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Impact of *in utero* Exposure of Triazophos on Reproductive System Functions in Female Albino Rats, *Rattus norvegicus*

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Abstract

Triazophos (TZ) at the level of Acceptable Daily Intake (ADI) (0.01 mg/kg bw/day) and 10×ADI (0.1 mg/kg bw/day) was given to the female albino rats to examine its effects on their reproductive system. In this study, the pregnant female albino rats were divided into three groups, each group comprising of six rats. The first group was gavaged with olive oil (control group), the second group was administered with ADI level of TZ (ADI group) and the third group was treated with 10×ADI level of TZ (10×ADI group). The dose was given through oral intubation until the end of pregnancy. At the end of gestational period, the reproductive parameters of dams were examined, however, the females which did not breed were dissected for uterine examination. The results of the present study revealed non-significant decrease in the body weight gain of females treated with ADI and 10×ADI in comparison with controls. The analysis of reproductive index depicts significant decrease in percentage pregnancy, litter size and pup survival, however, significant increase in gestational length of ADI group females as compared to controls. The examination of organ weight indicated significant decrease in weight of liver, however, significant increase in weight of thyroid, ovary, uterus and oviduct of 10×ADI group females in comparison to control. The levels of biochemical constituents viz. protein and Alanine transaminase (ALT) were significantly increased in plasma, however, the levels of Alkaline Phosphatase (AKP) significantly increased in ovaries of 10×ADI group females as compared to control. Histological studies of ovaries revealed significantly enhanced percentage of follicular atresia, however, significantly decreased diameter of ovarian follicles in 10×ADI group females. Histological examination of liver indicates distorted central vein, vacuolization of hepatocytes, dilated sinusoids with intense mono nuclear inflammatory cells and infiltration in ADI and 10×ADI group females as compared to controls. Hence, *in utero* intoxication of female rats with TZ at the level of ADI and 10×ADI has a potential to induce alterations at cellular and organ level.

Keywords: Females, *in utero*, Reproduction, *Rattus norvegicus,* Triazophos

1. Introduction

Nowadays, the production and preservation of agricultural commodities entirely depends upon the use of varied and large amount of pesticides[1]. Although efforts are being made for developing intensive alternative methods for controlling pest yet the chemical control using pesticides is found to be the most effective method and continues to increase day by day in several countries of the world[2]. During the last few decades, the utilization of these toxic substances has exceeded the tolerance level, thus, creating discrepancies in the environment[3].

In the past years, there has been a gradual increase in the application of Organophosphorus Pesticides (OPs) to enhance the agricultural production. Nevertheless, the persistence of residues of OPs in crops and environ-

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ment is expected to be hazardous to humans and other animals[4]. Triazophos (TZ), O,O-diethyl-1-H-1,2,4-triazol-3-yl phosphorothioate, a broad spectrum OP, being widely applied against a large number of pests and flies damaging agricultural and horticultural crops viz. rice, vegetables, sugarcane, potato, maize, fruits and coffee[5]. TZ is released in the environment either during its application as an insecticide or during its transportation and storage[6]. Human beings are exposed to TZ through different routes viz. food products, vegetables and drinking water. In the aforesaid samples, the maximum residue limit (MRL) of TZ is 0.3 mg/kg. However, according to Li *et al.*[7], the final residues of TZ analyzed in the samples of wheat grains, stems and leaves, and soil were 1.865, 44.506, and 0.973 mg/kg, respectively. The residues of TZ in vegetables, crops and vegetables and water may pose risks to the health of humans and other animals. Moreover, the severity of TZ intoxication may vary with dose, route and extent of exposure.

The residues of the pesticides can enter food chain and when their concentration exceeds the maximum limit, they can generate ill effects among humans and other animals[8]. OP insecticides elicit toxicity by irreversibly inhibiting cholinergic acetylcholinesterase (AChE) and disrupting function in the nervous system[9]. The chronic exposure to OP pesticides not only affects fertility in males[10] but also impairs reproductive system functions in females[4]. The pesticides influencing the endocrine system of the animals are considered as the endocrine disrupting compounds. These chemicals disturb the physiological processes including development, behaviour, reproduction and general metabolism of animals by interfering at multiple levels of endocrine pathways[11].

The reproductive effects associated with pesticide exposure in women are decreased fertility, spontaneous abortions, stillbirth, premature birth, low birth weight, developmental abnormalities, ovarian disorders, and disruption of hormonal function[11]. Besides impairing the structural and functional aspects of female reproduction, pesticides are altering the molecular, endocrinological, cytological and biochemical aspects as well, causing ovarian cycle irregularity and infertility.

TZ has also been shown to induce the toxicity in blood[13], liver and kidney of rats[14–16]. TZ at a dose level $1/10^{th}$ of LD_{50} has also resulted in ovarian changes and slight reduction in gestational length of female rats[13–17]. However, the data regarding the impact of ADI level and 10×ADI level of TZ on various morphological and physiological parameters of female albino rats is still lacking. Hence, the present study emphasizes the effect of TZ exposure at the dose level of ADI and 10×ADI on the reproductive, morphometric, biochemical and histomorphological parameters of female albino rats, *Rattus norvegicus*.

2. Materials and Methods

2.1 Chemicals

The commercial grade of Triazophos (purity 40%) as Hostathion 40 EC (emulsifiable concentrate) (Meghmani Organics Limited, Chharodi, India) was used for dosing female rats. Standard pelleted rat feed was obtained from Ashirwad Industries, Mohali, India. All the other chemicals required for this research work were of analytical grade and were acquired from Sigma-Aldrich, SD Fine-Chem Ltd. and Sissco Research Laboratories Pvt. Ltd. Mumbai, India.

2.2 Experimental Animals

The female albino rats aged 8–10 weeks and weighing 100–150 g were obtained from the Department of Livestock Production and Management, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India. Rats were accommodated in polypropylene cages under the environmentally controlled conditions. Rats were provided with food and water *ad libitum*. The methods and procedures used for handling the animals during the research work were in accordance with the guidelines of Control and Supervision of Experiments on Animals (CPCSEA), of India.

2.3 Treatment with Pesticide

Female albino rats were acclimatized for 10 days in laboratory conditions and were divided into three groups each consisting of six rats (n=6). The females in all the three groups were kept with untreated males in the ratio of 3:1. The females were examined for estrous cyclicity by preparing vaginal smear. The presence of sperms in the vaginal smear indicated pregnancy and that day was designated as gestational day 0 (GD 0) of pregnancy. The pregnant females were separated from males and housed in individual cages. The first group of rats was given olive

oil and served as control group and to the remaining two groups, TZ as Hostathion 40 EC at the level of acceptable daily intake (ADI) (0.01 mg/kg bw/day) and 10×ADI (0.1 mg/kg bw/day) was given until the end of pregnancy by oral intubation. After the completion of gestational period, various reproductive parameters viz. percentage of pregnancy, gestational length, litter size, pup survival, pup viability, sex ratio were examined, however, the female rats which did not breed were sacrificed for uterine examination.

2.4 Body Weight Analysis

The pregnant rats were weighed at weekly intervals using the following formula:

$$\text{Growth rate (g/day/100g bw)} = \frac{[\text{Current body weight (g)} - \text{Previous body weight(g)}] \times 100}{\text{Previous body weight (g)} \times \text{number of days}}$$

2.5 Blood Collection and Organ Weight Analysis

Rats were anaesthetized using diethyl ether and blood samples were collected directly from the heart in heparinized vials. The blood samples were centrifuged at 2300 r.p.m for 15 minutes to obtain supernatant as plasma. After collection of blood, the animals were dissected and various organs viz. liver, kidney, spleen, heart, lungs, adrenal, thyroid, parathyroid,ovary, uterus and oviduct were collected, cleaned off from adhering tissue, weighed separately and morphological data was recorded.

2.6 Biochemical Estimations

From the plasma, the biochemical constituents viz. proteins, acid phosphatase (ACP) and Alkaline Phosphatase (AKP), Aspartate Transferase (AST) and Alanine Transaminase (ALT), were determined by using standardized methods given by Lowry *et al.*[18], Bessey *et al.*[19], Bergmeyer[20], respectively.

For biochemical estimations, the ovaries were homogenized in 2 mL of phosphate buffer saline (PBS 0.1M, pH 7.4) and ovarian homogenate was centrifuged at 3000 r.p.m. for 10 mins. The supernatant was used for estimation of total soluble proteins, ACP and AKP by using the standardized methods given by Lowry *et al.*[18], Bessey *et al.*[19], respectively.

2.7 Histological Studies

For histological examination, the ovaries and liver of control and treated rats were fixed in alcoholic bouin's solution for 24 hours. The tissues were dehydrated in graded series of ethanol after complete fixation, then cleared in xylene and embedded in paraffin wax (melting point between 60-62°C).

The thick sections (5µm) were serially cut using a microtome and after performing the steps of de-waxing and dehydration in descending series of ethanol to water, the sections were stained in haematoxylin, using eosin as a counter stain. After this slides were cleared in xylene and then mounted in DPX. Slides were examined under light microscope (Olympus, Tokyo, Japan) for any marked histological changes.

2.8 Ethical Approval

The present research work was approved by Institutional Animal Ethics Committee (IAEC), GADVASU, Ludhiana, Punjab, India, vide approval number GADVASU/2014/IAEC/24/008, vide letter number IAEC/2014/179-212.

2.9 Statistical Analysis

Data was expressed as mean ± S.E. Statistical significance between the data of control and experimental animals was subjected to One-way Analysis Of Variance (ANOVA) together with student t – test ($p < 0.05$). Statistical analysis was done using the CPCS1 program.

3. Results

3.1 Body Weight Outcomes

The body weight gain of female albino rats was examined weekly during the gestational period. It was analysed that the body weight depicted non-significant ($p > 0.05$) decrease in ADI and 10×ADI group females as compared to control rats (Figure 1).

3.2 Reproductive Outcomes

In the present study, the females treated with 10×ADI level of TZ did not breed. However, the control and ADI group females parturited and the various reproductive parameters viz. percentage of pregnancy, gestational length, pup

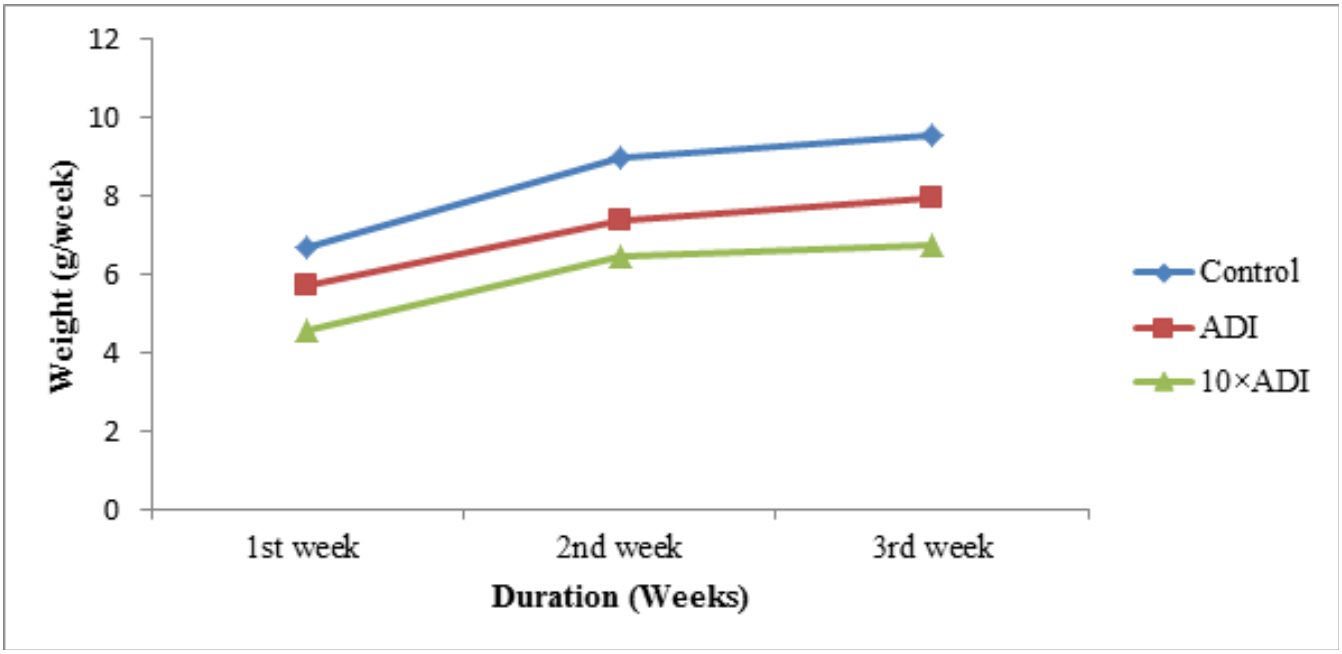


Figure 1. Weekly changes in body weight gain (g/week) of female albino rats
Key to abbreviations:
ADI= Acceptable Daily Intake

survival, pup viability, sex ratio were examined in them. The percentage of pregnancy was found to be significantly decreased ($p < 0.05$) in ADI group females as compared to control rats (Table 1). However, the gestational length was significantly increased ($p < 0.05$) in ADI treated females as compared to controls. Moreover, litter size, percentage of live pups and pup viability significantly decreased ($p < 0.05$) whereas percentage of dead pups after birth significantly increased ($p < 0.05$) in ADI group females as compared to control rats. It was also observed that sex ratio was significantly decreased ($p < 0.05$) in ADI group females where more females offsprings were born as compared to male offsprings (Table 1).

3.3 Organ Weight Outcomes

The ADI and 10×ADI of TZ treated rats which did not deliver pups were sacrificed for organ weight analysis. For comparison control rats were also dissected along with unbred mothers. The weight of vital organs viz. liver, kidney, spleen, heart and lungs, endocrine glands viz. adrenal, thyroid and parathyroid, and reproductive organs viz. ovary, uterus and oviduct was examined. In case of vital organs, the weight of liver was found to be significantly decreased ($p < 0.05$) in 10×ADI treated females in comparison to controls. However, the weight of kidney, spleen, heart and lungs depicted non-significant alteration ($p > 0.05$) in both ADI and 10×ADI group females as compared to control. In terms of endocrine glands, the weight of thyroid was significantly increased ($p < 0.05$) in both the ADI and 10×ADI group females as compared to control rats whereas the weight of adrenal and parathyroid indicated a non-significant increase ($p > 0.05$) in ADI and 10×ADI group females. The weight of reproductive organs viz. ovary, uterus and oviduct was significantly increased ($p < 0.05$) in 10×ADI treated females in comparison with control (Table 2).

3.4 Biochemical Observations

3.4.1 In Plasma

It was observed that there was a significant increase ($p < 0.05$) in level of plasma proteins in 10×ADI group females as compared to control group (Table 3). The activity of ACP and AKP increased non-significantly ($p > 0.05$) in plasma of ADI and 10×ADI group females as compared to control rats. The activity of Aspartate Transferase (AST) increased non-significantly in plasma of both the ADI and 10×ADI group, however, the activity of Alanine Transaminase (ALT) increased significantly

Table 1. Reproductive parameters of female albino rats

Parameters	Control group	ADI group	10×ADI group
Number of females paired	6	6	6
Number of females mated	6	4	6
Number of females pregnant	6	4	0
% pregnancy	100%	66%*	0%*
Gestational length	24.00±00.57 (23-24 days)	26.60±00.68* (26-29 days)	0*
Litter size	7-8	3-6*	_
% of live pups	100%	80%*	_
% of dead pups at birth	0	5%	_
% of dead pups after birth	0	20%*	_
Pups Viability	100%	80%*	_
Sex Ratio (Male:Female)	1:1	2:1*	_

Values are Mean±SE

*Significant difference at ($p<0.05$) between control and treated rats

Key to abbreviations: ADI = Acceptable Daily Intake, % = Percent

Table 2. Weights of different organs of female albino rats

Tissue/organ	Control group	ADI group	10×ADI group
Vital Organs			
Liver	4.80±0.310	4.73 ± 0.029	3.14±0.260*
Kidney	0.42±0.030	0.45±0.021	0.45±0.020
Spleen	0.37±0.004	0.35±0.018	0.32±0.020
Heart	0.42±0.004	0.38±0.014	0.43±0.017
Lungs	0.55±0.002	0.62±0.028	0.59±0.040
Endocrine glands			
Adrenal	0.020±0.001	0.022±0.002	0.024±0.001
Thyroid	0.013±0.019	0.018±0.002*	0.024±.005*
Parathyroid	0.043±0.001	0.047±0.001	0.049±0.001
Reproductive organs			
Ovary	0.036±0.001	0.039±0.001	0.048±0.009*
Uterus	0.100±0.004	0.150±0.012	0.220±0.012*
Oviduct	0.010±0.001	0.016±0.001	0.024±0.012*

Values are Mean±SE

*Significant difference at ($p<0.05$) between control and treated rats

Weights of organs are taken as g/100g bwt.

($p < 0.05$) in plasma of 10×ADI group females only, in comparison to controls (Table 3).

3.4.2 In Ovaries

In the present study, it was observed that there was a non-significant ($p > 0.05$) decrease in levels of ovarian proteins in ADI and 10×ADI group females as compared to control rats (Table 3). However, the levels of ACP increased non-significantly ($p > 0.05$) in ADI and 10×ADI treated females whereas the levels of AKP increased significantly ($p < 0.05$) in 10×ADI treated females as compared to controls (Table 3).

3.5 Histological studies

3.5.1 In ovary

The follicles of control and treated unbred rats were studied under the light microscope. The number of normal follicles were higher in control groups (Table 4, Figure 2. A, B) and their number decreased in the ovaries of ADI (Table 4, Figure 2. C, D) as well as in 10×ADI treated rats (Table 4, Figure 2. E, F). Moreover, significant increase ($p < 0.05$) in percentage of atresia was observed in ADI and 10×ADI treated females as compared to controls. Apart from this, the diameter of follicles was also noted at five stages of follicles categorized as primary, secondary, tertiary, early antral and antral follicles. It was observed that there was a significant decrease ($p < 0.05$) in diameter of follicles in ADI and 10×ADI treated females as compared to controls (Table 5).

Table 3. Biochemical constituents in the plasma and ovaries of female albino rats

Biochemical constituents	Control group	ADI group	10×ADI group
In Plasma			
Proteins (g/dL of sample)	12.63±0.64	15.23±0.39	17.67±0.59*
ACP (µ mole/mg protein)	2.05±0.11	2.43±0.19	3.65±0.25
AKP (µ mole/mg protein)	10.95±0.93	11.56±2.27	12.76±0.38
AST (IU/lt)	80.63±0.23	96.67±0.23	99.01±1.78
ALT (IU/lt)	67.08±1.37	74.51±0.59	76.51±1.36*
In Ovaries			
Proteins(mg/g wet wt)	3.36±0.13	3.05 ±0.26	2.81 ±0.23
ACP (µ mole/mg protein)	2.52±0.01	3.37±0.05	3.29±0.25
AKP (µ mole/mg protein)	6.27±0.23	6.88±0.07	8.08±0.38*

Values are Mean±SE
*Significant difference at ($p<0.05$) between control and treated rats

Table 4. Atresia (%) in ovaries of female albino rats

Follicles / Treatments	Control group	ADI group	10×ADI group
Total number of normal follicles	25	18	12*
Total number of atretic follicles	28	32	43*
Total number of follicles	53	50	55
% Atresia	52.83	64*	78.18*

Values are Mean±SE
*Significant difference at ($p<0.05$) between control and treated rats

Table 5. Follicular diameter (µm) of ovaries of female albino rats

Follicle Diameter (µm)	Control	ADI group	10×ADI group
Primary	66.45±1.90	58.69±0.83	47.50±1.02*
Secondary	142.78±1.05	132.36±2.59	112.69±1.26*
Tertiary	213.00±2.58	185.53±2.37	175.49±2.89
Early antral	319.16±0.99	283.02±1.32	260.58±2.91*
Antral	494.00±1.97	413.07±2.62*	368.04±2.63*

Values are Mean±SE
*Significant difference at ($p<0.05$) between control and treated rats

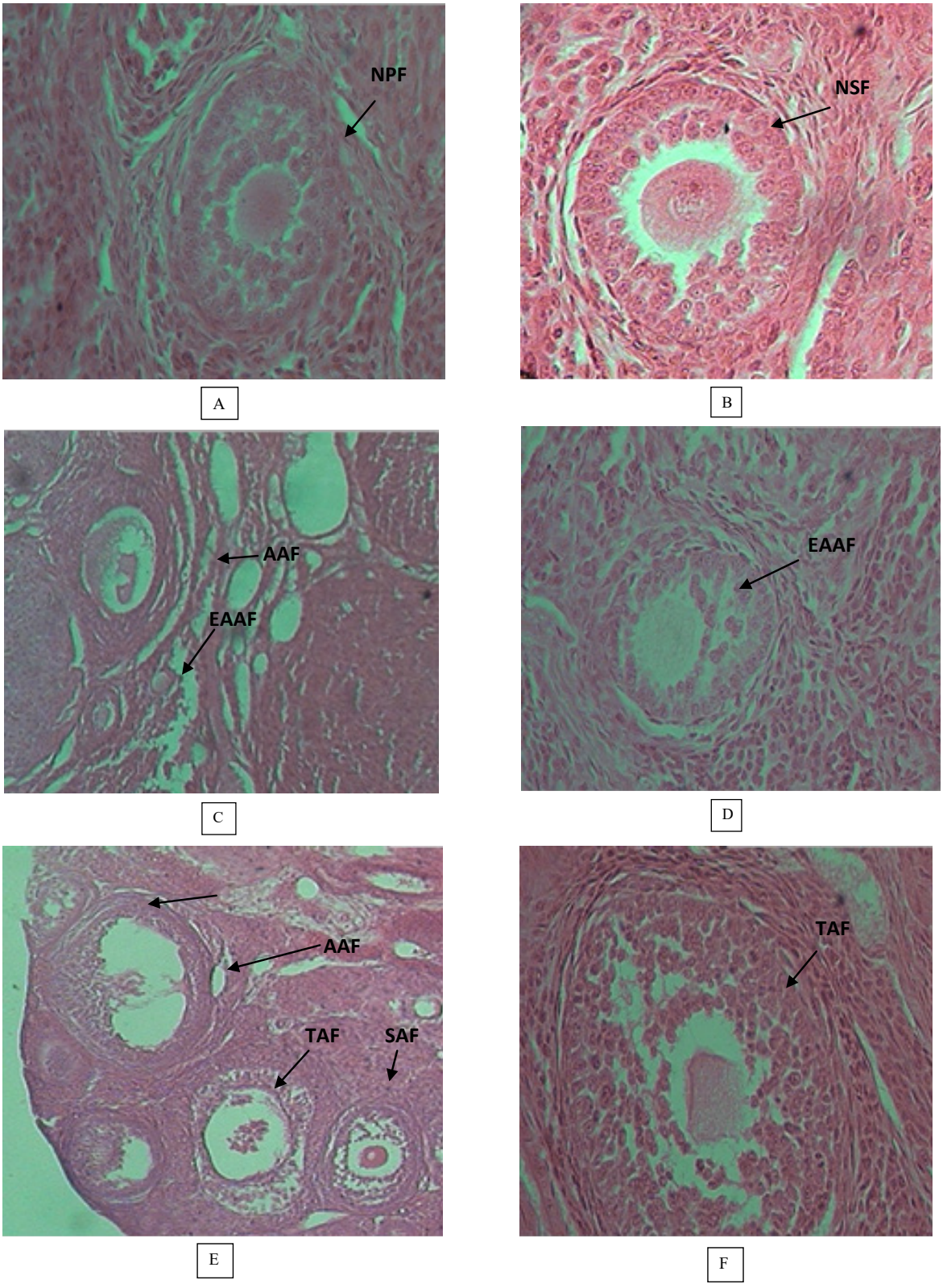


Figure 2. (A, B) T.S of ovary (control group) showing normal primary follicle (NPF) and normal secondary follicle (NSF) (arrow). Fig. A (40X), Fig. B (40X). (C, D) T.S of ovary (ADI group) showing atretic antral follicle (AAF) and early atretic antral follicle (EAAF) (arrow). Fig. C (10X), Fig. D (40X). (E, F) T.S of ovary (10×ADI group) showing atretic antral follicle (AAF), atretic primary follicle (APF), secondary atretic follicle (SAF) and tertiary atretic follicle (TAF) (arrow). (E) (10X), (F) (40X).

3.5.2 In liver

Light microscopy has revealed that in the control female rats' adjacent sinusoids radiate from the central vein, hepatocyte nuclei were round with dispersed chromatin, nucleoli was prominent, hepatocytes were organised into plates separated by vascular channels (sinusoids) and there was no infiltration of leucocytes in the sinusoids (Figure 3. A, B). However, the histological findings of ADI group females showed distorted central vein (Figure 3. C, D) whereas 10×ADI treated rats showed number of pathological alterations, including vacuolization of hepatocytes, dilated sinusoids in between the liver cords with intense mono nuclear inflammatory cellular and infiltration (Figure 3. E, F).

4. Discussion

In the present study, *in utero* exposure to the female albino rats with TZ at the level of ADI and 10×ADI has found to cause non-significant decline in the body weight. The findings of the present study are in agreement with the observations of Turner *et al.*[21] where treatment of dams with fenitrothion at the dose level of 20 and 25 mg/kg/day caused decrease in body weight gain between gestational day 12 and 21. It indicates that OPs have a potential to provoke decrease in body weight of treated animals either by causing suppression of food intake or by disturbing hormonal balance of animals.

Similar to the results of the present study, exposure to chlorpyriphos at the dose level of 5 and 10 mg/kg bw also caused significant decrease in percentage of pregnancy and pup viability[22]. The study conducted by Sharma *et al.*[13] indicated that the preconceptional exposure of TZ at dose level of 1/10th of LD_{50} has resulted in decreased gestational length, however, the outcomes of present study revealed that *in utero* exposure to TZ at the level of ADI has prolonged the length of gestation.

Likewise, the present study, Turner *et al.*[21] has also identified that *in utero* exposure to fenitrothion at the level of 25 mg/kg/day has caused significant decrease in percentage of live pups and litter size. Huang and Li [23] has observed that sex ratio of pups born to cypermethrin treated female rats was also reduced significantly, however, according to the study conducted by Sharma *et al.*[13], TZ exposure at the level of 1/10th, 1/20th and 1/40th of LD_{50} caused significant increase in sex ratio of treated female rats.

Similar to the findings of present study, Sharma *et al.*[13] also observed significant increase in the weight of liver when female albino rats were treated with different doses of TZ. However, the study conducted by Baligar and Kaliwal[24] indicated non-significant change in the weights of liver, lungs and spleen on exposure of female rats to 500, 600, 700 and 800 mg/kg/day mancozeb.

Similar to the results of present study, the intoxication of rats with alpha-cypermethrin and cypermethrin also caused significant increase in weight of thyroid, as evidenced by Seth *et al.*[25] and Sangha *et al.*[26], respectively.

The study conducted by Sharma et al.[17] indicated an increase in ovarian weight of female albino rats on treatment with 1/10th, 1/20th and 1/40th of LD_{50} of TZ. Significant increase in weight of ovaries and uterus was in corroboration with the findings of Nishi and Hundal[27] where female albino rats were treated with chlorpyriphos at the dose level of 0.1 and 2.5 mg/kg/day. Non-significant change was observed in weight of oviduct of the females exposed to methomyl and carbamate formulation[28]. However, study conducted by Baligar and Kaliwal[24] depicted non-significant increase in weight of ovary, uterus and oviduct of mancozeb treated females.

The present study depicted significant increase in the level of proteins in plasma, however, depressed level of proteins was found in the females treated with 1/10th of LD_{50} of TZ[13]. The present increase in the activity of ACP, AKP, AST and ALT in plasma is in agreement with the study conducted by Kaur and Dhanju[29] where females were exposed to three OPs viz. monocrotophos, methyl parathion and dimethoate at the level of 1/5th -1/8th of LD_{50}. Similarly, female exposure to 1/10th of LD_{50} of TZ also caused elevated levels of AST and ALT in plasma[13].

Similar to the outcomes of present study, there is non-significant alteration in ovarian protein content of females exposed to different concentrations of TZ[13]. However, organophosphates like methyl parathion, monocrotophos and dimethoate at 1/5th of LD_{50} caused significant decrease in concentration of ovarian proteins[30].

The enhanced levels of ACP and AKP in the present study depicts ovarian damage and subtle disturbance in hepatic dysfunctions and biliary secretions of animals[31]. As per the biochemical outcomes of the present study, it can be suggested that whenever an animal undergoes stress as a result of pesticide intoxication, there is reduced or enhanced synthesis of biochemical end products due to impaired tissue functioning.

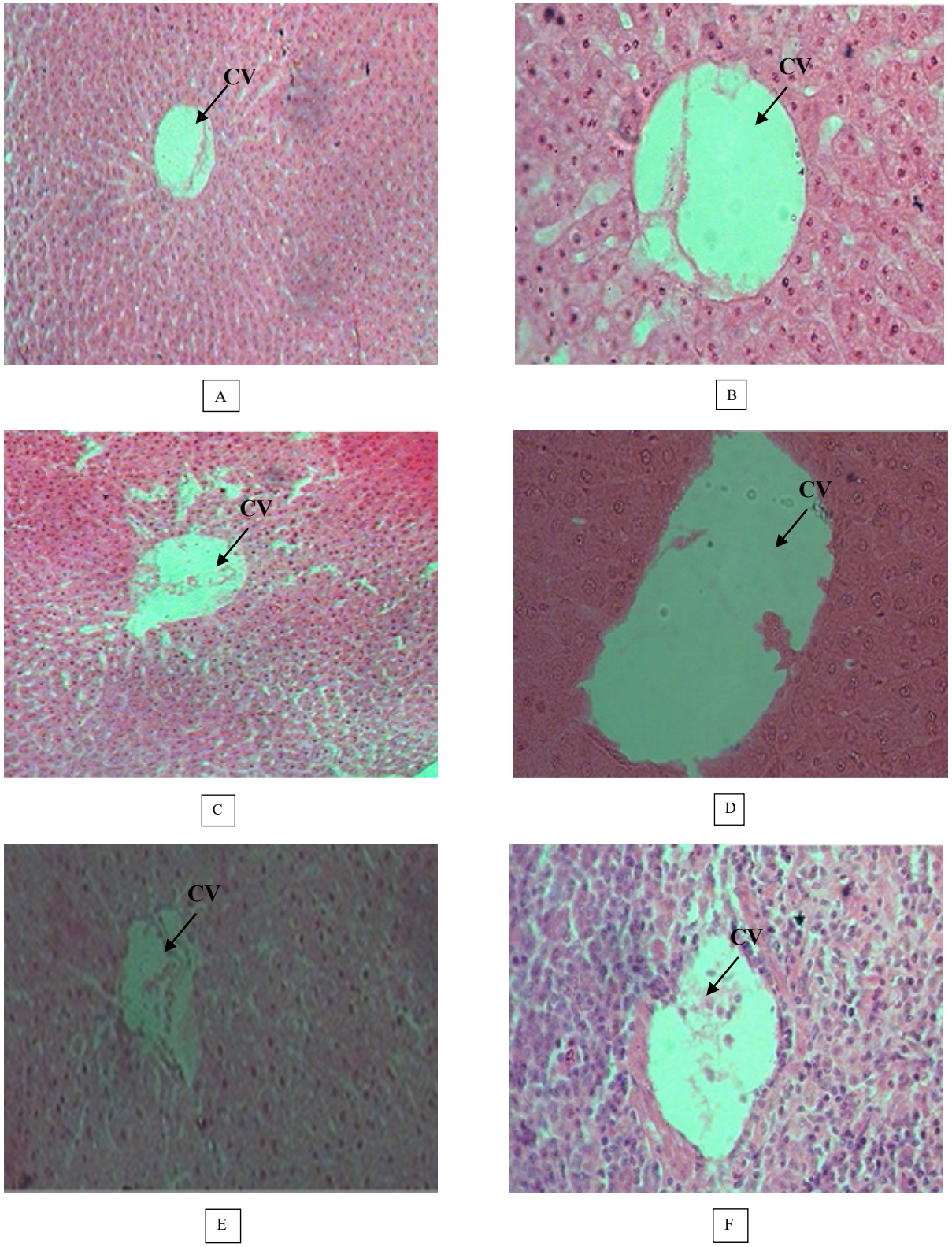


Figure 3. (A, B) T.S of liver (control) showing normal central vein (CV) with adjacent sinusoids radiating from centre (arrow). Fig. A (10X), Fig. B (40X). (C, D) T.S of liver (ADI group) showing distorted central vein (CV) (arrow). (C) (10X), (D) (40X). (E, F) T.S of liver (10×ADI group) showing more distorted central vein (CV) (arrow) and dilated sinusoids in between the liver cords with intense mono nuclear inflammatory cells and infiltration. (E) (10X), (F) (40X).

Our present findings of TZ effect on ovarian histomorphological sections revealed significant increase in percentage of atretic follicles. This observation is in agreement with the study conducted by Nishi and Hundal[27] in which female albino rats were exposed to chlorpyriphos at the dose level of 0.1 and 2.5 mg/kg/day. Similarly, Sharma et al.[17] also observed reduced diameter of tertiary and early antral follicles of females exposed to $1/10^{th}$ and $1/20^{th}$ of LD_{50} of TZ and decrease in antral follicle diameter in $1/10^{th}$ LD_{50} TZ-treated female rats. The aforesaid histological observations emphasizes the fact that OPs can cause damage to developing follicles, hence, in turn may cause damage to oocytes in developing follicles, embryo in the fallopian tube, luteal regression which leads to lack of luteal support to pregnancy.

The histological examination of liver conducted by Uzun and Kalender[32] also indicated vacuolization of hepatocytes, dilated veins, necrosis, hemorrhagic spots in the rats treated orally with chlorpyriphos at the dose level of 12 mg/kg bw. The aforesaid alterations in histo-architecture of liver indicates generation of reactive oxygen species in response to toxicity induced by TZ, posing damage to various components of cell membrane[13].

In conclusion, the exposure to TZ at the level of ADI and 10×ADI during gestational period has induced significant alterations in reproductive parameters viz. percentage of pregnancy, gestational length, litter size, pup viability, pup survival and sex ratio of offsprings born to treated female albino rats. Significant alterations in organ weight viz. liver, thyroid, ovary, uterus and oviduct emphasis the potential of TZ to induce toxicity at organ level. Furthermore, significant changes in biochemical profile viz. the levels of protein and ALT in plasma and the levels of AKP in ovaries and histoarchitecture of ovaries and liver confirms the toxic effects of TZ at cellular level. In view of the data of the present study, it can be interpreted that *in utero* exposure to TZ can severely impair reproductive system functions in female albino rats.

5. Acknowledgement

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6. Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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