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Ameliorative Effect Of Antioxidant(Vitamin C) Against Cypermethrin Induced Repro-Toxicity In Adult Male Rats

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Abstract: The aim of the present study was to evaluate the protective role of vitamin C on Cypermethrin induced reproductive toxicity in male rats. Healthy 40 male rats were randomly divided into 5 groups with 8 animals each. Group I served as control and administered with saline and group II and III were administered with Cypermethrin at a dose of 50mg and 100mg/kg BW respectively. Group IV administered with vitamin C alone at a dose of 60mg/kg BW orally and group V administered with both Cypermethrin(100mg/kg BW) and vitamin C(60mg/kg BW)for 60 days. After the treatment rats were sacrificed and isolated the reproductive organs immediately for biochemical analysis. The body weights of rats did not show any significant change in all groups. Whereas the weights of reproductive organs were significantly decreased in Cypermethrin administered groups. Treatment with Cypermethrin significantly decreased epididymal sperm count, viable sperms and motile sperms with a significant reduction in the testicular steroidogenic enzymes (3β-HSD and 17 β HSD) activities. Histoarchitechture of testis of rats administered with cypermethrin showed degenerative changes in seminiferous tubules with reduced size, increased interstitial spaces, and inhibited spermatogenesis. Where as co-administration of Vitamin C with Cypermethrin showed a significant improvement in the weights of reproductive organs, sperm count, sperm viability and motility. The co-treatment of vitamin C also significantly increased the activity levels of steroidogenic enzymes and it restored the structural integrity of testicular architecture in Cypermethrin intoxicated rats. The present study indicating the protective role of vitamin C against cypermethrin induced reproductive toxicity in male rats.

Key Words: Cypermethrin, Vitamin C, Male fertility, Spermatogenesis, Steroidogenesis.

1.Introduction

Infertility is a devastating problem of human reproduction, and male infertility contributes to 50% of all infertility cases (Whitfield et al., 2015). Several factors such as lifestyle factors, occupational agents, drugs, radiation and environmental pollutants have been attributed as reasons for suppressed male fertility (Tripathi et al., 2009).Exposure to environment pollutants interfere with normal function of the endocrine system (endocrine disruptors, EDCs) are the main factors for male infertility (Grey et al., 1999; Sharpe, 1993**)**.These chemicals can able to mimic, block the synthesis, release, transport, and binding or elimination of natural hormones (Caserta *et al.*, 2013a). Most of the environmental contaminants can act as EDCs. Among several pesticides including cypermethrin are one important compound (Hofmeister and Bonefeld-Jorgensen, 2004).

Published reports showed a clear correlation between pesticide exposure and reduced male fertility (Swan *et al.,* 2003; Lifeng *et al.,* 2006).The frequent use of pesticides to control pests in agriculture, household purpose is a common practice now a days . Several studies indicated that pesticides disrupt the normal function of the endocrine system in humans and wildlife (Colborn *et al.*,1993; Lintelmann *et al.*, 2003). Cypermethrin [(R,S)-α -cyano-3-phenoxybenzyl (1R,S)- cis,trans-3-(2,2- dichlorovinyl)-2,2-dimethyl-cyclopropane] (cyp) is a Type II synthetic pyrethroid that resemble pyrethrums structurally (Adelsbach and Tjeerdema, 2003). It is used as an insecticide in large-scale commercial agricultural applications, forestry as well as in consumer products for household domestic pest management. Chemically it is a potent neurotoxin for insects. An alpha-cyano group attached to the benzylic carbon which promotes the insecticidal properties. It is easily degraded on soil and plants but can be effective for weeks when applied to indoor inert surfaces. Exposure to sunlight, water and oxygen will accelerate its decomposition. Like other pyrethroids, cypermethrin is not target specific and its exposure causes several harmful effects in humans and wildlife (De Jager *et al.,* 2006). Cypermethrin initially thought to be safe for household application , a number of animal studies indicated that exposure to cypermethrin leads to neurotoxicity, immunotoxicity, genotoxicity and reproductive toxicity (Wang *et al*., 2009)

Cypermethrin can act as one of EDCs that interfere with androgen action and have a greater impact on male developmental programming, reproductive tract maturation, and one of the major pathway through which it operates is AR (Androgen Receptor) mediated signalling. Primary androgenic hormones, testosterone and its metabolite DHT (5-α-dihydrotestosterone) mediate their biological effects predominantly through binding of the AR and is expressed in many end-organs including the hypothalamus, pituitary, prostate and testes (Matsumoto *et al*., 2008). Cypermethrin is known to be anti androgenic, inhibits testosterone binding to AR and reduces the availability of testosterone and effects spermatogenesis (Eil and Nisula, 1990). Earlier studies reported that exposure of rodents to cypermethrin negatively affect sperm quality and quantity and disrupts testicular testosterone production (Sahaar *et al*., 2016) It has been shown that cypermethrin can able to modulate testicular pro- and anti-oxidant status there by provokes oxidative stress in rats (Sharma *et al*., 2014). Thus, it seems apparent that cypermethrin induced male reproductive toxicity at least in part mediate inhibition of testosterone biosynthesis and accelerates oxidative stress.

1.1 Antioxidant (Vitamin – C):

Ascorbic acid, often known as vitamin C, is an important endogenous water-soluble micronutrient and natural antioxidant that is crucial for a variety of biological processes and activities in many different types of animals (Mertoglu K, 2021). 2-oxo-L-threo-hexono-1,4-lactone-2,3-enediol is the chemical formula for vitamin C. According to (Corpe C.P,2010) vitamin C is a crucial component that is primarily obtained from food sources like fruits and vegetables. It is effectively and fully absorbed in the small intestine by a saturable active transport system. Dehydroascorbic acid, which is produced by oxidising vitamin C, is hydrolysed to produce diketogulonic acid, which is oxidised to produce oxalic and threonic acid. High vitamin C doses cause oxidation to carbon dioxide. The majority of vitamin C metabolites, including oxalate, and un metabolized vitamin C are eliminated in the urine.

As a hydrophilic vitamin that traps free radicals and shields biomembranes from peroxide,vitamin C is a crucial free radical scavenger in extracellular fluids. Hypochlorous acid, singlet oxygen, superoxide, hydroxyl, and water soluble peroxyl radicals are all efficiently scavenged by vitamin C.It is also said to be a very good donor of electrons, which means that it can quench the activity of free radicals like hydroxyl and super oxide radicals by giving them electrons.

Researchers have reported that vitamin C possesses heaptoprotective properties. This is supposed to be related to its antioxidant qualities.Vitamin C is known to protect spermatogenesis in the male reproductive system, where it plays a crucial role in raising of testosterone levels, reduces sperm agglutination, and is essential for semen integrity and fertility in both men and animals (Agarwal et al., 2005).
Contributing up to 65% of the total antioxidant capacity of seminal plasma found intra cellularly and extracellularly, it is a significant chain breaking antioxidant (Makker et al., 2009).Shrilatha and Muralidhara (2007) used a diabetic mouse model to demonstrate the protective impact of vitamin C on testicular oxidative stress, sperm oxidative stress, and genotoxic consequences.

Likewise, Naziroğlu (2003) deduced that vitamin C functioned as an antioxidant inside the reproductive environment.It was looked into how vitamin C protected against pesticides harmful effects on reproduction.Furthermore, it is said to neutralize ROS andVitamin C is known to safeguard spermatogenesis in the male reproductive system and is essential for the integrity and fertility of semen. Additionally, it is said to lessen oxidative DNA damage and, consequently, genetic alterations by neutralising ROS (Wang et al., 2008).

According to reports, vitamin C is linked to fertility, in male it increases testosterone levels, prevents sperm agglutination, preserves sperm integrity, and increases sperm mobility, all of which are crucial for sperm production and, ultimately, contribute to regular, appropriate, and successful spermatogenesis in a variety of organisms (Fernandes et al., 2011).Given that vitamin C has antioxidant properties, the current study aims to explore vitamin C's protective function on the cypermethrin induced reproductive toxicity in adult male rats.

2. Materials and Methods

2.1 Procurement and Maintenance of the animals

Male Wistar albino rats with a body weight of 190±10 g (90 days old) were purchased from authorized vendor (Sri Ragavendra Enterprises, Bengaluru, India). Rats were housed (four per cage) in clean polypropylene cages (18" x 10" x 8") containing paddy husk as bedding material and were provided with standard rodent chow (obtained from Sai Durga Agencies, Bengaluru, India) and tap water *ad libitum*. The rats were maintained under well-controlled laboratory conditions (temperature 22-25°C; 12:12 hr light: dark cycle). The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India (CPCSEA, 2003) and approved by Institutional Animal Ethical Committee of Sri Padmavati Mahila Visvavidyalayam, Tirupati(Regd.No.1677/PO/Re/S/2012/CPCSEA, Dated:21.12.2015; IAEC -39 D:6.5.16).

2.2 Experimental design

The animals were divided into five groups(Group I considered as control and remaining four groups considered as experimental groups). The rats in group I served as controls and administered with saline and standard rat feed. The rats in group II and III were administered with cypermethrin (50 and100 mg/kg bw) on daily dose by oral gavage. Rats in group IV were given in Vitamin - C (60 mg/kg bw) and the animals in group V received both cypermethrin(100 mg/kg bw) and Vitamin – C(60 mg/kg bw) for a period of 60 days.

After completion of the experimental period, rats were fasted overnight, weighed, and killed by cervical dislocation. Body weight, liver, kidney, spleen, testes, epididymis (caput, corpus, and cauda), vas deferens, seminal vesicles, prostate gland, and penis were immediately collected, washed with ice-cold saline, and weighed to the nearest milligram. Tissue somatic indices (TSI) was calculated by using the following formulas:

TSI = [weight of the tissue (g) / Body weight of the animal (g)] × 100

2.3 Chemicals

Cypermethrin (CYP), Vitamin-C (vit c), androstenedione, dihydroepiandrosterone, NAD and NADPH were purchased from Sigma Chemical Company, St Louis, MO, USA. All other chemicals used in study were of analytical grade and obtained from local commercial sources.

2.4 Sperm analysis

Sperm analysis was determined by the method of Belsey et al., (1980).Cauda part of epididymis was placed in a Petri plate contains 2.0 ml of physiological saline and collected the epididymal sperm suspension at 37°C. Sperm count was performed on the epididymal fluid using Neubauer Chamber. The number of sperm count was expressed as a percentage of the total sperm counted and presented in millions/ml.

2.5 Sperm motility

sperm motility was determined by Belsey et al., (1980) method. Following their isolation from the cauda epididymis, non-motile sperms were counted first, then motile sperms. Sperm motility was calculated as a proportion of the total number of sperm counted. Sperm motility was expressed as a percentage of the total sperm counted and were presented in millions/ml.

2.6 Sperm viability

The ratios of living to dead sperms were determined using 1% trypan blue (Talbot,1981). The dye (Trypan blue) will not be taken up by viable sperm since the cell membrane is intact. Sperm viability was calculated as a percentage of the total number of sperm counted. The number of viable sperm was expressed as a percentage of the total sperm counted and were presented in millions/ml.

2.7 Hypo-Osmotic Swelling (HOS) **test**

The hypo-osmotic swelling test is used to determine the integrity of the sperm membrane. When viable sperms are subjected to hypo-osmotic media, there is an influx of fluid that causes the tail to coil and observed under a phase-contrast microscope. The percentage of coiling was calculated (Jeyendran, R.S., 1992).

2.8 Steroidogenic enzymes assay

The testicular tissue was homogenized in ice-cold 20 mM Tris HCl buffer (pH 8.2). The microsomal fraction was separated and used as enzyme source. The activity levels of 3β-hydroxysteroid dehydrogenase (3β-HSD) (E.C. 1.1.1.51) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (E.C. 1.1.1.64) were measured by the method of Bergmeyer et al. 1974 &1965. The enzyme assays were made under the conditions following zero order kinetics after preliminary standardization regarding linearity with respect to time of incubation and enzyme concentration.

The reaction mixture in a final volume of 2.0 ml contained: 100 µ moles of sodium pyrophosphate buffer (pH 9.0), 0.5 µ moles of co-factor (NAD for 3β-HSD and NADPH for 17β-HSD), and 0.1 µ moles of substrate (dihydroepiandrosterone for 3β-HSD and androstenedione for 17β-HSD) and 25 mg equivalent of microsomal protein as enzyme source. The reactions were carried out in a quartz cuvette of 1.0 cm path at 23±1°C. The absorbance at 340 nm was measured at 20 sec intervals for 3 min in a UV-VIS spectrophotometer (Jeyendran *et al.,* (1992) against the reagent blank containing all the components, except the enzyme source. The enzyme activities were expressed in µ moles of NAD converted to NADH/mg protein/min (3β-HSD) and µ moles of NADPH converted to NADP/mg protein/min (17β-HSD).

2.9 Total protein estimation

Protein content in the enzyme source was estimated using bovine serum albumin as standard (Lowry, O.H.,1951).

3.0 Statistical Analysis

Data were statistically analysed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The values $P<0.05$ were considered significant. The data were presented as mean ± SD. All statistical tests were performed using Statistical Package for Social Sciences (SPSS), version 16.0

4. Results

4.1 Body weights of rats and Tissue Somatic Indices (TSI)

The body weights and indices of liver, kidney and spleen in experimental rats were not significantly changed from control rats (Table 1). The indices of testes and epididymis were significantly ($p< 0.05$) decreased in 50 mg/kgBW and 100 mg/kgBW cypermethrin treated rats. Whereas, co administration of vitamin-c and 100 mg/kgBW cypermethrin treated rats significantly ($p< 0.05$) increased the indices of testes and epididymis when compared to the control rats; however no significant ($p> 0.05$) changes were observed in vitamin-c alone treated rats over control rats (Table 1).

Table: 1. Protective role of Vitamin-C against cypermethrin induced toxicity on body weight and organ indices in albino rat

Organ	Control	Cyp 50mg/kg/BW	Cyp 100mg/kg/BW	Vitamin-c	100mg/kg/BW Cyp +Vit-c
Body weight (g)	198.03 ±2.11	185.4* ±1.98 (85.4)	177.11* ±2.0 (77.11)	199.1ns±1.09 (99.1)	191.03a±2.11 (91.03)
Testes(g)	2.84±0.25	2.68*±0.06 (-97.32)	2.27*±0.38 (-97.73)	2.88 ns ±0.70 (-97.12)	2.56a±0.23 (-97.44)
Epidydimis(g)	1.65±0.16	1.61*±0.06 (-98.39)	0.86*±0.42 (-99.14)	1.65 ns ±0.12 (-98.35)	1.06a ±0.12 (-98.94)
Spleen(g)	1.13±0.18	1.11*±0.18 (-98.89)	0.99*±0.21 (-99.01)	1.17 ns ±0.22 (-98.83)	1.08a±0.16 (-98.92)
Liver(g)	9.07±0.83	8.66*±0.86 (-91.34)	7.82*±.0.49 (-92.18)	9.08*±1.14 (-90.92)	8.88a±0.87 (-91.12)
Kidney(g)	1.74±0.23	1.60*±0.12 (-98.4)	1.50*±0.40 (-98.5)	1.75 ns ±0.78 (-98.25)	1.45a±0.08 (-98.55)

Values are mean± S.D.of 8 rats

Values in parenthesis are percentage change from control

Values with * significantly changed from control, p<0.05;

ns= not significant; a= change from group III

4.2 Sperm Analysis

The effects of cypermethrin, vitamin c, co- administation of vitamin-c and 100 mg/kgBW cypermethrin on epididymal sperm count, motility, viability, coiled sperm were represented in Table 2. A significant (p< 0.05) decrease in the sperm count ,motility , viability and HOS was observed in 50 mg/kgBW and 100 mg/kgBW cypermethrin treated group of rats when compared to controls. Whereas, supplementation of vit-c to 100 mg/kgBW cypermethrin significantly (p< 0.05) increased the sperm count, motility, viability and HOS coiling when compared to the cypermethrin treated group rats. On the other hand, no significant changes were observed in any of the above mention parameters vitamin –c alone supplemented rats (Table 2).

Table 2: Protective role of Vitamin-C against cypermethrin induced toxicity on Sperm quality and quantity

Parameter	Control	Cyp 50mg/kg/BW	Cyp 100mg/kg/BW	Vitamin-c	100mg/kg/BW Cyp +Vit-c
Sperm motility(%)	78.50±4.76	55.6*±1.51 (-44.4)	35.51*±4.85 (-64.49)	79.4ns±6.09 (-20.6)	64.67a±2.42 (-45.33)
Sperm viability(%)	83.83±3.49	62.83*±3.43 (-37.17)	61.67*±4.55 (-38.33)	83.50 ns ±5.32 (-16.5)	72.33a±9.93 (-27.67)
Sperm count (millions/ml)	61.17±3.10	60.95*±5.59 (-39.05)	51.87*±6.49 (-48.13)	62.76 ns ±5.02 (-37.24)	56.41a±5.01 (-43.59)
Sperm functional test(%) (HOS-Test)	71.50±5.89	49.50*±1.38 (-50.5)	44.50*±4.97 (-55.5)	52.50 ns ±10.3 (-47.5)	47.83a±4.17 (-52.17)

Values are mean± S.D.of 8 rats

Values in parathesis are percentage change from control

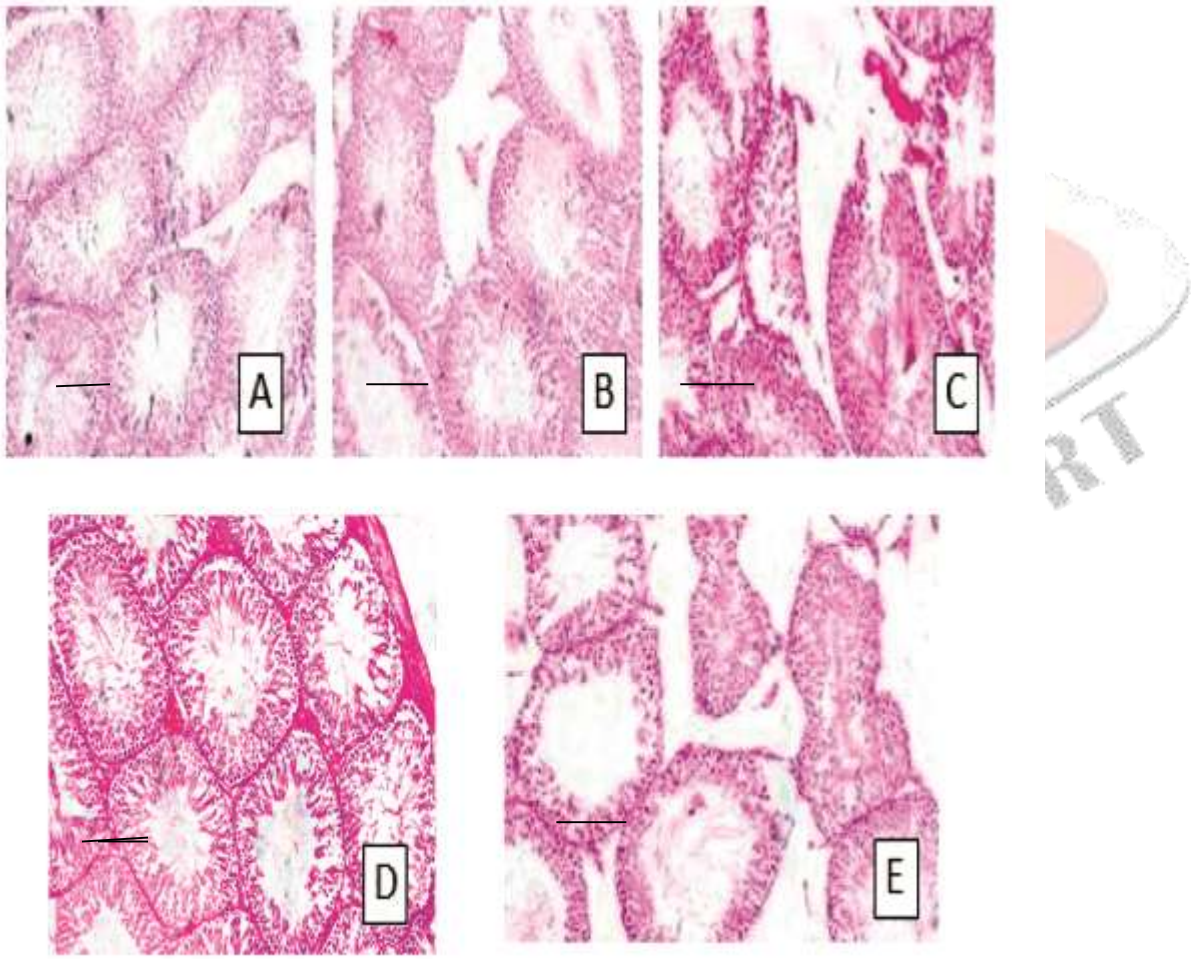
Values with * significantly changed from control, $p<0.05$;

ns= not significant; a= change from group III

4.3 Histopathological studies

Testes of a control rat showed seminiferous tubules with spermatids and mature spermatozoa with an outer membrane, theca albuginea alongside Leydig cells lying between seminiferous tubules (Figure 1A). Rats treated with 50 mg/kgBW and 100 mg/kgBW Cypermethrin exhibited clumped spermatozoa, degenerative changes in seminiferous tubules with increased lumen of seminiferous tubules, vacuoles and severe necrosis in seminoferous tubules. Beside these, increased size of lumen, degenerative changes in spermatids, atrophied seminiferous tubules, necrotic changes in theca albuginea and scattered spermatids in testes of high dose treated rats observed (Figures 1B&C). The treatment with 100 mg/kgBW Cypermethrin and vitamin c restore the changes after treatment with cyperemthrin (Figure 1E). Whereas the rats treated with vitamin C alone not showed any marked difference when compared with controls (Figure 1D).

Figure1: Protective effect of Vitamin-C against cypermethrin induced toxicity on testicular histology in adult male rats (Transverse Section of Testis)

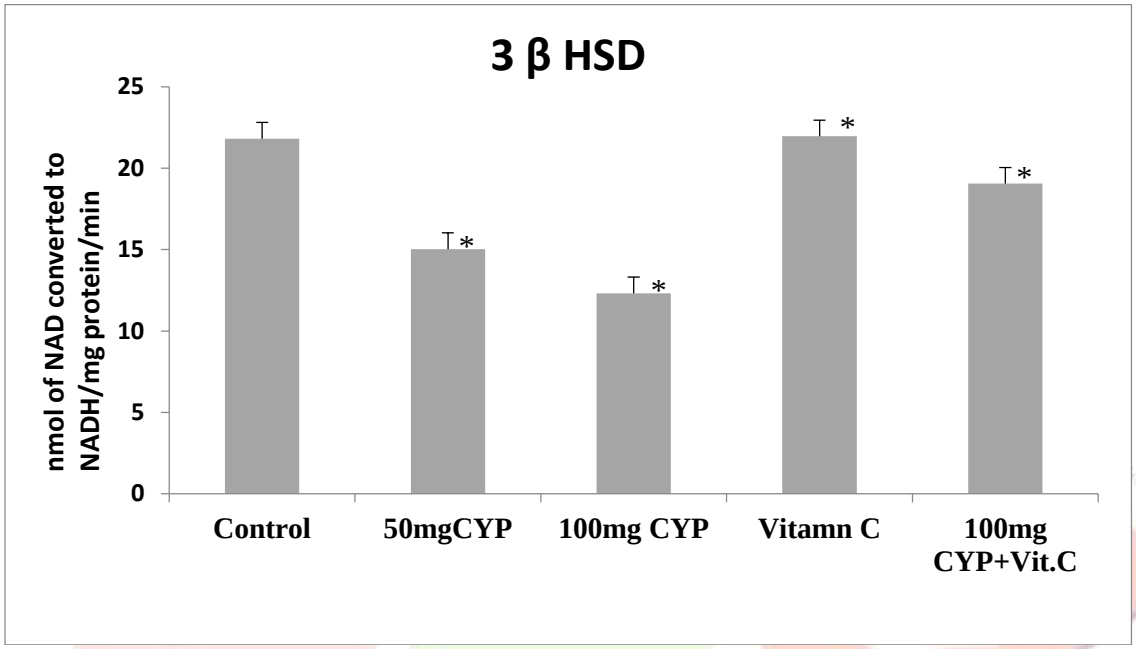


A: Control; B: Cyp 50mg/Kg BW; C:Cyp 100mg/Kg BW; D: Vitamin-C
E: 100mg/Kg BW + Vitamin-C ; Scale bar =25µm

4.4 Steroidogenic enzymes

The testicular 3β-HSD and 17β-HSD activity levels were significantly decreased($p<0.01$) in cypermethrin administered groups when compared to control rats (Figure.2a,2b) Whereas, co administration of Vitamin C showed significant increase($p<0.05$) in the activity levels of testicular steroidogenic enzymes.

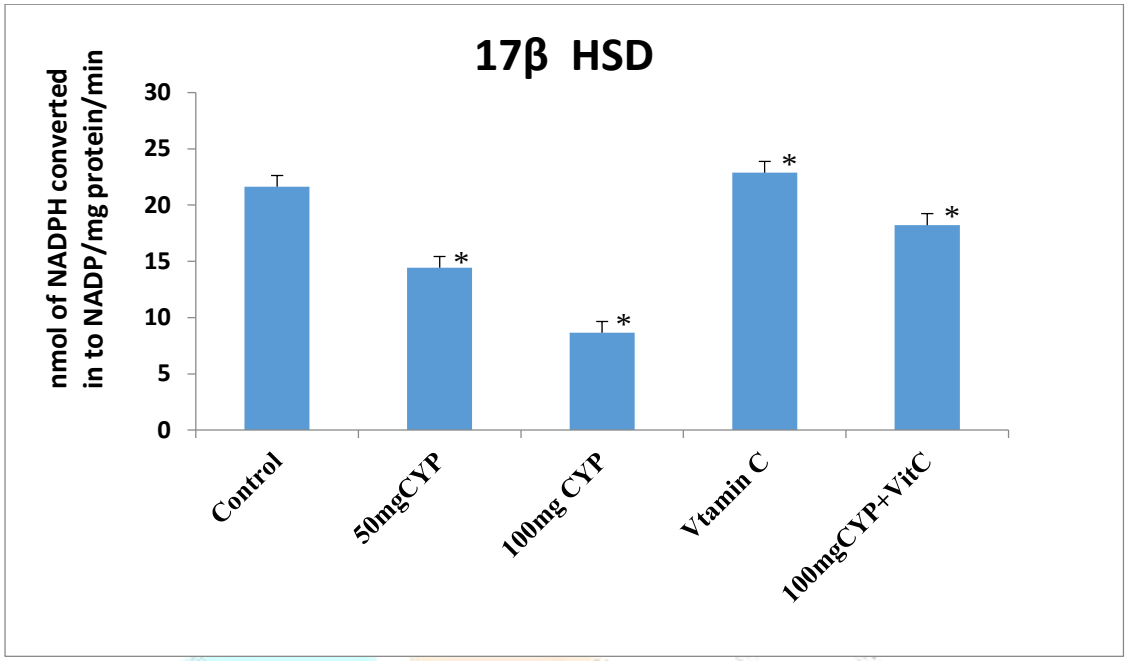
Figure 2a: Protective effect of Vitamin C against cypermethrin induced repro toxicity on steroidogenic enzymes (3 β HSD) in adult male rats.



Bars are mean ± S.D. of 6 rats

Bars with *significantly changed from control, $p<0.01$
For calculation of % change and evaluation of 'P' for treated rats untreated rat served as controls.

Figure 2b: Protective effect of Vitamin C against cypermethrin induced repro toxicity on steroidogenic enzymes(17β HSD) in adult male rats.



Bars are mean ± S.D. of 6 rats

Bars with *significantly changed from control, $p<0.01$
For calculation of % change and evaluation of 'P' for treated rats untreated rat served as controls.

5. Discussion

Environmental toxicants like pesticides exposure is a proven factor in impairment of male reproductive system and infertility. Though Cypermethrin considered to be safe for household application, a number of recent reports showed its reproductive toxicity in mammalian and non mammalian laboratory and wild life animal species (Hu et al., 2011).

A significant reduction in the relative weights of testis and epididymis in cypermethrin administered rats might be an indication of inadequate supply of androgens. Androgens play major role in maintenance of the structural and functional integrity of testis and accessory sex organs. Furthermore, the weight of testis depends on the mass of differentiated spermatogenic cells which has been used as an indicator to measure the damage of spermatogenesis (Schlappack, O.K., 1988). The present results are agreement with earlier studies (Lu Liu,2010; Wang, X.Z.,2009). Earlier studies have shown that several synthetic pyrethroid pesticides including cypermethrin exerted anti-androgenic activities (Zhang, J., 2008; Xu, L.C.,2008; Fang, LY.,2013). Exposure to cypermetrhin decreased the sperm count, motility and steroidogenic enzymes in laboratory animals (Vasudha, K., 2018). Testicular Leydig cells acts as a platform for testosterone biosynthesis. Several cascades of steps are involved in testicular steroidogenesis such as transport of cholesterol from outer to inner mitochondrial membrane, and conversion of cholesterol to testosterone via enzymatic catalysis by 3β- and 17β-hydroxysteroid dehydrogenases (Vasudha, Katragadda.,2020). Interestingly, a significant reduction in the expression of steroidogenic acute regulatory protein, which transfer cholesterol from outer to inner mitochondrial membrane of testis was observed in cypermethrin treated rats(Wang, H.,2010). Further, administration of cypermethrin deteriorated the activity levels of testicular steroidogenic marker enzymes 3β- and 17β-hydroxysteroid dehydrogenases in rats(Vasudha, Katragadda,2020). Plant based compounds were widely recognized in the recovery of chemical-induced reproductive toxicity in males(Sharma,P.,2014,2018; Pravallika, M.,2019).Co-administration of antioxidant Vitamin C significantly increased the weights of testis and epididymis in cypermethrin admnistered rats, might be attributed to the repair of spermatogenic and steroidogenic effects in cypermethrin intoxicated rats. The results indicated that vitamin c improved the sperm parameters in cypermethrin intoxicated rats. The improvement in sperm parameter may be due to the good antioxidant potential and facilitating the removal of ROS during post treatment regimen. The increase in steroidogenic enzymes may be due to increase of sperm viability and testicular mass and synchronism in all reproductive parameters. Co administration of Vitamin C has direct effect on damaged Leydig cells and restored the impairment of testicular integrity by

reducing oxidative stress and improved spermatogenesis and steroidogenesis in cypermethrin intoxicated rats.

By the results obtained from this study, it can be concluded as follows: cypermethrin intoxication a) provokes testicular oxidative damage, b) may exert toxic effects at the level of Sertoli cells and the Leydig cells and c) inhibits synthesis of 3β- and 17β-hydroxysteroid dehydrogenases biosynthesis. On the other hand, the antioxidant and steroidogenic effects of Vitamin C might be responsible for the enhanced spermatogenesis(enhancement in sperm quality and quantity), significant increase in the relative weights of testis and epididymis, significant elevation in testicular steroidogenesis in Vitamin C supplemented rats.

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