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RESEARCH ARTICLE

ZINC AND α-LIPOIC ACID AMELIORATES TESTICULAR DAMAGE AND OXIDATIVE STRESS IN CYPERMETHRIN-INTOXICATED RAT.

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Abstract

The current study was aimed to assess the protective role of zinc and lipoic acid on testicular damage and oxidative stress in Cypermethrin intoxicated male Wistar rats. Animals were exposed to cypermethrin at two different doses (40 and 80 mg/kg body wt.) for consecutive 14 days. Before the administration of cypermethrin rats were pre-treated with zinc (32.42mg/kg body wt.) and lipoic acid (35 mg/kg body wt.). At the end of the treatment, animals were sacrificed and testis, epididymis, prostate and seminal vesicles were removed and organ's somatic index, sperm characteristics, and various biochemical parameters were studied. Cypermethrin exposure resulted in a significant decrease in somatic index of testis, epididymis, prostate and seminal vesicles, sperm count, sperm viability and sperm hypo osmotic swelling counts, which were normalised by the pre-treatmentof zinc and lipoic acid. Testicular acid phosphatase was decreased and ascorbic acid was increased significantly by the administration of cypermethrin, which were regularized by the pre-treatment of zinc and lipoic acid. Treatment with cypermethrin resulted in alteration in MDA and release of testicular ROS which was also normalised by pre-treatment with zinc and lipoic acid.The findings of the presented study indicated that zinc and lipoic acid ameliorated cypermethrin-induced testicular damage by reducing oxidative stress.

Introduction:-

Pyrethroids are modified and improved synthetic compounds from natural components of pyrethrins extracted from *Chrysanthemum cinerariaefolium* (Larini, 1999; Oga et al., 2014). Among the various pesticides pyrethroid gain significant interest as it poses a broad spectrum of insecticidal action and have been used to control agricultural pests affecting corn, rice, cotton, beans, soybean, tomato and other crops (Larini, 1999) as well as in in formulation of household insecticides, shampoos, lotions etc (Pascotto et al.,2015). Pyrethroids are also used in the control and eradication of disease causing vectors such as ectoparasites in humans and animals (Abrasco et al., 2015).

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Cypermethrin is a synthetic pyrethroid commonly used in agriculture, veterinary and household insects or pests management (Solati et al., 2010). It remains in considerable residues both in food and environment and thus representing an important route of human exposure (Abrasco et al., 2015; Silva et al., 2015).These pesticides are preferentially used because of their target oriented mechanism of action and rapid biodegradability. A number of studies have shown that by generating free radicals cypermethrin causes harms to erythrocytes, liver, brain, sperm and other tissues (Yousef et al., 2003). Zinc is a vital trace element and controls cell proliferation, immune function and body defence (MacDonald, 2000; Prasad, 2008; Zheng et al., 2013). Through maintaining an adequate level of metallothione in zinc stimulates antioxidant system that inhibits oxidative stress mediated cell damage (Prasad, 2014; Cruz et al., 2015; Bogani et al., 2013).

Alpha (α)-lipoic acid is a natural molecule that helps in acyl-group transfer and acts as a coenzyme in the TCA cycle. Lipoic acid is beneficial when considered as a food supplement due to its antioxidant function and several studies have revealed its protective effects in aging, diabetes mellitus and vascular and neurodegenerative diseases through the reduction of free radicals (Packer et al., 1996; Packer et al., 2001;Yilmaz et al.,2002; Wollin and Jone, 2003). Pesticides are among the synthetic chemicals that have been identified as plausible contributors to increase these human reproductive disorders (Virtanen and Adamsson, 2012). Parallel with the increasing number of human studies, there are evidences in animal studies suggesting that pesticides, which are also considered as endocrine disrupting chemicals may contribute to male reproductive disorders (Bergman et al., 2013). During the past few decades, epidemiological data show rates of testicular germ cell cancer have increased among young men all over the world, suggesting a role for environmental factors such as endocrine disrupting chemicals (Kanetsky et al., 2009). The decline in sperm quality in several areas of the world stands out, with a consequent increase in the demand for assisted reproduction services (Giwercman and Giwercman, 2001).Cypermethrin is considered to be safe for household applications, although some studies reported the adverse effects of cypermethrin on male reproductive system of laboratory animals (Wang et al., 2010). Exposure to environmental toxicants may be responsible for decrease in libido, in the impairment of reproductive system and male sterility. Household use of synthetic pyrethroid cypermethrin may be a contributing factor for human male infertility. Recent studies reported that cypermethrin exposure decreased in the count of functional sperms in mice and rats (Hu et al., 2011). Viewing this background the present study was designed to search out the toxic potentials of cypermethrin in male reproductive toxicity and to evaluate the possible ameliorative role of zinc and lipoic acid on testicular damage and oxidative stress in cypermethrin intoxicated male Wistar rats.

Materials and Methods:-

Chemicals and reagents

Cypermethrin 10% emulsifiable concentrate (EC) was procured from UPL limited. Zinc sulphate ($ZnSO_4$), HCl, sulfo salicylic acid, dithionitrobenzoic, reduced glutathione (GSH), Tris-Hcl, pyragallol, sodium dodecyl sulfate, thiobarbituric acid, n-butanol-pyridine, and acetate buffer. *p*-nitrophenol phosphate, fructose, ferric chloride, glacial acetic acid, chlosterol, sodium chloride (NaCl), phosphate buffer, sodium hydroxide (NaOH), alanine, α-ketoglutaric acid. Glycerol, tetra sodium pyrophosphate (TNaPP), testosterone, di-hydroepiandrostreone (DHEA), RIPA lysis buffer, rhodamine-123, glucose-6-phosphate, Tris-buffered saline with Tween-20 (TBST). All chemicals used were of analytical grade and were purchased from Merck India Ltd, Himedia India Ltd, etc.

Animal care

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

Treatment protocol

Thirty six Wistar male albino rats weighing 130-150 g were acclimatized before the experimental procedure. The animals were housed in labelled cages, in a room designed for control of temperature (approximately 25±2°C), and light cycle (12 h light, 12 h dark). Animals were fed a standard laboratory pellets diet and water *ad libitum*. The experiment was conducted in accordance to the Institution's Animal Ethical Committee. After 10 days of acclimatization, the animals were randomly assigned to both the experimental groups and the control group, each containing six rats. Groups were designed as:

Group I: Control (5 ml /kg body wt.)
Group II: Zinc and lipoic acid (32.42 mg/kg body wt. and 35 mg/kg body wt.) control
Group III: Cypermethrin-treated (Low dose, 40mg/ kgbody wt.) group

Group IV: Zinc and lipoic acid + Cypermethrin-treated (Low dose, 40mg/kg body wt.) group
Group V: Cypermethrin-treated (High dose, 80mg/kg body wt.) group
Group VI: Zinc and lipoic acid + Cypermethrin-treated (High dose, 80mg/body wt.) group

The amount of $ZnSO_4.7H_2O$ consumed by each rat was 32.42 mg/kg body wt. It was calculated according to the concentration of $ZnSO_4.7H_2O$ at 227 mg/L in drinking water (Goel et al., 2006) and lipoic acid at the concentration of 35 mg/kg body wt. After one hour of pre-treatment with zinc and lipoic acid, cypermethrin were orally administered at the dose levels of 40 and 80 mg/kg body weight for 14 consecutive days. Weight of each animal was taken daily and the dose was adjusted accordingly.

Sample collection
On 15th day after sacrificed, serum and testis from control and treated rats were immediately collected. The other part of the testis was taken for histological analysis.

Estimation oftesticulo-somatic, epididymal-somatic, prostrato-somatic and seminal vesciculo-somatic index
Fats were removed from all testis, epididymis, prostrate, seminal vescicle of sacrificed male rats and their weights were taken to calculate testiculo-somatic, epididymal-somatic, prostrato-somatic, seminal vesciculo-somatic index using the following formula (Das et al., 2006):

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

$$\text{Epididymal} - \text{somatic index} = \frac{\text{Weight of epididymis}}{\text{Body weight}} \times 100$$

$$\text{Prostato} - \text{somatic index} = \frac{\text{Weight of prostate}}{\text{Body weight}} \times 100$$

$$\text{Seminal} - \text{vesciculo somatic index} = \frac{\text{Weight of seminal} - \text{vescicle}}{\text{Body weight}} \times 100$$

Sperm count
Caudal epididymal sperm suspension was diluted (1:100) with phosphate buffer. The count of spermatozoa were done by Neubauer haemocytometer chamber under light microscope and finally expressed as $\times 10^6$ ml^{-1} (WHO, 1999).

Sperm viability assay
To evaluate the sperm viability the eosin-nigrosin staining was done (WHO, 1999) by taking one drop of sperm suspension in two drops of 1% eosin Y. Then after 30 s 10% nigrosin were mixed well and a smear was prepared on a clean glass slide. The prepared slide was observed under the light microscope.

Hypo-osmotic swelling (HOS) test
Semen (0.1 ml) was added with 1ml of pre-warmed swelling solution (0.735 g sodium citrate dehydrate and 1.351 g fructose in 100 ml distilled water) and was mixed gently with the pipette. Then it was kept at 37°C for at least 30 min and examined the tail curling of sperm cells under microscope (Nikon Eclipse, LV100POL) (WHO,1999).

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

Determination of testicular ascorbic acid
Using 5% metaphosphoric acid-10% acetic acid solutions testicular tissues were homogenized and then centrifuged. After centrifugation a very small amount of concentrated bromine was given to the supernatant and kept for 10 min for complete oxidation and excess liquid bromine was removed.2 ml of tissue extract was added with 0.5 ml of dinitrophenylhydrazine-thiourea reagent (2.2%) and incubated at 37 °C for 3 h. Then 2.5 ml of 85% H_2SO_4 was slowly added in ice-cool condition and was mixed well for 30 min in room temperature and optical density was observed at 540 nm (MaitiChoudhury et al., 2011).

Estimation of Testicular Malondialdehyde

One ml (20mg/ml homogenate) was mixed with 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of acetate buffer (20% pH 3.5), and 1.5 ml of aqueous solution of thiobarbituric acid (0.8%) were taken. After heating at 95°C for 60 min, the red pigment produced was extracted with 5 ml of *n*-butanol- pyridine mixture (15: 1) and centrifuged at 5000 rpm for 10 min at room temperature. The absorbance of supernatants was estimated at 535 nm (Ohkawa et al., 1979).

Determination of intracellular reactive oxygen species (ROS) formation

To the sperm suspension, 1 µg ml^{-1} H_2DCFDA was added and incubated at 37°C for 30 min in the dark. Cells were washed with PBS followed by incubation. The cells were again washed thrice with fresh culture media. DCF fluorescence was measured at 485 nm excitation and 520 nm emission (Hitachi F-7000 fluorescence spectrophotometer) and was also detected by fluorescence microscopy (LEICA DFC295, Germany). The experiment was done in triplicate manner (Roy et al., 2008).

Results:-

In the present study it is observed that by treatment of cypermethrin absolute weight of the testes decreased significantly along with accessory reproductive organs, such as epididymis, prostate and seminal vesicles compared with that of the control group animals. Pre-treatment with zinc and lipoic acid prevented the weight loss by cypermethrin treatment (Figure 1-3).

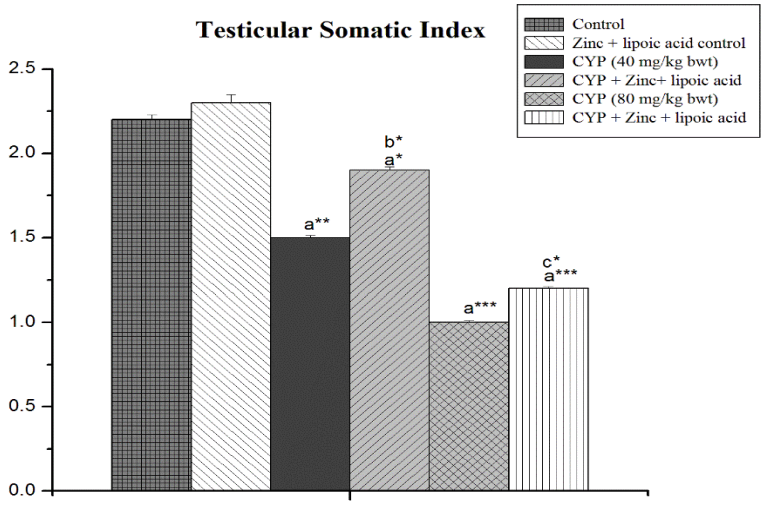

Figure 1:-The figure shows the effect of zincand α- lipoic acid on testiculo-somatic index in cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript **a,** Group-I versus all other groups; Superscript **c** Group-V versus Group-VI. Asterisk represents the different level of significance (* indicates p<0.05;** indicates p<0.01; *** indicates p<0.001).

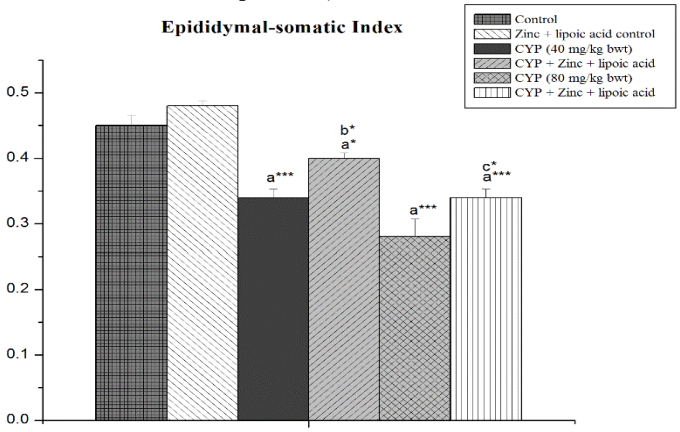

Figure 2:-The figure shows the effect of Zinc and α- lipoic acid on epididymal-somatic index in Cypermethrin induced male albino rat (N=6).Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups. Asterisk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

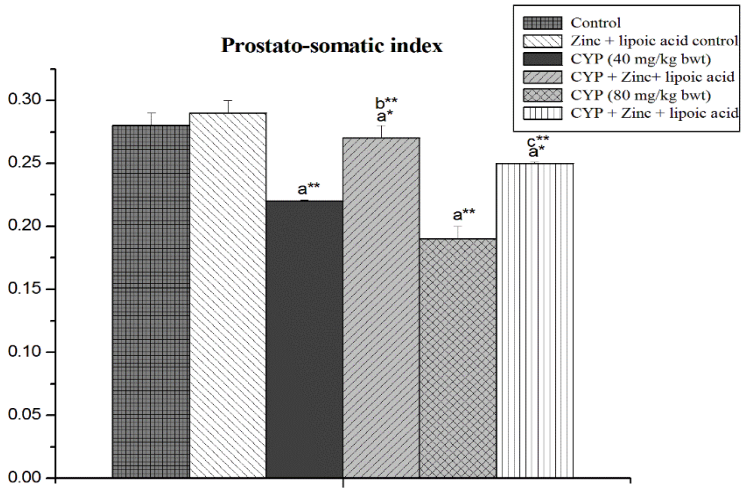

Figure 3:-The effect of zinc and α- lipoic acid on prostato-somatic index in cypermethrin induced male albino rat (N=6).Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups. Asterisk represents the different level of significance (*indicates p<0.05; **indicates p<0.01).

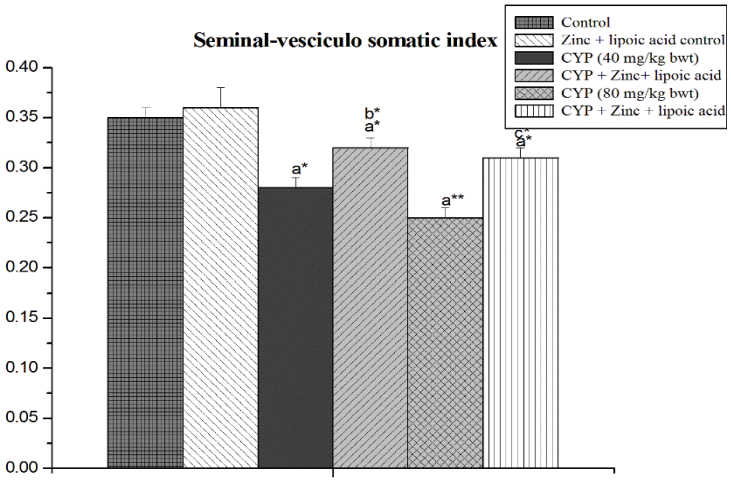

Figure 4:-The figure shows the effect of zinc and α- lipoic acid on seminal vesciculo-somatic index in cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups.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

Cypermethrin on sperm count and sperm viability

Sperm count of cypermethrin treated rat was found to be decreased significantly both in low and high doses. The animals which received the high doses of cypermethrin, showed maximum reduction in sperm count. Pre-treatment with zinc and lipoic acid increased the sperm count significantly in the recovery groups animal (Figure 5). Sperm viability of cypermethrin treated rat was decreased significantly both in low and high doses. Pre-treatment with zinc and lipoic acid increased the sperm viability significantly in cypermethrin treated animal (Figure 6).

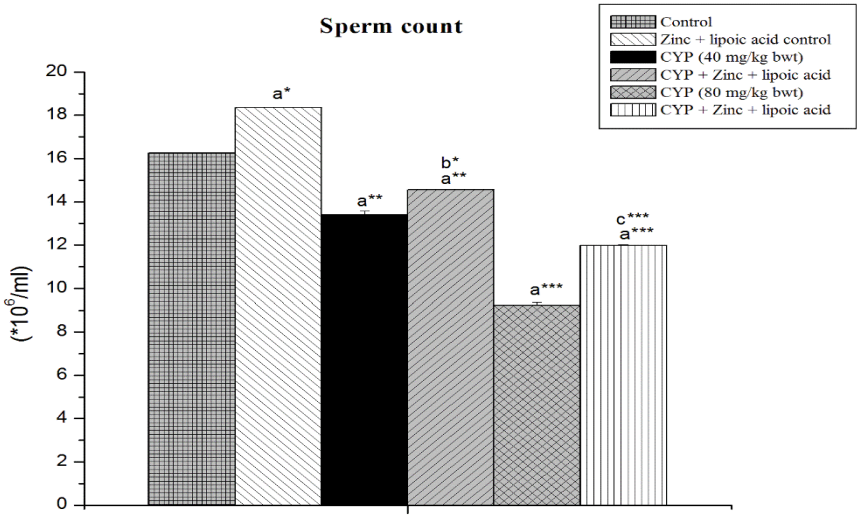

Figure 5:-The effect of zinc and α- lipoic acid on epididymal sperm count in cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisk represents the different level of significance (*indicates p<0.05; **indicates p<0.01;*** indicates p<0.001).

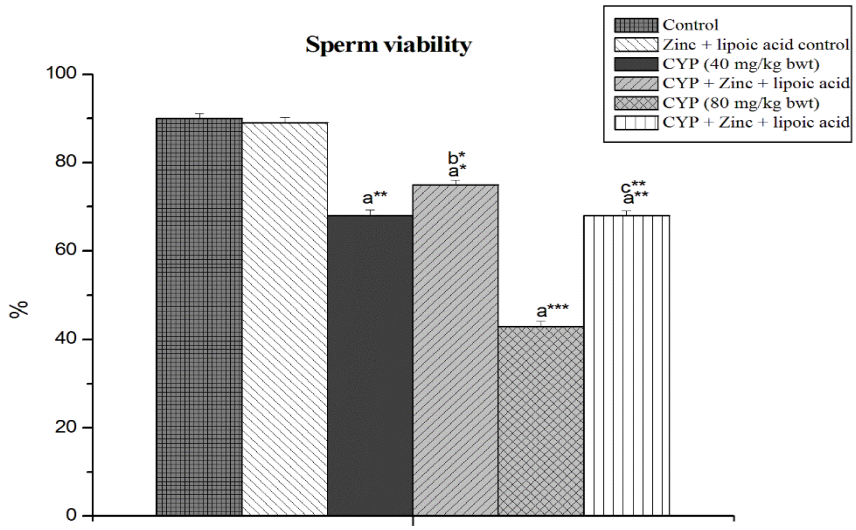

Figure 6:-The effect of zinc and α- lipoic acid on epididymal sperm viability in cypermethrin induced male albino rat (N=6). Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b Group-III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

Effect of cypermethrin on hypo-osmotic swelling

Treatment of cypermethrin in both low and high dose levels significantly decreased the hypo osmotic swelling in sperm plasma membrane. Pre-treatment with zinc and lipoic acid caused significant increase in hypo osmotic swelling in cypermethrin treated rats (Figure 7).

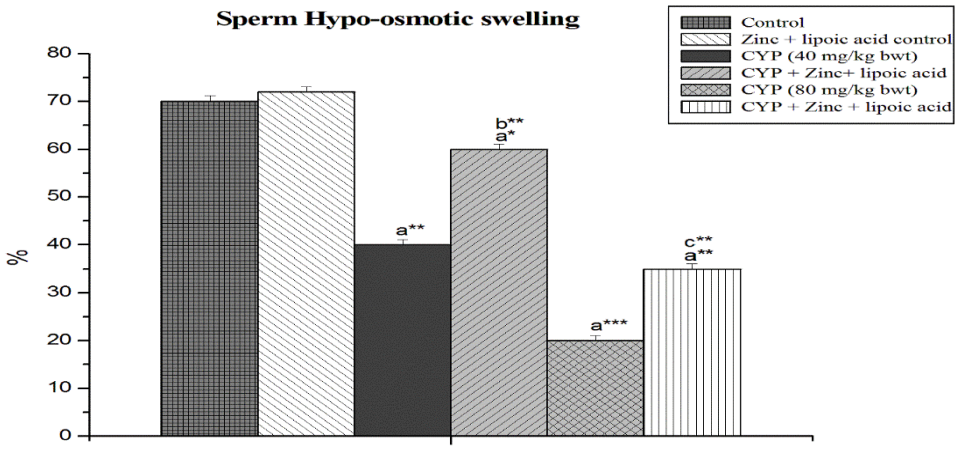

Figure 7:-The figure shows the effect of zinc and α- lipoic acid on hypo-osmotic swelling of rat spermincypermethrin induced male albino rat (N=6).Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisk represents the different level of significance (* indicates p<0.05;** indicates p<0.01; *** indicates p<0.001).

Effect of cypermethrin on testicular ACP

The testicular ACP decreased significantly in cypermethrin-treated groups. Pre-treatment with zinc and lipoic acid significantly increased testicular ACP in cypermethrin treated rats (Figure 8).

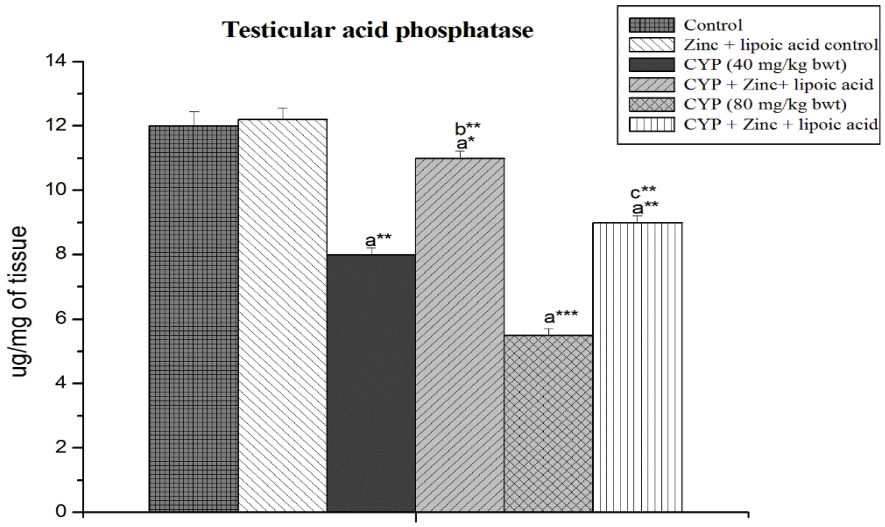

Figure 8:-The effect of zinc and α- lipoic acid on testicular acid phosphatase (ACP) in cypermethrin induced male albino rat. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisk represents the different level of significance (*indicates p<0.05, **indicates p<0.01, *** indicates p<0.001).

Testicular ascorbic acid

In the present study it is observed that cypermethrin intoxication caused significant increase in testicular ascorbic acid, which was decreased by pretreatment of zinc and lipoic acid (Figure 9).

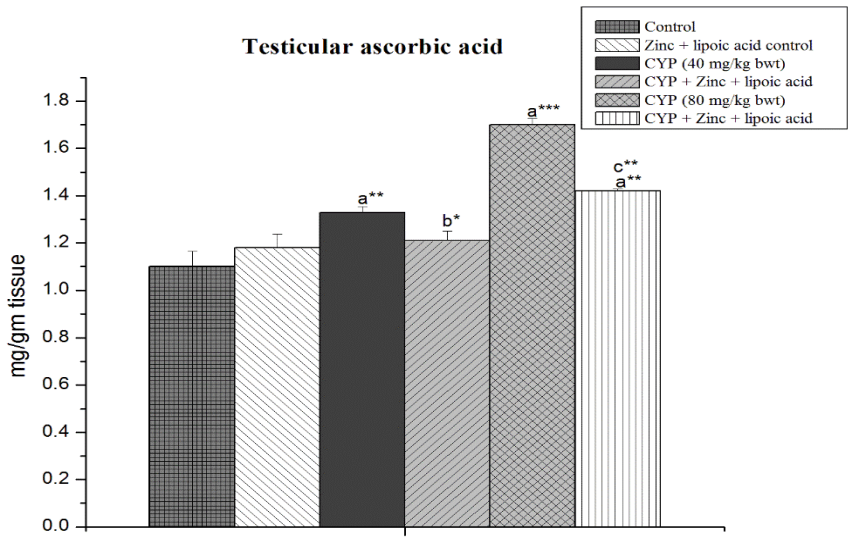

Figure 9:-The figure shows the effect of zinc and α- lipoic acid on testicular ascorbic acid in cypermethrin induced male albino rat (N=6). Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisk represents the different level of significance (* indicates $p<0.05$;** indicates $p<0.01$; *** indicates $p<0.001$).

Effect of cypermethrin on testicular MDA

The testicular MDA level increased significantly in cypermethrin-treated groups. Pre-treatment with zinc and lipoic acid caused significant decrease in MDA level in cypermethrin treated rats (Figure 10).

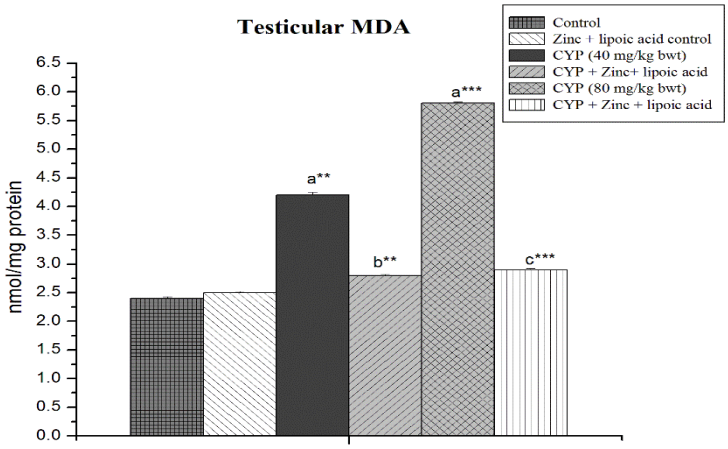

Figure 10:-The effect of zinc and α- lipoic acid on testicular malondialdehyde in cypermethrin induced male albino rat. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisk represents the different level of significance (** indicates $p<0.01$; *** indicates $p<0.001$).

Intracellular reactive oxygen species (ROS) formation

The Intracellular reactive oxygen species (ROS) formation was increased significantly in cypermethrin-treated groups. Pre-treatment with zinc and lipoic acid caused significant decrease in ROS formation in cypermethrin treated rats (Figure 11).

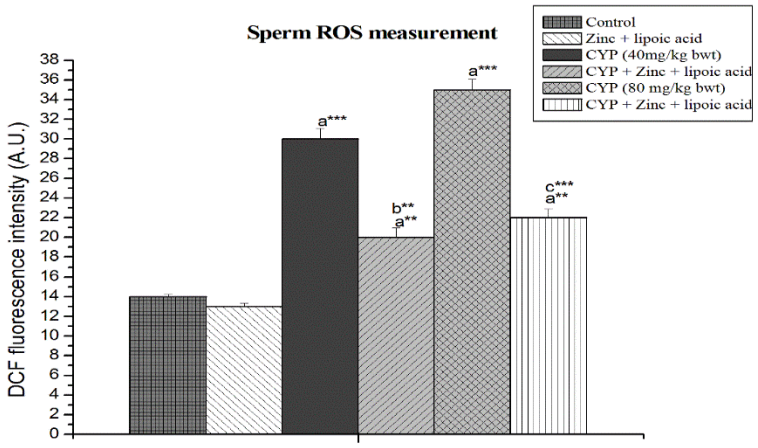

Figure 11:-The effect of zinc and α- lipoic acid on DCF fluorescence intensity in term of ROS production in cypermethrin induced male albino rat. Analysis is done by ANOVA followed by multiple comparison two-tail t-tests. Superscript a, Group-I versus all other groups; Superscript b Group-III versus Group-IV; Superscript c Group-V versus Group-VI. Asterisk represents the different level of significance (** indicates $p<0.01$; *** indicates $p<0.001$).

Discussion:-

The reduction in testicular weight may result in a decrease in sperm count as well as reduction in the weight of the seminal vesicles and ventral prostate, which may reflect an interference in androgen synthesis (Wang et al., 2009). In the combined pre-treatment with zinc and lipoic acid the diminution in testicular weight was observed significantly both in low and high doses of cypermethrin. These changes in the weights of the testis, prostate and epididymis indicate that cypermethrin exert toxic effects on these organs and zinc along with lipoic acid has significant protective role against cypermethrin intoxication.

Male fertility is highly correlated with sperm count. This study also displayed the adverse effect of cypermethrin on sperm count like the toxic effects of fenvalerate (Lifeng et al., 2006). Reduction in sperm count may be due to the disruption of spermatogenesis, change in male steroid hormone level and loss of fertility (MaitiChoudhury et al., 2011). Prominent swelling of sperm plasma membrane in hypo-osmotic medium is the indicator of intact plasma membrane.Cypermethrin causes membrane damage by many possible ways. It is observed that cypermethrin may get localised in the hydrophobic core of the membrane, where it increases lipid packing and consequently decreases membrane fluidity (Gabbianelli et al., 2002). Administration of cypermethrin had been shown to produce oxidative stress by generating free-radicles and eventually caused destruction of antioxidant defences (Atessahin et al., 2005). Pyrethroids are considered as lipophilic, they could penetrate easily to the cell membrane and cause membrane lipid peroxidation which may cause oxidative damage (Prasanthi et al., 2005). Zinc and lipoic acid due to their anti-oxidant and ameliorative functions may protect the sperm plasma membrane from the toxicity of cypermethrin.

Testicular acid phosphatase (ACP) activity is considered as functional enzymatic indicators of male reproduction or a biomarker for testicular toxicity (Kori-Siakpere et al., 2008). In the present study, there was significant decrease in ACP activity in the cypermethrin intoxicated animals, on the other hand, pre-treatment with zinc and lipoic acid prevented the toxic potentiality of cypermethrin and normalised the ACP activity (Figure 9) to a great extent. ACP is located in the subcellular organs like lysosome of the Leydig cells. It performs the synthesis of protein by carrying sex hormones. Alteration in the activity of ALP and ACP may be the useful tool in determining the spermatogenic function. A decrease in the ACP activity in cypermethrin treated animals indicated that produced a state of decreased steroidgenesis where inter and intracellular transport is reduced as a result in decreased steroidogenesis. Decrease in ACP enzymes activity shows decrease in the activity of phosphatase in the nucleus of the spermatocytes during spermatogenesis (Zhang and Sen, 2009). Decreased enzyme activity of testicular ACP of cypermethrin treated rats also reflect testicular degeneration, which may be a consequence of suppressed testosterone (Kaur et al., 1999).

Ascorbic acid, a micronutrient required for a wide variety of metabolic functions including male reproduction. Germ cell proliferation and differentiation is controlled by Sertoli cells through cell-cell communication and by forming

the blood-testis barrier (Angulo et al., 2011). Blood-testis barrier restricts direct entry of molecules from the plasma into the adluminal compartment of the seminiferous tubule. Sertoli cells are capable to transport both forms of ascorbic acid. Sertoli cells may regulate the amount of ascorbic acid in the adluminal compartment, as well as control the obtainability of ascorbic acid throughout spermatogenesis.Our findings showed an increase in testicular ascorbic acid which may be associated with the reduction in steroidogensis as ascorbic acid and NADPH are essential for testicular steroidogenesis (Maiti Choudhury et al., 2011) as well as cypermethrin exposure may impair sertoli cells by which normal ascorbic acid transport can be disrupted. Zinc and lipoic acid due to their anti-oxidant and ameliorative functions decreased ascorbic acid probably by protecting the sertoli cells (Angulo et al., 2011).

In present study, cypermethrin exposure increased MDA level in testis, indicating increase in oxidative stress in the tissue (Joshi et al., 2011). In the study, excess production of ROS in cypermethrin treated group was confirmed by fluorescence spectrophotometer using H_2DCFDA. Accumulation of cypermethrin in testicular tissue leads to membrane degeneration and excessive free radical formation. The excessive free radicals further damage the sperm membrane as well as the antioxidant defence system of the testicular tissue (Vaithinathan et al., 2009). Treatment of zinc and α- lipoic acid decreased the cypermethrin toxicity and restored the oxidative status of the testicular tissue.

Conclusion:-

The study showed that cypermethrin-induced testicular toxicity by altering somatic index of reproductive organs, sperm plasma membrane, decreasing sperm count, sperm viability, testicular acid phosphatase (ACP) activity, and increasing lipid peroxidation, ascorbic acid activity, ROS measurement in male rats and pre-treatment with zinc and α-lipoic acid has resulted in a pronounced ameliorating effect especially at the end of the experiment emphasizing their antioxidant role.

Acknowlegements:-

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