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Protective Effect of L-Carnitine against Deltamethrin Induced Male Reproductive Toxicity in Adult Rats Exposed at their Prepubertal Stage

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Abstract

The present study aims to investigate the protective role of L-Carnitine (LC) against Deltamethrin-induced testicular toxicity in male rats exposed during their early developmental period. DTM was administered at a dose of 6 mg/kg body weight to the prepubertal rat's Post-Natal Day (PND) 23 rats through oral route and exposure was continued until they reach PND 90. After completion of the exposure period, male rats were assessed for reproductive endpoints. Deltamethrin exposure caused a significant reduction in testosterone production and decreased the sperm parameters such as sperm count, sperm motility, sperm viability and sperm membrane integrity. Deltamethrin intoxication also reduced the reproductive organs' weight and testicular steroidogenic enzymes (3β-hydroxysteriod dehydrogenase and 17β-hydroxysteriod dehydrogenase) levels. Further DTM exposure induced the oxidative stress as evidenced by the significant reduction in the superoxide dismutase, catalase, and glutathione levels with significant elevation in the malondialdehyde. DTM also induced the significant sperm DNA damage. In addition, DTM exposure significantly declined the testosterone levels over the control group, indicating the impaired steroidogenesis. In addition to the compromised steroidogenesis DTM exposure also deteriorated the testicular architecture according to the histological observations. On the other hand, therapy with LC (100 mg/kg body weight) improved the biomass of reproductive organs, the features of the sperm, testicular steroidogenesis and testicular antioxidant enzymes levels. Concluding that DTM might disturb the testicular antioxidant level that eventually impaired the reproductive health of rats. However, LC supplementation mitigated the reproductive toxicity through its antioxidant property.

Keywords: Deltamethrin, L Carnitine, Prepubertal Rats, Reproductive Toxicity

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1. Introduction

Pyrethroids are well known for their cost effectiveness to the Dichloro-Diphenyl-Trichloroethane (DDT) in controlling the malaria[1]. They are neurotoxic compounds acts by interfere with neuronal ionic conductance in the targeted organisms[2]. Pyrethroids are two types based on their structural and useful properties. Type-I pyrethroids do not have the α-cyano moiety and produce the effects like tremors and sparring while Type-II pyrethroids contain the α-cyano group on their phenoxy benzyl moiety and produce the symptoms like salivation, choreoathetosis, and very few pyrethroids produces the mixed effects i.e., tremors and salivation[3]. These pyrethroids are derived from pyrethrins, a natural source obtained from the *Chrysanthemum cineraria folium* flowers that have been applied as insecticidal agents for more than one hundred years. Pyrethroids have high potency and greater environmental stability[4,5], they have the half-life range from 1-16 weeks[6]. Due to excessive usage of the pyrethroids in agriculture, they might enter into the surface water via agriculture runoff thereby escaping into the environment. Research studies reported the presence of pyrethroids in the food and water.

Exposure to pyrethroids results in abnormal facial sensation, tingling, paresthesia, and numbness. Further, pyrethroids are eye and skin irritants. Other toxic effects of pyrethroids include vomiting, salivation, fatigue, headache, and diarrhea. Pyrethroids can induce pulmonary edema and can trigger fatal lung swelling[7]. Deltamethrin is one of the pyrethroids widely employed in protecting the agricultural crops against the insects. During pyrethroids metabolism, Reactive Oxygen Species (ROS) are generated and they trigger several deleterious effects on male reproductive health[8].

Humans and wild life have been exposed to this pesticide through polluted food and water, and since DTM can readily absorbed through orally, it can cause deleterious effects on the health. Several studies have reported the toxic effects of DTM on male reproductive health. Most commonly during DTM metabolism, Reactive Oxygen Species (ROS) are produced and they play a key role in DTM induced male reproductive toxicity. Thus it may be hypotheses that testicular toxicity caused by DTM might be attributed to their mechanism of free radical production.

In the present study, 1/10th of LD50 of deltamethrin was calculated as 6 mg/kg and earlier studies have shown significant testicular toxicity at this dose according to the study done by Sharma *et al.,* in 2014. Pyrethroids are exclusively more toxic to sensitive populations like children due to their inability to inactivate the insecticides. Recent studies detected the pyrithroidal residues/metabolites in children's urine[9]. It indicates the possible health risk caused by the pyrethroids.

L-Carnitine is an endogenous antioxidant with a tri-methylated amino acid structure. It is endogenously synthesized from the methylation of dietary amino acids mainly from the lysine where it acts as a vehicle for transporting the acyl group into the mitochondria. Later, this acyl group converts as an acyl CoA and it acts (Acyl CoA) as a substrate for the process of oxidation to produce the energy required for the motility and respiration of sperm[10]. Generally, high levels of LC are found in the epididymis where its function is to assist the motility of sperm[11]. Carnitine has several beneficial effects like protecting cardiac cells against the ischemic injury that triggers the cardiac cell death. Moreover, carnitine is possessing an anti-apoptotic effect and it has been reported in several studies such as neuronal related studies[12,13] lymphocyte and hepatic and myocytes[14]. The anti-apoptotic ability of carnitine in different tissues indicates that carnitine could be helpful to inhibit the apoptosis of testicular germ cells apoptosis process. Several studies conducted on carnitine suggested that they are significantly elevated sperm quantity and quality, and they improved the sperm number in asthenozoospermia patients[15]. Latest studies suggested that this process of carnitine transport across the testicular epididymis is regulated by unique carnitine transporters that are present across the testicular seminiferous luminal epithelium as well as Sertoli cells[16]. Testicular carnitine is passively entered into the spermatozoa and found 2000-fold greater in epididymis than in systemic circulation[17]. Since the immature sperm uses more fatty acid for the oxidation process for energy metabolism, the main role of the carnitine is to transfer the long-chain fatty acid into the mitochondrial membrane matrix there by it facilitates the oxidation of fatty acid within the mitochondria and thus involves in the production of energy in the sperm. Further, supplementation of carnitine enhances the quality and quantity of sperm in the mice testis[18], which are physically insulted with X-irradiation and heat. It has also shown promising results in men suffering from idiopathic oligoasthenospermia. The beneficial effects produced by the LC might be due to the elevation of

mitochondrial fat oxidation that would result in increased sperm count and motility[11].

In view of the above studies, the current study was aimed to investigate whether DTM exposure in prepubertal male rats impairs the testicular function in their adult stage and if so, supplementation of L-Carnitine shields, the testis and its physiological function in adult rats exposed to DTM in their early stage.

2. Materials and Methods

2.1 Chemicals

L-Carnitine was procured from Himedia, and Deltamethrin powder with purity >98% was purchased from TCI Chemicals, INDIA. All chemicals used in the present experiment were analytical grade, purchased from the HI media laboratories (Mumbai, India).

2.2 Animals

Prepubertal male albino wistar rats (weight 30 g) were used in this study, they were purchased from the NIN, Hyderabad, Telangana, INDIA. Rats were maintained under a well-controlled laboratory at 22-25 °C temperature and relative humidity adjusted to 50 ± 5 °C throughout the experimental study. Rats were fed with laboratory pellets (Obtained from ICMR-NIN Hyderabad) and given portable water. All experimental studies conducted were in accordance with the current guidelines of the CPCSEA 2003 and permitted by the Institutional Animal Ethical Committe1837/PO/RcBiBt/S/15/CPCSEA.

2.3 Experimental Design

After completion of the acclimation period, all the male rats (40) were divided into 4 groups, each group consisting of ten animals (one control group and remaining three treated groups). Group I received peanut oil, Group II received L-Carnitine (100 mg/kg), Group III L-Carnitine (100 mg/kg), and DTM (6 mg/kg), Group IV DTM (6 mg/kg). All the rats used in the present study were fasted 12 hours before necropsy, weighed accurately to the nearest values, and humanly sacrificed. Animal individual organs such as seminal vesicles, testes, epididymis, ventral prostate, and vas deference were harvested, and adhesive tissues were removed. Reproductive organs were weighed using the electrical balance. Later, the Tissue Somatic Index (TSI) was calculated using the below formula:

TSI = (weight of the individual tissue/total weight of the rat) × 100

2.4 Semen Analysis

Cauda epididymis small section was isolated and sliced into a clean Petri plate, containing 2 ml of saline (NaCl 0.9%) at room temperature to make experimental sperm suspension. Sperm parameters like sperm progressive motility and count were done as suggested by Belsey *et al*[19]. Sperm viability was performed using the 1% trypan blue solution[20]. Sperm membrane integrity was assessed by treating the sperm with a hypo-osmotic solution and a number of coiled tails were counted under the microscopic observation[21]. Other sperm viz. sperm motility, sperm count and sperm viability were also evaluated.

The sperm count was stated as mn/ml. Daily production of sperm was estimated according to the method Blazak *et al*[22]. The Daily Sperm Production (DSP) was denoted as mn/g testis. Sperm morphological changes were observed as per the protocol given by Linder *et al*[23]. At least 100 sperm were evaluated for sperm morphological changes like defects in the sperm, tail defect, middle piece defect, and for any sperm head detachment. Sperm morphological abnormalities in the sperms were calculated using the light microscope (Model no. HV-12TR).

2.5 Sperm DNA Damage Studies

Comet assay was performed according to Daveedu *et al*[8]. To analyse the DTM induced DNA damage in the exposed rat sperm, experiments were performed in triplicate from every single slide. 100 cells were analyzed for evaluating the comet formation three slides were made for each animal comet study and analyzed for the comets. To reduce the experimental errors all the sperm samples were avoided from the light exposure. Comets were observed under the fluorescence microscope. Comet images were captured and evaluated using CASP lab software. In total 300 cells were examined from each animal. Finally, observed values were articulated as the percent number of comets per one hundred cells[24].

2.6 Estimation of Testicular Enzymes Steroidogenic Markers

A small piece of testis homogenate was finely made by 5% w/v ice cold 20mM Tris–HCl at pH 8.2 was added

to estimate the activity of the enzyme was measured (3β HSD) and 17β HSD. The content in the reaction mixture, 2mL for 3β HSD contains 0.1 M pyrophosphate buffer (pH 7.4), 0.08 μmol of dehydro-epiandrosterone, 100 μmol of NAD, and 20 mg enzyme source, whereas, for 17β HSD, the contents in the reaction mixture having the same concentration of buffer and enzyme microsomal source as similar to the 3β HSD excluding the changes in the substrate (0.08 μmol androstenedione) and cofactors (100 μmol NADPH). The sample reaction mixture deprived of substrates was considered a blank reagent. The sample absorbance was estimated spectrophotometrically at 340 nm. 3β- and 17β-HSDs were expressed as nmol of NAD reduced to NADH/mg protein/min and nmol of NADPH changed to NADP/mg protein/min.

2.7 Estimation of Oxidative Stress

A small segment of the testis was finely grinded in the ice-cold phosphate buffer (pH 7.0) using a sterilized mortar and pestle. A small portion of the homogenate was centrifuged at 10,000 rpm/30 min at a cold temperature. The obtained fraction was used for the estimation of enzyme quantification and also for assessing lipid peroxidation.

2.8 Enzymatic Antioxidants Quantification

Homogenate was used to evaluate the antioxidant enzymes. Superoxide dismutase levels were measured spectrophotometrically at 480 nm on the grounds of its capability to inhibit the epinephrine autoxidation at the alkaline medium. Catalase activity was estimated spectrophotometrically at 240 nm, where in its capability to decay to hydrogen peroxide was studied[25].

2.9 Non-Enzymatic Antioxidant Levels Estimation

Glutathione (GSH) assay in the testis was done according to the technique suggested by Beutler *et al*[26]. Elman's reagent was used (5,5′-dithio-bis- 2-nitrobenzoic acid: DTNB). Tissues were excised and extracted tissues were made immediately by adding trichloroacetic acid 12% (1:4 v/v), followed by centrifugation. Obtained aliquot was added to the 0.25 mM DTNB in 0.1MNaH2PO4 (pH 8.0), formed thiolate anion estimated spectrophotometrically at 412 nm.

2.10 Lipid Peroxidation (Lpx) Levels Estimation

The LPx (Lipid Peroxidation) levels of both testes were quantified in terms of the formation of Malondialdehyde (MDA) a degraded product of lipid peroxidation. It was estimated by using the reagent of Thio barbituric acid. The LPx intensities were measured as suggested by Ohkawa *et al*[27]. The reaction mixture containing 0.5 ml of homogenate was made in PBS solution (pH 7.2), 1.0 ml of TBA, and 1.0 ml TCA., the contents were finely mixed followed by boiling for 20 min, cooled and tubes were centrifuged at 1000 rpm/10 min. The prepared sample was subjected to spectrophotometric analysis at 535 nm. The amount of LPx produced is shown as μmoles of MDA/g wt of tissue. The concentration of protein in the testis was quantified using the BSA (Bovine Serum Albumin) a standard.

2.11 Quantification of Testosterone Levels in Serum

Testosterone levels were quantified in serum from the experimental groups, and they were analyzed using the ELISA kit procured from local ELISA kit suppliers. Testosterone levels estimated from all forty rats (40 testis) were taken to perform their quantification and it was carried out according to the protocol given by the suppliers. Test range sensitivities were between 0 and 16 ng/Ml. Testosterone levels were denoted as ng/ml.

2.12 Histological Studies of Testes

Animals' testis (Right) was collected from all experimental and control rats and their adhering tissues were carefully removed and they were kept immovable in a Boulin's solution for 1-day, fixed tissue sections were dehydrated by serial alcoholic treatment in ascending concentration manner. They were subjected to xylol washing followed by paraffin wax embedding. Sections having a thickness (3 µm) were gently colored with hematoxylin and eosin and examined using the microscope[28].

3. Results

None of the animals was excluded from the experiment, moreover, no mortality was observed in any of the control and experimental groups in the present study.

Table 1. Effect of L-Carnitine (LC) on body weights (g) and tissue somatic indices (W/W%) in deltamethrin treated groups

Parameter	Control	LA Supplement	DTM	LA + DTM
Body Weight	188.21[a] ±7.52	186.11[a] ± 5.26	155.49[b] ± 6.79	165.44[c] ±7.20
Testes	1.18[a] ± 0.01	1.19[a] ± 0.02	0.58[b] ± 0.02	0.920[c] ±0.11
Epididymis	1.005[a] ± 0.05	1.007[a] ± 0.11	0.703[b] ± 0.02	0.1.00[a] ± 0.01
Vas deference	0.162[a] ± 0.02	0.171[a] ± 0.01	0.78[b] ± 0.02	0.97[c] ± 0.02
Prostate gland	0.150[a] ± 0.01	0.152[a] ± 0.03	0.73[b] ± 0.02	0.11[c] ± 0.01
Seminal vesicle	0.444[a] ± 0.06	0.448[a] ± 0.07	0.310[b] ±0.02	0.392[c] ± 0.05
Brain	0.642[a] ± 0.02	0.639[a] ± 0.04	0.631[b] ± 0.03	0.626[c] ±0.03
Liver	3.205[a] ± 0.15	3.11[a] ±0.23	2.99[b] ±0.25	3.100[a] ±0.49
Kidney	0.624[a] ± 0.01	0.631[a] ± 0.08	0.615[a] ± 0.05	0.623[a] ±0.02

Data are expressed as mean ± S.D. of 10 individuals.
[a-c] Different superscripts in the same row indicate a significant difference ($p < 0.05$)



Figure 1. Macroscopic pictures of the testes in **(A)** Control **(B)** DTM-exposed group **(C)** and LC treated DTM-exposed **(D)** LC-treated group.

3.1 Body and Organ Weight Gain Profile

In animals treated with the DTM, group body weights and testicular weight declined significantly. ($P<0.05$) as shown in Table 1.

However, LC supplementation increased the weights of testis (Figure 1) in the deltamethrin and LC administered group compared with the deltamethrin only treated group.

3.2 Sperm Quality and Quantity

As shown in Table 2, Prepubertal rats exposed to the DTM showed a significant decline in the sperm parameters such as count, motility, and viability than the control and LC-treated groups ($P<0.05$). Further the mean serum values of testosterone were reduced in rats treated with DTM when compared with its corresponding control group a ($p<0$ .05). Meanwhile, hormonal levels in rats treated with DTM with LC supplementation group's hormonal levels were significantly improved than the rats administered with DTM only ($p<0$ .05)

Further, significant increase in the sperm head abnormalities such as pointed head, rod shaped head and headless sperm were noticed in deltamethrin exposed rats as compared to controls. Whereas, LC supplementation improved deltamethrin induced deteriorated sperm

Table 2. Effect of L-Carnitine (LC) on sperm parameters and testicular steroidogenesis in controls and deltamethrin exposed rats

Parameter	Control	LA	LA + DTM	DTM
Daily sperm count (millions/g testis)	$13.11^{a} \pm 1.14$	$13.87^{b} \pm 1.23$	$11.99^{b} \pm 0.25$	$8.10^{c} \pm 1.92$
Sperm count (millions/ml)	$71.12^{a} \pm 6.45$	$70.18^{b} \pm 3.44$	$59.39^{c} \pm 5.15$	$45.19^{d} \pm 4.23$
Sperm viability (%)	$70.12^{a} \pm 3.10$	$69.5^{b} \pm 3.12$	$60.48^{c} \pm 5.12$	$41.44^{d} \pm 2.11$
Sperm motility (%)	$66.88^{a} \pm 4.96$	$66.70^{b} \pm 6.12$	$52.13^{c} \pm 5.96$	$43.12^{d} \pm 6.99$
HOS Tail coiled sperm (%)	$62.56^{a} \pm 5.89$	$62.98^{b} \pm 7.15$	$59.15^{c} \pm 5.96$	$37.15^{d} \pm 3.22$
Testosterone (ng/ml)	$7.99^{a} \pm 0.201$	$7.12^{b} \pm 0.21$	$6.99^{c} \pm 0.53$	$4.55^{d} \pm 0.15$
3βHSD (nmol of NAD converted to NADH/mg protein/min)	$14.11^{a} \pm 2.55$	$15.00^{b} \pm 2.70$	$13.49^{c} \pm 1.10$	$6.15^{d} \pm 1.55$
17βHSD (nmol of NADPH converted to NADP/mg protein/min)	$11.12^{a} \pm 1.10$	$11.84^{b} \pm 1.20$	$10.01^{c} \pm 0.25$	$4.95^{d} \pm 0.69$

Data are expressed as mean ± S.D. of 10 individuals.
[a-d] Different superscripts in the same row indicate a significant difference ($p<0.05$)
Units for 3β-hydroxysteroid dehydrogenase (HSD) = μ moles of NAD converted to NADH/mg protein/min;
Units for 17β-hydroxysteroid dehydrogenase (HSD) = μ moles of NADPH converted to NADP/mg protein/min.

quality and quantity in rats. No changes were observed in selected sperm variables in LC alone injected male rats as compared to the controls Figure 2.

3.3 Sperm DNA Damage

Sperm DNA damage results were shown in (Figure 3), these results indicated that DNA damage in the sperm tail was increased in the DTM exposed groups than in the control and only LC administered groups.

The results of comet assay indicated that deltamethrin exposure at a dose of 6 mg/kg negatively affected the integrity of sperm DNA in rats. Conversely, LC supplementation reduced the sperm DNA injury in deltamethrin exposed rats.

3.4 Antioxidant Enzymes, GSH and Lipid Peroxidation

Antioxidant enzymes such as catalase and super oxide dismutase levels were significantly decreased in the DTM-exposed groups in comparison with the untreated and LC+DTM group ($P<0.05$), and MDA levels were significantly increased in the DTM-exposed group.

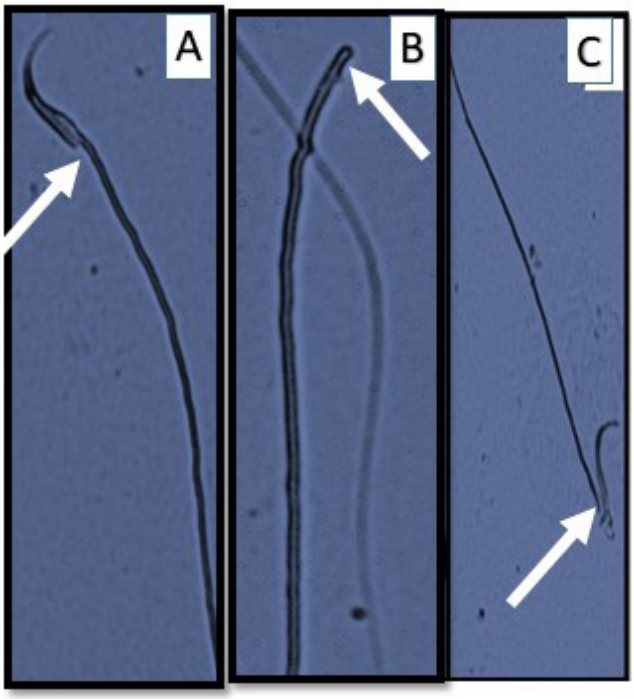


Figure 2. Sperm morphological abnormalities were observed in s of rats exposed to DTM during the early life exposure period Sperm head showing hook **(A),** Rod shaped **(B)** and pin shaped **(C)**.

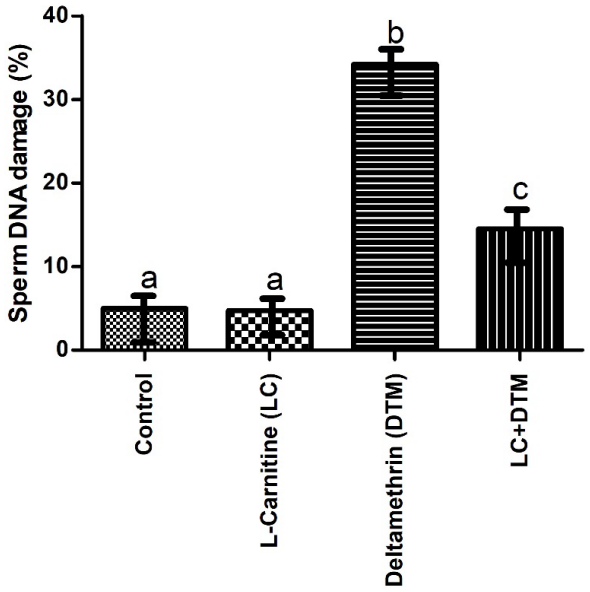


Figure 3. Effect of LC on deltamethrin induced sperm DNA damage.

On the other hand, LC supplementation to the DTM decreased the MDA formation ($P<0.05$) (Table 3).

3.5 Histopathological Studies

The testicular structures and seminiferous tubules in control and LC-administered groups displayed a regular morphology with an oval shape appearance. They were separated by interstitial connective tissue in addition to encircled by a connective tissue capsule (*Tunica albuginea*). Seminiferous tubules in the rats treated with DTM displayed severe deformities and a considerable proportion of degenerating germ cells, Sertoli, and Leydig cells were found. However, compared to the DTM group, the LC-supplemented group showed less damage and milder germ cell loss. Leydig cells in the DTM group that was 90 days old had severe damage, and the germ cells were depleted. LC + DTM showed improvement in the testicular architecture.

Table 3. Effect of L-Carnitine on testicular oxidative stress parameters in deltamethrin exposed adult rats

Parameter	Control	LA	LA + DTM	DTM
Super oxide dismutase (SOD)	$18.69^{a} \pm 1.81$	$18.20^{a} \pm 1.52$	$12.88^{b} \pm 0.99$	$9.89^{c} \pm 1.20$
Catalase	$12.18^{a} \pm 1.66$	$12.88^{a} \pm 0.96$	$8.95^{c} \pm 0.86$	$8.29^{d} \pm 0.29$
Glutathione (GSH)	$13.93^{a} \pm 05.15$	$13.29^{a} \pm 0.31$	$8.69^{c} \pm 0.39$	$6.80^{d} \pm 1.25$
Lipid peroxidase (LPx)	$8.97^{a} \pm 0.35$	$8.25^{a} \pm 0.15$	$13.21^{c} \pm 1.52$	$15.55^{d} \pm 1.22$

Data are expressed as mean ± S.D. of 10 individuals.
[a-d] Different superscripts in the same row indicate a significant difference ($p< 0.05$)

4. Discussion

Deltamethrin (DTM), is most used to control pests, which invade agricultural crops. However, several studies reported that uncontrolled usage of DTM will enter the environment followed by groundwater. Upon consumption of water polluted with DTM, it might result in the serious health issues including reproductive problems. Further, it could also trigger reproductive damage to the testicles and thus decrease the male infertility[29,30]. L-Carnitine is an excellent antioxidant and several studies proved their beneficial effects including improved male reproductive functions.

This investigation sought to define the mechanism by which LC shields young rats from DTM toxicity during spermatogenesis. According to earlier research, DTM impairs male reproductive performance by reducing testis mass, and declining sperm parameters like count, motility viability and viability[29,30]. In the current study, prepubertal rats exposed to DTM had significantly lower testicular and body weights. Rats treated with DTM still had the least body weight when equated to the other treatment groups, despite the typical weight rise associated with puberty. Semen was taken for this study and promptly analyzed in warmed physiological saline[31]. However, supplementation of LC reduced sperm abnormality and

improved sperm count, motility, and viability. Rats fed with LC after receiving DTM demonstrated improved spermatogenic characteristics as well as higher weights in the body and testes. A handful of studies suggested that DTM has the ability to trigger testicular toxicity by disturbing the function of the different enzymes for sperm maturation and it could also dysregulate subsequent patterns. It's plausible that the development of oxidative stress has an impact on these cascade processes[32]. The equilibrium between the generation of ROS and the protective antioxidant system deteriorates under oxidative stress. Due to the rich concentration of lipids over the sperm membrane, any excessive formation of free radicals triggers lipid peroxidation, the testis is the main organ that is affected by oxidative stress[33]. Since MDA is the final byproduct of lipid peroxidation, it is regarded as a good ROS indicator, rats given DTM treatment had significantly higher MDA concentrations than rats in the control group. The spermatogenetic process may fail if testosterone levels are reduced[33]. In line with the earlier study[30], rats treated with DTM displayed a drop in testosterone levels. Two possibilities can be used to explain this drop in hormone levels: either DTM caused severe damage to Leydig and Sertoli cells as a result of enhanced ROS creation[34], because LC has anti-oxidative qualities that guard against the damage of Leydig and Sertoli cells brought on by oxidative stress, it can boost testosterone hormonal levels[35].

The seminiferous tubules of rats exposed to DTM during their prepubertal stage had a substantial impairment and changed spermatids. These results were comparable to those reported by Desai *et al.,* who showed that testicular damage caused by DTM reduced male reproductive performance.[30]

The seminiferous epithelium was significantly protected by the supplementation of LC to the DTM-exposed group. The improvement in sperm quality was evident in the present study, in addition, the decreased germ cell due to DTM exposure was reduced upon LC supplementation and the defense of spermatogonia cells that resume the spermatogenesis these positive effects were consistent with earlier data[36,37].

5. Conclusion

The current investigation highlights that deltamethrin potentially disrupts the male reproductive system in adult rats when they were exposed during their early life developmental stage. Administration of L-Carnitine has ameliorated toxic effects caused by deltamethrin by inhibiting oxidative stress, encouraging the development of spermatids, and reducing the hormonal disturbance brought on by deltamethrin. Further L-Carnitine improves sperm parameters through its antioxidant nature. In conclusion, DTM exposure is one of the serious public health concerns, exclusively in the context of its raising metabolites presence in the urine sample of sensitive populations like children, we recommend the public to minimize the rampant usage of DTM.

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