.50	0.084
	28	141.00 ± 1.48	136.79 ± 1.40	134.49 ± 1.59	127.62 ± 1.75	0.407

Values are specified as Mean ±SEM. *, **, *** showing significance variance at p< 0.05, p<0.01, p<0.001, respectively. **a** represents Value vs control, **b** indicates Value vs group 1and **c** signifies Value vs. Group 2.

Body mass index:

There was no significant decrease recorded in the body mass index when comparison was held between control and treatment groups. When comparison was held between control and group 1 there was no significant decrease (p=0.909) recorded. When comparison was made between control and group 2 no change (p=0.763) was observed. (p=0.7260 showed between control and group 3. Non-remarkable change (p=0.852) was observed when comparison was held between group 1 and 2. Group 1 and 3 showed no remarkable decline (p=0.813). while group 2 and 3 showed no significant difference (p=0.961).

Table 2: Mean ± SEM body mass index(kg/m^2) among adult male rats in control and treated groups.

Parameter	Control	Group 1	Group 2	Group 3	p- value statistics
Body mass index(kg/m^2)	8.18 ± 0.56	8.05 ± 0.51	7.97 ± 0.75	7.77 ± 0.45	0.982

Values are specified as Mean ±SEM. *, **, *** showing significance variance at p< 0.05, p<0.01, p<0.001, respectively. **a** represents Value vs control, **b** indicates Value vs group 1and **c** signifies Value vs. Group 2.

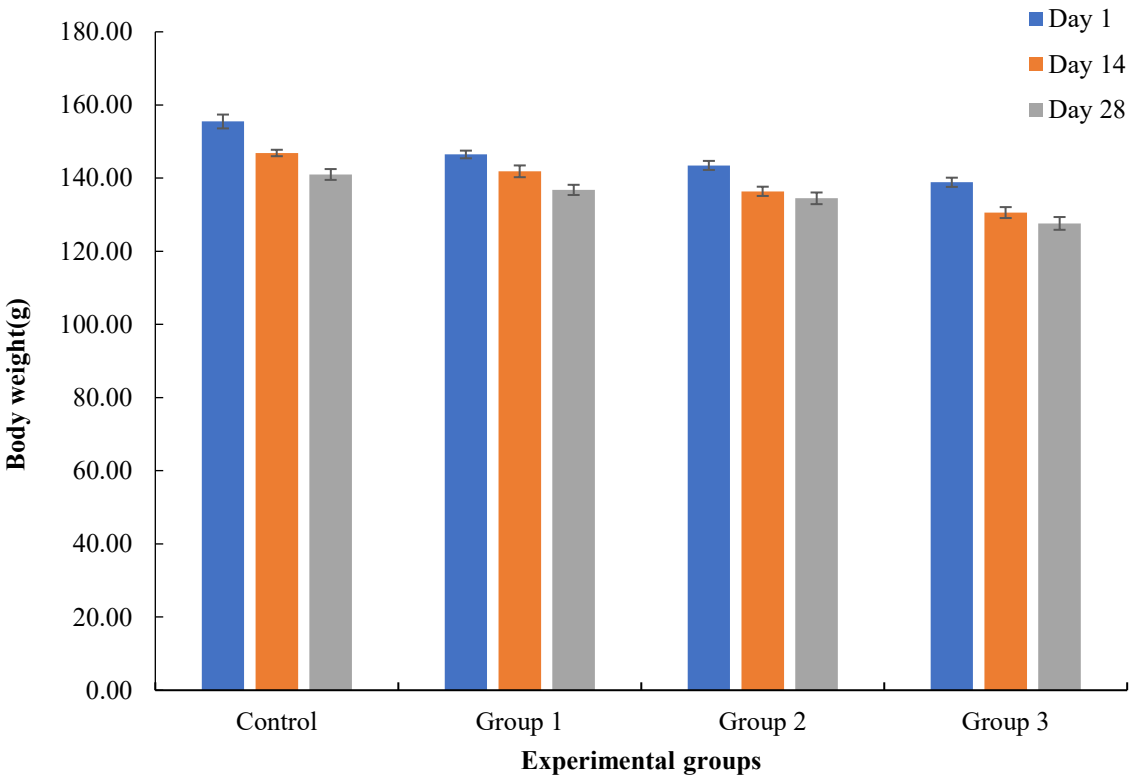


Figure 4: Comparison of body weight (g) among adult male rats in control and treated groups at day 1, 14 and 28.

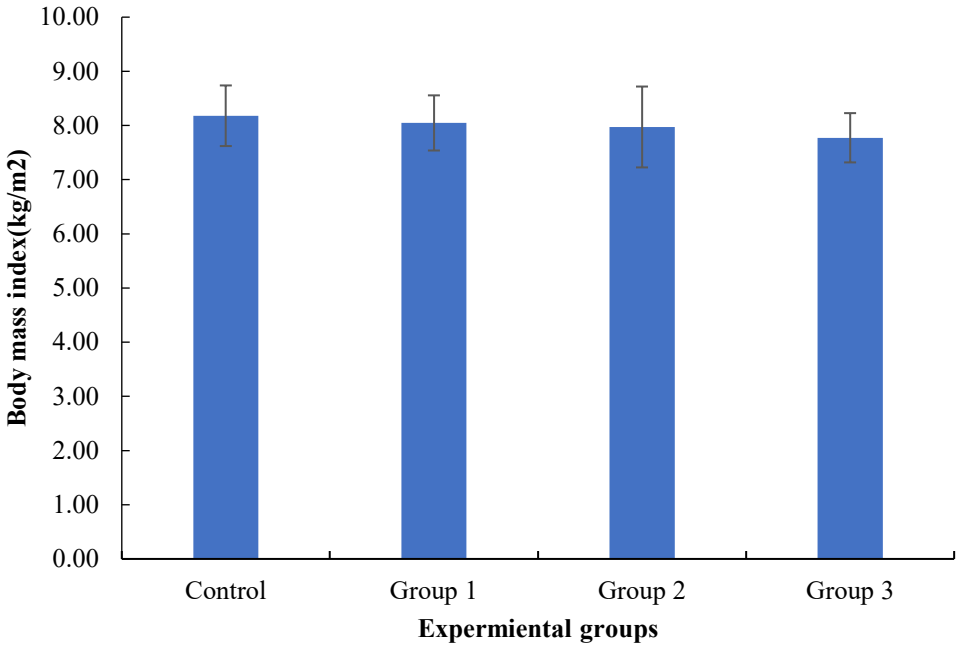


Figure 5: Comparison of body mass index among adult male rats in control and treated groups.

Blood glucose level:

At day 1, highly significant decrease ($p<0.001$) was noted in the blood glucose level among all the groups when compared with the control. When comparison was made among groups a highly significant decrease ($p<0.001$) was noted. At day 14, all the groups showed highly significant decrease in the blood glucose level in comparison with the control. While a significant decrease ($p<0.01$) was shown when group 1 and 2 was compared. Highly significant decrease in blood glucose level was shown when group 1 and 2 were compared with group 3. At day 28, highly significant decrease ($p<0.001$) was noted in the blood glucose level among all the groups when compared with the control. While there was no significant change when group 1 and 2 were compared. While group 3 showed highly significant decrease ($p<0.001$) in blood glucose level when compared with group 1 and 2.

Table 3: Mean ± SEM blood glucose level(mg/dl) among adult male rats in control and treated groups.

Parameter		Experimental groups			
	Days	**control**	**Group 1**	**Group 2**	**Group 3**	**P-value statistics**
Glucose level (mg/dl)	1	108 ± 0.71	105.6 ± 0.81 a***b***c***	101.8 ± 0.86 a***b***c***	94.40 ± 1.12 a***b***c***	0.000
	14	108.6 ± 1.21	103.4 ± 0.40 a***	100 ± 0.89 a***b**	92.40 ± 1.28 a***b***c***	0.000
	28	109 ± 1.22	102.8 ± 0.58 a***	98.4 ± 0.67 a***	90.60 ± 0.24 a***b***c***	0.000

Values are specified as Mean ±SEM. *, **, *** showing significance variance at p< 0.05, p<0.01, p<0.001, respectively. a represents Value vs control, b indicates Value vs group 1and c signifies Value vs. Group 2.

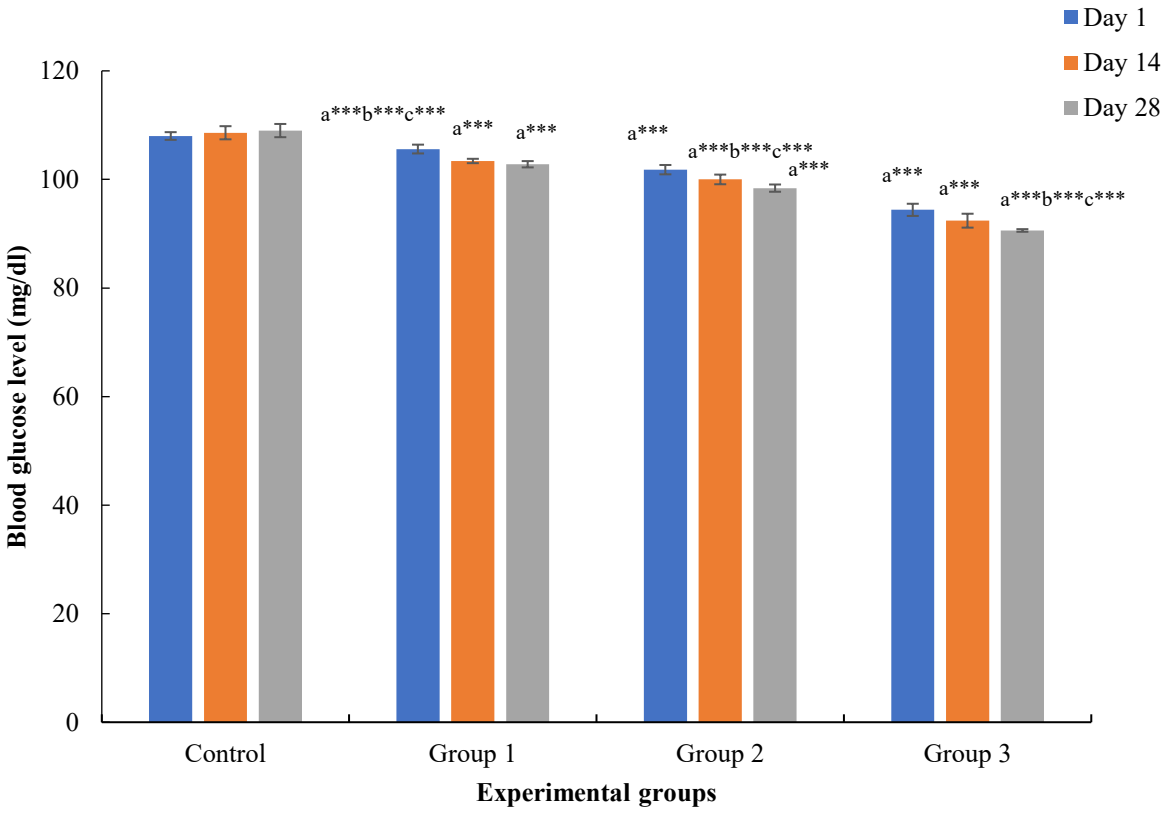


Figure 6: Comparison of blood glucose level (mg/dl) among adult male rats in control and treated groups.

Testis weight:

No remarkable change was noted in the testicular weight (right and left) in group 1 and 2 when compare with the control ($p<0.001$) while when the comparison was made between control and group 3, a highly significant decrease ($p<0.001$) in testicular weight was noted. When group 2 was compared with group 1, a non-significant change ($p<0.378$) in both the testicular weights was noted. Highly significant decrease ($p<0.001$) was observed in testicular weight when group 2 was compared with group 3.

Epididymis weights:

There was non-significant ($p=0.274$) decrease in the left and right epididymal weights when group 1 and 2 was compared with control. While when group 3 was compared with control a significant decrease ($p<0.05$) was shown in case of right epididymis weight. When group 2 and 3 were compared with the control a negligible decrease was noted in both right and left testis weights. When the comparison was made between group 2 and 3 a non-significant decrease ($p<0.07$) in the epididymal weight was noted**.**

Accessory organ weight:

No remarkable change in weight of prostate ($p=0.466$) was detected among all groups in comparison with control. When group 1 was compared with control no-significant change was observed in the seminal vesicle weight. While group 2 and 3 showed a remarkable change ($p<0.05$) in seminal vesicle when compared to control.

Table 4: Mean ± SEM testicular, Epididymis, and Accessory Organ weights after 28 days of exposure among adult male rats.

Parameters	Control	Group 1	Group 2	Group 3	P-value Statistics
Testis weights (R)	3.45 ± 0.00	3.46 ± 0.01	3.46 ± 0.01	3.42 ± 0.01 ^{a***b***c***}	0.000

Testis weights (L)	3.47 ± 0.01	3.46 ± 0.01	3.44 ± 0.01 ^{a***}	3.39 ± 0.00 ^{a***b***c***}	0.000
Epididymis weights(R)	0.54 ± 0.02	0.52 ± 0.02	0.47 ± 0.03	0.45 ± 0.02^{a*}	0.074
Epididymis weights(L)	0.52 ± 0.04	0.51 ± 0.03	0.48 ± 0.02	0.44 ± 0.02	0.287
Prostate weights(g)	0.58 ± 0.04	0.56 ± 0.05	0.52 ± 0.07	0.48 ± 0.02	0.466
Seminal vesicle(g)	0.76 ± 0.04	0.72 ± 0.07	0.62 ± 0.04 ^{a*}	0.58 ± 0.02 ^{a**b*}	0.051

Values are specified as Mean ±SEM. *, **, *** showing significance variance at p< 0.05, p<0.01, p<0.001, respectively. a represents Value vs control, b indicates Value vs group 1and c signifies Value vs. Group 2.

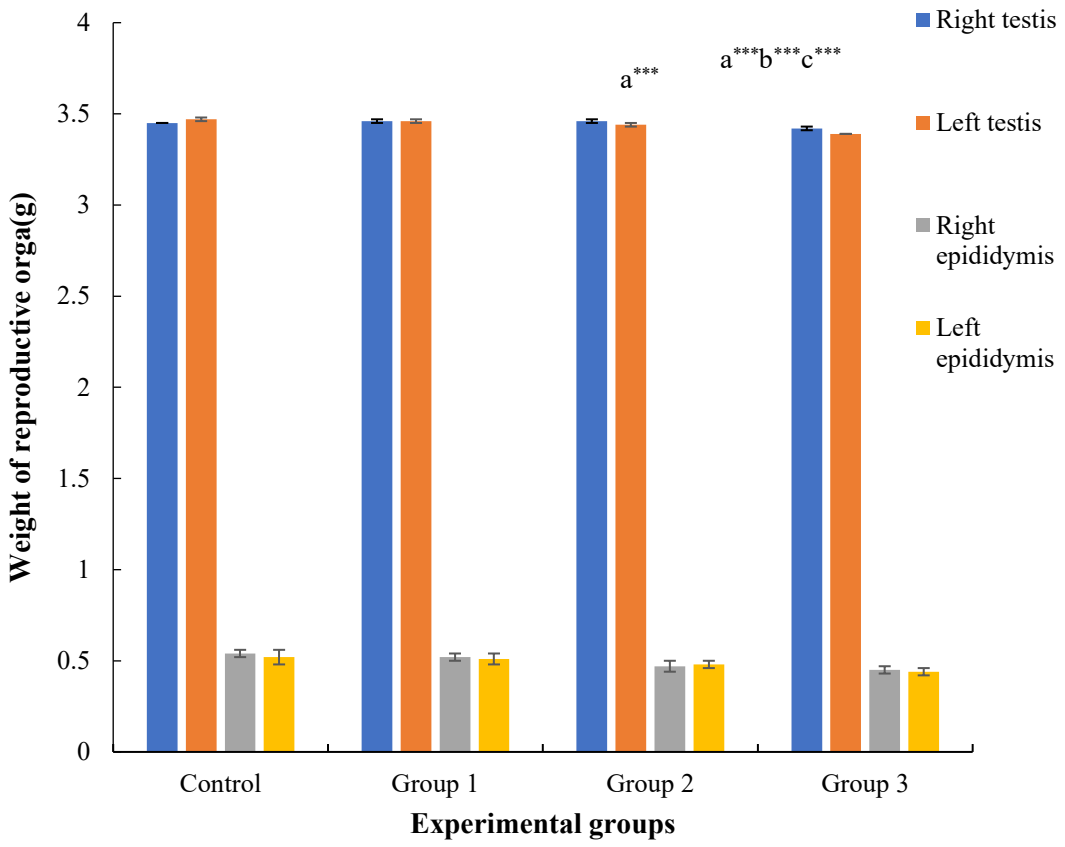


Figure 7: Comparison of weight testis (left and right) and epididymis (left and right) among adult male rats in control and treated groups.

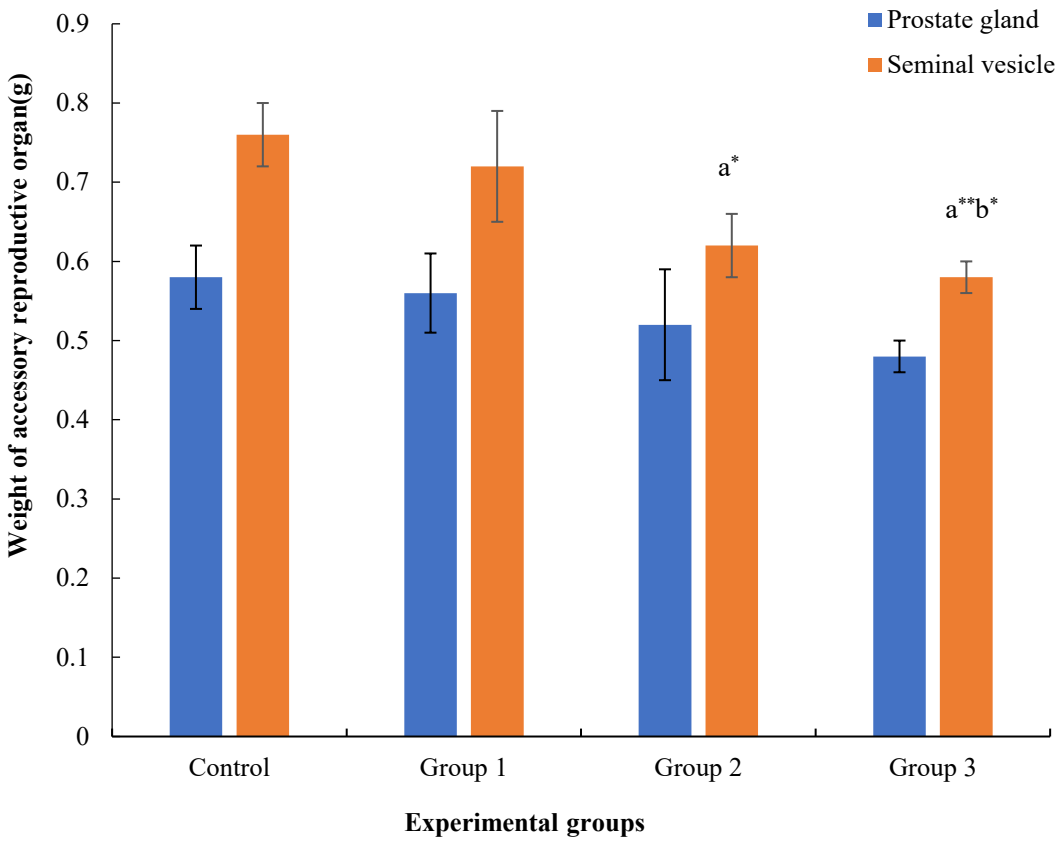


Figure 8: Comparison of accessory reproductive organs weight (seminal vesicle and prostate glands) among adult male rats in control and treated groups.

Body organ's weight:

Weight of Brain(g):

There was no remarkable increase in the weight of the brain was noted when comparison was held between control and treatment groups. When comparison was made between control and group 1, no remarkable increase (p=0.687) was noted. when comparison was made between group 2 and control, no significant change (p=0.605) was noted. When comparison was held between control and group 3, no remarkable change (p=0.458) was shown. Negligible increase (p=0.908) was noted when comparison was held between group 1 and 2. When group 1 and 3 were compared, no remarkable increase (p=0.730) was noted. When comparison was made between group 2 and 3, no significant increase (p=0.818) was observed.

Weight of Heart(g):

No significant increase in the weight of the brain was noted when comparison was held between control and treatment groups. When comparison was held between control and group 1, no significant increase (p=0.987) was noted. when comparison was observed between group 2 and control, no remarkable change (p=0.933) was noted. When comparison was noted between control and group 3, no remarkable change (p=0.638) was noted. Little increase (p=0.946) was noted when comparison was held between group 1 and 2. When group 1 and 3 were compared, no significant increase (p=0.650) was noted. When comparison was made between group 2 and 3, no remarkable increase (p=0.698) was noted.

Weight of Liver(g):

There was no remarkable increase recorded in the weight of liver when comparison was held between control and treatment groups. When comparison was made between control and group 1 there was non-remarkable increase(p=0.254) recorded. When comparison was held between control and group 2 no significant increase (p=0.0.115) was observed. (p=0.069) between control and group 3. Non-remarkable change (p=0.634) was noted when comparison was made between group 1 and 2. Group 1 and 3 showed no significant increase (p=0.455). while group 2 and 3 showed no remarkable difference (p=0.782).

Weight of Pancreas(g):

No significant increase in the weight of the pancreas was noted when comparison was made between control and all the treatment groups. When comparison was held between control and group 1, no remarkable increase (p=0.424) was recorded. No significant change (p=0.093) was noted when comparison was made between group 2 and control. When comparison was held between control and group 3, no remarkable change (p=0.064 was recorded. No remarkable increase (p=0.348) was noted when comparison was made between group 1 and group 2. When group 1 and 3 were compared, no change (p=0.260) was noted.

When comparison was held between group 2 and 3, no remarkable increase (p=0.842) was observed.

Weight of kidneys(g):

There was no significant difference noted in the weight of the left and right kidneys when comparison was recorded between control and all the treatment groups. There was no remarkable increase noted in the both the kidneys when comparison was held among groups. For left kidney (p=0.869) was recorded. While for right kidney, (p=0.717) was noted.

Table 5: Mean ± SEM body organs (brain, heart, liver, pancreas, and kidneys) weights after 28 days of exposure among adult male rats.

Parameters	Experimental groups				
	Control	Group 1	Group 2	Group 3	P-value statistics
Weight of brain(g)	1.74 ± 0.16	1.78 ± 0.17	1.79 ± 0.22	1.82 ± 0.15	0.893
Weight of heart(g)	0.63 ± 0.17	0.63 ± 0.19	0.64 ± 0.20	0.68 ± 0.21	0.958
Weight of liver(g)	4.05 ± 0.40	5.20 ± 0.44	5.67 ± 0.62	5.95 ± 0.65	0.258
Weight of pancreas(g)	0.30 ± 0.15	0.38 ± 0.17	0.47 ±0.18	0.49 ±0.19	0.208
Weight of kidney(g) (L)	0.51 ± 0.09	0.58 ± 0.10	0.58 ± 0.10	0.59 ± 0.11	0.869
Weight of kidney(g) (R)	0.51 ± 0.12	0.56 ± 0.09	0.60 ± 0.10	0.65 ± 0.13	0.717

Values are specified as Mean ±SEM. *, **, *** showing significance variance at $p< 0.05$, $p<0.01$, $p<0.001$, respectively. **a** represents Value vs control, **b** indicates Value vs group 1and **c** signifies Value vs. Group 2.

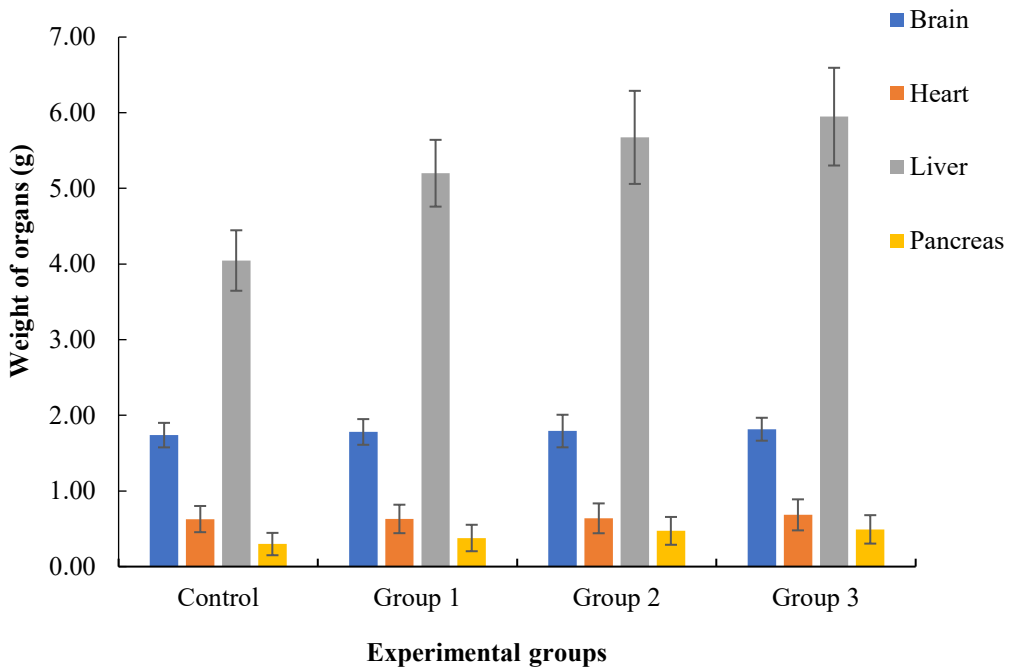


Figure 9: Comparison of organs weight (brain, heart, liver, and pancreas) among adult male rats in control and treated groups.

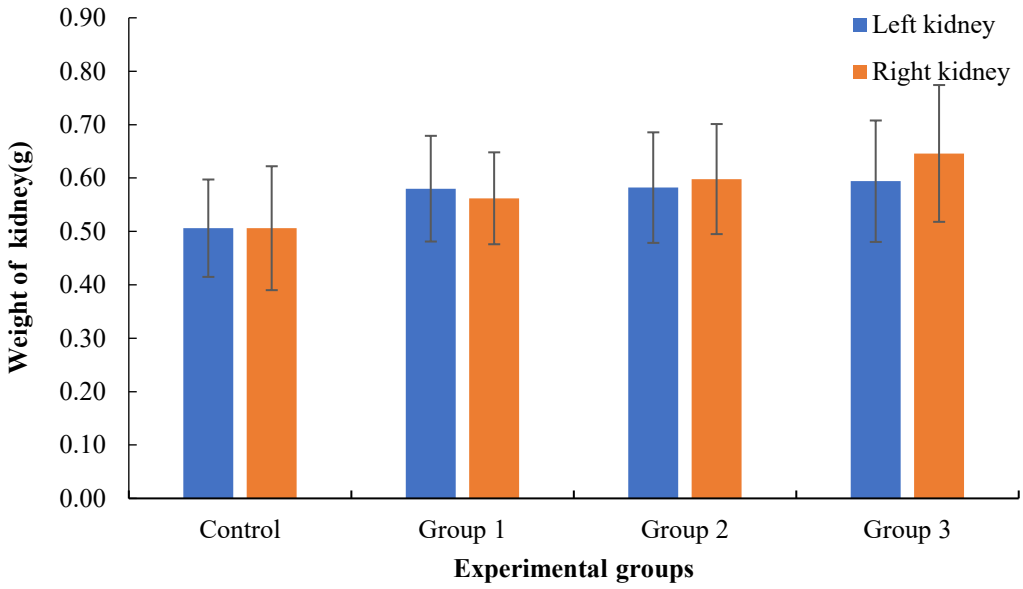


Figure 10: Comparison of organ weight (left and right kidney) among adult male rats in control and treated groups.

Sodium dismutase (SOD):

A highly significant increase ($p<0.001$) was observed in SOD levels among all the groups when compared with the control. There was no remarkable change observed ($p=0.971$) in SOD level when group 1 was compared with group 2. Group 3 shows non-significant increase ($p=0.899$) when compared with group 2. When the comparison is held between group 1 and group 3 a negligible increase ($p=0.870$) was observed.

Peroxidases (POD):

significant increase was observed in group 1 when compared with the control($p<0.001$), negligible increased was observed in POD levels when comparison was made between group 2 and control ($p=0.023$). when comparison was made between group 3 and control, no significant change was observed ($p=0.743$), there was no significant difference observed in POD levels when group 2 and 3 were compared ($p=0.047$). levels of POD were not significantly increased ($p=0.236$) when group 1 and 2 were compared.

Reactive oxygen species (ROS):

A significant rise in the ROS level ($p< 0.01$) was noticed in group 1 when comparison was made with control. Non-significant increase ($p=0.665$) in the ROS levels was seen in group 2 when compared with control. While significant rise ($p<0.001$) was observed when comparison was held between group 3 and control. A significant increase ($p<0.001$) in ROS level was observed when comparison was held between group 3 and group 2. Group 3 showed significant rise ($p<0.001$) in ROS level when comparison was made with group 1. Non-significant changes ($p=0.033$) in ROS level were observed when group 1 and 2 were compared.

Catalase (CAT):

A highly remarkable Increase in CAT levels was observed in the control group ($p<0.01$) when comparison was made with all the treatment groups while CAT levels were significantly rise in the group 3 ($p<0.05$) when a comparison was made with group 2. A non-significant

increase in CAT levels in the group 1 was seen (p=0.086) in comparison with group 2. when a comparison was made with group 3, a significantly rise in the group 1 (p<0.05) was found.

Table 6: Mean ± SEM biochemical parameters after 28 days of exposure among adult male rats:

Antioxidants/oxidants	Control	Group 1	Group 2	Group 3	P-value statistics
Sodium oxide dismutase (nmol min−1 mg−1 protein)	12.64 ± 3.77	19.94 ± 2.55 a***	23.03 ± 3.40 a***	29.04 ± 7.78 a***	0.000
Peroxidases (nmol min-1 mg-1 protein)	27.26 ± 2.52	31.53 ± 4.21 a***	35.18 ± 4.18	37.73 ± 4.65	0.206
Reactive oxygen species (U mg-1 tissue)	1.08 ± 0.15	1.12 ± 0.12 a**	1.13 ± 0.15	1.19 ± 0.24 a***b***c***	0.000
Catalase (U mg−1 protein)	13.32 ± 0.53	23.13 ± 0.56 a**	25.05 ± 0.67 a**	30.02 ± 0.89 a**b* c*	0.000

Values are specified as Mean ±SEM. *, **, *** showing significance variance at p< 0.05, p<0.01, p<0.001, respectively. **a** represents Value vs control, **b** indicates Value vs group 1and **c** signifies Value vs. Group 2.

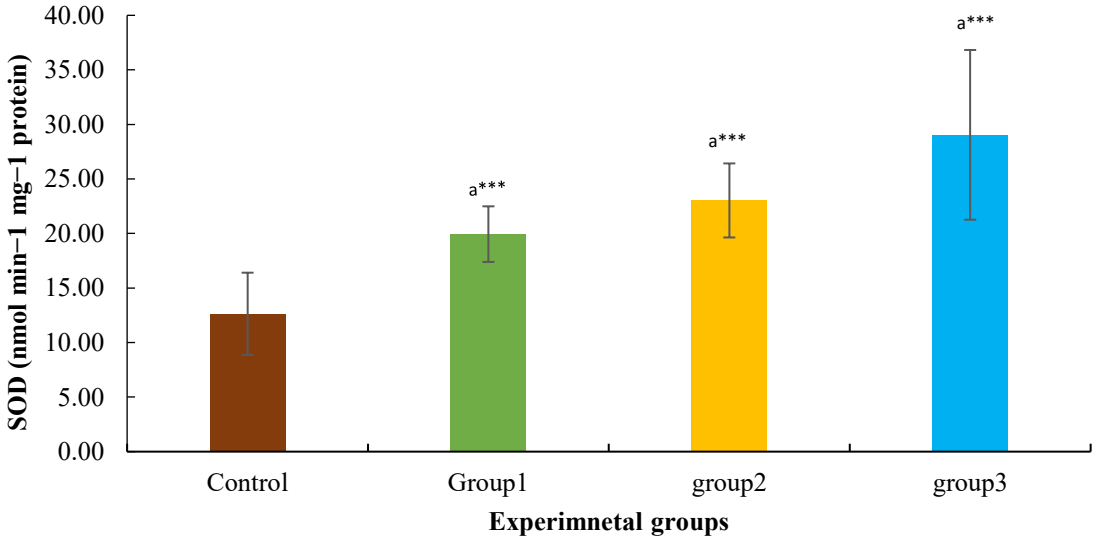


Figure 11: Comparison of blood plasma concentration of Superoxide dismutase (nmol min-1mg-1 protein) in control and treated groups.

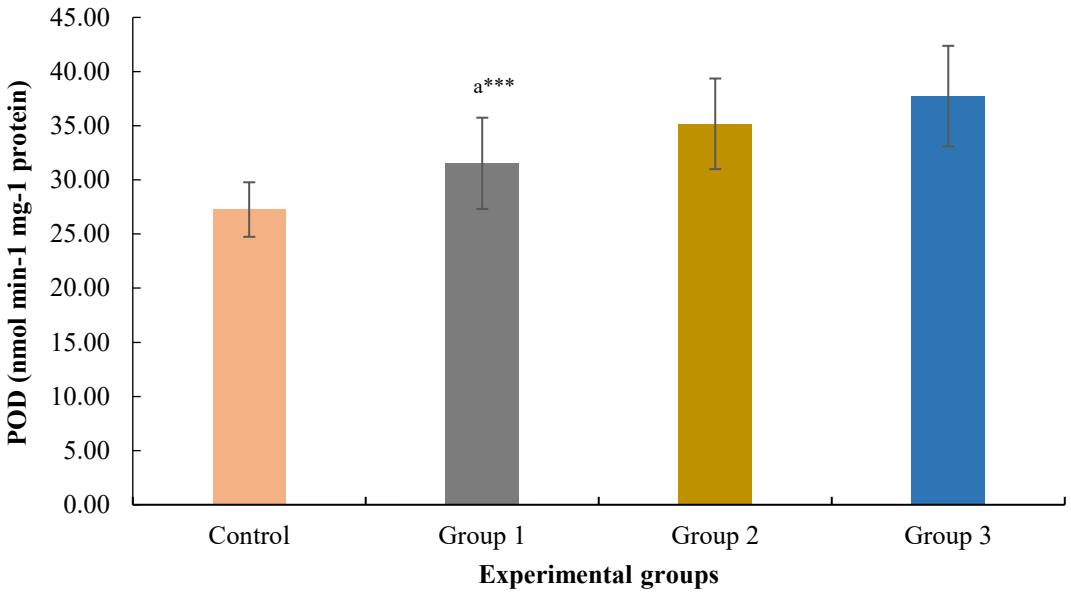


Figure 12: Comparison of blood plasma concentration of Peroxidase (nmol min-1 mg-1 protein in control and treatment groups.

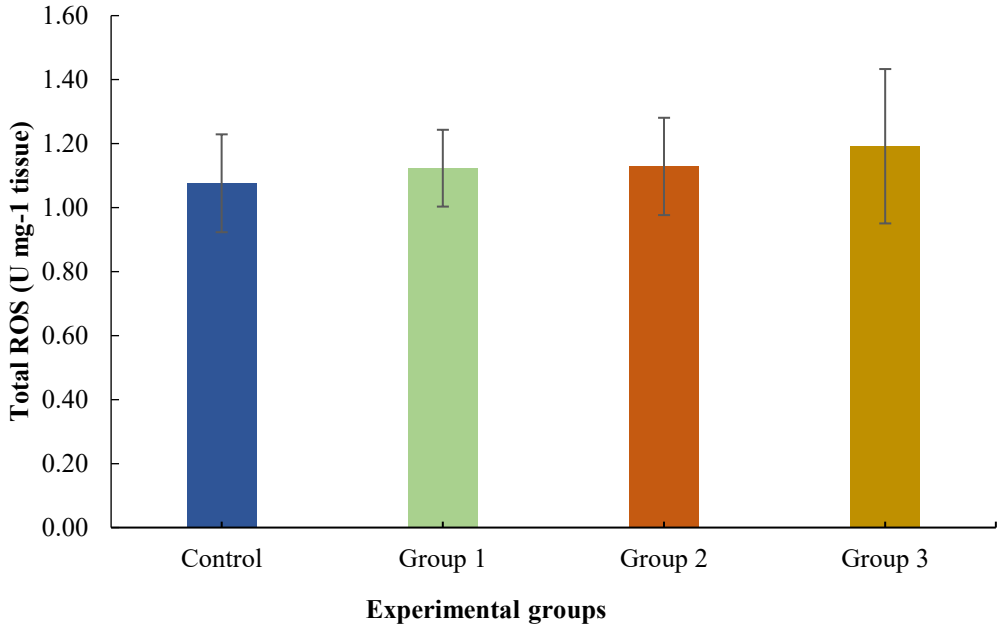


Figure 13: Comparison of blood plasma concentration of Reactive oxygen species (U mg-1 tissue) in control and treated groups.

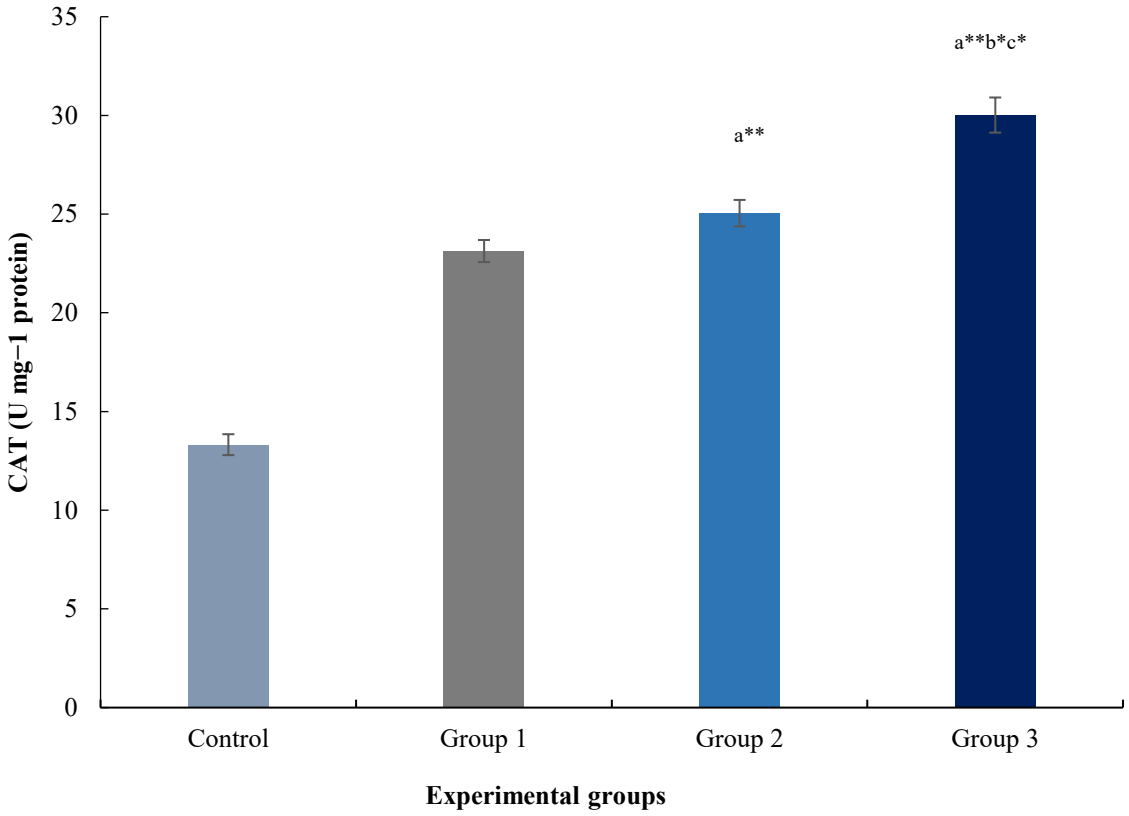


Figure 14: Comparison of blood plasma concentration of Catalase (U mg−1 protein) in control and treated groups.

Histopathological results:

In control group, seminiferous tubules were closely placed to each other, and held tightly with the presence of intact cellular organization. Many fresh spermatozoa were present in the luminal compartment. The Sertoli cells were tightly bound to each other. The rounded and oval shaped Leydig cells are enriched in the spermatozoa. Large blood vessels were seen around seminiferous tubules.

In the pyriproxyfen treated groups, testicular organization of seminiferous tubules was adversely affected. Normal organization of seminiferous tubules were absent. Sperm counts were decreased at high doses and in the lumen of seminiferous tubules vacuolization were visible clearly. Degeneration and scattering of spermatozoa were seen in rats that were treated with high doses of pyriproxyfen. Clumped spermatozoa were also observed at high doses.

There was notable increase in the tubular diameter when the comparison was made between control and pyriproxyfen treated groups. No remarkable difference seen at low doses of pyriproxyfen while at high doses, a remarkable increase was noted. When the comparison was made between control and group 1, no notable increase ($p<0.149$) was seen. Control and group 2 showed non-significant change ($p<0.146$) in tubular diameter. Similarly, no remarkable increase ($p<0.992$) in the tubular diameter was seen when group 1 was compared with group 2. While group 3 and group 2 showed significant increase($p<0.001$) in the tubular diameter. When comparison was made between control and group 3 a highly significant increase ($p<0.001$) was observed. A highly significant increase ($p<0.001$) was noted when comparison was made between group 1 ang group 3.

A remarkable increase in the lumen diameter was observed in the pyriproxyfen treated groups when compared with the control. No remarkable difference was noted at low doses of pyriproxyfen while high dose showed remarkable decrease in the lumen diameter. When the comparison was made between control and group 1, no notable increase ($p<0.111$) was noted Control and group 2 showed non-significant increase ($p<0.030$). Similarly, no remarkable change ($p<0.557$) was observed when group 1 was compared with group 2. Group 3 and group 2 showed non-significant increase($p<0.008$) in the lumen diameter. When comparison was made between control and group 3 a highly significant increase ($p<0.001$) was observed. Significant increase ($p<0.001$) was measured when group 1 and 3 was compared.

A considerable decrease was noted in the epithelial height between control and pyriproxyfen treated groups. A non- significant change ($p<=0.155$) was measured in epithelial

height when group 1 was compared with the control. When comparison was made between control and group 2 a highly significant decrease ($p< 0.001$) was observed. While control and group 3 showed highly significant decrease ($p< 0.001$) in the epithelial height. When comparison was made between group 1 and 2 a significant decrease ($p< 0.01$) was measured. No remarkable difference ($p<=0.186$) was noted between group 2 and 3. A highly significant decrease ($p< 0.001$) was measured between group 1 and 3.

Table 7: (Mean ± SEM) Evaluation of seminiferous tubule diameter (tubular, lumen), and epithelial height of testis after 28 days of exposure among adult male rats.

Parameters	**Control**	**Group 1**	**Group 2**	**Group 3**	**P-value statistics**
Tubular diameter(µm)	455.60 ± 6.37	540.63 ± 6.90	541.19 ± 8.50	797.84 ± 8.25 ^{a***b***c***}	0.000
Lumen diameter(µm)	200.15 ± 3.80	266.98 ± 4.65	281.65 ± 5.87	321.56 ± 5.00 ^{a***b***}	0.000
Epithelial height (µm)	380.90 ± 8.33	333.02 ± 8.13	241.90 ± 5.26 ^{a***b***}	190.49 ± 4.73 ^{a***b***}	0.000

Values are specified as Mean ±SEM. ***, **, ***** showing significance variance at **p< 0.05, p<0.01, p<0.001** respectively. **a** represents Value vs control, **b** indicates Value vs group 1 and **c** signifies Value vs group 2.

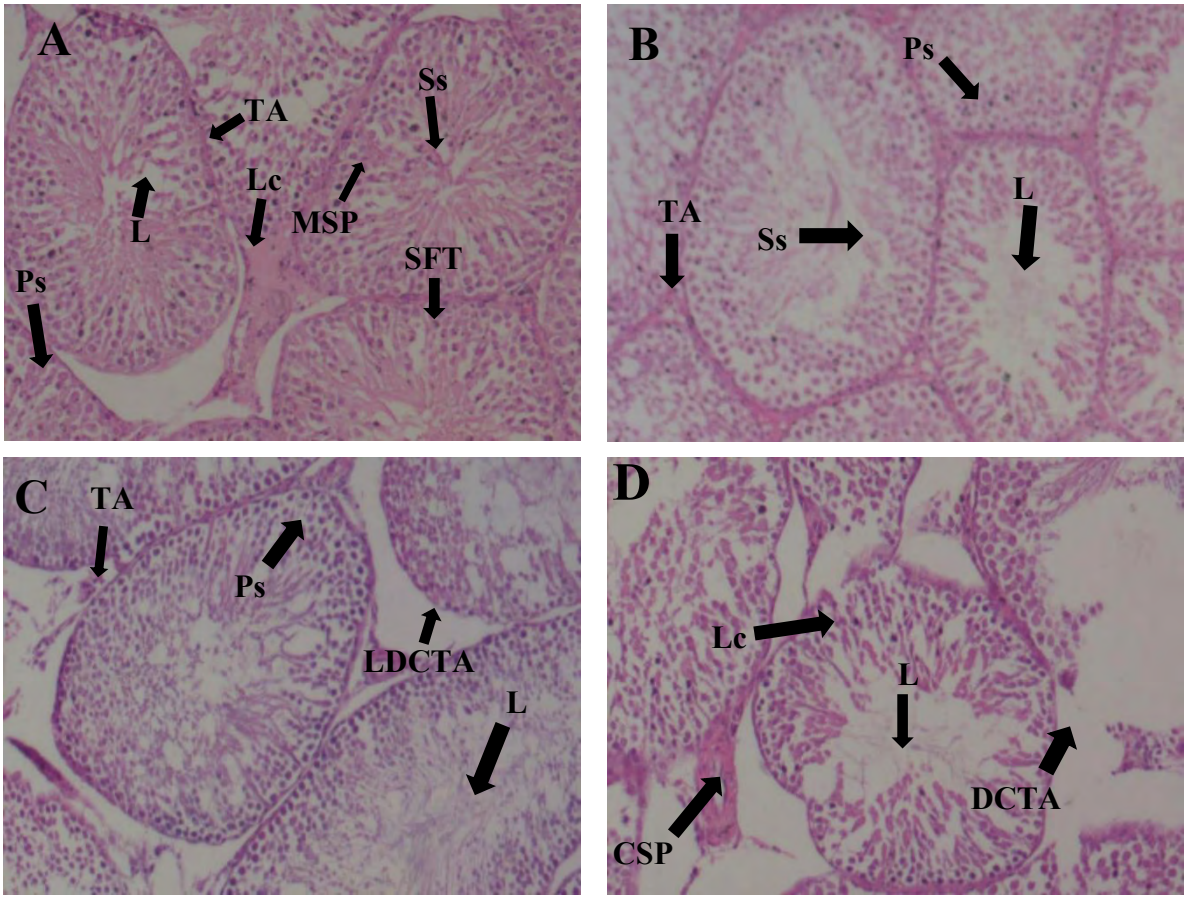


Figure 15: Photomicrograph of seminiferous tubules of testis of adult male rats. (A) control., after 28 days of the experiment, rat's testis having mature spermatozoa (MSP), normal testis, the normal seminiferous tubules (SFT), normal basement membrane of tunica albuginea (TA), reduced lumen (L), high number of Leydig cells (Lc), spermatozoa both kinds, primary spermatozoa (Ps) and secondary spermatozoa (Ss). (B) Group 1., number of sperms (S.) decline, and having Leydig cells that contained normal tunica albuginea (TA). (C) Group 2., showed little increased in lumen (L), primary spermatozoa (Ps), there was little degenerative changes in the tunica albuginea (LDCTA), Leydig cell was there (L). (D) Group 3., showed degenerative changes in the tunica albuginea (DCTA), increased lumen (L), decreased epithelium, increased in the tubular diameter, clumping of spermatozoa (CSP).

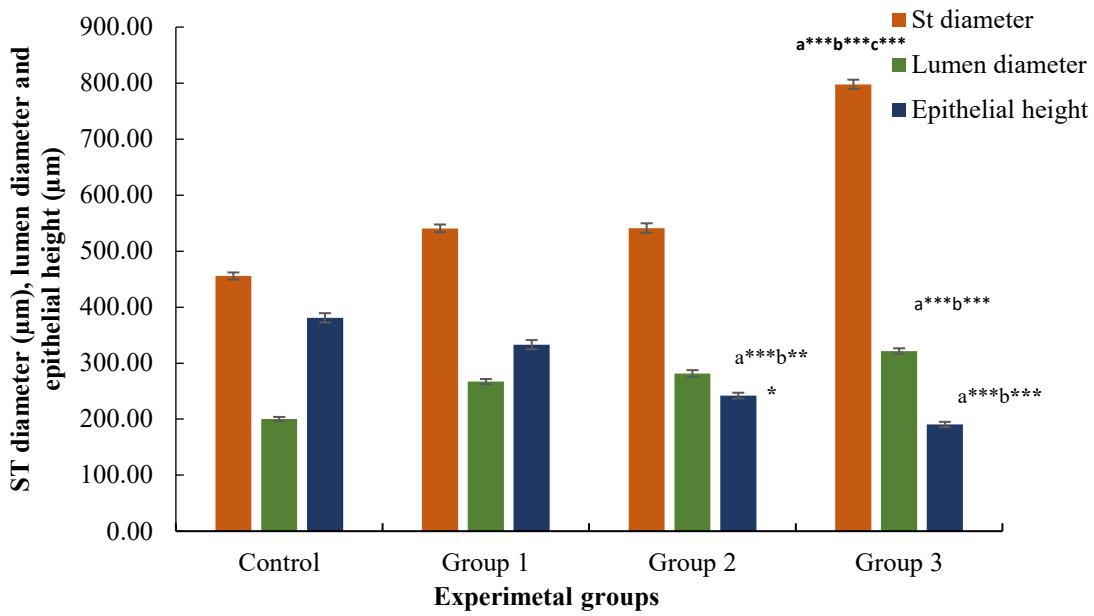


Fig 16: Mean ± SEM seminiferous tubular diameter (µm), lumen diameter and epithelial height (µm) of testis after 28 days of exposure among adult male rats.

Caput epididymis:

In the control group histo-morphological analysis of caput showed, larger tubular diameter and thin lumen and thin pseudostratified epithelium. The epididymal tubules were closely organized and enclosed by stroma. Lumen contained large number of spermatozoa, Caput tubular diameter, lumen diameter and epithelial height showed significant decrease in pyriproxyfen treated groups when compared with the control.

There was significant decrease ($p<0.005$) in the tubular diameter of caput when comparison was made between control and group 1. When group 2 and group 3 were compared with control a highly significant decrease ($p<0.001$) was measured. When comparison was held between group 1 and group 2 no remarkable difference ($p=0.306$)) was noted. When group 1 and group 3 was compared a highly remarkable decrease ($p<0.001$) was noted. A notable decline ($p<0.001$) was noted when group 2 and 3 was compared.

A highly significant decrease ($p<0.001$) in the lumen diameter of caput was measured when comparison was made between control and all the treatment groups. When comparison

was made between group 1 and group 2 a remarkable decrease ($p<0.005$) was noted. When group 1 and group 3 was compared a highly remarkable decrease ($p<0.001$) was observed. when group 2 and 3 was compared a significant decrease ($p<0.005$) was noted.

A non- remarkable decrease ($p=0.039$) in the epithelial height of caput was observed in group 1 when compared with control. A substantial decline ($p<0.001$) was observed in the epithelial height between control and group 2 and group 3. A non-substantial difference($p=0.035$) was noted when comparison was held between group 1 and group 2. When group 2 and 3 was compared a significant decline ($p<0.01$) was observed. A highly remarkable decrease ($p<0.001$) was noted when group 1 and 3 was compared.

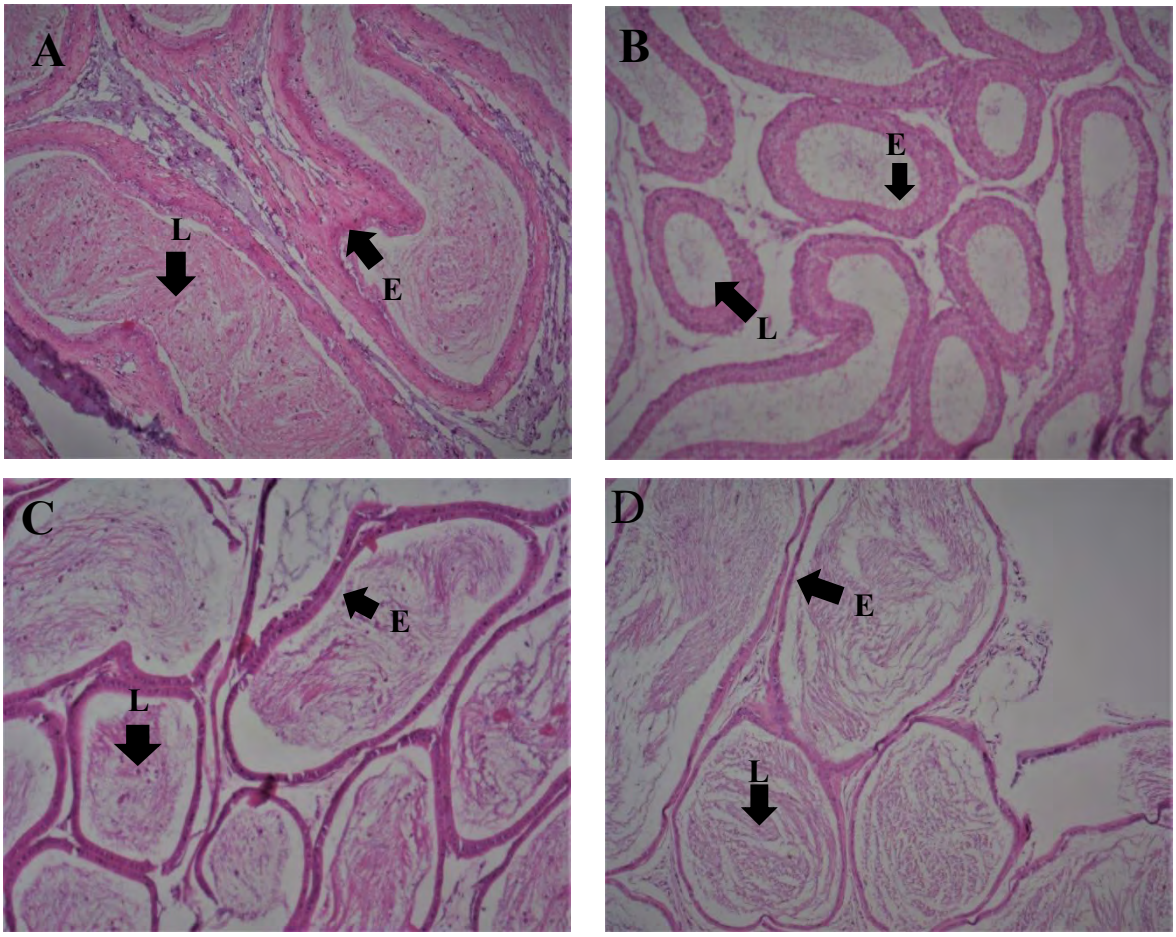


Figure 17. Photomicrograph of caput epididymis of adult male rats. (A) control., after 28 days of the experiment, rat's testis having normally organized seminiferous tubules (SFT), having lumen (L), Leydig cells are increased in number (Lc), substantial number of spermatozoa are present. (B) Group 1., decrease in number of sperms (S) decreased lumen (L), decreased in the tubular diameter. (C) Group 2., showed little change in lumen

(L), the tubular diameter was further reduced. (D) Group 3., showed degeneration of spermatozoa, decreased lumen (L), decreased epithelium, disorganized epididymal tubules.

Corpus epididymis:

In the control group, histomorphological analysis of caput showed, increased tubular diameter, and decreased pseudostratified epithelium. Stroma enclosed the closely arranged epididymal tubules. Large number of spermatozoa were present in the lumen.

Corpus tubular diameter showed non-significant decrease in pyriproxyfen treated groups when compared with the control while lumen diameter and epithelial height showed significant change in pyriproxyfen treated groups when compared with the control at high doses.

There was no significant decline in the tubular diameter of corpus at low and high doses of pyriproxyfen in comparison with control. The lumen diameter and epithelial height of corpus showed highly significant increase at high dose of pyriproxyfen while at low doses no significant change was measured.

No remarkable decline was observed when comparison was made between control and all the treatment groups. When group 1 was compared with control no significant decline ($p=0.063$) was measured. Group 2 showed no remarkable change ($p=0.008$) in comparison with control. Similarly group 3 and control showed non- significant results ($p=0.003$). When comparison was held among treatment groups no substantial decrease ($p=0.744$) was observed as comparison between group 2 and 3. Similar non-significant results ($p=0.263$) obtained when group 1 and 3 was compared. Non-substantial results ($p=0.428$) obtained when group 1 and group 2 was compared.

There was no significant change ($p=0.233$) observed in the lumen diameter of corpus when comparison was held between group 1 with control. While group 2 ($p<0.001$) and group 3 ($p<0.001$) showed highly significant increase in the lumen diameter in comparison with control. When the comparison is made between group 1 and group 2 a significant increase ($p<0.01$) was obtained. While group 1 and group 3 showed highly significant ($p<0.001$)

increase. Non-substantial increase (p=0.018) was noted when group 2 and group 2 was compared.

Non-significant increase (p=0.858) in the epithelial height of corpus was obtained when group 1 was compared with control. No remarkable change (p=0.039) was noted when group 2 and control was compared. While highly significant increase (p<0.001) was obtained when comparison was held between group 3 and control. When the comparison was made between group 1 and 2, non-substantial results (p=0.059) was obtained. Group 1 and group 3 showed highly significant increase (p<0.001) in the epithelial height. When comparison was made between group 2 and group 3, no significant increase (p=0.006) was noted.

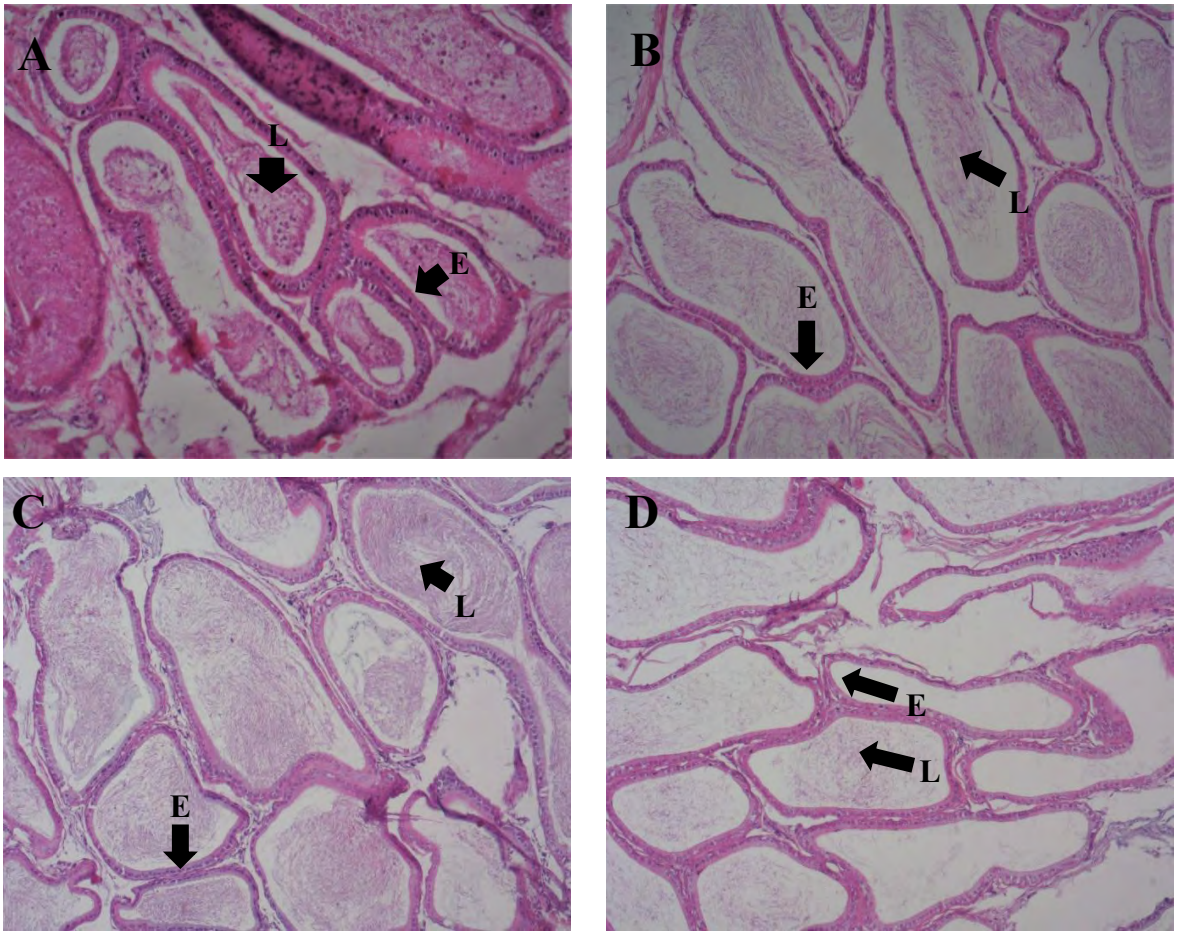


Figure 18: Photomicrograph of seminiferous tubules of corpus epididymis of adult male rats. (A) control., after 28 days of the experiment, rat's corpus showed normal morphology. Closely arranged tubules, lumen flushed with spermatozoa. (B) Group 1., decrease in number of sperms (S.), and decreased in lumen diameter. (C) Group 2., showed little decreased in lumen (L), there was little degenerative changes in the

spermatozoa. (D) Group 3., showed degenerative changes in spermatozoa in lumen, increased epithelium, decreased tubular diameter.

Cauda epididymis:

Epididymis of cauda in case of control group showed normal morphology with closely arranged tubules. tubules are surrounded by stroma with thick and pseudostratified at basal epithelium. The spermatozoa are occupied in the lumen. Epididymis has two types of cells. Principal cells and basal cells. Principal cells have short lumen and spread from basal lumen while basal cells are those cells that placed at basal lamina.

There was a significant decrease in the tubular diameter and epithelial height of caput observed at high dose of pyriproxyfen when compared with the control. While lumen diameter showed significant increase at high dose of pyriproxyfen. There was no considerable change noted at low doses of pyriproxyfen.

There was no considerable decline in the tubular diameter noted when the comparison was made among group 1($p=0.707$) and group 2 ($p=0.033$) with control. While when group 3 was compared with control a highly significant decline ($p<0.001$) was noted. When group 1 and group 2 was compared no remarkable change ($p=0.078$) in the tubular diameter was noted. While when comparison was held between group 1 and 3 a considerable decrease ($p<0.001$) was noted. A non-substantial decline ($p=0.104$) was observed when group 2 and 3 were compared.

No notable change ($p=0.477$) in the lumen diameter of cauda was observed when group 1 was compared with control. While group 2 ($p<0.01$) and group 3 ($p<0.001$) showed remarkable increase in the lumen diameter in comparison with control. There was non-significant increase ($p=0.008$) in lumen diameter when comparison was made between group 1 and group 2. While group 1 and group 3 showed remarkable change ($p<0.001$) in lumen diameter. When comparison was held between group 2 and group 3 a highly substantial increase ($p<0.001$) was measured. A highly significant decrease ($p<0.001$) in the epithelial height of cauda was measured when comparison was held between control and all the treatment groups. When the comparison was held among group 1 and group 2 a highly considerable

decline ($p<0.001$) was noted. When group 1 and 3 was compared a highly significant devaluation ($p<0.001$) of epithelial height was noted. While when comparison was built between group 2 and group 3 no remarkable change ($p=0.824$) was observed.

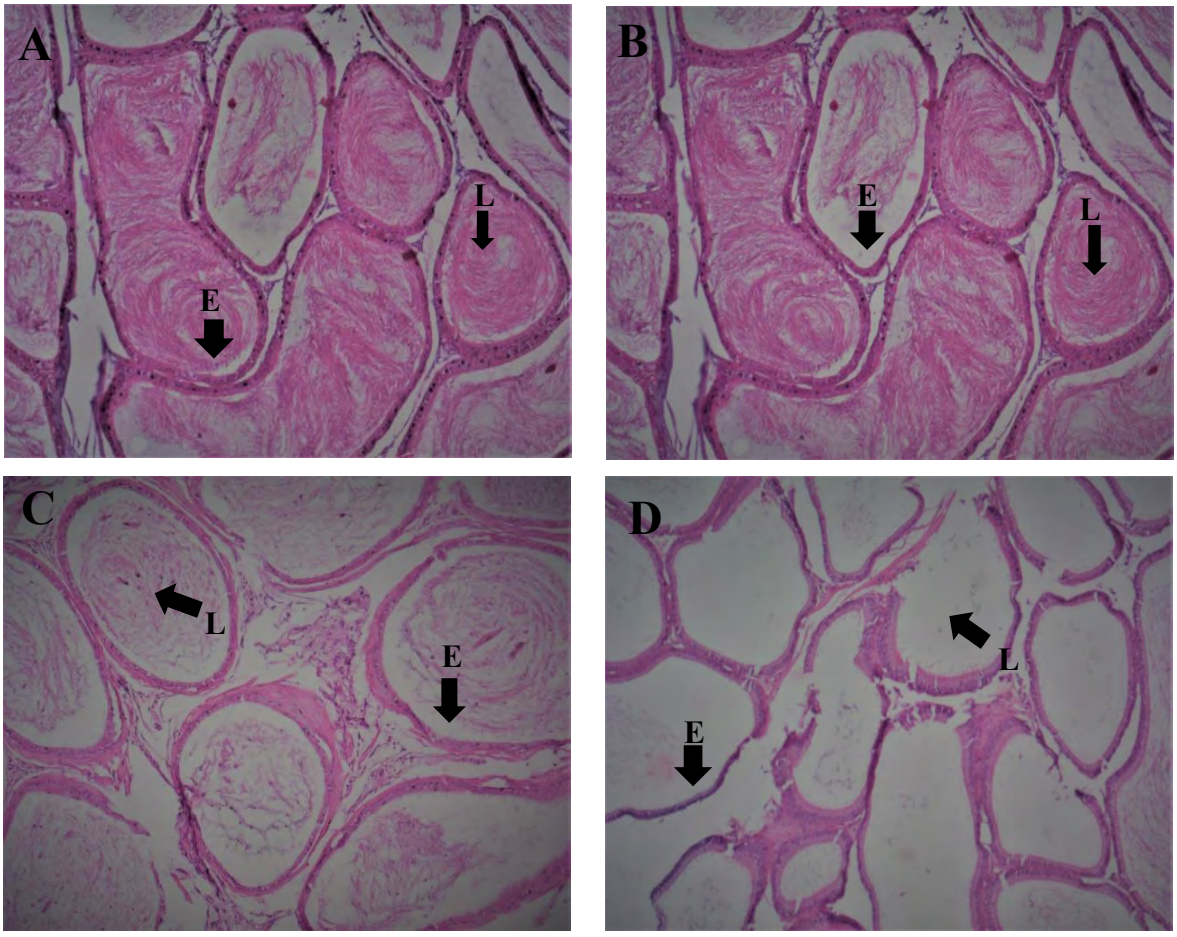


Figure 19: Photomicrograph of seminiferous tubules of caudal epididymis of adult male rats. (A) control., after 28 days of the experiment, rat's cauda showed normal morphology with closely arranged tubules, lumen flushed with spermatozoa. (B) Group 1., showing increased lumen diameter, decreased number of spermatozoa. (C) Group 2., showed little increased lumen (L), there was some degenerative changes in the spermatozoa. (D) Group 3., showed increased lumen (L), decreased epithelial height (H), increased tubular diameter.

Table 8: Mean ± SEM of ductular diameter (µm), diameter of lumen (µm) and height of epididymis cell (µm) of the epididymis after 28 days of exposure among adult male rats.

Parameters	Experimental groups				
	Control	**Group 1**	**Group 2**	**Group 3**	**P-value statistics**
Caput epididymis					
Tubular diameter(µm)	741.43 ± 8.06	619.96 ± 5.70	575.61 ± 5.23 a***	433.97 ± 6.17 a***b***c**	0.000
Lumen diameter(µm)	635.94 ± 7.96	451.93 ± 5.44 a***	335.54 ± 5.41 a***b***	255.53 ± 5.49 a***b**c**	0.000
Epithelial height (µm)	78.36 ± 3.45	68.15 ± 1.87	57.77 ± 1.92 a***	45.21 ± 1.46 a***b***c**	0.000
Corpus epididymis					
Tubular diameter(µm)	681.61 ± 8.08	586.90 ± 6.30	546.58 ± 5.60	529.97 ± 7.67	0.015
Lumen diameter(µm)	538.09 ± 7.13	485.90 ± 6.69	342.95 ± 6.65 a***b**	238.79 ± 5.73 a***b***c**	0.000
Epithelial height (µm)	68.15 ± 1.92	71.70 ± 2.18	109.26 ± 5.80	163.61 ± 6.64 a***b***	0.000
Cauda epididymis					
Tubular diameter(µm)	609.05 ± 7.73	591.47 ± 6.29	508.64 ± 6.16	432.32 ± 6.67 a***b***	0.001
Lumen diameter(µm)	250.70 ± 6.47	275.82 ± 6.13	370.51 ± 5.36 a**	528.29 ± 5.46 a***b***c***	0.000
Epithelial height (µm)	79.84 ± 2.41	68.15 ± 1.92	51.09 ± 1.57 b*	50.47 ± 1.67 a***b***	0.000

Values are specified as Mean ±SEM. ***, **, ***** showing significance variance **at p< 0.05, p<0.01, p<0.001,** respectively. **a** represents Value vs control, **b** indicates Value vs group 1 **c** signifies Value vs group 2.

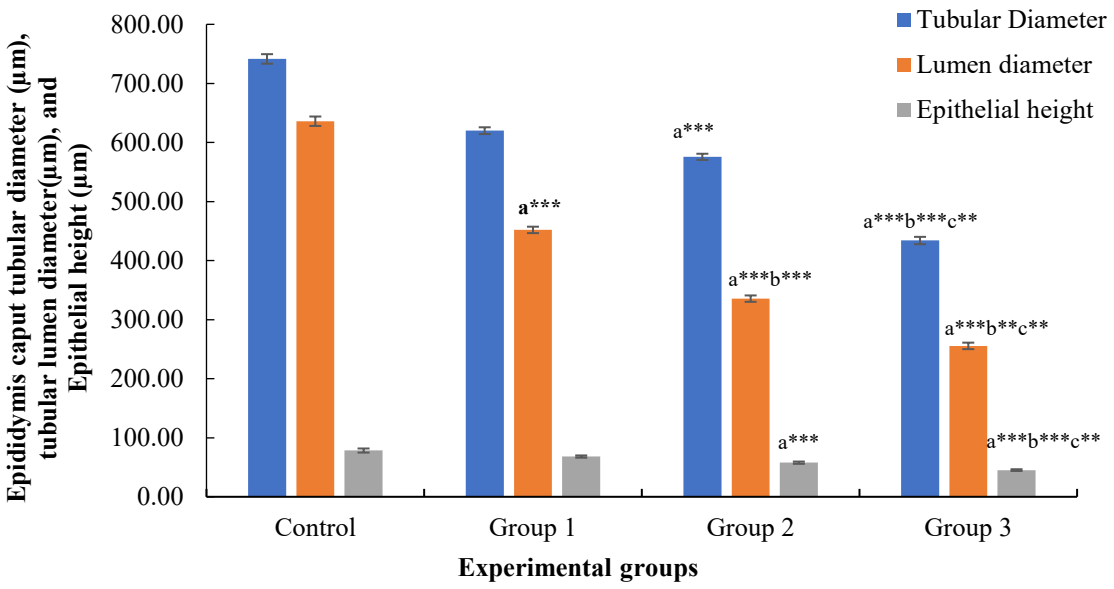


Fig 20: Mean ± SEM caput tubular diameter (µm), lumen diameter (µm) and epithelial height (µm) after 28 days of exposure among adult male rats.

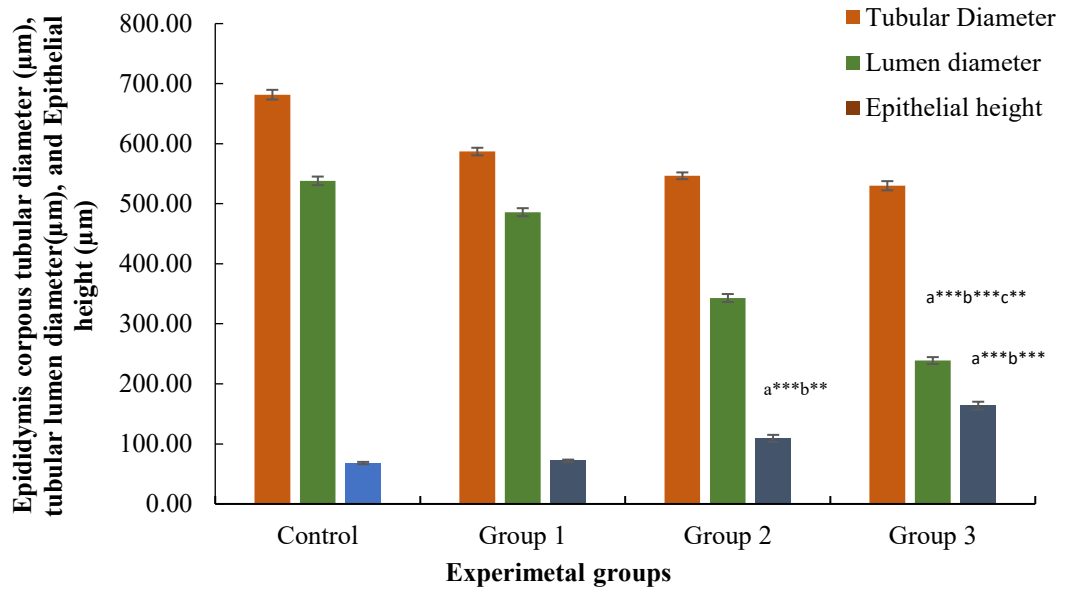


Fig 21: Mean ± SEM corpus tubular diameter (µm), lumen diameter (µm) and epithelial height (µm) after 28 days of exposure among adult male rats.

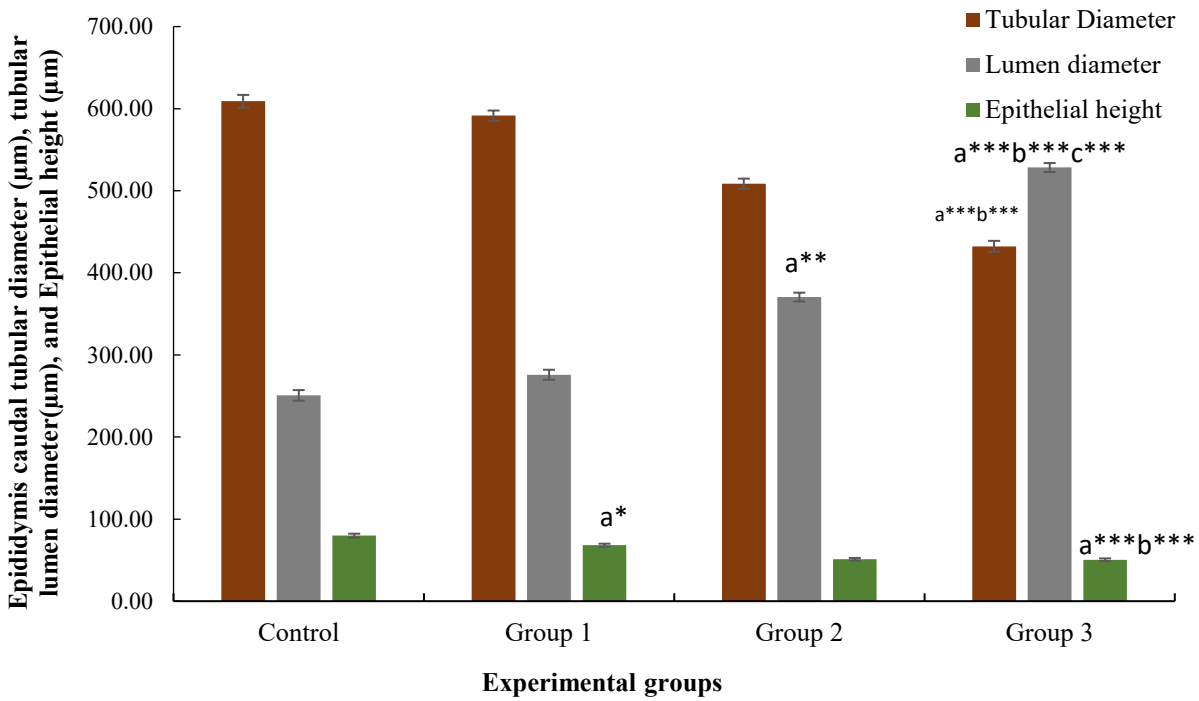


Fig 22: Mean ± SEM cauda tubular diameter (µm), lumen diameter (µm) and epithelial height (µm) after 28 days of exposure among adult male rats.

Discussion:

Numerous pesticides have been and widely used throughout the world with few regulations and restrictions. The major influence of pesticides is relatively good for the human healthiness but when majority of the insecticides as well as pesticides are improperly used on the vegetables and on the other crops such as cereals, they significantly increased the fertility risks and abnormal sperm count. pesticides contain numerous substances that can be pointed out as endocrine disruptors (Vinaggard *et al.,* 2000; Anderson *et al.,* 2002; Kojima *et al.,* 2004; Lemaire *et al.,* 2006). One of the types of pesticide is bio-pesticide. It is originated from the biological sources such as viral, fungal, and bacterial sources, Or from the bio-chemical sources such as insect growth regulators (IGRs). One of the endocrine disruptors is pyriproxyfen, that is the juvenile hormone agonist (Jha), so stops the expression of that genes that are needed for the process of metamorphosis in insects (Dhadialla *et al.,* 1998).

There has been a lot of data recorded related to the endocrine disruption effect of pyriproxyfen in variety of the invertebrate organisms, as in Daphnia, PPF effect fertility, reproduction and have several ecological effects (Ginjupalli *et al.,* 2015). But the pyriproxyfen reproductive toxicity is limited in vertebrates (Maharjan *et al.,* 2018). Therefore, it is crucial to perform prolonged testing and determine the pyriproxyfen toxicity owing to low concentrations of long duration exposure to reduce danger to human health. Keeping these facts in mind, current study was conducted to determine the toxic risk assessment of pyriproxyfen on the reproductive system of adult male Sprague Dawley rats. For this reason, some biomarkers were used to determine the toxicity risks. Body weight, body mass index, blood glucose levels, and weight of various reproductive organs and visceral organs weight are the common biomarkers in this regard. The antioxidant study and histopathological changes were noted to check the reproductive toxicity. In the clinical assessment, during the period of the dose administration, no death was reported in any group. The hair loss was noticed, mostly from the back area, and the first few dosages were associated with the loose stools. This can be because of consuming a lot of water while taking the dose. From the few days after the dose administration to the end of the experiment, yellow whitish discoloration was observed. No other notable change was observed. In the current experimental research, no significant decline was noted in the body weight at day 1($p=0.142$), 14($p=0.084$) and 28($p=0.407$) when the control and experimental

groups were compared. There was slight decline in the control group was recorded at day 1, 14 and 28. This decline in the control group may be due to placebo. The decline in the experimental and control group was due to the dose administration, this is according to the previous study (Koyama *et al.,* 1989). One of the tools that is used to determine the obesity and to check changes in the body weight is body mass index (BMI), so it is the effective tool for determining the effectiveness of the weight loss therapy (Knutsen *et al.,* 2017). The results of the current experimental research showed that, there was no significant reduction($p=0.982$) in the mass index noted in any of the group in comparison with the control group. In our current study, highly significant decrease($p<0.001$) in the blood glucose level was observed in all the treatment groups when compared with the control group at day 1, 14 and 28. This decrease in the blood glucose level may be due to the avoidance of food intake during placebo or may be due to the outside stress or response to the outside stress during the PPF administration. The current study is according to the previous study where changes in the blood parameters in rats were observed (Roshanravan *et al.,* 2021).

The results of the current study showed that, no significant decline in the weight of the testis was recorded at low doses while at high dose there was highly significant decrease($p<0.001$) shown when compared with the control. The decrease in the testis weight is due the decline in the size of seminiferous tubules and decrease in the number of mature spermatozoa. The weight of testis decreases because of decline in the elongated spermatids number and decline in the population of germ cells (Sanchez and Pena, 2004). The weight of left and right epididymis showed highly significant decrease($p<0.001$) at high doses while at low dose no significant decrease was shown. This may be due to the reason that at low dose there was little degenerative changes in the epididymis was recorded but at high dose, there was greater degenerative changes in tunica albuginea, decline in the DSP and decrease in the number of mature spermatozoa. The accessory organ weight showed non-significant change($p=0.466$) in the weight of prostate in all groups when compared with the control. The weight of seminal vesicle showed remarkable decrease($p<0.005$) in group 2 and group 3 when compared with the control. This result was according to the previous findings, that showed the weight of accessory organs (seminal vesicle and prostate gland), and the weight of androgen responsive organs decline significantly in rats and dogs when PPF was administrated. (S.M. and Lee, 2004). The current study showed that there was no significant increase noted in the

weights of body organs such as brain(p=0.893), heart(p=0.958), liver(p=0.258), pancreas(p=0.208), and left kidney(p=0.869) and right kidney(p=0.717). when comparison was made between control and pyriproxyfen treatment groups. The previous study showed that when the mammals are exposed to any toxicant, their body organs such as kidney, testis, liver, pancreas, ovaries, and lungs showed abnormal weight (Badr *et al.*,2020).

Toxicity testing is an important component of environmental monitoring to protect aquatic life and human health. The first response to environmental stressors is oxidative stress (Livingstone, 2001) that is the complex mechanism involved in the physiological processes. Oxidative stress occurs when there the level of ROS exceeds the scavenging activities of antioxidant molecules. The development of the embryo, and the bio-chemical and physiological functions are affected by the variation between ROS formation and antioxidant formation (Wu *et al.,* 2016). The result of our current study shows significant increase in the reactive oxygen species in the treatment group when compared with the control group. This result is according to the current studies that indicated, the elevated level of ROS in PPF-treated *Danio rerio* embryos suggested that the drug may operate as inducer of oxidative stress (Maharjan *et al.,* 2018; Lajmanovich *et al.,* 2019).

Two vital antioxidant enzymes that plays significant role in the first line of defense of the organism are Superoxidase and the peroxidase that work against oxidative stress (Slaninova *et al.,* 2009; Yang *et al.,* 2014). SOD is required for superoxide radical catalysis to produce H_2O_2 and O_2. The formation of H_2O_2 produced during the catalysis process was deteriorated by the activity of catalase allowing it to be detoxified (Adeyemi *et al.,* 2015). In current study, level of SOD and CAT increased significantly($p<0.001$) in all the treatment group when compared with the control group. The higher level of SOD and POD activity in this study may be because of protection of the organism by the harmful effects of free radicals that were formed due to damage caused by oxidative stress (Gu *et al.,* 2010). The previous study indicated that when Pyriproxyfen was administrated to *Danio Rerio*, the level of SOD and POD activity showed substantial increase in the treatment groups in comparison with the control group (Maharjan *et al.,* 2018).

Peroxidase (POD) is another main antioxidant enzyme that plays significant role in mechanism of cell defense. The fatty acids are oxidatively degenerated in to LPO in the plasma membrane that results in cell damage. The current study showed that, POD level was not significantly increased($p=0.206$) in the treatment groups when compared with the control group. There was significant increase($p<0.001$) in the POD level observed between group 1 and control group. The same increased was noted in the LPO levels in the embryos of zebrafish that were exposed to the cypermethrin (Shi *et al.,* 2011).

The most efficient parameter for the determination of the toxicity in the reproductive system of the rats is Histology (Creasy *et al.,* 2001; Lanning *et al.,* 2002). The insecticides directly affect the testis and interrupt the activities of androgen hormones that leads to the infertility issues. the sexual behavior is affected by the toxins that has direct action on the anterior pituitary may have indirect action on the testis (Amann *et al.,* 1982).

Current study indicated the shrinkage of seminiferous tubules and changes in the tunica albuginea, degenerative changes take place in the structure of the testis. The decline in numbers of sperms cells, the increase in the lumen and tubular diameter and decreased epithelial height in all the experimental groups when compared with the control group. There was significant increase in the tubular and lumen diameter and increase in the epithelial height in group treated with high dose of PPF when compared with the control group while the groups that were treated with low dose of PPF shows little degenerative changes. This is according to the current study (Shahid *et al.,* 2019) that showed that in mice when high concentration of pyriproxyfen was applied, there was higher interstitial spaces and vacuolation was noted, while little degenerative changes were observed at low doses of pyriproxyfen.

In the current study, the caput epididymis showed reduction in the tubular diameter, lumen diameter, decreased number of spermatozoa, degenerative changes in the epithelium that turns reduction in the epithelium. There was significant reduction ($p<0.001$) in the tubular, lumen, and epithelial height at the high dose of PPF while at low dose there was no remarkable decline noted. Similar findings according to the previous study (Fattahi *et al.,* 2009). Showed that diazinon treatment may cause seminiferous tubule degeneration, so inhibiting spermatogenesis.

In the corpus epididymis, the lumen diameter decreased non-remarkable($p=0.015$) at high dose of PPF while at low dose no significant change was noted. There was non-remarkable change noted in the tubular diameter at low and high doses of pyriproxyfen while epithelial height showed significant increase ($p<0.001$) at high dose while low dose showed non-remarkable change in the epithelial height. Same study was done by many other researchers that observed that histopathology of the tissue of testis, showed decrease in spermatogenesis and minor to major degenerative changes in the albino rats when they were treated with chlorpyrifos (Joshi *et al.,* 2007; Kashoury *et al.,* 2010).

In the cauda epididymis, there was non-remarkable changes observed at the low doses of PPF while at high dose, there was substantial increase($p<0.01$) in the lumen diameter at high dose administration of PPF. while the tubular diameter and epithelial height showed remarkable decrease($p<0.001$) at high administration of PPF, this may be due to the high level of ROS. This study is according to the previous study by Najafi *et al.,* 2010.

Conclusions:

The present study concludes that, pyriproxyfen causes antioxidant changes in the adult male Sprague Dawley rats. In the testicular tissues, histopathological changes are induced by the pyriproxyfen at different doses. Because of this finding, it is suggested that the usage of pyriproxyfen should be done with the proper cautions. There should be the proper knowledge of hazardous effects caused by PPF before usage, so to achieve this goal further research should be done to know the molecular process of the pyriproxyfen interaction with the germ cells.

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