SUBMISSION OF FINAL REPORT FOR MAJOR RESEARCH PROJECT

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PROJECT TITLE

Assessment of the toxic effects of synthetic pyrethroid Cypermethrin on biochemical, hormonal and oxidative stress parameters of reproductive system and its alleviation through supplementation of Zinc and α-lipoic acid in animal.

Submitted To

UNIVERSITY GRANT COMMISSION

Submitted By

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10. Objectives of the Project

Cypermethrin, a synthetic pyrethroid insecticide, is effectively used in our country for the management of pests. The acceptability of this pyrethroid in our community is being increased gradually and is causing toxicological adverse effects on different system including reproductive system over the last decades. Some studies are also going on the preventive measures produced by different antioxidants and metals to overcome these types of health hazards. On that basis, the present project has been designed. The major objectives of this project are-

To evaluate the toxic effects of cypermethrin on reproductive system of male and female Wistar rat in oral route.

To assess the mode of action of cypermethrin as endocrine disruptors, this may cause the disruption of male and female hormonal function, if any.

To study the biochemical and hormonal parameters of reproductive system in cypermethrin -treated rats.

To investigate whether there is any relation between cypermethrin and oxidative stress in reproductive system.

To study the histopathological changes in reproductive system of male and female rat by oral route.

To explore the attenuating effect of α- lipoic acid and mineral Zn in reducing reproductive toxicity.

To establish if the antioxidant agent, α- lipoic acid and mineral Zn mitigate the oxidative stress, the cypermethrin- toxicity will be reduced.

11. Methodology

Thirty six albino (Wistar) mature male (130 ± 10 g) rats were used in the present investigation. The animals were kept in the standard laboratory conditions with a standard laboratory food. Water was given ad libitum. The study was approved by the Institution's animal ethical committee.

Thirty six albino mature male rats were divided in six groups, each group consisting of six rats. The groups and treatments were designed as:

1. Group I: Saline control
2. Group II: Zinc(1-2mg/kg body wt) and α- lipoic acid(35 mg/kg body wt.) control
3. Group III: Cypermethrin-treated (Low dose, 40mg/body wt.) group
4. Group IV: Zn+ α- lipoic acid+ Cypermethrin-treated (Low dose, 40mg/body wt.) group
5. Group V: Cypermethrin-treated (High dose, 80mg/body wt.) group
6. Group VI: Zn+ α- lipoic acid+ Cypermethrin-treated (High dose, 80mg/body wt.) group

After one hour of the treatment of Zinc (1-2mg/kg body wt) and α- lipoic acid(35 mg/kg body wt.), Cypermethrin was administered at two dose levels.

Study of Sperm biology parameters

1) Estimation of Testicular index:

Testes of sacrificed male albino rat (Wister) were dissected from its body and all fats were removed from the testes. Then their weights were taken. Testicular index was measured.

2) Estimation of Indices of different male accessory sex organs:

Epedidymis, prostate gland and seminal vesicles of sacrificed male albino rat (Wister) were dissected from its body and all fats were removed from the accessory sex organs. Then their weights were taken and indices were measured.

3) Epididymal sperm motility analysis (WHO, 1999):

Assessment of epididymal sperm motility was done by the method of WHO, 1999.

4) Epididymal sperm morphology analysis (WHO, 1999):

Assessment of epididymal sperm motility was done by the method of WHO, 1999.

5) Epididymal sperm count (WHO, 1999):

After liquification of semen, specimen mixed thoroughly.Specimen was drawn upto 0.5 mark of WBC pipette and then the diluents upto 11 mark , mixed by pipette mixture.(dilution is 1:20). This diluting semen specimen is used to charge the hemocytometer chamber and the sperms were counted.

6) Study of Sperm membrane integrity by Hypoosmotic swelling test (WHO, 1999):

About 1 ml was warmed by swelling solution (sodium citrate dihydrate and fructose in 100 ml distilled water) in a closed Eppendorf tube at 37°C for about 5 minutes. Then liquefied semen was added and mixed gently with the pipette, Kept at 37 °C for at least 30 minutes and examined the sperm cells with a phase-contrast microscope. The number of swollen cells in a total of 200 spermatozoa was counted in duplicate and the mean percentage was calculated.

8) Sperm apoptosis study by flow cytometric method (Cai et al., 2000; Shikone et al., 1994):

In sperm cells PBS was added with ice chilled ethanol, they were Kept in 40C for 1-1.5 hrs. and they were kept for the centrifugation at 1000rpm for 7 min. The supernatant was discarded and the staining solution (PBS: dye: RNAse) was added and it was kept at 370C for 30 min. The cells were then analyzed by flow cytometry and the percentage of cells in the subG1, G0/G1, S and G2/M phases were determined.

B. Experiments on biochemical, hormonal and oxidative stress parameters of male reproductive system

1) Seminal Plasma fructose Concentration (Karronen et. al , 1995)

Fructose in seminal fluid was measured following the method of Karronen et. al, 1995.

2) Testicular and Adrenal cholesterol (Zlatkis et al,1953)

Tissue is homogenized with 0.5% FeCl3. Then centrifuged at 2000 rpm for 10mins. 6ml glacial acetic acid was taken with 0.1 ml supernatant, 0.1ml working standard, 0.1ml distilled water. 4 ml color reagent was added to each test tube. Finally reading is taken at 570 nm.

3) Testicular and adrenal Ascorbic Acid (Roe et al., 1943)

The testicular and adrenal tissue was homogenized using 2.5 mL of 5% metaphosphoric acid–10% acetic acid solutions for the assay of ascorbic acid. After extraction, the mixture was centrifuged. Supernatant was taken and a very small drop of concentrated bromine was added. Tube was shaken and kept for 10 min for complete oxidation to dehydroascorbic acid. Excess liquid bromine was removed by passing air. With 2 mL of tissue extract, 0.5 mL of dinitrophenylhydrazine– thiourea reagent (2.2% 2,4-DNPH in 100 of 10(N) H2SO4–5% thiourea) was added and this was incubated at 37°C for 3 h. Then 2.5 mL of 85% H2SO4 was slowly added by placing the tube in ice bath. It is mixed well in room temperature for half an hour for color development. Optical density was observed at 540 nm.

4) Testicular protein (Lowry et al , 1951)

Tissue homogenate was mixed with 5 ml. of Protein reagent, 0.5ml Folin reagent and normal saline. Five standard solutions were prepared for standared curve. All samples were mixed and incubated at 37o C. And OD was taken at 660 nm.

5) Testicular glutamate oxaloacetate transaminase (GOT) and glutamate Pyruvate transaminase (GPT) (Reitman and Frankel , 1957)

One ml Buffered substrates (SGOT/SGPT ml) were given in each test tube and waited for 5 minutes at 37˚C. After that 0.2 ml tissue homogenate was added to 'Test' marked test tube. Then incubated at 37˚C for 60 minutes. After that 1 ml DNPH solution was added to each test tube. Then 0.2 ml tissue homogenate was added to 'Control' marked test tube. Then mixed and waited for 20 minutes. After that 10.0 ml 0.4 (N) NAOH was added to each test tube and mixed and waited for 10 minutes. Then reading was taken at test against control at 520 nm. Another two test tubes were taken and marked as "standard" and "blank". Then 0.2 ml working pyruvate standard solution was added to standard marked test tube. After that 0.8 ml and 1 ml buffered substrate Then 0.2 ml water

was added to each test tube. After that 1 ml DNPH solution was added to each test tube and mixed well and waits for 20 min at room temperature. Then 10 ml 0.4 (N) NAOH solution was mixed and waited for 10 min. The reading was taken at standard against blank at 520 nm.

6) Testicular acid phosphatase (Moss et al .,1984)

0.8 ml of sodium acetate was taken in a test tube. then 1ml homogenized tissue, NaF and 0.3 ml distilled water and 0.3 ml $CoCl_2$ were added. Kept for 30 min in ice cooled water. Then PNPP was mixed and incubate at 370C for 30 min. After that NaOH and redistilled water was added and centrifuged. Supernatant was taken, amount of PNP liberated was measured at 410 nm. Value of sample was obtained from standard curve and expressed as microgram of PNP liberated / mg of tissue / hour of incubation.

7) Testicular and adrenal Δ53β-HSD (Talalay et al., 1962)

Homogenates are centrifuged in cold centrifuge at 10000 rpm (-4°c) for 30-45 min. Supernatant collected in a dry test tube and storage in ice container. For the measurement of Δ53β-HSD, 1ml supernatant, 0.9 ml double distilled water, TNaPP and DHEA were mixed. It was waited for stable reading. Then 0.1 ml NAD was added and six readings were taken at 30 sec interval at 340 nm.

8) Testicular and adrenal 17β-HSD (Jarabak et al., 1962)

Homogenates are centrifuged in cold centrifuge at 10000 rpm (-4°c) for 30-45 min. Supernatant collected in a dry test tube and storage in ice container. For the measurement of 17β HSD, 1ml supernatant, 0.9 ml double distilled water, TNaPP and testosterone were mixed. It was waited for stable reading. Then 0.1 ml NAD was added and six readings were taken at 30 sec interval at 340 nm.

9) Testicular and adrenal Glucose -6- P- dehydrogenase

Testicular and adrenal Glucose -6- P- dehydrogenase were estimated by the method of Bergmeyer et al. (1965).

C. Estimation of hormonal parameters

1) Serum testosterone level (ELISA Method by using kit)

2) Serum LH level(ELISA Method by using kit)

3) Serum FSH level(ELISA Method by using kit)

D. Estimation of oxidative stress parameters

1) Testicular Malon dialdehyde (Ohkawa et al., 1979)

0.5ml sample (tissue concentration 50 mg /m), 0.5 ml normal saline [0.9%], 2 ml TBA – TCA mixture were mixed. It was boiled for 10 min and was cooled at room temperature. And centrifuged in 4000 r.p.m for 10 min. Supernatant was poured in cuvette. Reading was taken at 535 nm.

2) Testicular Catalase (Luck et al., 1971)

Homogenates (50 mg /mL) in 0.5 M Tris-hydrochloric acid (HCL) buffer solution (pH-7.0) were centrifuged at 10,000 g at 4oC for 10 min in spectrophotometric cuvette, 0.5 mL of hydrogen peroxide and 2.5 mL of distilled water were mixed and absorption was noted at 240 nm and reading was noted at 30 sec interval.

3) Testicular Glutathione peroxidase (Flohe and Gunzler,1984)

Peroxidase activity was determined by the method of Flohe and Gunzler,1984 .

4) Testicular Super-oxide dismutase (Kono et al., 1978)

Homogenates (50 mg /mL) in ice-cold 100 Mm Tris-cacodylate buffer were centrifuged at 10,000 g for 20 min at 4oC. The SOD activities of the supernatants were estimated by measuring the percentage inhibition of the pyragallol autooxidation by SOD (Marklund S, Marklund G, 1974).

In a spectrophotometric cuvette, 2 mL of buffer, 100 µl of 2 mM pyragallol and 10 µl of supernatant were poured and the absorbance was noted in a spectrophotometer at 420 nm for 3 min.

5) Testicular Reduced Glutathione (Moron et al., 1979)

200µl sample mixed with sulfosalicylic acid and centrifuged for 10 min at 3000 rpm. The supernatant was added with DTNB and was shaken well. Reading was taken at 412-420nm.

6) Testicular Glutathione -S-transferase (Habig et al., 1974)

Activities of Testicular Glutathione-S-transferase in the testicular tissues were individually measured spectrophotometrically (Habig WH, Pabst MJ, 1974) using 1-chloro 2,4-dinitrobenzene as substrate. The assay mixture contained 1mM CDBN in ethanol; 1 M reduced glutathione, 100 Mm potassium phosphate buffer (pH-6.5) and supernatant of tissue homogenate. The formation of the adduct of CDNB, S-2,4-dinitrophenylglutathione was monitored by measuring absorbance at 340 nm against blank.

D. Histological study

Histological studies were done for Testicular tissue by conventional histological method with Haematoxylin and Eosin.

Study of female parameters

Thirty six albino (Wistar) mature female (130 ± 10 g) rats were used in the present investigation. The animals were kept in the standard laboratory conditions with a standard laboratory food. Water was given *ad libitum*. The study was approved by the Institution's animal ethical committee.
Thirty six albino mature female rats were divided in six groups, each group consisting of six rats. The groups and treatments were designed as:

1. Group I: Saline control
2. Group II: Zinc (1-2mg/kg body wt) and α- lipoic acid (35 mg/kg body wt.) control
3. Group III: Cypermethrin-treated (Low dose, 34.33mg/body wt.) group
4. Group IV: Zn+ α- lipoic acid+ Cypermethrin-treated (Low dose, 34.33mg/body wt.) group
5. Group V: Cypermethrin-treated (High dose, 51.5mg/body wt.) group

6. Group VI: Zn+ α- lipoic acid+ Cypermethrin-treated (High dose, 51.5mg/body wt.) group

After one hour of the treatment of Zinc (1-2mg/kg body wt) and α- lipoic acid (35 mg/kg body wt.), Cypermethrin was administered at two dose levels.

A. Study of estrous cycle:

The estrous cycle was examined daily by the modified method of Marcondes et al., 2002 with slight modifications and identified under a microscope using a vaginal smear flushed with normal saline for 14 days.

B. Experiments on biochemical, hormonal and oxidative stress parameters of female reproductive system

2) Ovarian and adrenal cholesterol (Zlatkis et al.,1953)

Tissue is homogenized with 0.5% $FeCl_3$. Then centrifuged at 2000 rpm for 10mins. 6ml glacial acetic acid was taken with 0.1 ml supernatant, 0.1ml working standard, 0.1ml distilled water. 4 ml color reagent was added to each test tube. Finally reading is taken at 570 nm.

3) Ovarian and adrenal ascorbic acid (Maiti Choudhury et al.,2011)

The ovarian and adrenal tissue was homogenized using 2.5 mL of 5% metaphosphoric acid–10% acetic acid solutions for the assay of ascorbic acid. After extraction, the mixture was centrifuged. Supernatant was taken and a very small drop of concentrated bromine was added. Tube was shaken and kept for 10 min for complete oxidation to dehydro ascorbic acid. Excess liquid bromine was removed by passing air. With 2 mL of tissue extract, 0.5 mL of dinitrophenylhydrazine–thiourea reagent was added and this was incubated at 37°C for 3 h. Then 2.5 mL of 85% H_2SO_4 was slowly added by placing the tube in ice bath. It is mixed well in room temperature for half an hour for color development. Optical density was observed at 540 nm.

4) Ovarian glutamate oxaloacetate transaminase (GOT) and glutamate Pyruvate transaminase (GPT)(Goel,1988)

One ml Buffered substrates (GOT/GPT) were given in each test tube and waited for 5 minutes at 37˚C. After that 0.2 ml tissue homogenate was added to 'Test' marked test tube. Then

incubated at 37˚C for 60 minutes. After that DNPH solution was added to each test tube. Then 0.2 ml tissue homogenate was added to 'Control' marked test tube. Then mixed and waited for 20 minutes. After that 10.0 ml 0.4 (N) NaOH was added to each test tube and mixed and waited for 10 minutes. Then reading was taken at test against control at 520 nm. Another two test tubes were taken and marked as "standard" and "blank". Then 0.2 ml working pyruvate standard solution was added to standard marked test tube. After that 0.8 ml and 1 ml buffered substrate Then 0.2 ml water was added to each test tube. After that 1 ml DNPH solution was added to each test tube and mixed well and waits for 20 minutes at room temperature. Then 10 ml 0.4 (N) NaOH solution was mixed and waited for 10 minutes. The reading was taken at standard against blank at 520 nm.

5) Ovarian acid phosphatase (Vanha-Pertulla and Nikkanen,1973)

The acid phosphatase activity was measured in sodium acetate buffer at pH 4.5 using *p*-nitrophenol phosphate as a substrate. Amount of PNP liberation was considered spectrophotometrically at 420 nm.

6) Ovarian and adrenal $\Delta^5$3β-HSD(Talalay , 1962)

Homogenates are centrifuged in cold centrifuge at 10000 rpm (-4°c) for 30-45 min. Supernatant collected in a dry test tube and storage in ice container. For the measurement of $\Delta^5$3β-HSD, 1ml supernatant, 0.9 ml double distilled water, TNaPP and DHEA were mixed. It was waited for stable reading. Then 0.1 ml NAD was added and six readings were taken at 30 sec interval at 340 nm.

7) Ovarian and adrenal 17β-HSD (Jarabak et.al., 1962)

Homogenates are centrifuged in cold centrifuge at 10000 rpm (-4°c) for 30-45 min. Supernatant collected in a dry test tube and storage in ice container. For the measurement of 17β HSD, 1ml supernatant, 0.9 ml double distilled water, TNaPP and testosterone were mixed. It was waited for stable reading. Then 0.1 ml NAD was added and six readings were taken at 30 sec interval at 340 nm.

C. Estimation of hormonal parameters

1) Serum LH level (ELISA Method by using kit)
2) Serum FSH level (ELISA Method by using kit)
3) Serum estradiol level (ELISA Method by using kit)

D. Estimation of oxidative stress parameters

1) Ovarian malon dialdehyde (Ohkawa et al., 1979)

MDA assay was measured by the method of Ohkawa et al., 1979. One ml tissue homogenate was mixed with 8.1 % sodium dodecyl sulfate, acetate buffer (20 % pH 3.5), and 1.5 ml of thiobarbituric acid (0.8 %) were taken. After heating at 95°C for 60 min, the red pigment produced was extracted with 5 ml *n*-butanol-pyridine mixture (15: 1) and centrifuged at 5000 rpm for 10 min at room temperature. The absorbance of supernatants was estimated at 535nm.

2) Ovarian catalase (Aebi, 1974)

Catalase was estimated by the method of Aebi (Aebi,1974).The reaction mixture consisted of H_2O_2, double distilled water and 40µl of homogenate (in 0.05M tris HCl) and was taken in a cuvette. After mixing, readings were noted at 240nm at 30 sec interval.

3) Ovarian glutathione peroxidase (Rotruck et al., (1973)

Peroxidase activity was determined by the method Rotruck et al., (1973)**.**

4) Ovarian superoxide dismutase (Marklund and Marklund , 1974)

Superoxide dismutase was measured by the method of Marklund and Marklund (Marklund and Marklund, 1974). At first 50 Mm Tris HCl, 10 mM pyrogallol in the presence of EDTA and 20 µl of homogenate were poured in a spectrophotometric cuvette and the reading was measured in the spectrophotometer at 420 nm for 3 min.

5) Ovarian reduced glutathione (Griffith, 1981)

At first 200µl sample mixed with sulfosalicylic acid and centrifuged for 10 min at 3000 rpm. The supernatant was added with DTNB and was shaken well. Reading was taken at 412-420nm.

6) Ovarian glutathione -s-transferase (Habig et al., 1974)

Activities of ovarian glutathione-S-transferase in the Ovarian tissues were individually measured spectrophotometrically (Habig et al., 1974) using 1-chloro 2,4-dinitrobenzene as substrate. The

assay mixture contained 1mM CDBN in ethanol; 1 M reduced glutathione, 100 Mm potassium phosphate buffer (pH-6.5) and supernatant of tissue homogenate. The formation of the adduct of CDNB, S-2,4-dinitrophenylglutathione was monitored by measuring absorbance at 340 nm against blank.

E. Histological study

Histological studies were done for ovarian tissue by conventional histological method with Haematoxyline and Eosin.

F. Statistical analysis

The experimental results were expressed as Mean ± Standard error of mean (SEM). Statistical analysis of the collected data were done by Analysis of variance (ANOVA) followed by Multiple comparison t-test. Difference was considered significant when $p<0.05$.

12. Achievements from the project

A. Results of Sperm Biology

1) Effect on Testiculo-Somatic index:

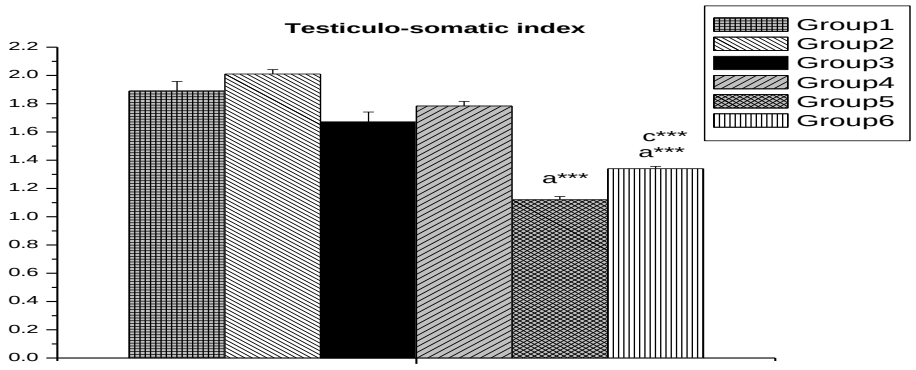


Figure-1: The figure shows the effect of Zinc and α- lipoic acid on **Testiculo-Somatic index** in Cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (*** indicates $p<0.001$).

2) Effects on Indices of different male accessory sex organ

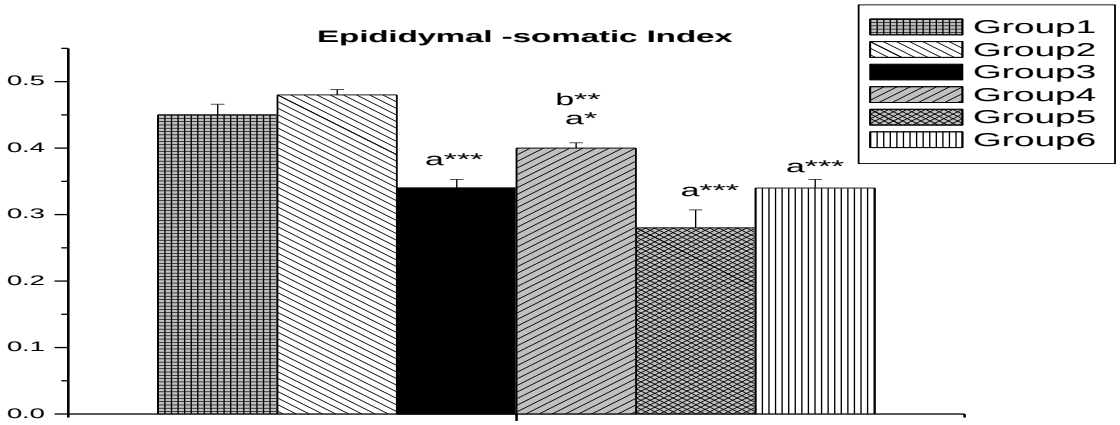


Figure-2: The figure shows the effect of Zinc and α- lipoic acid on **Epididymal--Somatic index** in Cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV. Asterisks represents the different level of significance (* indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001).

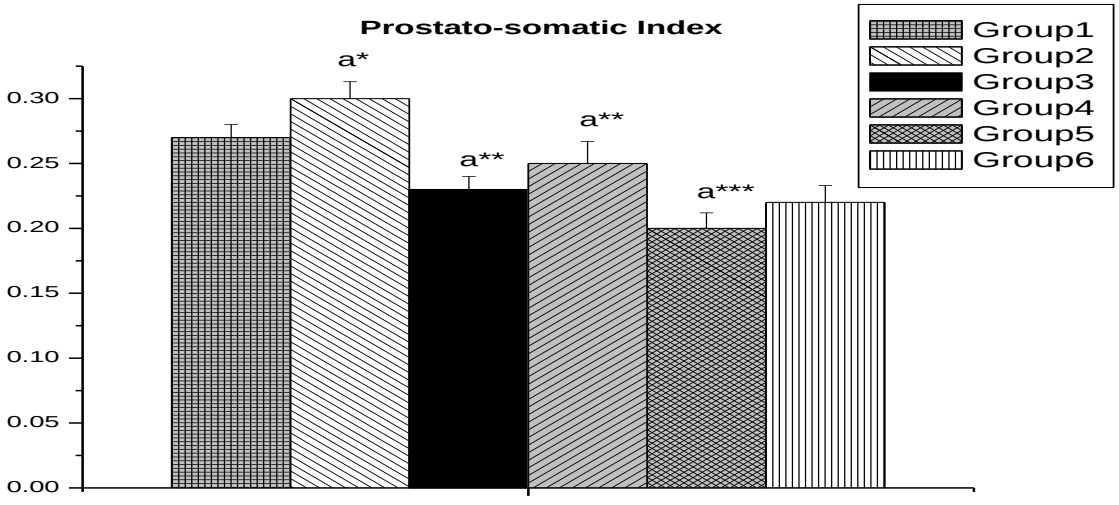


Figure-3: The figure shows the effect of Zinc and α- lipoic acid on **Prostrato--Somatic index** in Cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus

all other groups. Asterisks represents the different level of significance (* indicates p<0.05,** indicates p<0.01 ,*** indicates p<0.001).

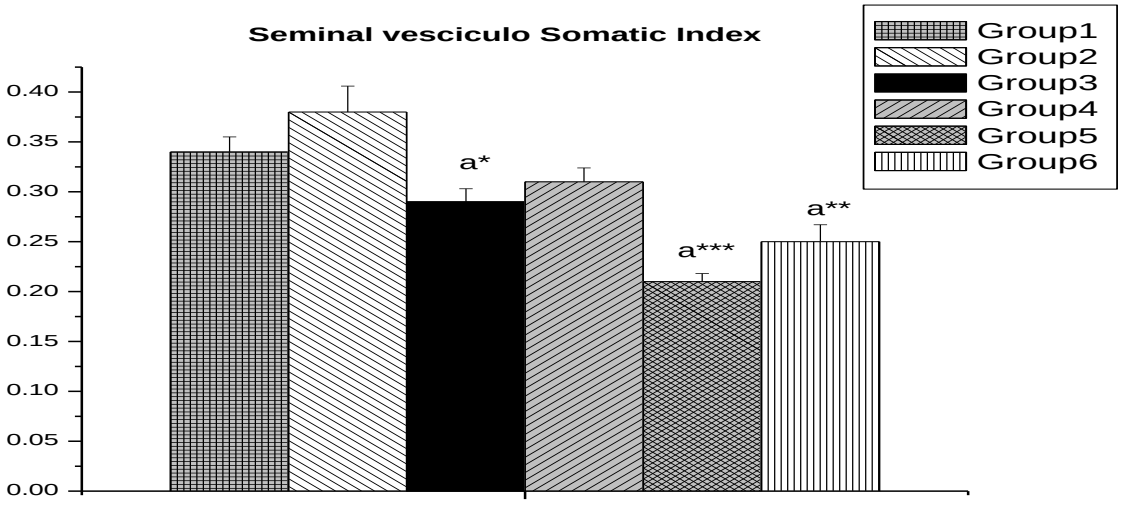


Figure-4: The figure shows the effect of Zinc and α- lipoic acid on **Seminal vesciculo--Somatic index** in Cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Asterisks represents the different level of significance (* indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001).

3) Effect on Epididymal sperm motility:

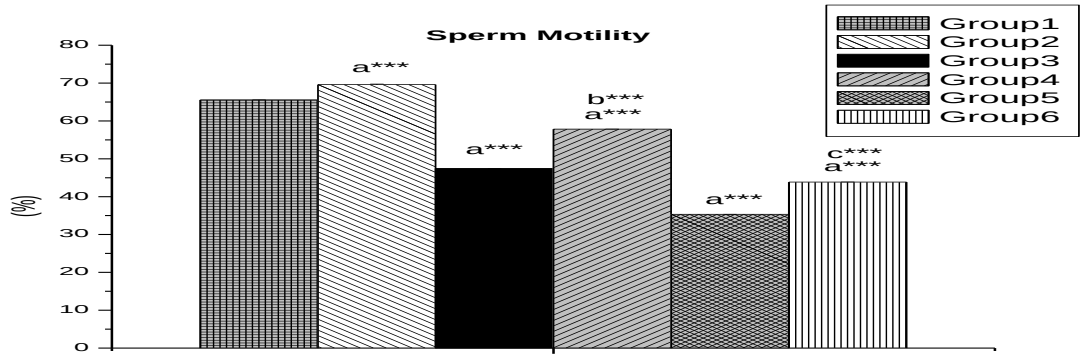


Figure-5: The figure shows the effect of Zinc and α- lipoic acid on **Epididymal sperm motility** in

Cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (*** indicates p<0.001).

4) Effect on Epididymal sperm morphology analysis

Table 1 shows the effect of Zinc and α- lipoic acid Cypermethrin on **Epididymal sperm morphology** in Cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (*** indicates p<0.001).

Group	**Saline Control (%)**	**Zn+ Lipoic acid control (%)**	**Cyp (Low) (%)**	**Cyp(L)+ Zn+ Lipoic acid(%)**	**Cyp (High) (%)**	**Cyp(H)+Zn + Lipoic acid(%)**
Tailless head	4.1±0.2	5.8±0.90	6.1±0.17	4.81±0.15	6.2±0.74	5.2±0.78
Headless tail	4.7±0.5	3.8±0.83	6.3±0.13	4.92±0.16	8.6±0.62	5.1±0.58
Bent tail	5.2±0.32	5.1±0.92	7.1±0.21	6.83±0.14	9.6±0.43	5.6±0.38

Curve tail	6.2±0.3	4.2±0.84	8.4±0.32	6.73±0.26	10.2±1.3	7.9±0.42
Bent mid piece	4.1±0.4	5.7±0.62	6.3±0.26	0.41±0.02	9.83±0.21	6.21±0.23
Coiled tail	0.3±0.02	4.6±0.53.	0.72±0.03	0.41±0.02	1.8±0.09	0.6±0.02
Looped tail	0.12±0.02	0.4±0.06	0.23±0.03	0.19±0.008	1.62±0.12	1.2±0.03
Rudimentary tail	0.23±0.13	0.09±0.006	0.73±0.08	0.31±0.06	2.5±0.72	0.36±0.04
Curve mid piece	6.24±0.72	0.3±0.08	8.21±0.45	6.72±0.32	11.2±0.13	8.2±0.63
Total	**33.9±2.61**	**30.0±4.8[a]***	**44.09±1.6[a]***	**37.04±1.42[a]***b***	**61.55±4.36 a***	**40.37±3.48[a]**c***

Values represents Mean± SEM (N=6).

5) Effect on Epididymal sperm count:

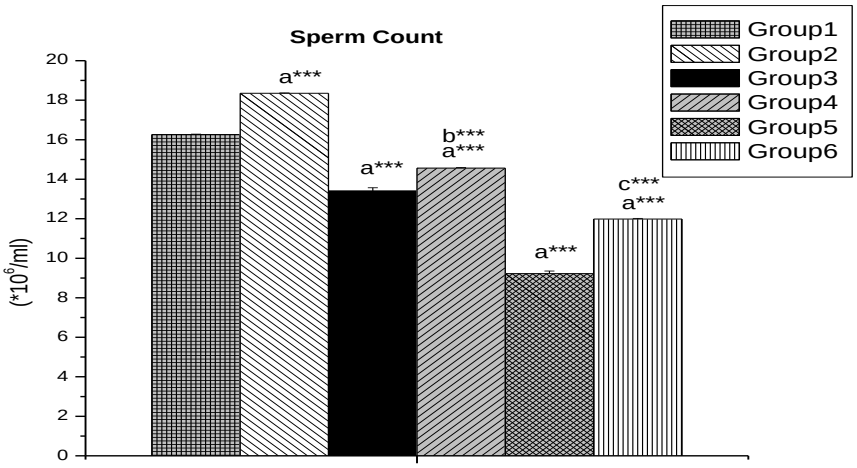


Figure-6: The figure shows the effect of Zinc and α- lipoic acid on **Epididymal sperm count** in Cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (*** indicates p<0.001).

6) Effect on Sperm membrane integrity by Hypo-osmotic swelling test:

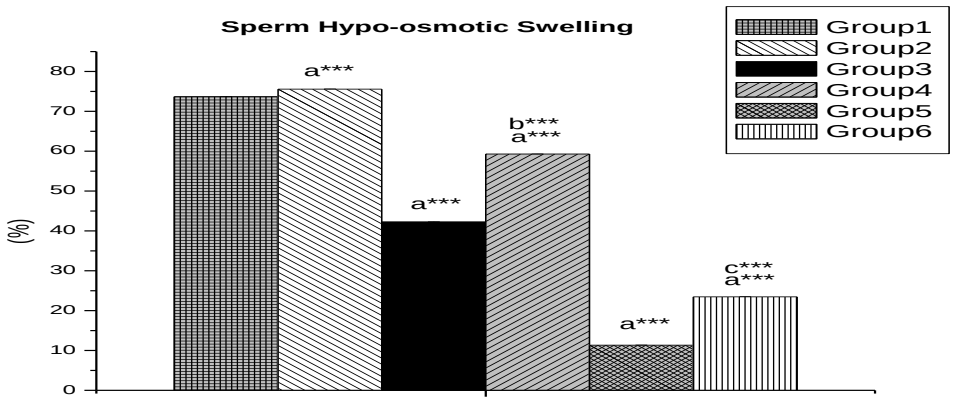


Figure-7: The figure shows the effect of Zinc and α- lipoic acid on **Hypo-osmotic swelling of rat sperm** in Cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,**

Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (*** indicates p<0.001).

7) Effect on Sperm apoptosis:

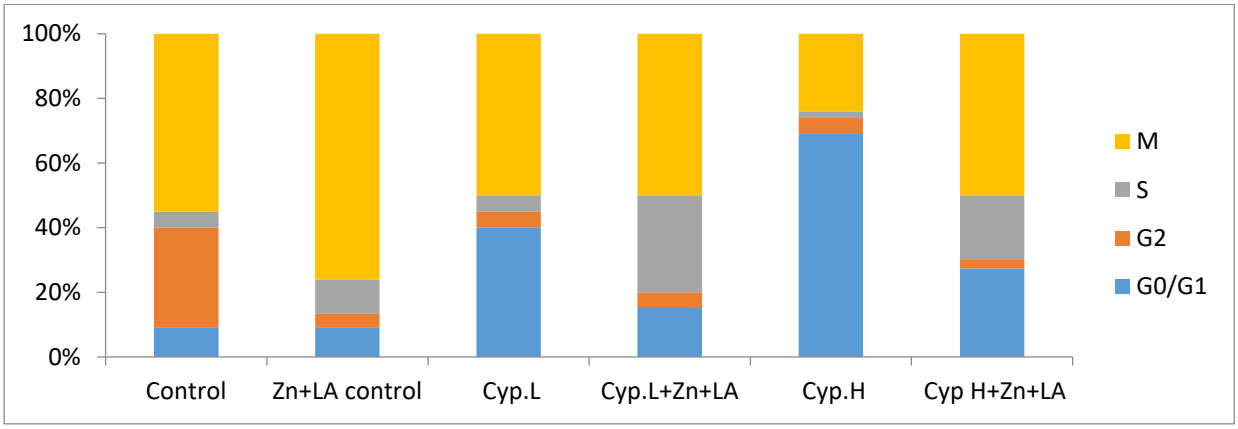


Figure-8 shows the effect of Zinc and α- lipoic acid on **Cell cycle arrest of rat sperm** in Cypermethrin induced male albino rat (N=6). Results are expressed as Mean (%).

B. <u>Effects of zinc and alpha-lipoic acid on biochemical, hormonal and oxidative stress parameters of male reproductive system after the administration of Cypermethrin in oral route</u>

1) Effect on Seminal Plasma fructose Concentration:

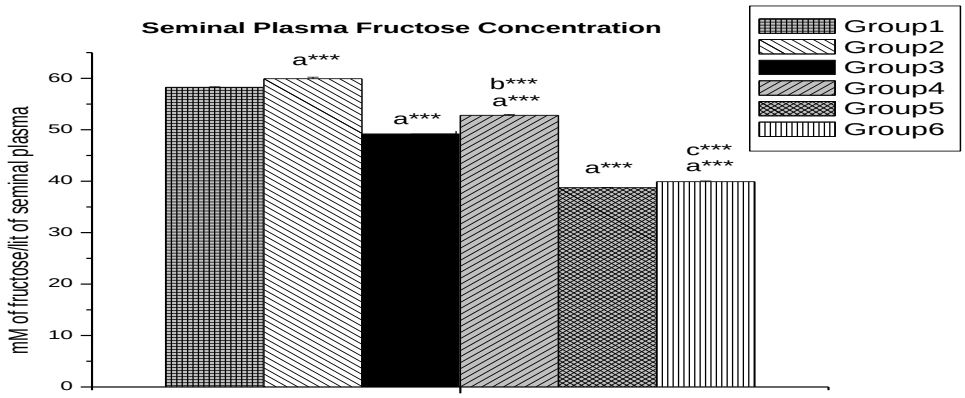


Figure-10: The figure shows the effect of Zinc and α- lipoic acid on **Seminal Plasma fructose Concentration** in Cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (*** indicates p<0.001).

2) Effect on Testicular cholesterol:

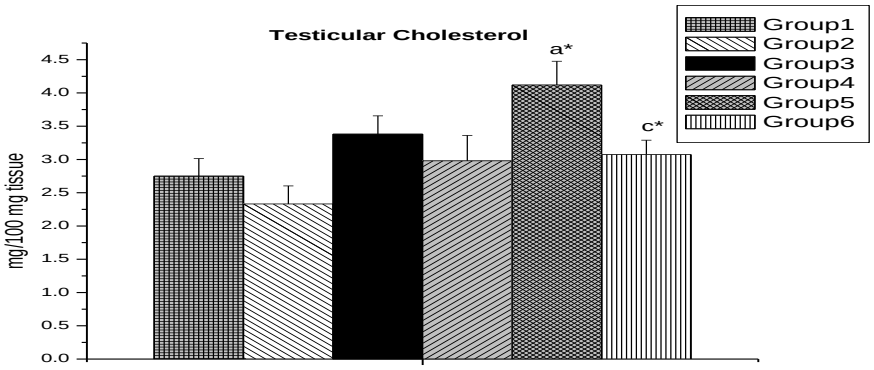


Figure-11: The figure shows the effect of Zinc and α- lipoic acid on **Testicular cholesterol** in Cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05).

3) Effect on Adrenal cholesterol:

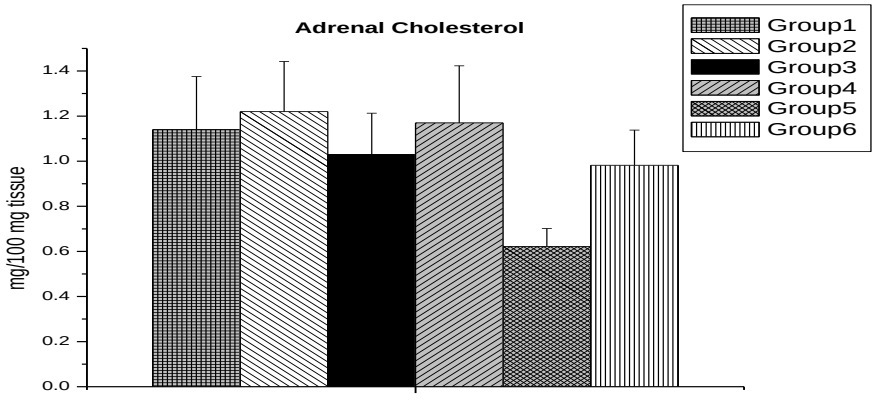


Figure-12: The figure shows the effect of Zinc and α- lipoic acid on **Adrenal cholesterol** in Cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates $p<0.05$,** indicates $p<0.01$; ,*** indicates $p<0.001$).

4) Effect on Testicular Ascorbic Acid :

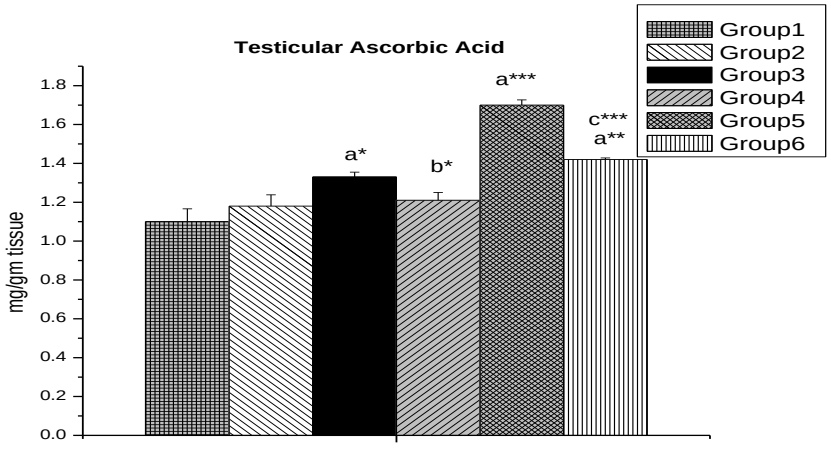


Figure-13: The figure shows the effect of Zinc and α- lipoic acid on **Testicular Ascorbic Acid** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by

ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001).

5) Effect on Adrenal Ascorbic Acid:

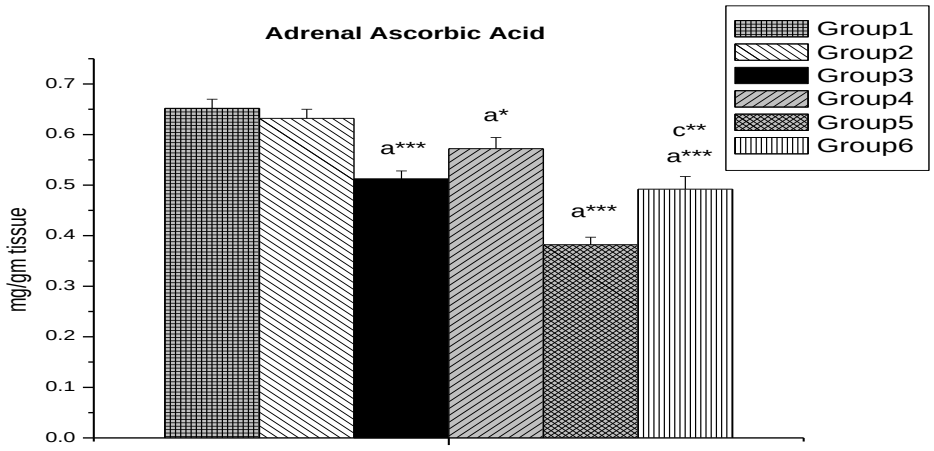


Figure-14: The figure shows the effect of Zinc and α- lipoic acid on **Adrenal Ascorbic Acid** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05, ** indicates p<0.01 ,*** indicates p<0.001).

6) Effect on Testicular protein:

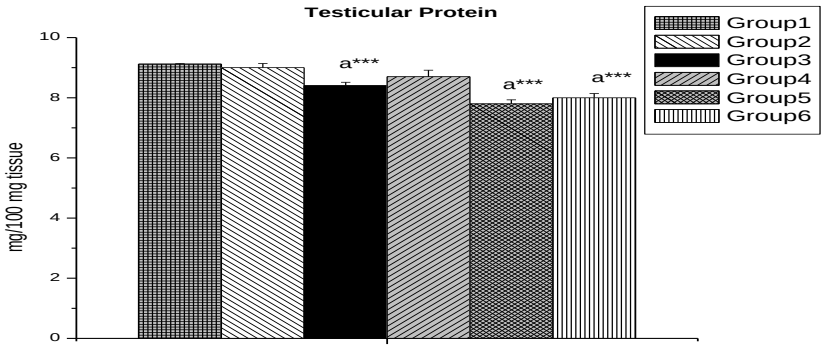


Figure-15: The figure shows the effect of Zinc and α- lipoic acid on **Testicular protein** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups. Asterisks represents the different level of significance (*** indicates p<0.001).

7) Effect on Testicular Glutamate Pyruvate Transaminase (GPT)

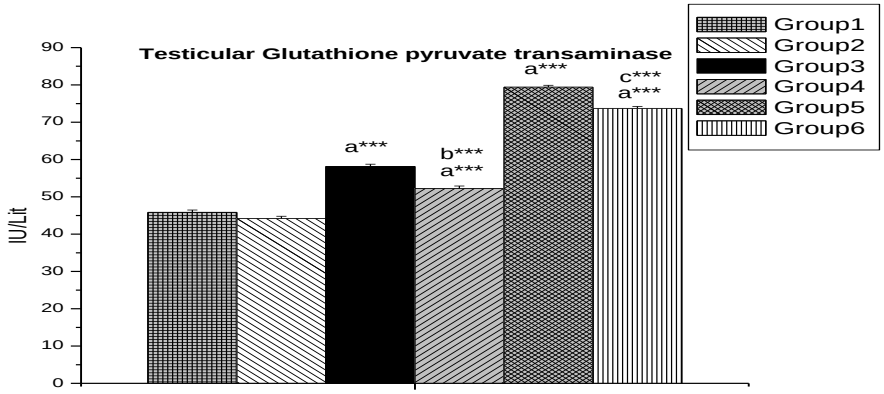


Figure-16: The figure shows the effect of Zinc and α- lipoic acid on **Testicular Glutamate Pyruvate Transaminase (GPT)** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (*** indicates p<0.001).

8) Effect on Testicular Glutamate Oxaloacetate Transaminase (GOT)

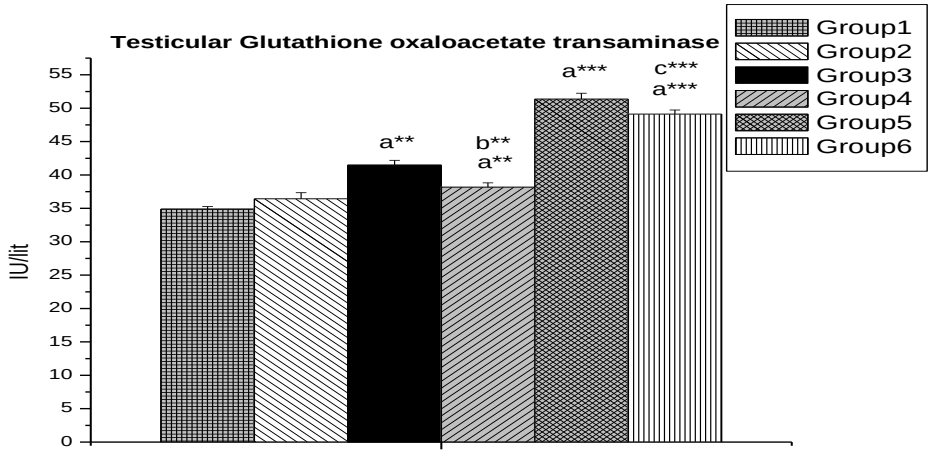


Figure-17: The figure shows the effect of Zinc and α- lipoic acid on **Testicular Glutamate Oxaloacetate Transaminase (GOT)** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (** indicates p<0.01 ,*** indicates p<0.001).

9) Effect on Testicular Acid Phosphatase(ACP)

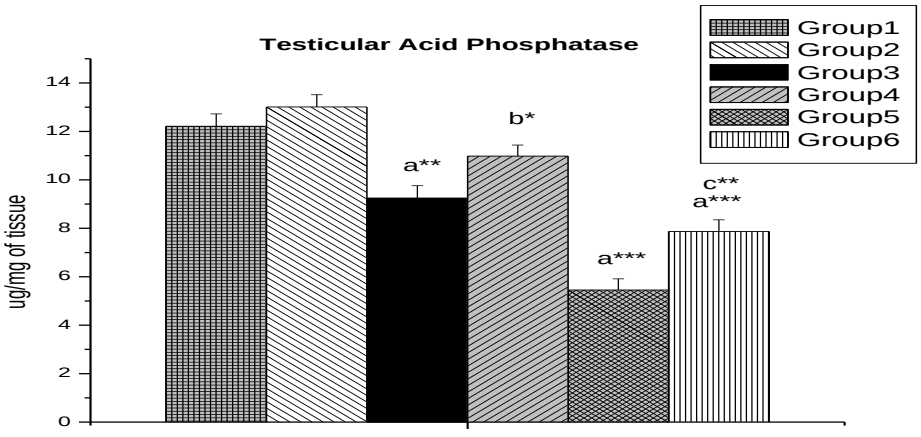


Figure-18: The figure shows the effect of Zinc and α- lipoic acid on **Testicular Acid Phosphatase(ACP)** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests.

Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05,** indicates p<0.01; ,*** indicates p<0.001).

10) Effect on Testicular $\Delta^5$3β-HSD

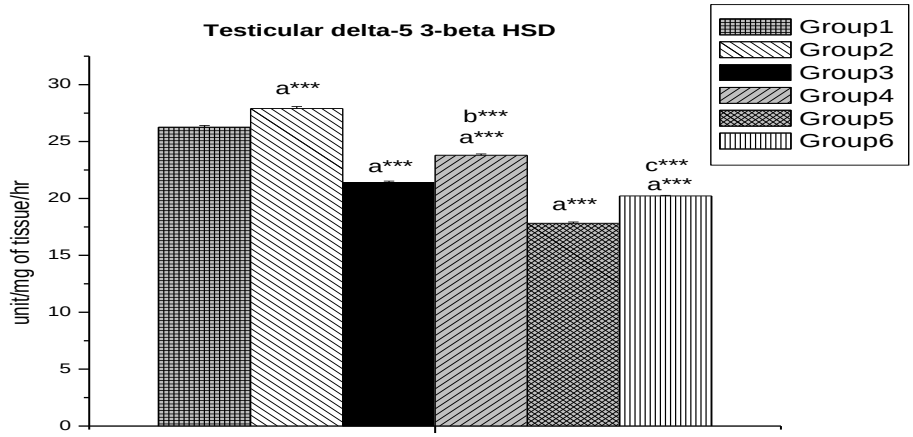


Figure-19: The figure shows the effect of Zinc and α- lipoic acid on **Testicular** Δ^5**3β-HSD** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (*** indicates p<0.001.

11) Effect on Testicular 17β-HSD

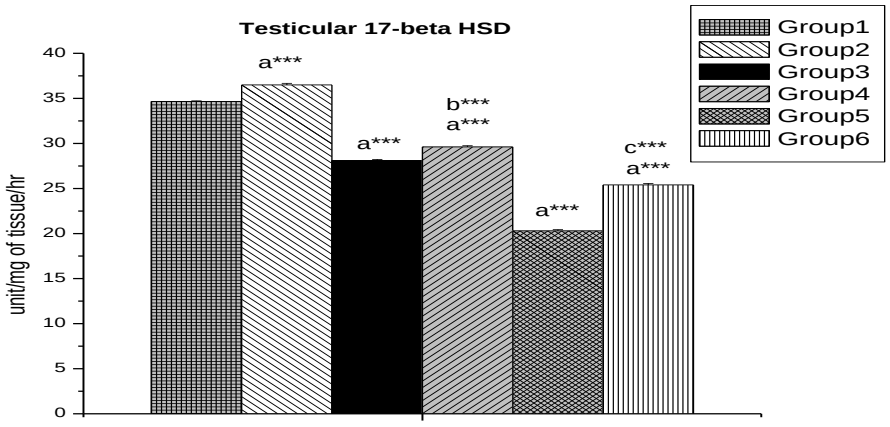


Figure-20: The figure shows the effect of Zinc and α- lipoic acid on **Testicular 17β-HSD** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (*** indicates p<0.001).

12) Effect on Adrenal $\Delta^5$3β-HSD

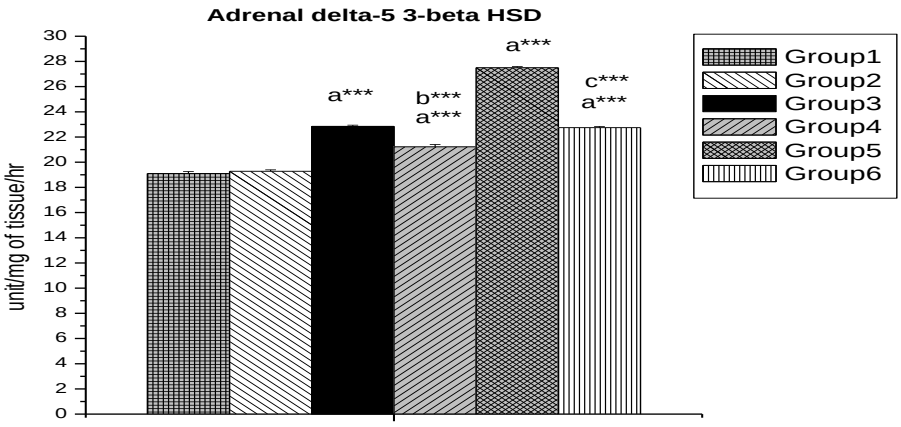


Figure-21: The figure shows the effect of Zinc and α- lipoic acid on **Adrenal $\Delta^5$3β-HSD** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (*** indicates p<0.001).

13) Effect on Adrenal 17β-HSD

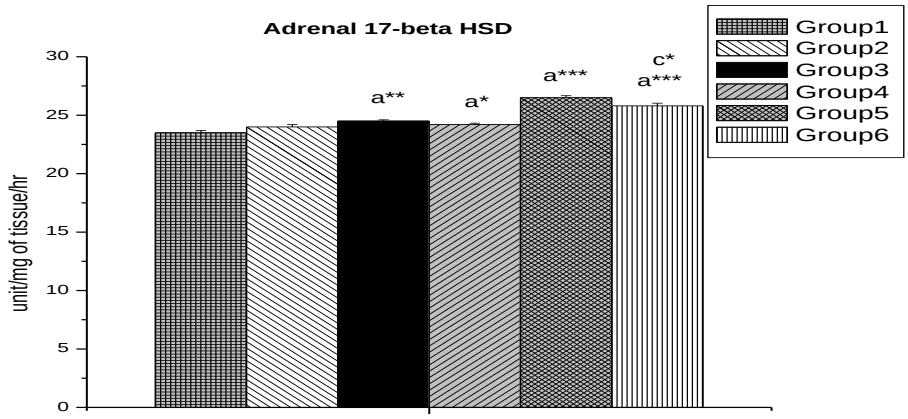


Figure-22: The figure shows the effect of Zinc and α- lipoic acid on **Adrenal 17β-HSD** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001).

14) Effect on Testicular Glucose -6- P- dehydrogenase

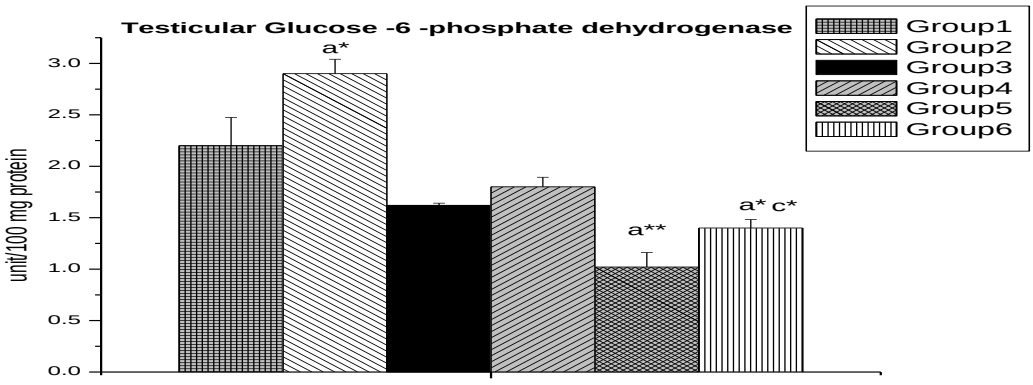


Figure-23: The figure shows the effect of Zinc and α- lipoic acid on **Testicular Glucose -6- P-dehydrogenase** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,**

Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05, ** indicates p<0.01).

15) Effect on Adrenal Glucose -6- P- dehydrogenase

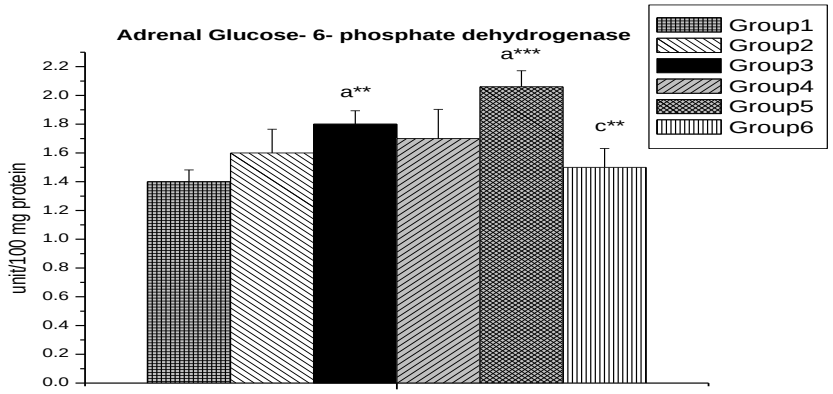


Figure-24: The figure shows the effect of Zinc and α- lipoic acid on **Adrenal Glucose -6- P-dehydrogenase** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (** indicates p<0.01 ,*** indicates p<0.001).

C. Estimation of hormonal parameters

1) Effect on Serum testosterone level

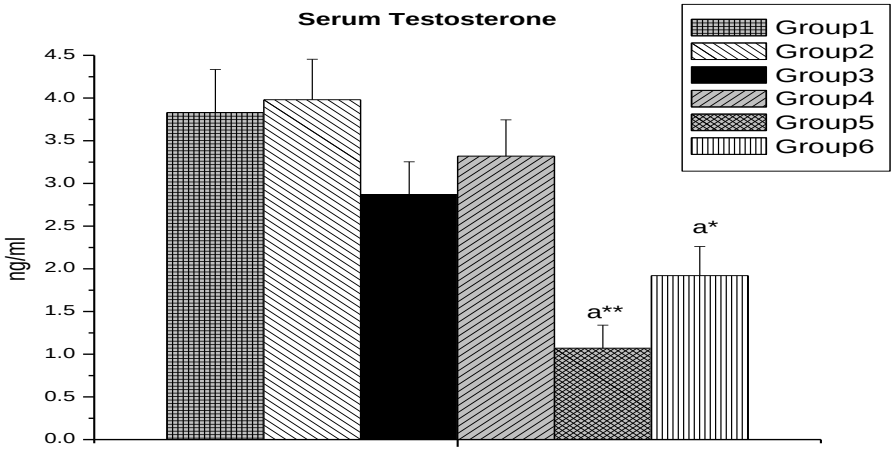


Figure-25: The figure shows the effect of Zinc and α- lipoic acid on **Serum testosterone level** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups. Asterisks represents the different level of significance (* indicates $p<0.05$,** indicates $p<0.01$).

2) Effect on Serum LH level

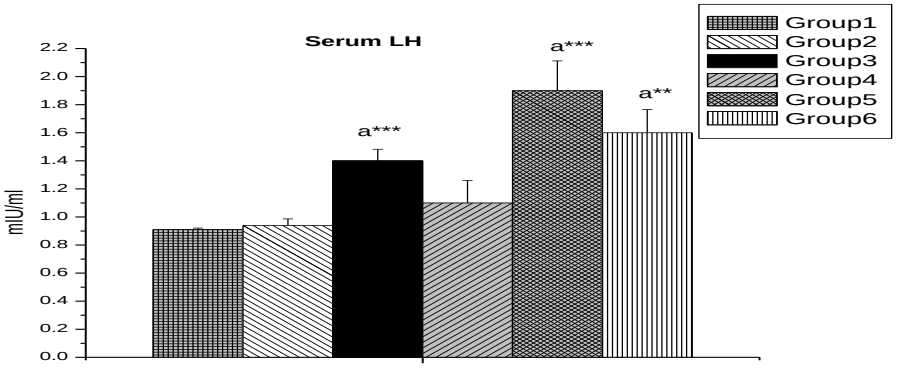


Figure-26: The figure shows the effect of Zinc and α- lipoic acid on **Serum LH level** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups. Asterisks represents the different level of significance (** indicates $p<0.01$,*** indicates $p<0.001$).

1) Effect on FSH level

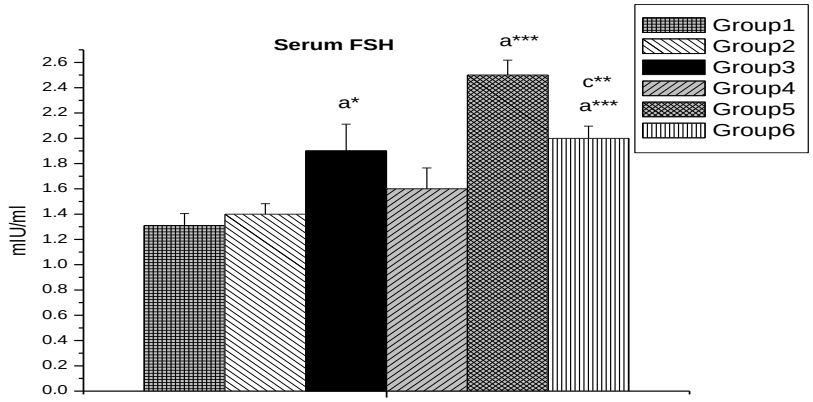


Figure-27: The figure shows the effect of Zinc and α- lipoic acid on **Serum FSH level** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05, ** indicates p<0.01 ,*** indicates p<0.001).

2) Oxidative stress parameters-

1) Effect on Testicular Malon-di-aldehyde

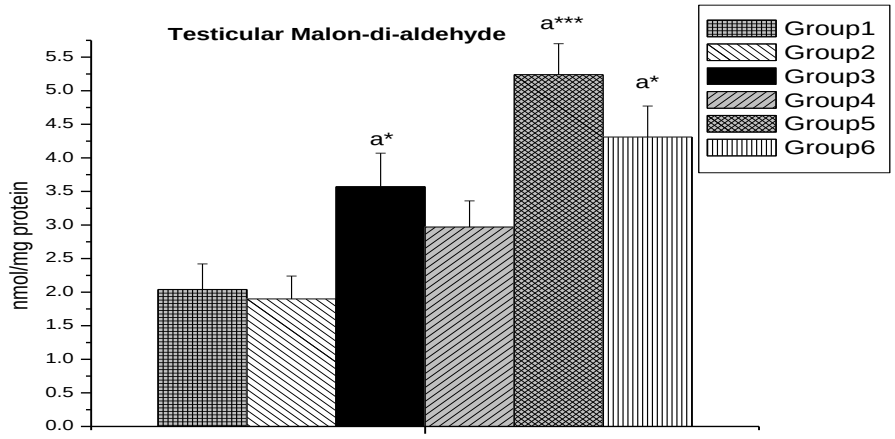


Figure-28: The figure shows the effect of Zinc and α- lipoic acid on **Testicular Malon-di-aldehyde** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups. Asterisks represents the different level of significance (* indicates p<0.05, *** indicates p<0.001).

2) Effect on Testicular Catalase

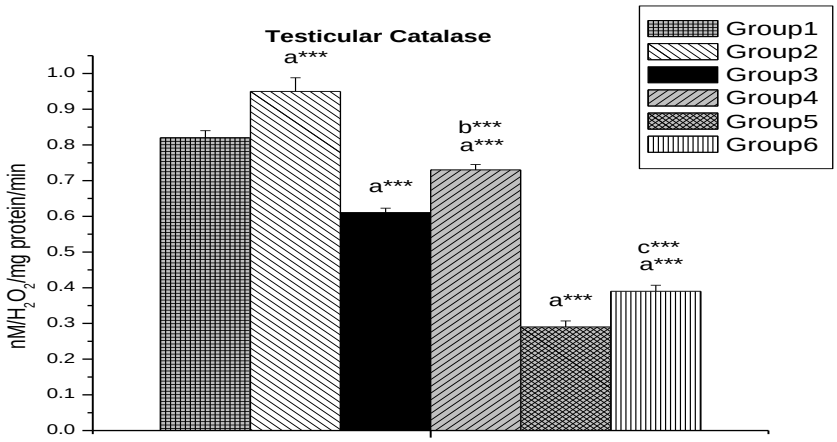


Figure-29: The figure shows the effect of Zinc and α- lipoic acid on **Testicular Catalase** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (*** indicates p<0.001).

3) Effect on Testicular Glutathione peroxidase

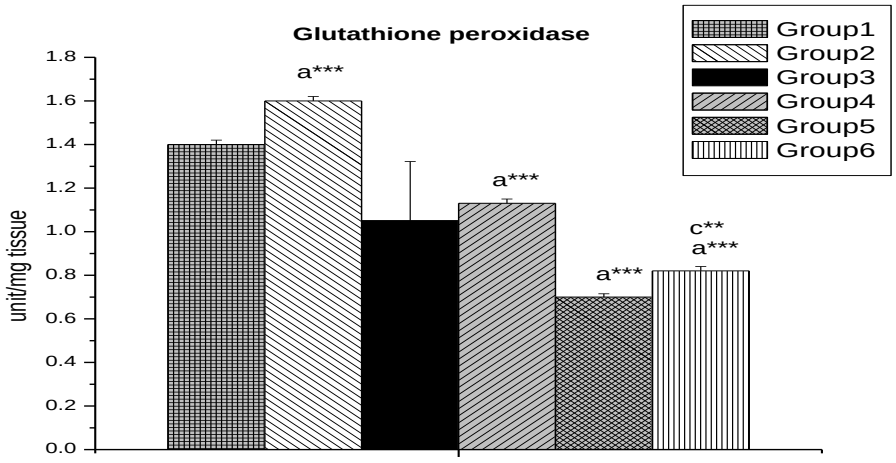


Figure-30: The figure shows the effect of Zinc and α- lipoic acid on **Glutathione peroxidase** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (** indicates p<0.01, *** indicates p<0.001).

4) Effect on Testicular Super-oxide dismutase

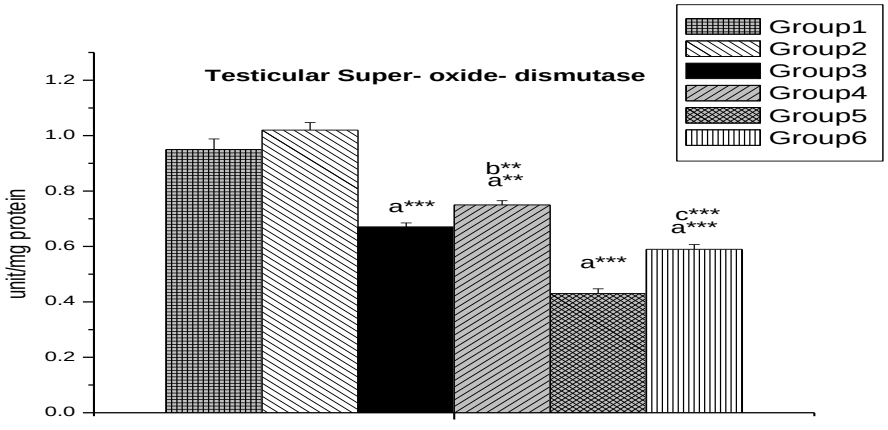


Figure-31: The figure shows the effect of Zinc and α- lipoic acid on **Super-oxide dismutase** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by

ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (** indicates p<0.01; ,*** indicates p<0.001).

5) Effect on Testicular Reduced Glutathione

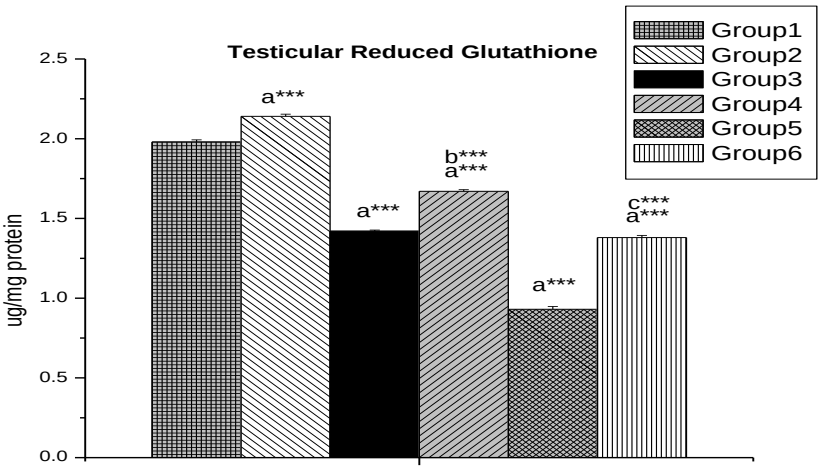


Figure-32: The figure shows the effect of Zinc and α- lipoic acid on **Reduced Glutathione** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (,*** indicates p<0.001).

6) Effect on Testicular Glutathione -S-transferase

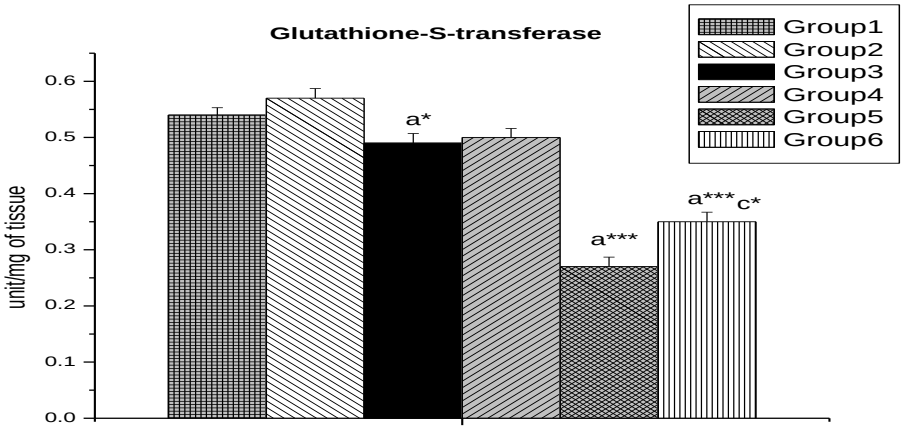


Figure-33: The figure shows the effect of Zinc and α- lipoic acid on **Glutathione -S-transferase** in Cypermethrin induced male albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05,*** indicates p<0.001).

D. <u>Results of Estrous Cycle</u>

Group I: Control (5 ml /kg body wt.)

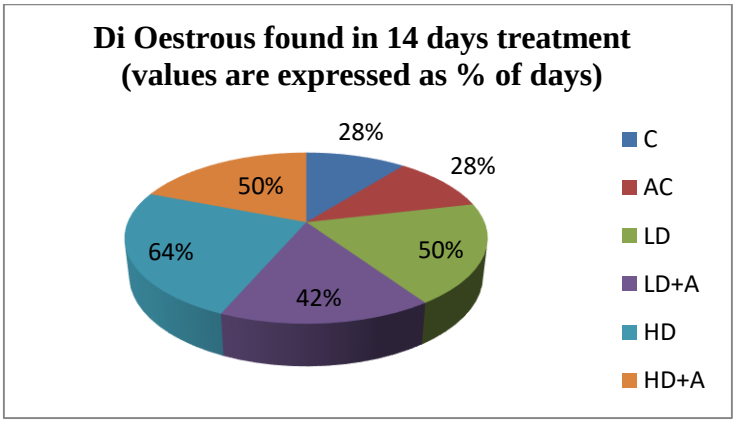
Group II: Zinc (227 mg/l in drinking water) and α- lipoic acid (35 mg/kg body wt.) control

Group III: Cypermethrin-treated (Low dose, 34.33mg/body wt.) group

Group IV: Zinc + α- lipoic acid+ Cypermethrin-treated (Low dose, 34.33mg/body wt.) group

Group V: Cypermethrin-treated (High dose, 51.5mg/body wt.) group

Group VI: Zinc + α- lipoic acid+ Cypermethrin-treated (High dose, 51.5mg/body wt.) group



C=Control group, AC= Zinc+ lipoic acid control, LD=Low Dose, LD+A =Low Dose +Zinc+Lipoic acid control, HD= High dose, HD + A =High dose+ Zinc+ Lipoic acid.

Table 1: Day wise changes in oestrous cycle during treatment

Group	Days													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
C	M	D	D	P	E	M	M	D	P	E	E	M	D	P
AC	E	M	D	P	P	E	M	D	D	P	E	M	D	P
LD	E	M	D	D	P	E	M	D	P	E	D	D	D	D
LD+A	D	P	E	M	D	D	P	E	M	D	P	E	D	D
HD	P	E	M	D	D	P	ER	D	D	D	D	D	D	D
HD+A	M	D	D	P	E	M	D	D	P	E	M	D	D	D

Figure 1 shows the day wise changes in Oestrus cycle in different group during treatment with cypermethrin. Here, M=Met oestrus, D=Dioestrus, E=Oestrus, P= Pro oestrus.

E. <u>Effects of zinc and alpha-lipoic acid on biochemical, hormonal and oxidative stress parameters of female reproductive system after the administration of cypermethrin in oral route</u>

16) Effect on Ovarian cholesterol:

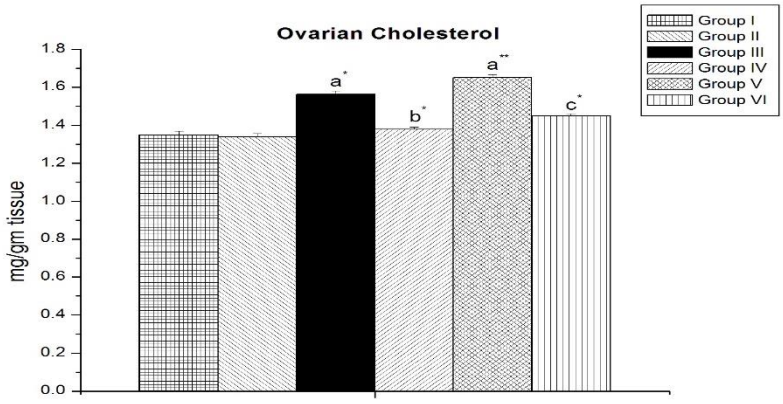


Figure 2: The figure shows the effect of zinc and α- lipoic acid on ovarian cholesterol in cypermethrin induced female albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05; ** indicates p<0.01).

17) Effect on Adrenal cholesterol:

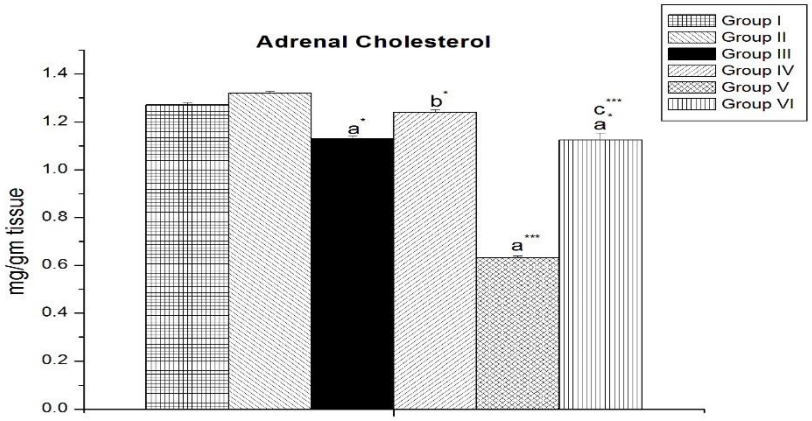


Figure 3: The figure shows the effect of zinc and α- lipoic acid on adrenal cholesterol in cypermethrin induced female albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05; *** indicates p<0.001).

18) Effect on Ovarian ascorbic acid :

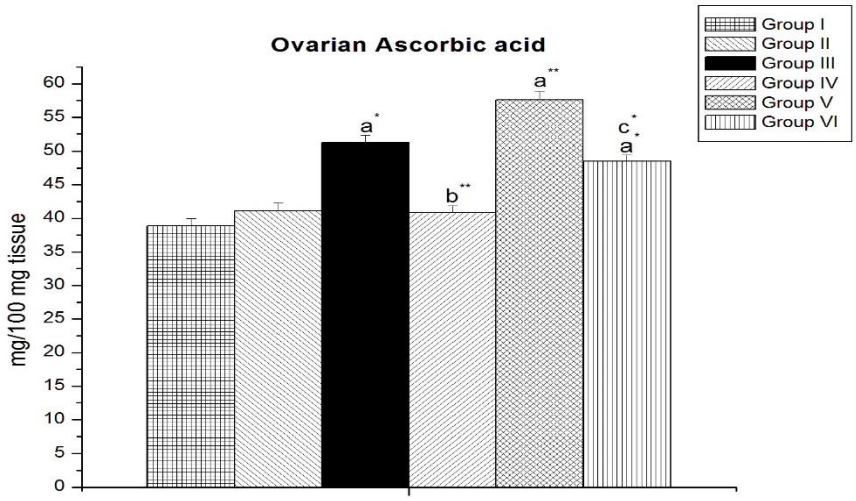


Figure 4: The figure shows the effect of zinc and α- lipoic acid on ovarian ascorbic acid in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates $p<0.05$, ** indicates $p<0.01$).

19) Effect on Adrenal ascorbic acid:

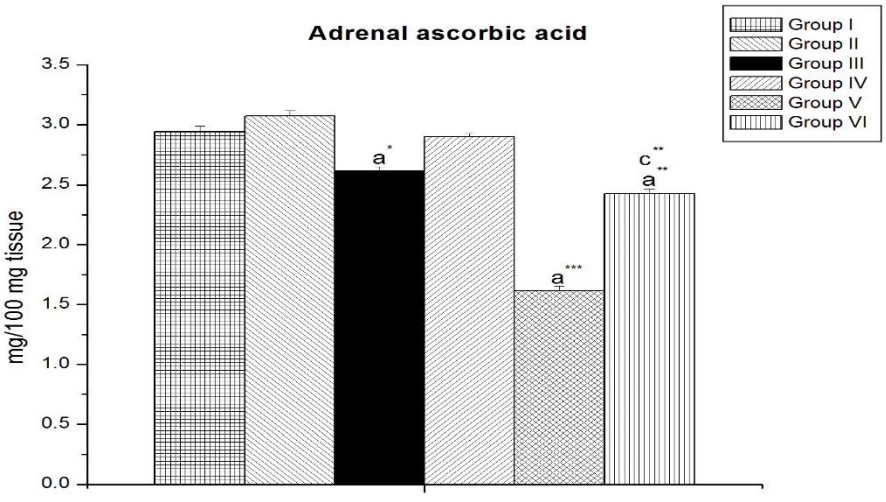


Figure 5: The figure shows the effect of zinc and α- lipoic acid on adrenal ascorbic acid in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001).

20) Effect on Ovarian glutamate pyruvate transaminase (GPT)

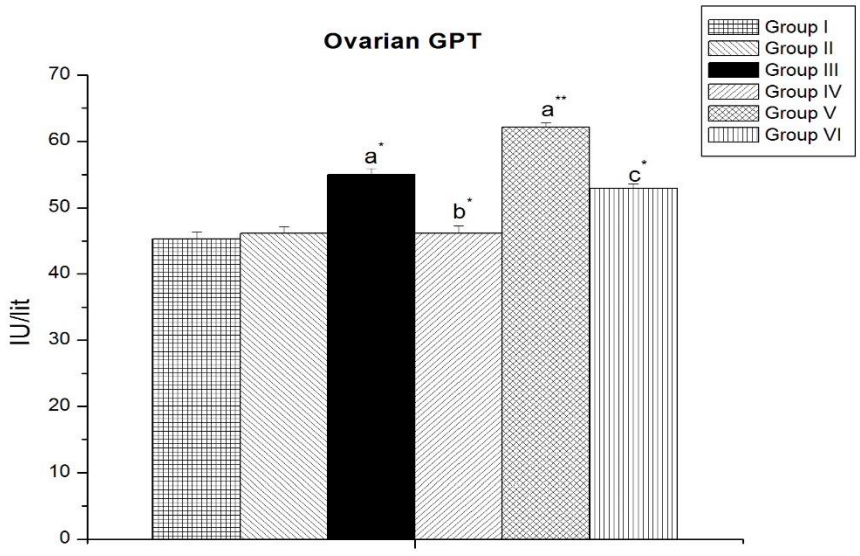


Figure 6: The figure shows the effect of zinc and α- lipoic acid on ovarian glutamate pyruvate transaminase (GPT) in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05, ** indicates p<0.01).

21) Effect on Ovarian glutamate oxaloacetate transaminase (GOT)

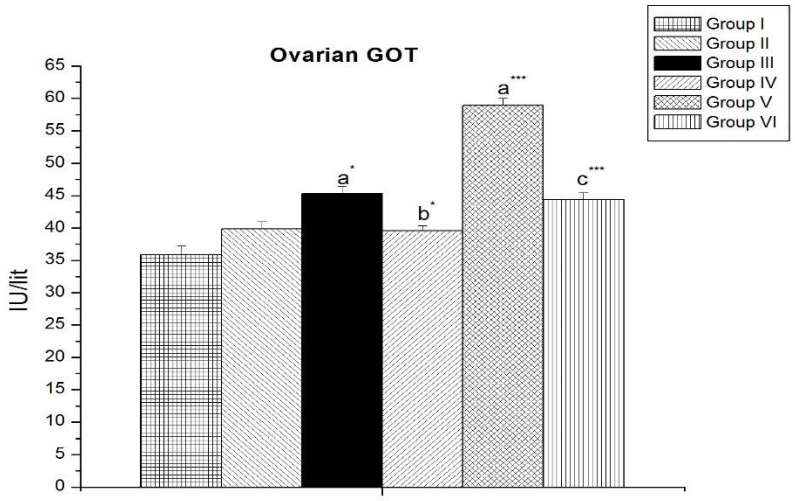


Figure 7: The figure shows the effect of zinc and α- lipoic acid on ovarian glutamate oxaloacetate transaminase (GOT) in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates $p<0.05$, *** indicates $p<0.001$).

22) Effect on Ovarian acid phosphatase(ACP)

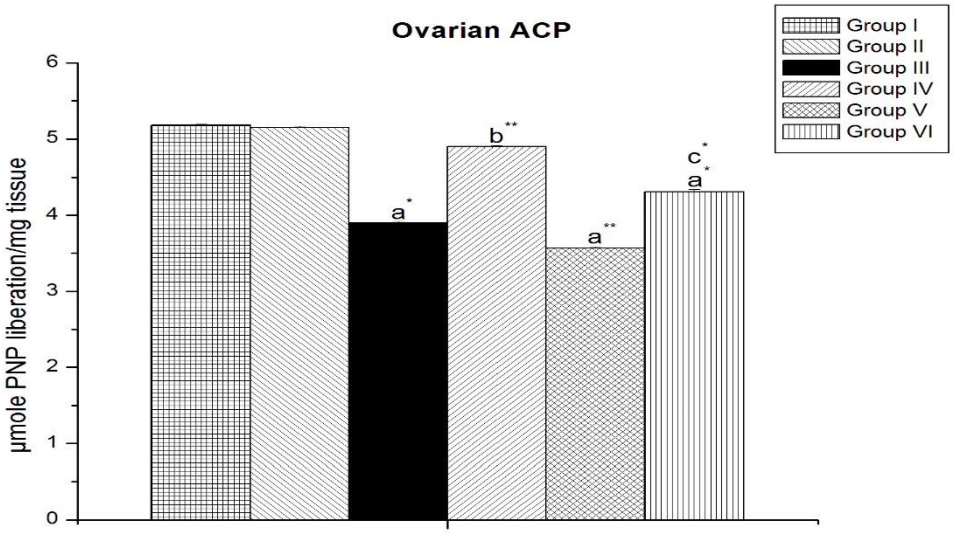


Figure 8: The figure shows the effect of zinc and α- lipoic acid on ovarian acid phosphatase (ACP) in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates $p<0.05$,** indicates $p<0.01$).

23) Effect on Ovarian $\Delta^5$3β-HSD

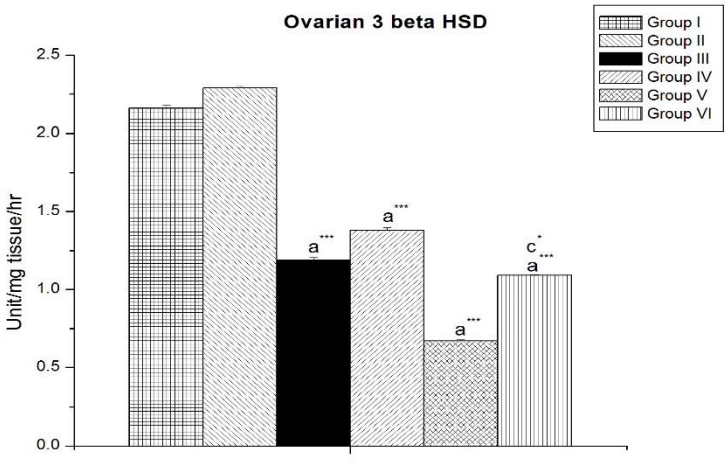


Figure 9: The figure shows the effect of zinc and α- lipoic acid on ovarian $\Delta^5$3β-HSD in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is

done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates $p<0.05$;*** indicates $p<0.001$).

24) Effect on Ovarian 17β-HSD

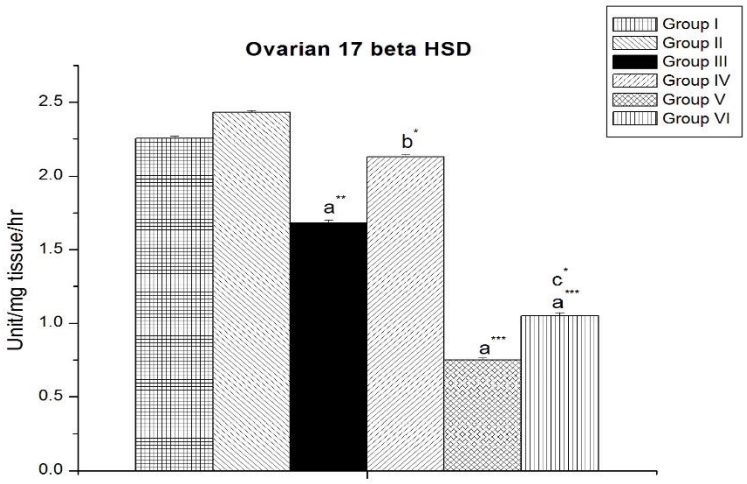


Figure 10: The figure shows the effect of zinc and α- lipoic acid on ovarian 17β-HSD in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates $p<0.05$,** indicates $p<0.01$; *** indicates $p<0.001$).

25) Effect on Adrenal $\Delta^5$3β-HSD

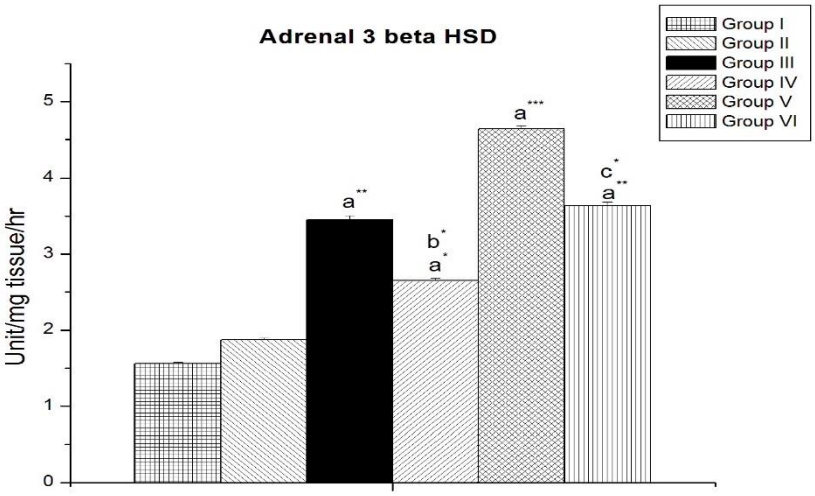


Figure 11: The figure shows the effect of zinc and α- lipoic acid on adrenal $\Delta^5$3β-HSD in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05,** indicates p<0.01; *** indicates p<0.001).

26) Effect on Adrenal 17β-HSD

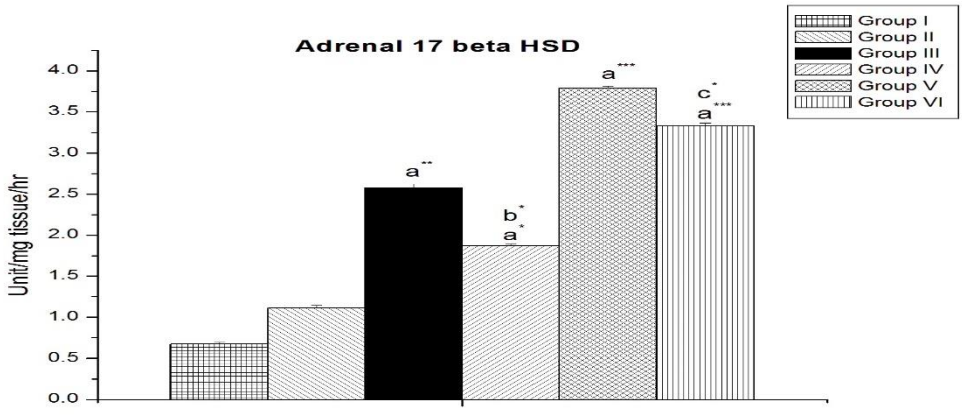


Figure 12: The figure shows the effect of zinc and α- lipoic acid on adrenal 17β-HSD in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

F. Estimation of hormonal parameters

Table 2 shows the effect of zinc and α- lipoic acid on reproductive hormone levels in cypermethrin induced female (mature) rats

	LH (mIU/ml)	**FSH (mIU/ml)**	**Estrogen (ng/ml)**
Group-I	0.75±0.013	0.88±0.007	36±1.18
Group-II	0.77±0.014	0.91±0.006	35.16±1.19
Group-III	0.63±0.006 a^{*}	0.726±0.007 a^{*}	27.41±0.77 a^{**}
Group-IV	0.73±0.008 b^{**}	0.855±0.011 b^{*}	34.58±0.73 b^{**}
Group-V	0.47±0.007a^{***}	0.59±0.01 a^{***}	16±0.53 a^{***}
Group-VI	0.68±0.01a^{*} c^{***}	0.78±0.013 a^{*} c^{**}	29.83±0.94 $a^{*}c^{***}$

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

G. Oxidative stress parameters-

7) Effect on Ovarian malondialdehyde

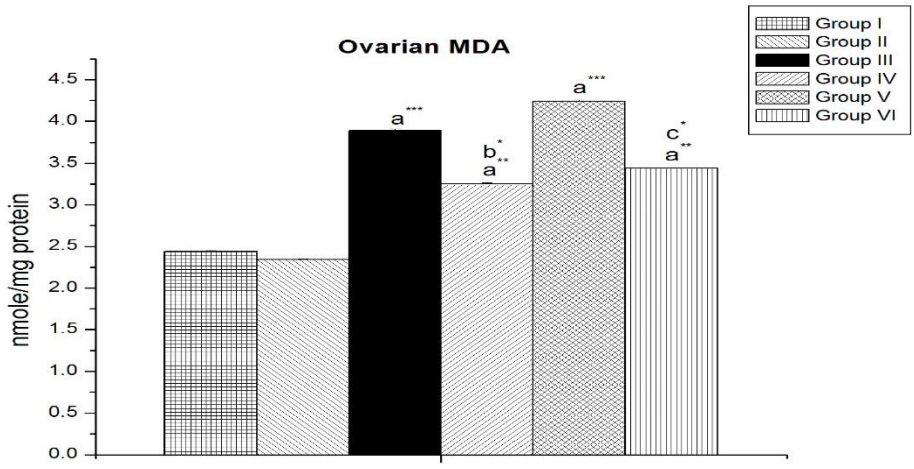


Figure 13: The figure shows the effect of zinc and α- lipoic acid on ovarian malon-di-aldehyde in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups. Asterisks represents the different level of significance (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

8) Effect on Ovarian catalase

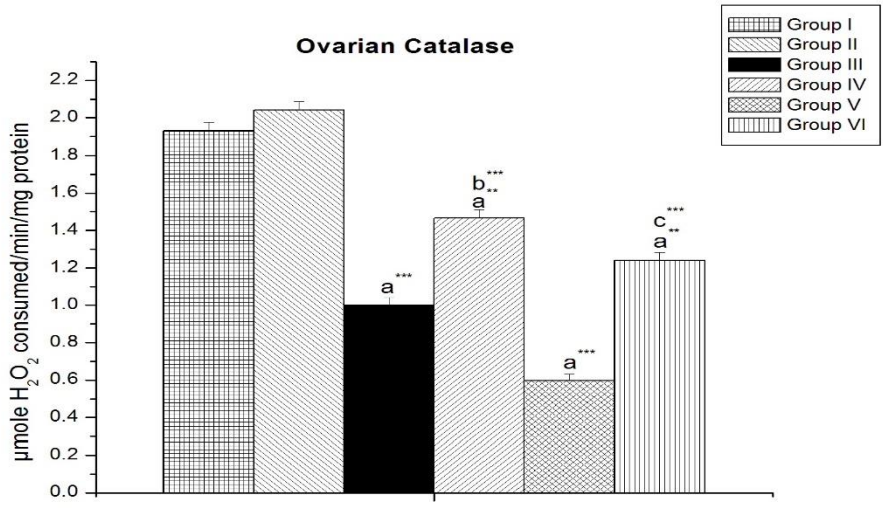


Figure 14: The figure shows the effect of zinc and α- lipoic acid on ovarian catalase in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-

I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (** indicates p<0.01;*** indicates p<0.001).

9) Effect on Ovarian glutathione peroxidase

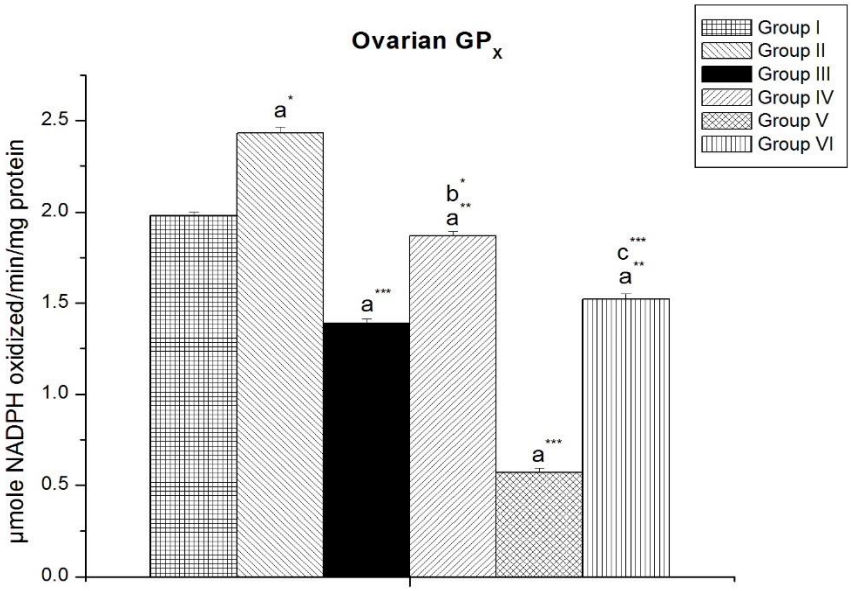


Figure 15: The figure shows the effect of zinc and α- lipoic acid on ovarian glutathione peroxidase in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05,** indicates p<0.01, *** indicates p<0.001).

10) Effect on Ovarian superoxide dismutase

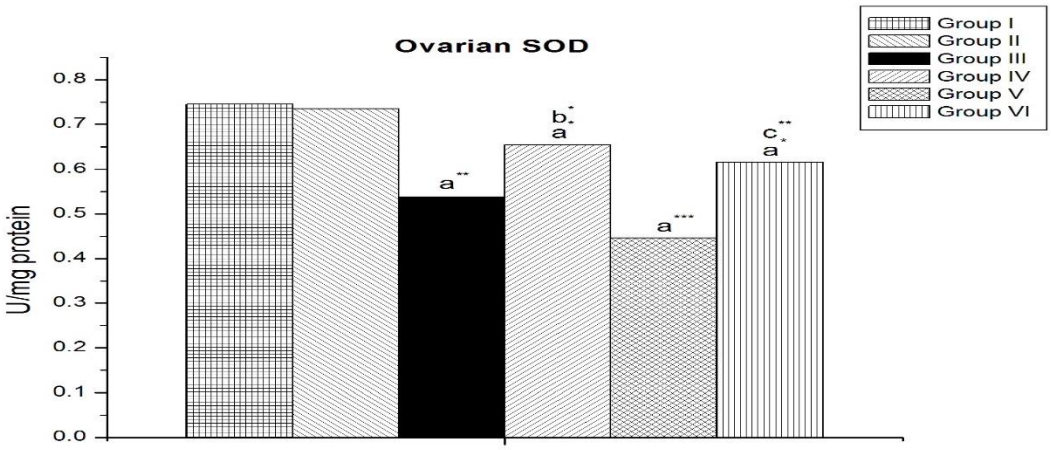


Figure 16: The figure shows the effect of zinc and α- lipoic acid on superoxide dismutase in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05,** indicates p<0.01, *** indicates p<0.001).

11) Effect on Ovarian reduced glutathione

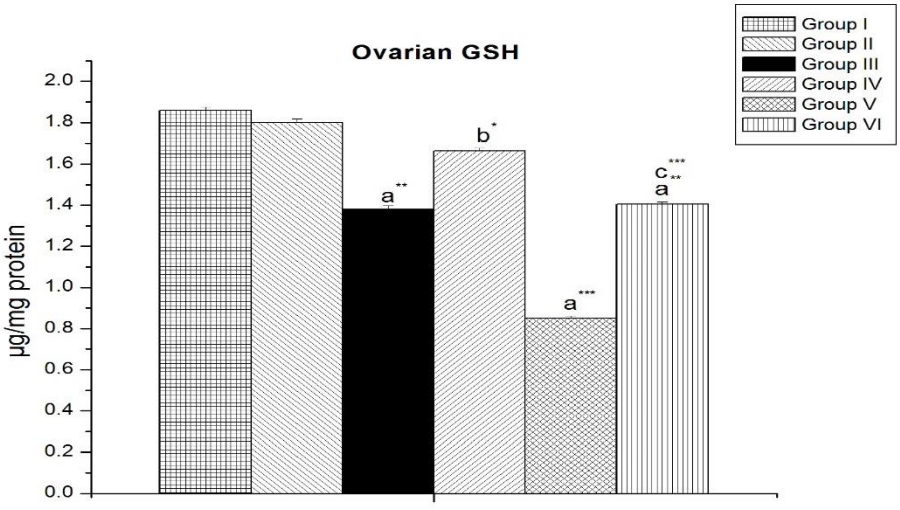


Figure 17: The figure shows the effect of zinc and α- lipoic acid on ovarian reduced glutathione in cypermethrin induced female albino rat. Results are expressed as

Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **b** Group-III versus Group-IV; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05,** indicates p<0.01, *** indicates p<0.001).

12) Effect on Ovarian glutathione -S-transferase

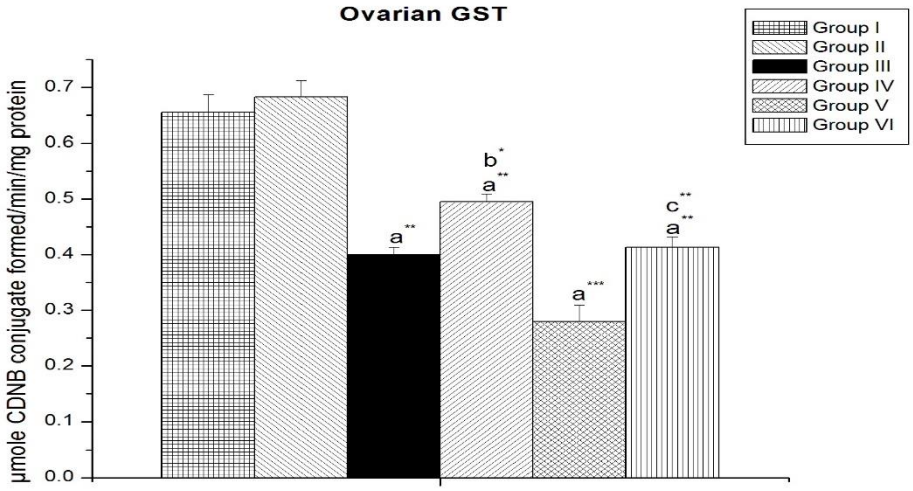


Figure 18: The figure shows the effect of zinc and α- lipoic acid on ovarian glutathione -S-transferase in cypermethrin induced female albino rat. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisks represents the different level of significance (* indicates p<0.05,** indicates p<0.01, *** indicates p<0.001)).

C) <u>Histological study</u> of ovarian tissue by Haematoxylin and Eosin.

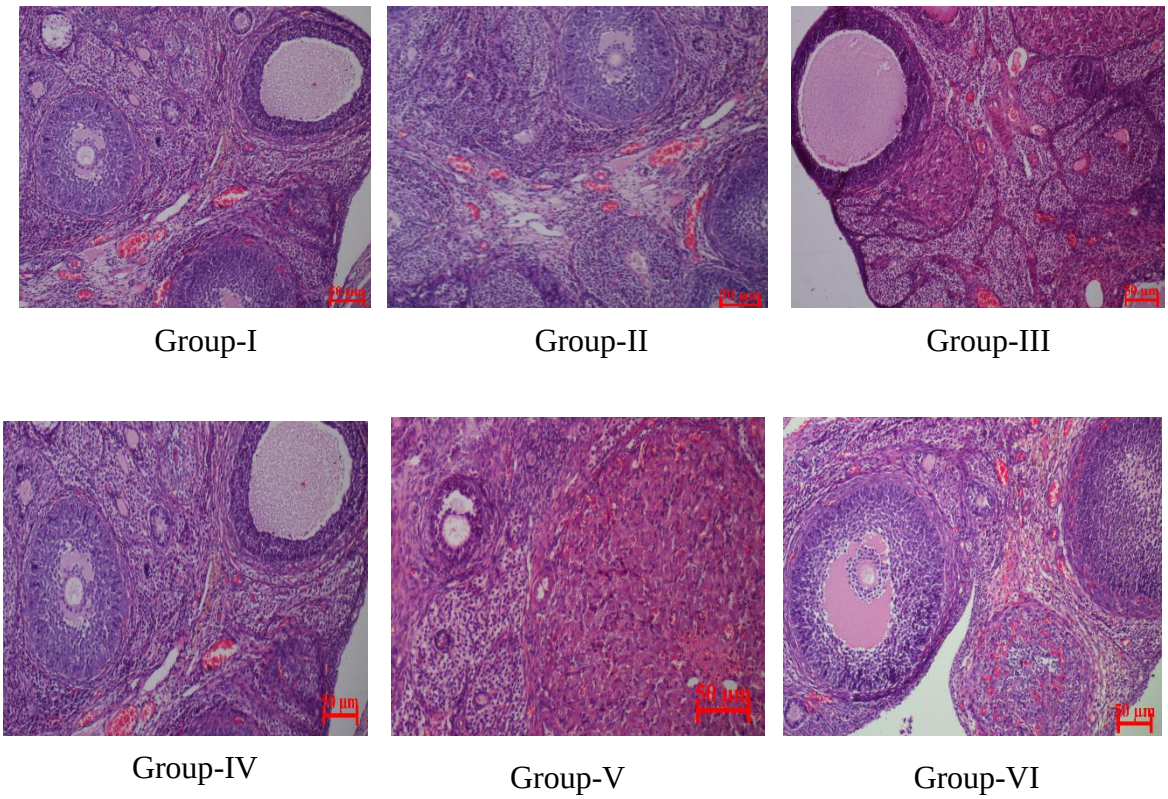


Figure 19: The figure shows the effect of zinc and α- lipoic acid on Ovarian histology in Cypermethrin induced female albino rat.

The prolonged and indiscriminate use of cypermethrin is reported to cause both acute and chronic toxicity in non target species including humans.

In this study, the evaluation of testicular tissue, adrenal tissue and seminal vesicular tissue as well as serum using biochemical assay indicated that acute oral doses of cypermethrin induce dose dependent biochemical changes in reproductive tissue.

Figure-1 shows the effect of zinc and α- lipoic acid on **Testiculo-somatic index** in cypermethrin induced male albino rat. The Testicular index has been decreased significantly ($p<0.001$) in cypermethrin high dose treated group. The reduction in the weight of the testis may be due to decreased number of germ cells, inhibition of spermatogenesis and steroidogenic enzyme activity (Takahashi and Oishi, 2001). Zinc and Lipoic acid combination ameliorated the cypermethrin induced reduction in the testicular weight.Figure-2, -3, -4 show the effect of Zinc and α- lipoic acid on **Epedidymal--somatic index, Prostrato--somatic index, Seminal vesciculo--somatic**

index in Cypermethrin induced male albino rat. These three indices were decreased significantly ($p<0.05$, $p<0.001$) in cypermethrin-treated groups in a dose dependent manner. The decrease in the weight of epididymis may indicates cypermethrin toxicity on the organ. Reduction in the weight of the seminal vesicles and ventral prostate, which may reflect an interference with androgen output (Wang et al., 2009). Zinc and α- lipoic acid restored it to a good extent.

Figure -5, 6 and 7 show the effects of zinc and α- lipoic acid on **Epididymal sperm motility Epididymal sperm count**, **Hypo-osmotic swelling of rat sperm** respectively in cypermethrin induced male albino rat. Comparing with the control group in cypermethrin-treated group epididymal sperm motility, sperm count and hypo-osmotic swelling of rat sperm decreased significantly ($p<0.001$). Treatment of zinc and α- lipoic acid decreased the cypermethrin toxicity and tried to restore the normal oxidative status of the testicular tissue. The reduction in sperm count may be due to an adverse effect of cypermethrin on spermatogenesis. In this study sperm motility decreased may be due to altered fructose synthesis and secretion by the accessory glands. The HOS of sperm in cypermethrin treated group was reduced significantly indicating that the pesticide may probably cause injury to the sperm plasma membrane. Co-administration of Zinc and Lipoic acid significantly improved the evaluated parameters though not all were identical to control levels. Zinc and Lipoic acid combination ameliorated the Cypermethrin induced reduction in the reproductive organs weights, sperm count, motility and hypoosmotic swelling of sperm.

Table-1 shows the effect of Zinc and α- lipoic acid Cypermethrin on **Epididymal sperm morphology** in Cypermethrin induced male albino rat. In this study alteration of sperm cell morphology caused by Cypermethrin can be grouped into primary or secondary abnormalities according to the classification by Noarkes et al. (2004). The occurrence of rudimentary tail sperm abnormality was observed higher in rats in Cypermethrin treated group relative to the control. Curved mid-piece sperm cell abnormality had the highest occurrence, followed by, curve tail, bent mid piece, bent tail, head less tail, tail less head of all the secondary sperm abnormalities observed. But treatment of zinc and α- lipoic acid decrease sperm abnormality significantly ($p<0.001$). It may be due to combined ameliorative effect of zinc and α- lipoic acid in reproductive system.

Figure-8 shows the effect of zinc and α- lipoic acid on **Cell cycle arrest of rat sperm** in Cypermethrin induced male albino rat. The figure depicts that significant increase in G_O/G_1 phase compared to control indicated that induction of apoptosis by Cypermethrin treatment. In this study

it was found that zinc and lipoic acid treatment leads to suppression of apoptosis induced by Cypermethrin.

Figure-9 shows the effect of zinc and α- lipoic acid on **sperm nuclear fragmentation** in Cypermethrin induced male albino rat. Cypermethrin treated group showed **sperm nuclear fragmentation.** Zinc and α- lipoic acid prevented it.

Figure-10 shows the effect of Zinc and α- lipoic acid on **Seminal Plasma fructose Concentration** in Cypermethrin induced male albino rat. Fructose production is usually employed as a marker of seminal vesicle function due to its inverse relationship with spermatozoa motility. Fructose levels in seminal vesicle homogenates were significantly higher in cypermethrin treated group compared to control rats. Zinc and α- lipoic acid restored it to a good extent.

In figure-11 **Testicular cholesterol** has been increased significantly ($p<0.05$) in high dose whereas in figure-12 it is seen that Adrenal cholesterol level is decreased in cypermethrin induced male albino rat. The testicular cholesterol conc. increased for the diminished steroidogenesis in testicular tissue. Adrenal cholesterol level is decreased due to the increased adrenal steroidogenesis. Co-administration of zinc and α- lipoic acid significantly improved testicular steroidogenesis.

Figure-13 shows the significant accumulation of **testicular ascorbic acid** in Cypermethrin induced male albino rat and the figure-14 shows the diminution of **adrenal ascorbic acid due to the active use of it in** adrenal steroidogenesis. The **treatment** of zinc and α- lipoic acid caused marked decrease in cypermethrin toxicity that result in improved steroidogenesis in testicular tissue of male albino rat.

Testicular protein concentration has been decreased in figure-15 in cypermethrin induced male albino rat and zinc and α- lipoic acid helped to normalize it.

Testicular Glutamate Pyruvate Transaminase (GPT) (Figure-16) and **Testicular Glutamate Oxaloacetate Transaminase (GOT) (**Figure-17) levels were increased in cypermethrin induced male albino rat in a dose-dependent manner which were altered after the treatment of zinc and α-lipoic acid.In testis increased testicular GPT and GOT levels suggest that cypermethrin causes testicular damage. Increase of transaminase activity along with the decreased of content of free

radical (O_2·) scavengers are probably the consequence of cypermethrin induced pathological changes in testis.

Figure-18 shows the effect of Zinc and α- lipoic acid on **Testicular Acid Phosphatase(ACP)** in Cypermethrin induced male albino rat. Cypermethrin toxicity decreased **Testicular Acid Phosphatase** level in a dose dependent manner, which signify less testicular activity; that was restored by Zinc and α- lipoic acid significantly.

Figure-19: The figure shows the effect of Zinc and α- lipoic acid on **Testicular $\Delta^5$3β-HSD** in Cypermethrin induced male albino rat. Testicular Δ^5 3β HSD conc. decreased significantly ($p<0.05$) whereas **Adrenal $\Delta^5$3β-HSD (** Figure-21) increased significantly ($p<0.05$, $p<0.001$) in cypermethrin-treated groups in a dose dependent manner. Figure-20 shows the effect of Zinc and α- lipoic acid on **Testicular 17β-HSD** in Cypermethrin induced male albino rat. Testicular 17β HSD conc. decreased significantly ($p<0.05$) whereas **Adrenal 17β-HSD (** Figure-22) increased significantly ($p<0.05$, $p<0.001$) in cypermethrin-treated groups in a dose dependent manner. Zinc and α-lipoic acid treatment decreased the cypermethrin toxicity. Likewise **Testicular Glucose-6-P-dehydrogenase (** Figure-23) decreased and **Adrenal Glucose -6- P- dehydrogenase (** Figure-24**)** in Cypermethrin induced male albino rat as testicular steroidogenesis was reduced which was like to be compensated by the adrenal steroidogenesis.

Figure-25 shows the effect of Zinc and α- lipoic acid on **Serum testosterone level** in Cypermethrin induced male albino rat. **Serum testosterone** decreased significantly ($p<0.01$) in high dose cypermethrin-treated group compared to the control group. Treatment of zinc and α- lipoic acid increased the **Serum testosterone level** and tried to restore the normal hormonal status of the testicular tissue. Male reproductive dysfunction may be due to the alteration of steroid hormone level i.e. due to the low level of testosterone.

Figure-26, 27 show the effect of Zinc and α- lipoic acid on **Serum LH level** in Cypermethrin induced male albino rat. **Serum LH, FSH** increased significantly ($p<0.01$,) in high dose cypermethrin-treated group compared to the control group. Treatment of zinc and α- lipoic acid decreased the **Serum LH, FSH level** significantly.

Figure-28 shows the effect of Zinc and α- lipoic acid on **Testicular Malon-di-aldehyde** in Cypermethrin induced male albino rat. The Testicular MDA level increased significantly ($p<0.05$,

p<0.001) in cypermethrin-treated groups in a dose dependent manner. Zinc and α- lipoic acid treatment decreased the cypermethrin toxicity. In this study treatment of cypermethrin is associated with oxidative stress which may be the cause of reduced testicular steroidogenesis. Oxidative stress in testis is proved by the high level of MDA which is the indicator of lipid peroxidation.

Figure -29,-30,-31,-33 show the effects of zinc and α- lipoic acid on **Testicular Catalase(CAT), Glutathione peroxidase, Super-oxide dismutase (SOD), Glutathione-S-transferase (GST)** in Cypermethrin induced male albino rat. These antioxidant enzymes decreased significantly (p<0.001) in high dose cypermethrin-treated group compared to the control group. Treatment of zinc and α- lipoic acid decreased the cypermethrin toxicity and restored the normal oxidative status of the testicular tissue. **Glutathione-S-transferase** (GST) itself acts as a protein and it bind diverse group of chemical and its protective role against lipid peroxidation. The present results showed that cypermethrin causes a significant (p<0.001) decrease in the activity of GST in testicular tissue.

Figure-32 shows the effect of Zinc and α- lipoic acid on **Reduced Glutathione** in Cypermethrin induced male albino rat. The Testicular GSH conc. has been decreased significantly (p<0.001) in high dose cypermethrin-treated group. GSH conjugation is recognized as a major detoxication mechanism in reproductive tissue. Administration of zinc and α-lipoic acid reduced cypermethrin mediated oxidative stress significantly.

The decreased GSH, CAT and SOD activities and increased GSSG and MDA level in testis suggest that cypermethrin caused testicular damage. Zinc and α- lipoic acid were used to prevent oxidative damage by interrupting the propagation of the oxidation of polyunsaturated fatty acids. Zinc and α- lipoic acid assisted to normalize the reproductive parameters at a good extent. These observations might also indicate that zinc and lipoic acid has therapeutic effects on CYP-induced male reproductive toxicity.

Figure-34 shows the effect of zinc and α- lipoic acid on **testicular histology** in Cypermethrin induced male albino rat. Cypermethrin impairs the testicular histology by altering the different stages of spermatogenesis. Vacuolation occurs and regression of the interstitial cells also were seen. Zinc and α- lipoic acid normalized the histological changes effectively at cypermethrin low dose treated group compared to cypermethrin high dose treated group.

In this study, the evaluation of ovarian tissue, adrenal tissue as well as serum using biochemical assay indicated that acute oral doses of cypermethrin induce dose dependent biochemical changes in reproductive tissue.

Figure-1 shows the effect of zinc and α- lipoic acid on **oestrous cycle** in cypermethrin induced female albino rat. In this study, estrous cycle have been observed in the cypermethrin exposed female mature rats as an indicator about the toxic effects of cypermethrin on the female reproductive function.

In figure-2 **ovarian cholesterol** has been increased significantly ($p<0.05$) in high dose whereas in figure-3 it is seen that adrenal cholesterol level is decreased in cypermethrin induced female albino rat. The ovarian cholesterol conc. increased for the diminished steroidogenesis in ovarian tissue. Adrenal cholesterol level is decreased (figure-3) due to the increased adrenal steroidogenesis. The significant rise in ovarian cholesterol content of cypermethrin treated rats promotes the less utilization of cholesterol towards the biosynthesis of ovarian steroid hormones. Thus it shows the malfunctioning of ovarian steroidogenesis in cypermethrin treated rats (**MaitiChoudhury et al., 2016**). Co-administration of zinc and α- lipoic acid decreased the cypermethrin toxicity and returned the steroidogenic status of the ovary.

Figure-4 shows the significant accumulation of ovarian ascorbic acid in cypermethrin induced female albino rat whereas figure-5 shows the diminution of adrenal ascorbic acid due to the active use of it in adrenal steroidogenesis. The treatment of zinc and α- lipoic acid caused marked decrease in cypermethrin toxicity that result in improved steroidogenesis in ovarian tissue of female albino rat.

Ovarian glutamate pyruvate transaminase (GPT) (Figure-6) and ovarian glutamate oxaloacetate transaminase (GOT) **(**Figure-7) levels were increased in cypermethrin induced female albino rat in a dose-dependent manner which were altered after the treatment of zinc and α- lipoic acid. In ovary increased ovarian GPT and GOT levels suggest that cypermethrin causes ovarian damage. Increase of transaminase activity along with the decreased of content of free radical ($O_2^{\cdot}$) scavengers are probably the consequence of cypermethrin induced pathological changes in ovary. GOT level is significantly increased in cypermethrin dose group with compare to control. This may be due to the early apoptosis or necrosis of ovarian tissue which enhances transaminase activity by cypermethrin toxicity. In case of lipoic acid, zinc and both the lipoic acid and zinc treated groups GOT level is relatively low. This effect suggests that there may be a neutralizing

effect of lipoic acid and zinc as antidote. Ovarian GPT level is also increased significantly in cypermethrin dose group comparing with control group. It may be due to tissue damage and less membrane permeability of ovary by the toxicity of cypermethrin. It results increased liberation of pyruvate transferase. When we apply lipoic acid and zinc as antidote therapy; ovarian GPT level is significantly decreased comparing with cypermethrin treated group. Hence, there may be a positive role of zinc and lipoic acid to neutralize cypermethrin induced transaminase activities.

Figure-8 shows the effect of zinc and α- lipoic acid on ovarian acid phosphatase (ACP) in cypermethrin induced female albino rat. Cypermethrin toxicity decreased ovarian acid phosphatase level in dose dependent manners, which signify less ovarian activity; that was restored by zinc and α- lipoic acid significantly. Activities of phosphatases enzymes have been shown to rise when ovarian steroidogenesis is increased. From our result it is confirmed that after cypermethrin exposure ovarian steroidogenesis was decreased; but pretreatment with zinc and lipoic acid this process has restored to the normal status. Zinc markedly increased the ACP activity and this occurred concomitantly with the appearance of spermatids and mature sperm cells (**Guha and Vanha Perttula, 1983**).

Figure-9 shows the effect of zinc and α- lipoic acid on ovarian $\Delta^5$3β-HSD in cypermethrin induced female albino rat. Ovarian Δ^5 3β HSD conc. decreased significantly ($p<0.05$) whereas adrenal $\Delta^5$3β-HSD **(**Figure-11) increased significantly ($p<0.05$, $p<0.001$) in cypermethrin-treated groups in a dose dependent manner. Figure-10 shows the effect of zinc and α- lipoic acid on ovarian 17β-HSD in cypermethrin induced female albino rat. Ovarian 17β HSD conc. decreased significantly ($p<0.05$) whereas adrenal 17β-HSD **(**Figure-12) increased significantly ($p<0.05$, $p<0.001$) in cypermethrin-treated groups in a dose dependent manner. Zinc and α-lipoic acid treatment decreased the cypermethrin toxicity. The Ovarian Δ5 3β HSD and 17β HSD level were decreased significantly in cypermethrin treated group. In antidote group both the enzyme level s were increased compared to cypermethrin treated group. This may be due to action of zinc and lipoic acid that restored the effect of cypermethrin. Ovarian steroidogenesis is controlled by rate limiting enzymes- Δ5 3β HSD & Δ5 17β HSD. The less activity and concentration of these enzymes may be related to decreased steroidogenesis; induced by cypermethrin intoxication.

Data regarding serum hormone level (LH, FSH, estrogen) were documented in table-2. A significant reduction in the serum hormone level (LH, FSH, estrogen) were observed in

cypermethrin treated groups compared to control and protective effect was prominent in zinc and α- lipoic acid treated group in combination with cypermethrin. The diminution of the secretion of LH and FSH might be due to direct action of cypermethrin on anterior pituitary gonadotrophs, responsible for the secretion of LH and FSH; or hypothalamic neurons, responsible for the secretion of gonadotropin-releasing hormone (GnRH) that describes trophic action on anterior pituitary gonadotrophs (**Samarawickrema et al., 2008**).

Figure-13 shows the effect of zinc and α- lipoic acid on ovarian malondialdehyde in cypermethrin induced female albino rat. The ovarian MDA level increased significantly ($p<0.05$, $p<0.001$) in cypermethrin-treated groups in a dose dependent manner, indicating increase in oxidative stress in the tissue. Pesticide induced increase in LPO has previously been reported (**Giray et al., 2001**) zinc and α- lipoic acid treatment decreased the cypermethrin toxicity. In this study treatment of cypermethrin is associated with oxidative stress which may be the cause of reduced ovarian steroidogenesis. Normal cellular functioning depends on a balance between ROS produced and antioxidant defense mechanisms present in the cell.

Figure-14 showed the activities of CAT in the cypermethrin treated low and high dose groups, which were significantly ($p<0.001$) reduced compared to the control group. However, the activity of CAT was significantly increased by zinc and α- lipoic acid supplementation. The activities of glutathione peroxidase (figure-15) in the cypermethrin treated group were significantly decreased. However, pretreatment with zinc and α- lipoic acid improved the activity of antioxidant enzymes to the normal status. The activity of SOD in cypermethrin treated low and high dose groups was significantly decreased (figure-16). The findings showed that zinc and α- lipoic acid treatment markedly enhanced SOD level. Antioxidant enzymes such as SOD and CAT are considered to be a primary defense that prevents biological macromolecules from oxidative damage. According to the present data, the activities of CAT, SOD and GPx in ovaries of cypermethrin treated rats were significantly decreased. These results strongly suggested that cypermethrin has the capability to induce free radicals and oxidative damage as evidenced by perturbations in various antioxidant enzymes (**Salama et al., 2005**). Depletion of antioxidant enzyme activity could be due to the direct effect on the enzymes by cypermethrin -induced ROS generation, direct inhibition of the enzymes by methomyl, depletion of the enzymes substrates and down-regulation of transcription and

translation processes (**Garg et al., 2008**). Treatment of zinc and α- lipoic acid decreased the cypermethrin toxicity and restored the normal oxidative status of the ovarian tissue.

From this figure-17 it is observed that GSH were decreased significantly in cypermethrin treated groups compared to the control rats. The observations suggest that pretreatment with zinc and α-lipoic acid improved the antioxidant defense of the animal and showed ameliorating effect on cypermethrin toxicity. Glutathione an important antioxidant molecules, which in conjugation with GPx plays a significant role in protecting cells against oxidative stress of xenobiotics by scavenging ROS. The Ovarian GSH conc. has been decreased significantly ($p<0.001$) in high dose cypermethrin-treated group. The decrease in GSH may be due to enhanced utilization of GSH for detoxification of cypermethrin-induced free radicals (**Raina et al., 2009**). Administration of zinc and α-lipoic acid reduced cypermethrin mediated oxidative stress significantly.

GST level was declined significantly in case of cypermethrin treated group compared to control, it was improved when pretreatment of zinc and α- lipoic acid were done (figure-18). The present results showed that cypermethrin causes a significant ($p<0.001$) decrease in the activity of GST in Ovarian tissue. Cypermethrin administration to rat showed reduced activity of GST. GST catalysis the conjugation of the reduced GSH to electrophiles and protects cellular components from oxidative damage **(Hayes and Pulford, 1995).** GST is known to bind strongly to hydrophobic compounds like pyrethroids.

Histological examination of the ovary showed significant alterations in the architecture of graffian follicles after cypermethrin treatment compared to the control rats (figure-18). A significant protection was found after antidote supplementation. Histopathological study in cypermethrin treated group suggest that the pesticide may lead to disorders of the follicular maturation, structural degenerations of ovary most probably by promoting the oxidative stress induced damage of the tissues of ovary. Pretreatment of antidote restored towards normal architecture of ovary to a good extent.

13. Summary of the findings

Pyrethroids have appeared as a new class of pesticides. However, the extensive use of pyrethroids increase the risk of intoxication to non-target organisms such as birds, animals but have better selectivity to target species than organophosphates. Cypermethrin, a synthetic type II α-cyano-ectoparasiticide pyrethroid, is used broadly as an insecticide in agriculture, forestry, animal

husbandry and public health sector but reported to induce several health problems. The widespread use of Cypermethrin necessitates an in deph-understanding of the ecological and health effects of their use. Zinc, a key constitute or cofactor of many mammalian proteins, is intensively being studied in recent years for its protective efficiency in various models of animal toxicity. So, in this study Cypermethrin induced male and female reproductive toxicity were explored in Wistar rat and mitigation of these toxicities by zinc was also scrutinized. Cypermethrin was administered orally alone at 40 and 80 mg/kg body wt. for male rat and 34.33 and 51.5 mg/kg body wt. for female rat or combined with zinc (32.42 mg/kg body wt.) and lipoic acid (227mg/kg body wt.) for consecutive 14 days. Steroidogenic and spermatogenic disarrays were detected by significant alterations in sperm motility, count, viability, hypo-osmotic swelling, sperm DNA fragmentation as well as in testicular antioxidant status, serum testosterone and pituitary gonadotrophins after Cypermethrin exposure. Testicular damage was also noticed in testicular histo-architecture, in testicular steroidogenic enzymes activities and in the expression of StAR and P450 scc. In Cypermethrin-treated mature female rats, ovarian toxicity was also found by a change in ovarian biomarkers, steroidogenic enzymes activities and their expression as well as in hormonal level and ovarian histology. Zinc and alpha-lipoic acid also alleviated the male and female reproductive toxicity by restoring the hypothalamo-pituitary gonadal axis towards normalcy in Cypermethrin intoxicated rat. Pretreatment with zinc and lipoic acid significantly exhibited the protective effect on male and female reproductive toxicity by normalizing the hypothalamo-pituitary gonadal axis in Cypermethrin induced rat.

14. Contribution to the society

Conclusively, the present study suggests that Cypermethrin plays a destructive impact on reproductive system functions in male and female rats, while zinc and alpha-lipoic acid supplementation has beneficial effects on Cypermethrin intoxication. Zinc and alpha-lipoic acid benefit in reducing the oxidative stress, hence testicular hypofunction would encourage its supplementation as an add-on therapy and this is the main contribution to the society. This could open new horizon to its usage as a complementary approach to pesticide intoxication.

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