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RESEARCH ARTICLE

Investigations of Hemato-Biochemical, Histopathological, Oxidative Stress and Reproductive Effects of Thiram in Albino Rats

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ABSTRACT

Thiram, being a dithiocarbamate fungicide, is frequently used to protect a variety of food crops (vegetables, ornamentals and fruits) and seeds and it may remain in various products of plants, water and in soil after applications. The current study was executed to record the toxic effects of thiram in male albino rats. A total of 15 adult male albino rats were obtained from local market and were placed in wire cages in three groups (A-C). Thiram was administered daily to all rats of group B (50 mg/kg bw) and C (75 mg/kg bw) for 21 days and different tissues (blood, testes and liver) were harvested for determination of hematological, reproductive and oxidative stress parameters. Results revealed significantly lower values of red blood cell counts and hemoglobin quantity while increased values of total white blood cell counts and neutrophil percentage. The different serum biomarkers including alkaline phosphatase, alanine aminotransferase, urea, creatinine, triglyceride, glucose and cholesterol significantly escalated in thiram treated rats. Results showed significantly lower levels of antioxidant enzymes (peroxidase and catalase) while higher values of oxidative stress biomarkers such as reactive oxygen species (ROS) and thiobarbituric acid substance (TBARS) in liver of treated albino rats. Histopathological investigations indicated different pathological lesions including degeneration and damage of spermatogonia, cellular debris in lumen of seminiferous tubules, necrosis of spermatids, decrease in frequency of seminiferous tubules with normal spermatozoa, arrest of process of spermatogenesis and inflammatory materials in testes of rats. This work shows that thiram causes disruption of normal functions of blood-biochemistry, antioxidant enzymes and reproductive tissues through induction of oxidative stress.

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INTRODUCTION

Pesticides and fungicides are widely used in agriculture system to protect crops and plants from various pests and the related diseases (Ahmad *et al*., 2021; Akram *et al*., 2022). About 70% of pesticides are being utilized by developed countries and these are entering into the food chain which cause hazardous effects to aquatic and terrestrial life due to their toxic nature. An uncontrolled exposure of fungicides can lead to contamination of environment as they may persist in soil, water and plants (Chiaia-Hernandez *et al*., 2017). Thiram (fungicide) is a multipurpose chemical that is used in the rubber industry as a vulcanizer accelerator, in agricultural

fields as a fungicide and in medicine to treat human scabies (Chen *et al*., 2019). Thiram exposure is linked to a number of toxicological effects, such as thyroid gland disruption (Chen *et al*., 2018), induction of tibial dyschondroplasia in a variety of avian species (Zhang *et al*., 2018), reproductive toxicity in rats (Mishra *et al*., 1998), and an increased mortality rate in the *Daphnia magna* (Belaid and Sbartai, 2021). Thiram disrupts the normal functions of arylamine N-acetyltransferase-1 leading to abnormal digestion of xenobiotic substances. It is recorded that thiram induces reproductive disorders leading to abnormal development of fetus (Liu *et al*., 2022). Thiram is also well known for inducing morphological and biochemical changes in chickens and causes a metabolic cartilage disease known as tibial dyschondroplasia in rapidly developing poultry (Waqas *et al*., 2020).

Intoxication with pesticides and increased generation of reactive oxygen species (ROS) and free radicals have been found to be strongly associated. An increase in ROS and free radical levels that surpass the ability of cells to neutralize them identifies oxidative stress (Kurpios-Piec *et al*., 2015). Several diseases including nephrotoxicity, (Merdana *et al*., 2021) nephrotoxicity and renal disorder, (Mohammed *et al*., 2021) rheumatoid arthritis, cardiovascular disease, cancer and dementia are primarily caused by the pathogenesis of these reactive species (Thind and Hollomon, 2018). The exact mechanism of toxicity of thiram in different animals is not yet clearly known. However, previous studies reveal that pesticides can cause renal damage, inflammation and nephrotoxicity in rats as a model (Behling *et al*., 2006). Dithiocarbamates readily degrade in an acidic atmosphere to carbon disulphide (CS2) and their associated amine (Xu *et al*., 2011). The widespread use of thiram exposure is currently causing countless abnormalities throughout the world. Scanty information is available on the hepatotoxicity of thiram in liver, testes and its effects on blood biochemistry. However, different toxic effects on developing chickens have been reported (Liu *et al*., 2022). Chemical poisoning, including exposure to pesticides, can cause permanent heart damage, including myocardial necrosis, tachycardia, cardiomyopathy, heart failure, and even cardiac arrest (Jiang *et al*., 2022). The present study investigated the toxic effects of thiram on albino rats, its hematological parameters, serum biochemistry, biochemical analysis in liver and histopathological study in testes of albino rats.

MATERIALS AND METHODS

Ethical statement: The current experimental trial was designed and executed according to the guidelines of bioethics committee of Islamia university of Bahawalpur regarding use and welfare of laboratory animals.

Chemicals: Thiram was purchased from Sigma Aldrich, USA. Other chemicals were obtained from Merck (Germany) and Sigma Aldrich company. For serum analysis different commercial kits were purchased from Randox company (Pvt.) Pakistan. All Chemicals used were of analytical grade.

Animal and experimental study design: A total of 15 albino rats were purchased from the local market. The animals had free access to clean drinking water and standard commercial pellet rat diet. The wire cages had proper ventilation and separation. After one week of acclimatization period, the albino rats were divided into 3 equal groups. One was the control group (A) while the other two were treated groups (B and C). The group A was the control group and kept on standard diet and clean water while the groups B and C were treated with 50 mg/kg and 75 mg/kg body weight of albino rats. The doses of thiram used in current study were selected on the basis of already published literature (Salam *et al*., 2021).

Sample preparation and storage: Blood was collected in EDTA tubes for hematological study and blood without anticoagulant was collected in falcon tubes and was centrifuged at 5000 rpm for 15 minutes for the separation of serum. Blood cells were settled down and serum was separated in serum cups and stored below 4°C for further analysis. Liver from each rat was separated during necropsy and was placed in chilled normal saline for further process.

Hematological analysis: Different hematological parameters include white blood cells count (10^3/mm^3), total number of erythrocytes (10^6 /mm^3), total proteins (g/dl), hemoglobin concentration (g/dl), hematocrit (%), neutrophil (%), lymphocytes (%) and monocytes (%) were studied using hemocytometer by following the standard protocols (Hussain *et al*., 2019).

Serum biochemistry: Centrifuged serum blood samples were used to measure alkaline phosphatase (ALP; IU/l), alanine aminotransferase (ALT; IU/l), urea (mg/dl), creatinine (mg/dl), triglycerides (mg/dl), glucose (mg/dl), and cholesterol (mg/dl) by using commercially available kits in a chemistry analyzer (Randox company Pvt.) (Hussain *et al*., 2021).

Antioxidative enzymes and oxidative stress biomarkers: The status of different antioxidative enzymes like peroxidase (POD) and Catalase (CAT) was determined in homogenate of liver tissues (Beers and Sizer, 1952). Oxidative stress biomarkers including reactive oxygen species (ROS) and Thiobarbituric acid reactive substances (TBARS) were measured in homogenates of liver tissues with certain modifications as previously described (Iqbal *et al*., 1996).

Histopathology: For histological analysis, testes of all treated and untreated albino rats were obtained after dissection and were preserved in 10% formalin solution, dehydrated in ethanol, cleared with xylene, embedded in paraffin, and finally stained with eosin and hematoxylin. All the sections of testes were carefully observed under light microscope (Khan *et al*., 2022).

Statistical analysis: The results obtained from the experimental work are represented as mean ± standard error (SE). All the data collected from each group was statistically analyzed by one-way ANOVA using IBM SPSS version 20 ($P<0.05$).

RESULTS

Physical Parameters: No mortality, clinical and behavioral alterations were observed in treated rats. All the treated rats remained normal throughout the trial.

Hemato-biochemical analysis: Results on various hematological parameters of control and treated albino rats with 50 mg/kg body weight and 75 mg/kg body weight are shown in Fig. 1. The total erythrocyte counts significantly decreased in the albino rats of group treated with 10 mg/kg body weight of thiram as compared to the control group. Whereas the total number of white blood cells increased significantly with the increase in dose of thiram. The hemoglobin concentration showed a significant decrease in both treated groups as compared to the values of the control group. The results of percentage of hematocrit also showed a significant decrease in rats of the group C after 21 days of trial treated with 75 mg/kg of body weight. The results on monocytes, lymphocytes and neutrophil count showed a significant increased value in both treated groups of albino rats as compared to the control group. The values of leukocytes, neutrophil, lymphocyte and monocyte increased significantly with the increase in dose of thiram while red blood cell counts, hemoglobin and hematocrit showed significant decreased values as compared to the control group.

Results regarding serum analysis of albino rats treated with different doses of thiram are shown in Fig. 2. The concentrations of various serum biochemistry profiles like alanine aminotransferase (ALT) and alkaline phosphatase (ALP) increased significantly after 21 days of research in groups B and C treated with 50 mg/kg and 75 mg/kg body mass respectively. The total protein contents decreased significantly with the increase in concentration of thiram as compared to the control group. A noticeable increase was recorded in results of triglycerides in the group treated with 50 mg/kg of thiram after exposure of 21 days. The quantity of urea and glucose increased significantly in both the treated groups of albino rats whereas the cholesterol level decreased significantly in the group C treated with 75 mg/kg of thiram as compared to the control group.

Antioxidative enzymes: Results on antioxidative enzymes showed significantly reduced quantity of peroxidase and catalase enzymes in homogenates of liver tissues of treated albino rats (Fig. 3). The effect of thiram on the activity of peroxidase showed a significant decrease in the liver tissue of the treated groups. The results obtained on the catalase activity in liver also showed a significant decrease in the groups treated with 50 mg/kg body weight and 75 mg/kg body weight of thiram as compared to the control groups.

Oxidative stress biomarkers: The results on effects of thiram oxidative stress biomarkers are shown in Fig. 3. The contents of ROS increased significantly in the thiram treated groups. Results obtained showed a greater difference in the values of ROS contents in albino rats of group C treated with 75 mg/kg body weight as compared to the control group. The quantity of thiobarbituric acid reactive substances also increased significantly in the liver tissues of albino rats.

Histopathological studies: The testes of group A untreated rats exhibited normal histological structure including germinal epithelium and seminiferous tubules. Mild to moderate histological changes were observed in testes of albino rats of group B like necrosis of germinal epithelium, degeneration, and damage of spermatogonia, cellular debris in lumen of seminiferous tubules (arrows) and admixture of necrotic cells in lumen of seminiferous tubules (Fig. 4). The results on severity of various microscopic alterations are indicated in Table 1. At microscopic levels, different histoarchitectural changes in testes like sloughed cells in seminiferous tubules, hypospermatogenesis, necrosis of spermatids, germ cell depletion in seminiferous epithelium, decrease in frequency of seminiferous tubules with normal spermatozoa, necrosis of spermatogonia and Sertoli cells, arrest of process of spermatogenesis, reduced diameter of seminiferous tubules, inflammatory processes, necrosis of epithelia of seminiferous tubules, edema (Fig. 4) and partial germ cell arrest were observed in albino rats of group C treated with thiram.

DISCUSSION

Thiram and various other insecticides are persistently applied in agro-production sector, veterinary practices and public health management to control insects and fungi

Table 1: Severity of gross and microscopic alterations in testes of albino rats treated with different doses of thiram.

Histopathological lesions	A (0.0 mg/kg)	B (50 mg/kg)	C (75 mg/kg)
Reduction in volume	-	+	++
Reduction in size	-	+	++
Testicular weight	-	+	++
Necrosis of spermatogonia and Sertoli cells	-	+++	++++
Arrest of process of spermatogenesis	-	++++	++++
Inflammatory processes	-	+++	++++
Reduction in diameter of seminiferous tubules	-	++	+++
Sloughed cells	-	++	+++
Severe degeneration and damage of spermatogonia	-	+++	+++
Necrosis of spermatids	-	+++	++++
Hypospermatogenesis	-	+++	++++
Increase cellular debris in lumen of seminiferous tubules	-	++	++++
vacuolation of epithelia of seminiferous tubules	-	+++	++++
Escalated germ cell depletion in seminiferous epithelium	-	+++	++++
Partial germ cell arrest	-	+++	++++
Decrease in frequency of normal seminiferous tubules	-	+++	++++
Disorganization of spermatogonia and Sertoli cells	-	+++	++++

Normal (-), Mild (+), Moderate (++), Severe (+++), Very severe (++++).

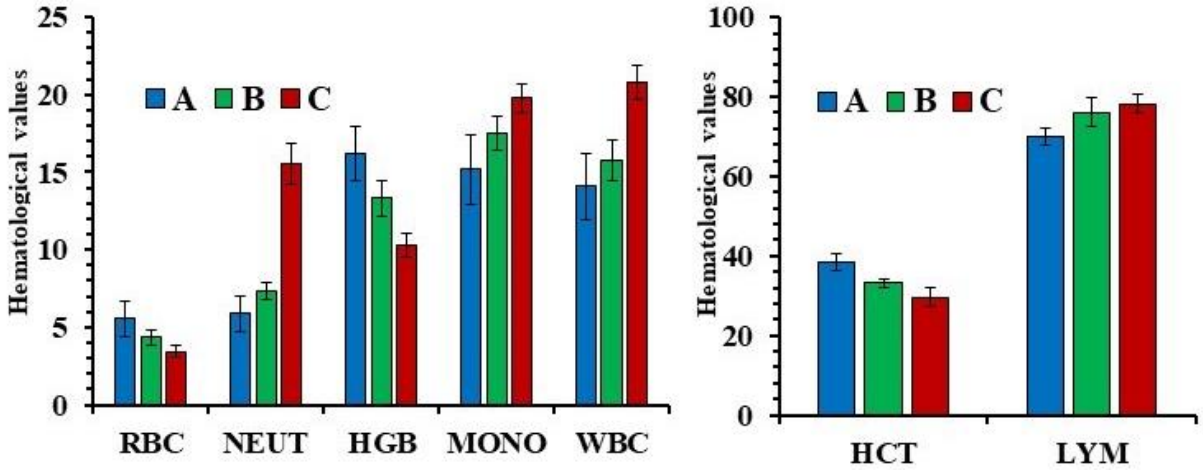

Fig 1: Hematological attributes including red blood cell (RBC), neutrophil (NEUT), hemoglobin (HGB), monocytes (MONO), white blood cell (WBC), hematocrit (HCT) and lymphocyte (LYM) of albino rats exposed to different concentrations of thiram.

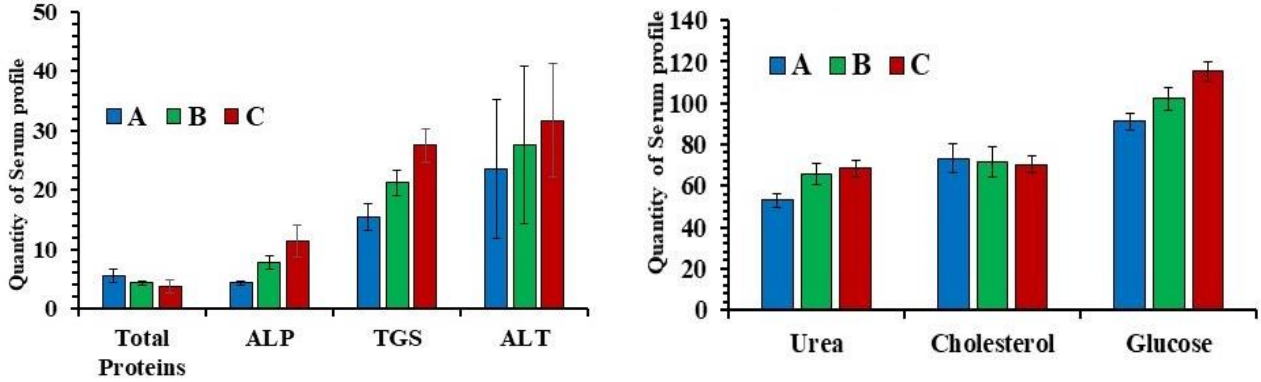

Fig 2: Comparisons of different serum biochemical attributes of albino rats exposed to different concentrations of thiram.

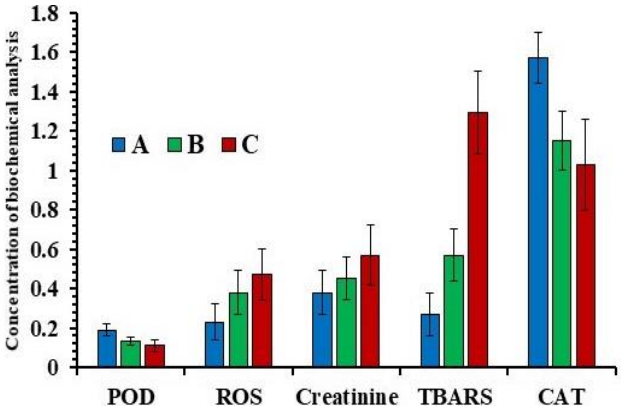

Fig 3: Comparisons of different antioxidant enzymes and oxidative stress in liver along with creatinine profile in kidneys of albino rats exposed to different concentrations of thiram.

(Mujahid *et al*., 2021; Enwemiwe *et al*., 2022; Liu *et al*., 2022). Studies have shown that thermal decomposition and combustion of pesticides have led to serious threats to the environment, human health and other target and non-target animals (Sankowska *et al*., 2017). Therefore, it is necessary to monitor toxicological impacts of such chemicals (Jabeen *et al*., 2021; Salam *et al*., 2023). Therefore, our study provides the adverse effects of thiram (an organic dithiocarbamate fungicides) on blood, serum biochemistry, oxidative stress, antioxidative status and histopathological changes in testes of albino rats.

Hematopoietic and leukocytic biomarkers are known as the best indicators for screening of toxicological effects of synthetic and natural toxicants leaking to the aquatic and terrestrial environment (Namratha *et al*., 2021; Akram *et al*., 2022). Numerous studies have demonstrated that fungicides and pesticides can induce the disorders in hematopoietic system of the rodents by influencing the number of erythrocytes, hemoglobin, hematocrit, and leukocytic counts (Kasmi *et al*., 2018; Namratha *et al*., 2022). In the current study it is noted that albino rat treated with thiram had lower values of erythrocytes, hematocrit and hemoglobin concentration while higher values of total leukocyte counts, neutrophil and monocyte. The decrease in the number of red blood cells, hemoglobin and hematocrit are suggestive of toxic effects of thiram on blood forming tissues of albino rats leading to development of anemia. The lower values of red blood cells in this study could also be due to destruction of red blood cells in the microvascular system. The hematological disorders in albino in this study can also be related to over production of free radicals. Similar observations have been reported in previous studies that difenoconazole which is also a fungicide decreases Hb and RBCs count due to hemolysis and shrinkage of cells in rats (Kasmi *et al*., 2018). Recently the published data on toxicity of thiram in male albino rats has also reported decreased number of erythrocytes (Bawane *et al*., 2023). The decrease in hemoglobin concentration might be due

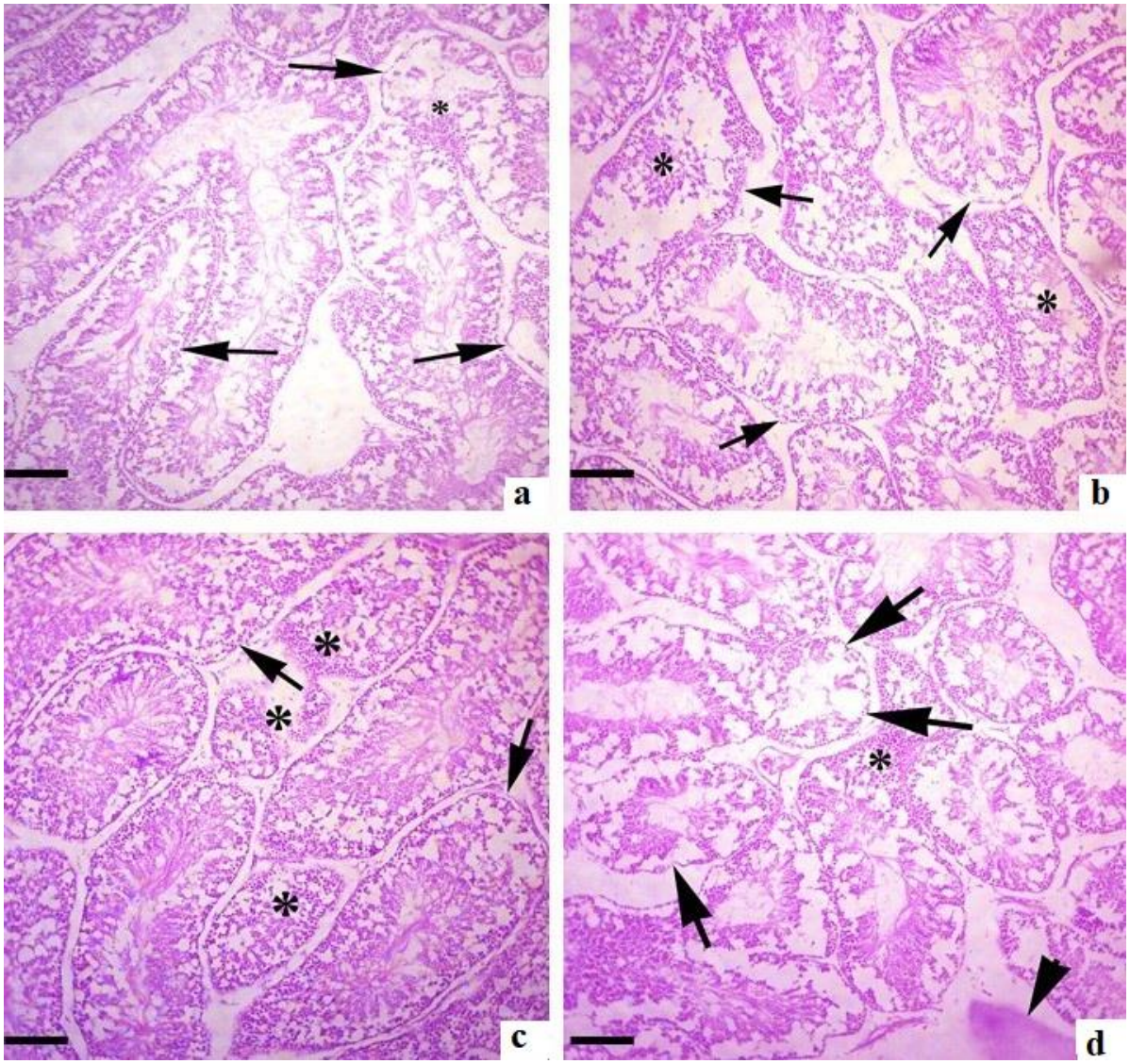

Fig. 4: Photomicrograph of testes showing different microscopic lesions like necrosis of germinal epithelium (arrows) and admixture of necrotic cells (*) in lumen of seminiferous tubules in male albino rats (a-b) of group (B) and necrosis/sloughing of germinal epithelium (arrows), edema (arrow head) and admixtures of necrotic cells in lumen of seminiferous tubules in male albino rats (c-d) of group (C) administered thiram. H and E stain; 400X.

to iron deficiency which is necessary for Hb synthesis and this decrease may occur at any stage of maturation of erythrocyte. The increased values of hematological parameters like total leukocyte counts, neutrophil and monocytes might be due to injurious stimulation/injuries caused by thiram resulting in stimulation of immune system. The rise in white blood cells number may be attributed due to the toxic effect of thiram which increases the number of B cells and T cells. T cells are responsible for phagocytic activity to the sites of inflammation or damage while B cells work on immunoglobin production (Aulbach and Weiss, 2022).

Hyperglycemia was observed in treated albino rats in our study. It is reported that pesticides raise the blood glucose levels by preventing body cells from absorption and utilization of glucose (Badr, 2020). In addition, increased quantity of glucose in our research work could also be due to glycogenolysis in muscle and liver along with disruption of carbohydrate metabolism in liver leading to glucagon and adrenocorticotropic hormone release or abnormal functioning of insulin (Han *et al*., 2014).

In the current experimental work, the intoxication of albino rats with thiram showed a significant increase in the level of triglycerides. This increase seems to be an imbalance synthesis of triglycerides. An increase in the serum cholesterol level in the present study due to exposure of thiram can corelates with the liver toxicity due to the accumulation of thiram fungicide in the liver of albino rats that was also associated with the disturbance in the metabolism of lipids. The increased quantity of triglycerides in albino rats in this study can be related to disorders of permeability of cell membranes of liver. Similar to our study, hypercholesterolemia has been reported in other studies due to toxicity caused by pesticides (Lal *et al*., 2022).

The liver is essential for maintaining the homeostasis of the body (Javed *et al.*, 2021; Wu *et al*., 2021). Different liver functions tests such as ALP and ALT are thought to be useful indicators of liver damage. In our research work, the values of ALP and ALT were increased significantly in albino rats when treated with thiram indicating liver damage. Several reports showed that the increase in dose of fungicide may cause liver damage and increase the

quantity of ALP and ALT (LaRocca *et al*., 2020; Kwon *et al*., 2021). Hence, it is concluded from the results that exposure to thiram exerts a significant toxic effect on hematology and serum profile of albino rats.

Thiram induces oxidative stress by generating free radicals and ROS (Salam *et al*., 2021). It is easily absorbed in the body and metabolizes to diethyldithiocarbamate and carbon disulfide, threatening both human health and the natural ecosystem (Guo *et al*., 2020). In the current study, increased contents of ROS and TBARS are suggestive of induction of oxidative stress due to exposure to thiram in albino rats. It has been recorded that thiram enters in animal body and causes congestion of liver sinuses and necrosis eventually leading to damages in the liver by the transfer of transaminases from the liver to blood stream ultimately causing increased levels of ALT and AST in blood (Waheed *et al*., 2020). Several reports showed that ROS causes protein destructions, lipid peroxidation, increased level of collagen that leads to fibrosis (Kurpios-Piec *et al*., 2015). TBARS is the common way to determine lipid peroxidation in cells and tissue (Bhutta *et al.*, 2022). Similar to previous findings, our results showed an increase in the level of TBARS when albino rats were treated with thiram, which gives a clear indication of liver injury. Our results showed a significant decrease in the activity of catalases and peroxidases enzymes and this decrease might be due to over generation of free radicals, hepatolysis and mitochondrial damage (Javed *et al.*, 2021; Kiran *et al.*, 2022; Wand *et al*., 2023). Previously, up to our knowledge no data is available on the antioxidant status in liver tissue of albino rats exposed to thiram.

Histopathological studies have primarily been used as a biomarker for investigation of toxicity caused by the various environmental pollutants including pesticides (Hussain *et al.*, 2019; Pasha *et al*., 2022). Different fungicides are known to induce histological changes in testes of rats (Prathima *et al.*, 2023). In the present study, there are several histological changes observed in testes of albino rats after exposure to thiram including decrease in diameter of seminiferous tubules, seminiferous tubules are thick layers of different shape of cells surrounding the lumen, during infection Sertoli cells can not differentiate in meiosis for the sperm production, results in reproductive failure or there may be necrosis factor. The inflammatory reactions, arrest of spermatogenesis, edema, sloughed cells and congested blood vessels were observed in testes of albino rat. The testicular changes in current study might be related to low ATP production and disruption of endocrine functions leading to substantial reduction of sperm motility (Heikal *et al*., 2014; Zubair *et al.*, 2022). Moreover, the histopathological changes in testes of albino rats could be due to lower values of LH and acquisition of increasing Ca2+ motility through the particular b-defensin which is absorbed from the main cells (Klüver *et al*., 2006). The degenerative and necrotic ailments and arrest of spermatogenesis in testes of male albino rats in this study may also be due to rapid production of free radicals/increased oxidative stress (Sharma *et al*., 2014).

Conclusions: In this study, we were able to observe that most commonly used fungicide thiram induced hematological, serological, biochemical and histopathological changes in albino rats. The exposure to thiram even 50 and 75 mg/kg of body weight caused damage to liver, testes as well as several changes in hematology and serum analysis. Hence, it is proved that thiram is toxic to albino rats.

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Authors contribution: RH, SA, and MR involved in research planning and execution. Data were obtained by SA, RM and MR. ZI, MIU, NI and MWN were involved in data analysis and manuscript preparation.

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