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Early exposure to deltamethrin deteriorates testicular and post-testicular sperm maturation events in rats

Daveedu Thathapudi¹, Naresh Dumala^{2✉}, Shailendra Singh², Anil Kumar Adimulapu³, Sainath Sri Bhashyam^{1✉}¹Department of Biotechnology, Vikrama Simhapuri University, Nellore–524320, Andhra Pradesh, India²KL College of Pharmacy, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Guntur–522302, Andhra Pradesh, India³School of Pharmacy, The Assam Kaziranga University, Koraihowa, Jorhat–785006, Assam, India

ABSTRACT

Objective: To explore the effects of deltamethrin exposure during prepubertal stage on male reproductive parameters in rats.**Methods:** Forty male Wistar rats, aged 23 postnatal days, were randomly divided into four groups of ten rats each. The control group received tap water, while the vehicle control group was administered with 1 mL of peanut oil. Experimental groups received deltamethrin 3 mg/kg and 6 mg/kg, respectively. All treatments were administered orally. At postnatal day 91, all the rats were sacrificed to assess the histology of testes and serum testosterone levels. We also evaluated the binding of deltamethrin to 3 β -hydroxysteroid dehydrogenase (HSD), a key enzyme involved in steroidogenesis, using *in silico* docking and molecular dynamics simulations to assess the stability and binding energy of the 3 β -HSD and deltamethrin complex over 100 ns time frame.**Results:** Deltamethrin-exposed rats showed significant weight loss, followed by a marked reduction in serum testosterone levels. They also demonstrated the suppression of testicular steroidogenic enzymes like 3 β -HSD and 17 β -HSD levels, which were accompanied by sperm morphological abnormalities when compared with the control group rats. Moreover, molecular docking studies indicated that deltamethrin interacts with 3 β -HSD competitively with pregnenolone which may be the reason for impaired steroidogenesis.**Conclusions:** Early life exposure to deltamethrin may induce reproductive toxicity at adult stage. Further exploration studies are warranted to record conclusive evidence for the effects of deltamethrin early exposure.**KEYWORDS:** Deltamethrin; 3 β -HSD; Reproductive toxicity; Pesticide pollution; Prepubertal exposure; Toxicity; Climate action

1. Introduction

Pesticides, including insecticides, herbicides, fungicides, and

rodenticides, are extensively used worldwide in agriculture and vector control. Their widespread application poses risks, particularly to children, who encounter pesticides in various environments, including homes, schools, and neighborhoods[1]. India is the fourth largest producer of the pesticides and the most widely used pesticides in India are insecticides, which account for more than 65% of all pesticide usage[2]. Pesticides are widely employed for both agricultural and non-agricultural uses, which may cause

Key Points

Question: What is the effect of prepubertal exposure to deltamethrin on rats testicular and post-testicular sperm maturation events at adulthood?**Findings:** Deltamethrin exposure caused decrease in testicular steroidogenic enzymes like 3 β -hydroxysteroid dehydrogenase (HSD) and 17 β -HSD levels, testosterone levels and caused significant sperm morphological abnormalities when compared with the control group rats.**Meaning:** Exposure to deltamethrin unintentionally or occupationally during early life stages may impair sperm maturation events during adulthood which in turn manifest reproductive toxicity and failure.

✉To whom correspondence may be addressed. E-mail: nareshpharma2020@gmail.com; drsainath@simhapuriuniv.ac.in

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negative impact on human health and the environment. A substantial portion of insecticides and herbicides applied to control target populations unintentionally disperse into the environment, affecting the air, water, and soil. Insecticides that are both synthetic and natural are causing serious threats to human health[3]. Pyrethroids are a class of synthetic insecticides derived from natural pyrethrins, known for their high toxicity to insects while exhibiting low toxicity to mammals and birds. This selective toxicity is primarily due to their mechanism of action, which targets the sodium channels in insect neurons, leading to neurotoxicity in insects but minimal effects in mammals and birds, whose sodium channels respond differently to these compounds[4]. Further, pyrethroids can also target the voltage-sensitive calcium and sodium ion channels, thus causing the excess release of neurotransmitters that finally leads to toxicity[5]. Exposure to pyrethroids during pregnancy and childhood has been linked to adverse effects on neuronal development, raising significant public health concerns. Research indicates that prenatal exposure can disrupt neurodevelopmental processes, potentially leading to behavioral issues and cognitive deficits in children[6].

Deltamethrin (DTM) is a widely utilized synthetic pyrethroid insecticide all over the world and its chemical structure is shown in Figure 1. It is one of the strongest insecticides now available and is frequently used to manage a variety of ectoparasites, including lice, flies, and ticks, while also protecting fish, fruits, and vegetables from pests. Its application spans multiple domains, from aquaculture to agriculture and livestock management, showcasing its versatility and efficacy[7,8]. The ingestion of DTM-contaminated food and drink poses significant health risks to humans. This pyrethroid insecticide has been linked to both acute and chronic toxicity, affecting various bodily systems[9]. Initially, DTM was thought to be the least hazardous to mammals, but investigations have shown that it has a variety of toxic effects on both mammals and non-mammalian organs. DTM exposure has been shown to induce oxidative stress, leading to DNA damage and cell death across various organisms. Research indicates that exposure to deltamethrin can significantly disrupt cellular functions, primarily through the generation of reactive oxygen species (ROS) and subsequent mitochondrial stress[10,11]. Further, DTM has been associated with adverse health effects, including potential carcinogenicity, particularly concerning liver and thyroid cancers[12–14]. DTM can disrupt testicular steroidogenesis by inhibiting key enzymes involved in testosterone production[15,16]. However, many of these studies were conducted at the adult stage. Studies on DTM-mediated reproductive toxicities linked with its exposure during pre-, and peripubertal stages which are often considered as key stages in postnatal development were obscuring. Additionally, during this time morphological, endocrine, and immunological developments take place. Most importantly, testicular activities, such as steroidogenesis and spermatogenesis, have not yet fully evolved in the peripubertal stage and the animals

are particularly vulnerable to endocrine disruption during the peripubertal stage, a critical period marked by rapid growth and significant changes in the reproductive system. This sensitivity makes them more susceptible to the adverse effects of hazardous substances, such as pesticides and industrial chemicals, which can interfere with normal endocrine function and lead to reproductive health issues[17,18].

While several researchers have investigated DTM-mediated reproductive toxicity in adult male rats, studies examining its effects on rats exposed during the juvenile stage are limited. Therefore, this study was undertaken to address this gap. The objective of this study was to evaluate the potential reproductive toxicity of DTM on enzymes involved in steroidogenesis using both *in vivo* and *in silico* approaches.

2. Methods

2.1. Chemicals

Pure DTM powder (>98%) was purchased from TCI Chemicals Pvt. Ltd. India. All other chemicals were procured from Himedia laboratory (Mumbai, India) with the analytical-grade substances for the current investigation.

2.2. Animals

Prepubertal male albino Wistar rats [(25±5) g body weight] of age 23 postnatal days (PND) were procured from National Animal Resource Facility for Biomedical Research (NIRFBR)-Hyderabad, India and were fed with standard rat diet. During housing and experimentation, rats were provided with unlimited access to water.

2.3. Experimental design

Prepubertal male rats used in the current study were acclimatized for seven days prior to the commencement of the experiment. All rats ($n=40$) were divided equally into four (control, vehicle control, low dose and high dose) groups, each group containing 10 rats. Animals in the control group were given tap water and the vehicle control group animals were administered with peanut oil (1 mL). Low dose group rats received DTM 3 mg/kg body weight (in 1 mL of peanut oil) and rats in high dose group received DTM 6 mg/kg body weight (in 1 mL of peanut oil). All rats received their respective treatments orally and the doses were ingested by using a suitable intubation cannula. DTM treatment was initiated from PND 30 and continued till PND 90. After the last dose, rats were fasted overnight, weighed, and humanely sacrificed with a high dose of anesthesia.

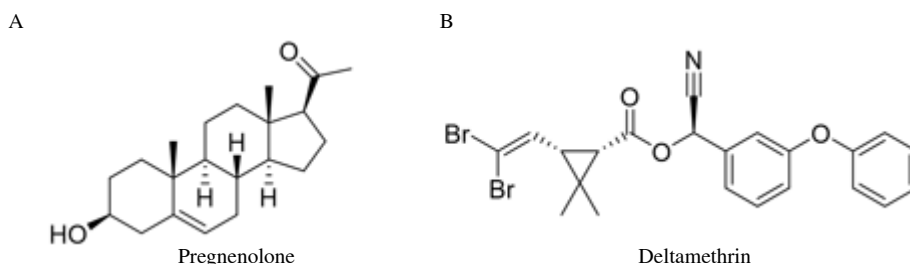


Figure 1. Chemical structures of pregnenolone (a bioligand for the 3β -HSD enzyme) (A) and deltamethrin (a pyrethroid insecticide) (B) which are used for molecular docking and molecular dynamics simulation studies. 3β -HSD: 3β -hydroxysteroid dehydrogenase.

2.4. Blood sampling and testes harvesting

Before performing necropsy, blood was collected 24 hours after administration of the last dose by cardiac puncture. For hormonal investigation, serum was carefully isolated and stored at -20°C . Testes adherent tissues were removed, and the organs were weighed to the nearest values.

2.5. Histology

Each testis from rats in the control and experimental groups was fixed for 24 hours in Bouin's solution, dehydrated with a series of alcoholic solutions with increasing concentrations, washed in xylol, and then embedded in paraffin wax. For histological analysis, hematoxylin and eosin Y stains were used to impart colour to the sectioned testicular tissue specimens. The Hovers microscope (Model No. HV-12TR) was used to observe histological alterations and photomicrographs were acquired with a higher magnification using a $40\times$ objective lens.

2.6. Testicular steroidogenic enzyme activity level estimation

A small piece of the testis was finely homogenized in ice cold Tris-HCL containing 0.1% of acetyl trimethyl ammonium bromide (pH: 6.8). Microsomal fraction was taken from the homogenate post centrifugation and used as an enzyme source. Testicular enzymes such as 3β -hydroxysteroid dehydrogenase (3β -HSD) and 17β -hydroxysteroid dehydrogenase (17β -HSD) were estimated as per the procedure mentioned in Bergmeyer[19]. Initial conditions for enzymatic quantification were set to zero and next to the first order kinetics with respect to the linearity with the differences in the enzymatic concentrations and incubation. The amount of the protein concentration/enzymatic source was employed to estimate the testicular enzymes level as described by Lowry *et al*[20] and bovine serum albumin protein was used as a standard protein. Spectrophotometric analysis was used to quantify the sample absorbance at 340 nm and 3β HSD, 17β HSD enzyme activity levels were estimated by following the procedure of Prathima *et al*[21].

2.7. Serum testosterone levels

Blood samples were collected from all rats by cardiac puncture using a heparinized syringe. Serum was separated by centrifuging the blood at a relative centrifugal force (RCF) of approximately $755\times g$ for 10 min and was subsequently stored at -20°C . Serum testosterone levels were quantified from the treatment and control groups by using testosterone enzyme-linked immunosorbent assay (ELISA) kit with sensitivity ranges from 0.2-16 ng/mL (DiaMetra, Italy). The assay was performed as per the instructions of ELISA kit and the concentration of testosterone was expressed in ng/mL.

2.8. Semen morphology analysis

The epididymis and vas deferens of the rats were carefully dissected from the testes, and sperm were collected by cutting, squeezing, or flushing the epididymis. Samples were fixed with Hancock's solution after semen collection, and smears were prepared. Giemsa's dye was applied to stain the slides, which were then examined under a $400\times$ magnification. Each rat's 500 spermatozoa were analyzed and classified as normal or abnormal based on the exact sperm morphological standards. The head and tail deformities were separated from the morphological abnormalities. As part of the evaluation of the sperm tail, mid-piece sperm abnormalities were taken into consideration. The proportions of sperms with normal and unusual shapes were also estimated.

2.9. Molecular docking

The 3β -HSD sequence was retrieved from the UniProt database with ID: P22072, and the retrieved sequence was modelled using software modeler by the chosen human 17β -HSD as template (PDB: 1JTV). Modelled 3β -HSD was docked with the pregnenolone and DTM as ligands (for which chemical structures are shown in Figure 1) using AutoDock Vina as a molecular docking tool. Binding interactions between ligands (pregnenolone and DTM) and 3β -HSD enzyme were visualized using BIOVIA Discovery Studio v21.1.0.20298.

2.10. Molecular dynamics simulations

Molecular dynamics simulations were performed for the 3 β -HSD and DTM, pregnenolone complexes from the docking studies using GROMACS version 2021.3[22]. The receptor topology was created using “pdb2gmx” script; CHARMM27 forcefield was assigned together with TIP3 TIP water model. Ligand topology was obtained using swissparam webserver[23]. The processed protein and ligand were joined together to obtain the protein-ligand complex. The complex was then solvated in a cubic periodic box (90 Å, 90 Å, 90 Å) using the water model extended simple point charge (SPC). The system was made neutral by adding sodium ions. The complex's energy was minimized for 50000 steps using the steepest descent algorithm, with a parameter to stop the minimization when the maximal force was less than 10.0 kJ/mol. With that done, the equilibration of the system is next where the ligand in the system was restrained. The equilibration was performed utilizing two successive steps. A thermostat environment was created with a constant temperature of 300 K and a constant pressure of 1 atm throughout the course of the molecular dynamics simulations. NVT equilibration was performed first then followed by NPT which were carried out for 50000 steps each. Finally, 100 ns simulation was carried out on the equilibrated system. The root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), and number of hydrogen bonds (Hbond) of the protein-ligand complexes were calculated using the GROMACS commands, gmx rms, gmx rmsf, gmx gyrate and gmx hbond, respectively.

2.11. Molecular mechanics Poisson–Boltzmann surface area (MM–PBSA) analysis

The MM-PBSA study was utilized using gmx_mmpbsa version v1.6.3 based on MMPBSA.py v.16.0 to estimate the solvent accessible surface area (SASA), Van der Waal energy, electrostatic energy, polar solvation and the binding free energy (ΔG binding) for the pregnenolone, DTM and 3 β -HSD enzyme bound systems.

2.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 10.4.1. All data were first tested and confirmed to conform to a normal distribution. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons *post-hoc* test. Results are expressed as mean $\pm$ standard deviation (mean $\pm$ SD), and differences were considered statistically significant at $P < 0.05$.

2.13. Ethics statement

The current research work was performed in accordance with the present guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, INDIA (CPCSEA 2003) and experimental procedures of this study were approved by the Institutional Animal Ethical Committee (Ref. No. 1837/PO/RcBiBt/S/15/CPCSEA) on 10 March 2017. The national norms and standards on animal welfare and institutional guidelines were strictly followed in performing the experiments on rats.

3. Results

3.1. Animal observation

The clinical signs and symptoms of all the animals included in the current investigation, including urine, salivation, sluggish behavior, and vocalization, were closely monitored. Neither the control nor the treated group rats showed any outward symptoms of clinical toxicity. However, the DTM-treated groups lost weight and the same was significantly decreased when compared with their respective control group animal weight. The testis, epididymis, and other ancillary organs such the cauda, carpus, and caput weights in the DTM-exposed groups were reduced than the control groups, similarly weight of the prostate gland, seminal vesicles, and vas deferens was also found to be decreased.

3.2. Histology

Studies on the testicles' histopathology were carried out on both untreated and treated rats and histological alterations in the testes are depicted in Figure 2. Intact basement membrane containing various stages of germ cells and sperms surrounding the intestinal lumen were visible in the testicular transverse section of the control group and the vehicle control group (Figure 2A, 2B). In contrast, the altered testicular architecture was observed in DTM-exposed animals (Figure 2C), and damaged epithelium, a lumen devoid of sperm were also noted (Figure 2D). Conversely, the testis sections from the rats that were exposed to the vehicle exhibited no structural irregularities.

3.3. Testicular steroidogenic enzyme and serum testosterone levels

Testicular steroidogenic enzyme levels after PND 90 were analyzed in both control and treated group animals and presented in Figure 3. When compared to the control group, both low and high

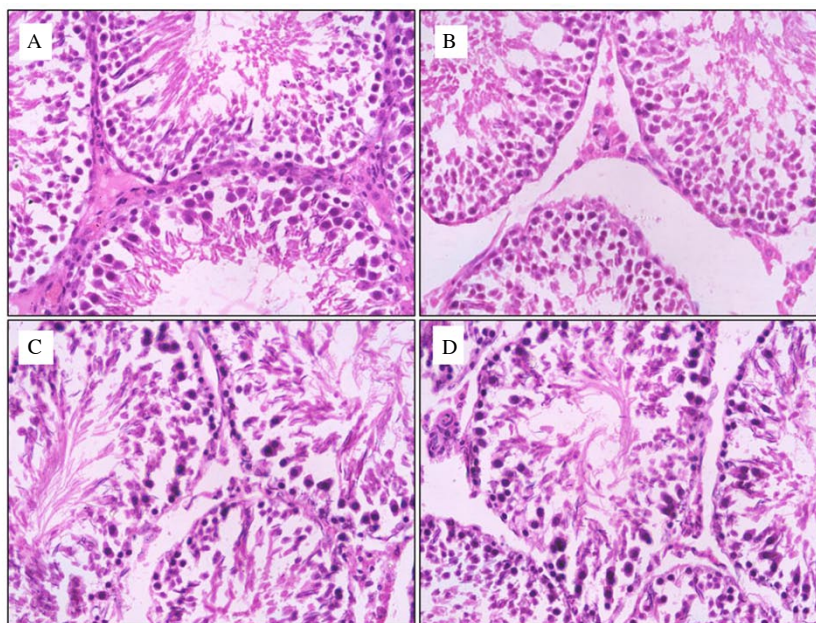


Figure 2. Transverse sections of the testis from the control (A), vehicle control (B) and experimental (C and D) group rats. The control group receives tap water, while the vehicle control group is administered with 1 mL of peanut oil. Experimental groups receive deltamethrin 3 mg/kg and 6 mg/kg, respectively. A and B photomicrographs show the compact arrangements in the seminiferous tubules with normal spermatogenesis. C and D are the photomicrographs showing disorganized and ruptured epithelium with lumen empty of sperm.

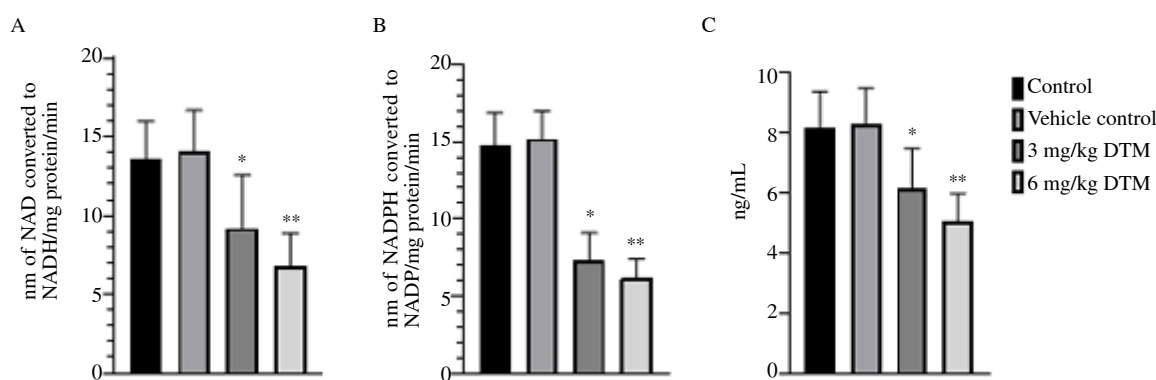


Figure 3. Effect of deltamethrin (DTM) pre-pubertal exposure on testicular steroidogenic enzyme A) 3β-HSD, B) 17β-HSD, and C) testosterone levels of rats. * $P < 0.01$ and ** $P < 0.001$ indicate significant differences compared to the control group. 3β-HSD: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17β-hydroxysteroid dehydrogenase.

dose of DTM exposure groups showed significantly lower levels of 3β-HSD and 17β-HSD ($P < 0.05$) (Figure 3A, 3B). Further, DTM-exposed groups also demonstrated a significant dose dependent reduction in the serum testosterone levels ($P < 0.05$) at low and high concentrations (Figure 3C).

3.4. Sperm morphology analysis

All the animals in the control group had sperm structures that were obviously in their usual morphological shape, while rats subjected to DTM treatment showed significant morphological changes. Sperm morphological abnormalities observed in rats exposed to DTM are depicted in Table 1. Normal morphology of sperm was significantly decreased in DTM intoxicated groups with 3 mg/kg exposure,

meanwhile these values were further reduced ($P < 0.001$) in DTM at their high dose (6 mg/kg). Sperm abnormalities in the head and tail were also studied, where the abnormalities in the sperm head were significantly increased ($P < 0.001$) in the DTM treated rats, similarly morphological abnormalities of sperm tail were also analyzed, the abnormal tails were significantly increased ($P < 0.001$) in DTM exposed groups than that of the control groups.

3.5. Molecular docking

The molecular docking interactions between 3β-HSD and pyrethroid, DTM (PubChem CID, 40585) and natural ligand, pregnenolone (PubChem CID, 31402) were analyzed in the present study. Where the modelled 3β-HSD protein interacted with

Table 1. Morphological abnormalities observed in sperm cells post pre-pubertal exposure to deltamethrin (DTM) in rats.

Parameters	Control	Vehicle control	Deltamethrin	
			3 mg/kg b.w.	6 mg/kg b.w.
Normal morphology, %	85.05±6.72	83.01±8.21 ^{ns}	62.02±7.87 [*]	44.19±5.36 [*]
Abnormal head, %	1.94±0.59	2.07±0.34 ^{ns}	2.99±0.29 [*]	3.64±0.31 [*]
Abnormal tail, %	0.96±0.19	1.04±0.23 ^{ns}	3.99±0.36 [*]	4.70±0.14 [*]

Data are expressed as mean±SD. The control group receives potable water; the vehicle control group receives peanut oil (1 mL); b.w.: body weight. Significant difference when compared to the control group at ^{*} $P < 0.001$, and ns = not significant ($P > 0.05$).

Table 2. Binding energy and its components determined by MM-PBSA analysis for pregnenolone, deltamethrin (DTM)-bound systems with 3β-HSD enzyme.

Ligand bound to 3β-HSD	ΔVDWAALS	ΔEEL	ΔEGB	ΔESURF	ΔGGAS	ΔGSOLV	ΔTOTAL
Pregnenolone	-34.71±2.83	-20.74±5.96	27.72±4.17	-4.69±0.36	-55.45±6.38	23.03±4.09	-32.42±4.14
Deltamethrin	-47.40±3.51	-8.88±3.70	29.35±3.07	-5.95±0.42	-56.28±4.99	23.40±2.88	-32.89±3.21

Note: Energies are in kcal/mol (mean±SD). MM-PBSA: molecular mechanics Poisson-Boltzmann surface area; ΔVDWAALS: van der Waals interaction energy; ΔEEL: electrostatic interaction energy; ΔEGB: polar solvation energy (Generalized Born); ΔESURF: non-polar solvation free energy; ΔGGAS: gas-phase energy (ΔVDWAALS + ΔEEL); ΔGSOLV: solvation energy (ΔEGB + ΔESURF); ΔTOTAL: total binding energy (ΔGGAS + ΔGSOLV). 3β-HSD: 3β-hydroxysteroid dehydrogenase.

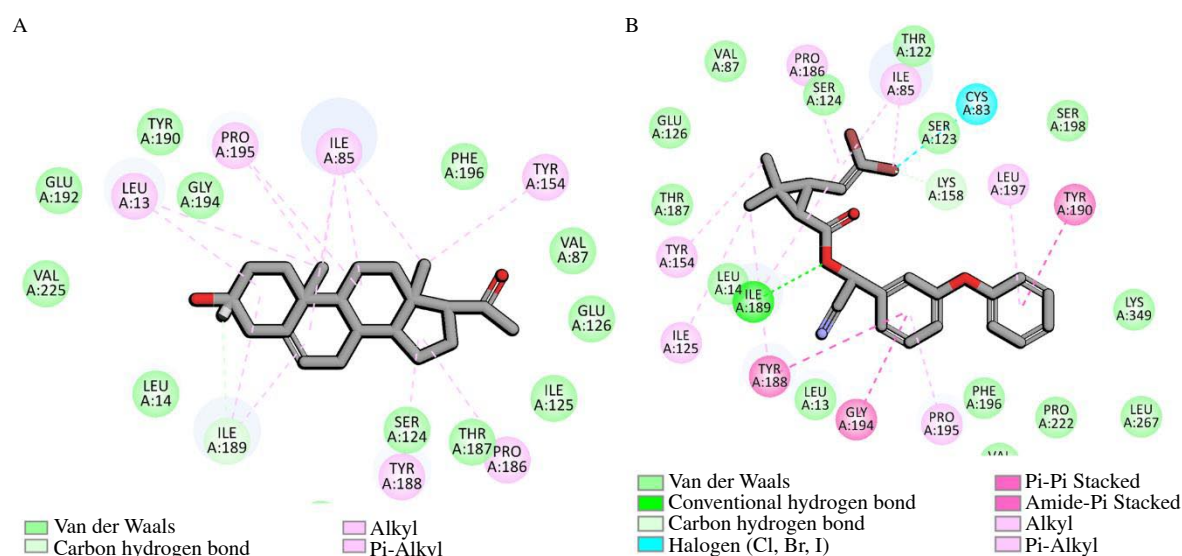


Figure 4. 3β-hydroxysteroid dehydrogenase (3β-HSD) residues that are participating or interacting with its bioligand, pregnenolone are LEU 13, ILE 85, TYR 154, PRO 186, TYR 188, ILE 189, PRO 195 (A); and insecticide, DTM are CYS 83, ILE 85, ILE 125, TYR 154, LYS 158, PRO 186, TYR 188, ILE 189, TYR 190, GLY 194, PRO 195, LEU 197 (B). The binding interactions are visualized using BIOVIA Discovery Studio.

pregnenolone and the DTM with a binding affinity of -7.2 kcal/mol and -8.3 kcal/mol, respectively.

Furthermore, the CDocker results in Biovia Discover Studio showed the molecular interactions of pregnenolone and DTM with the ligand binding site predicted for the 3β-HSD and are presented in Figure 4. Pregnenolone occupied the binding pocket and interacted with amino acid residues by forming hydrogen bond (H-bond) interaction, pie-pie interaction, and pie-alkyl interaction. Pro A186, Tyr A188, IleA85 were the interacting amino acids through pie alkyl, while Leu A13, Tyr A154 and Pro A195 were interacting with alkyl interaction and Ile189 form H-bond interaction along with pie alkyl interaction. In case of pesticide DTM, the Cyano group orients the molecule in L-shape and projects phenoxy phenyl ring in hydrophobic region, while bromo vinyl di methyl cyclopropyl group has adopted the same symmetry as pregnenolone. The amino

acid residues Ile A85, Pro A195 were interacting by pie alkyl, Pro A186, Tyr A188 and Gly 194 show pie pie stacking, while Tyr A154, Ile A125 form alkyl interactions, the oxygen near to cyano group form H-bond interactions with Ile A189. All these interactions were similarly observed in pregnenolone suggesting that DTM can be a potential competitor to pregnenolone for binding at the site of 3β-HSD enzyme. Apart from additional amino acid interaction for DTM like CysA83 (halogen interaction), Leu A197 (pie alkyl) and Tyr A190 (pie-pie stacking) were also observed.

3.6. Molecular dynamics simulations

The molecular dynamics simulation studies conducted for pregnenolone and DTM assessed the protein-ligand stability for 100 ns time by calculating RMSD. After initiation of simulation,

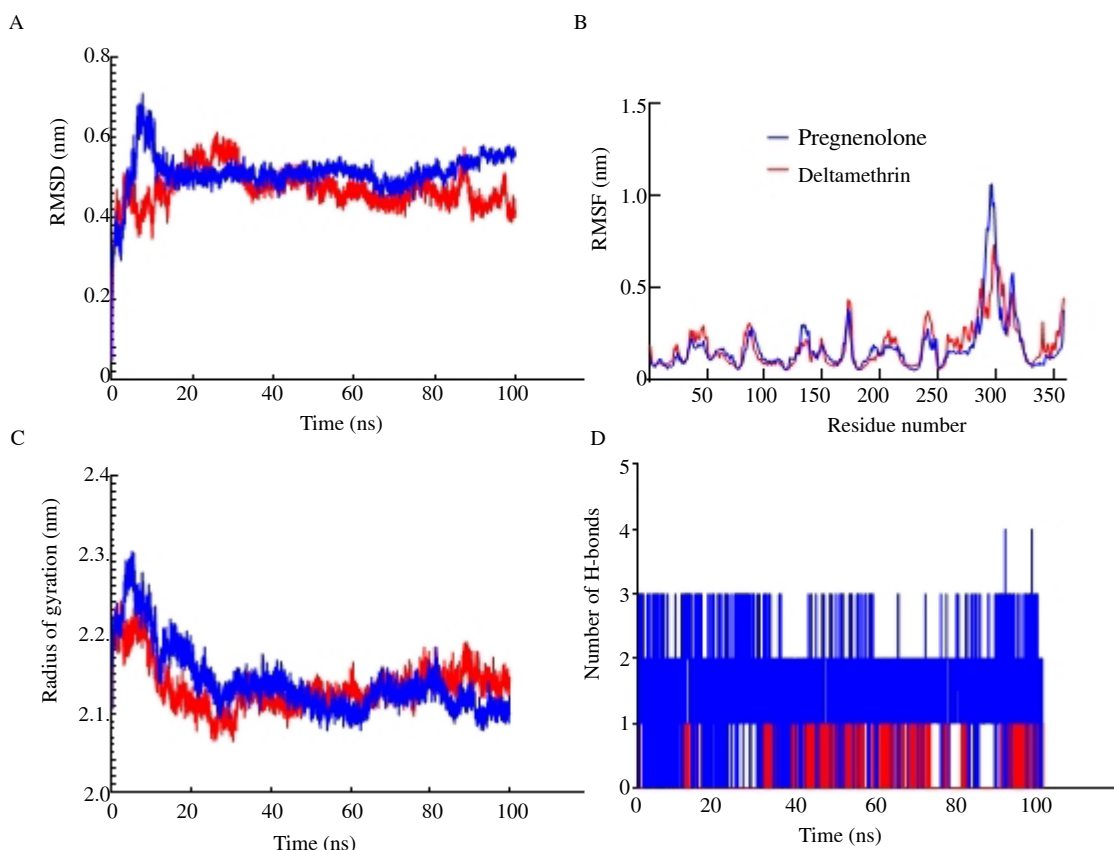


Figure 5. Graphical representations of root mean square deviation (RMSD) (A), root mean square fluctuation (RMSF) (B), radius of gyration (Rg) (C), and number of hydrogen bonds (H-bonds) (D) obtained from 100 ns molecular dynamics simulations of 3β -hydroxysteroid dehydrogenase (3β -HSD) complexes with pregnenolone and deltamethrin (DTM).

the pregnenolone and DTM were stabilized after 20–30 ns indicating the stable complex (Figure 5A). The RMSF values, which measure the per-residue fluctuation of the protein upon ligand binding, indicate rather small values for the binding site residues (270–300) for pregnenolone and amino acid residue 260–330 for DTM in the protein ligand complex (Figure 5B). The radius of gyration observed for pregnenolone and DTM (2.0 nm – 2.3 nm) in Figure 5C confirms the occupancy and stability of the protein-ligand complex. The number of H-bonds between the pregnenolone, DTM and 3β -HSD enzyme was monitored as a function of time (100 ns), as shown in Figure 5D. Initially, the number of H-bonds fluctuated, reflecting the system's equilibration phase. After ~20 ns, the number stabilized around an average of 3 ± 1 , indicating a stable interaction between the pregnenolone, DTM and the 3β -HSD enzyme binding pocket. These observations are consistent with the low RMSD values observed suggesting a well-defined binding mode.

3.7. MM-PBSA analysis

The binding free energy values determined by MM-PBSA analysis for pregnenolone and DTM are shown in Table 2. The binding free energy for the complex 3β -HSD and pregnenolone was (-32.42 ± 4.14) kcal/mol, while that of 3β -HSD and DTM

complex was (-32.89 ± 3.21) kcal/mol (Δ TOTAL). However, the Δ G solvation for 3β -HSD and pregnenolone complex was (23.03 ± 4.09) kcal/mol and 3β -HSD and DTM complex was (23.40 ± 2.88) kcal/mol. Similarly, Δ GGAS, Δ ESURF, and Δ EGB for 3β -HSD and pregnenolone complex was (-55.45 ± 6.38) , (-4.69 ± 0.36) and (27.72 ± 4.17) kcal/mol, while that of 3β -HSD and DTM complex was (-56.28 ± 4.99) , (-5.95 ± 0.42) and (29.35 ± 3.07) kcal/mol, respectively. The binding free energy results from the MM-PBSA studies are comparatively higher for 3β -HSD and DTM complex than 3β -HSD and pregnenolone complex suggesting the superiority of DTM towards the binding site.

4. Discussion

DTM is extensively used in agriculture, particularly in developing regions for pest control in crops and livestock. Its application in African livestock systems is increasing, driven by the need for enhanced productivity and food security[24]. DTM is highly toxic to non-target organisms, including aquatic life and beneficial insects like bees[25]. Studies indicate that even low concentrations (0.01 ppm) of DTM can significantly reduce dung beetle populations and disrupt ecosystem functions, such as dung

decomposition[24]. The compound degrades moderately in soil and sediments, but its residues can persist and affect local biodiversity[25]. Acute exposure to DTM can lead to severe health effects in humans and animals, including neurotoxicity and reproductive issues[9]. Research shows that DTM can disrupt microbial activity in soil, further impacting ecological balance[26]. However, sufficient evidence linking early life exposure and associated adverse reproductive health outcomes at the adult stage led us to initiate this study, and we made an attempt to fill this knowledge gap in the present work.

DTM is a lipophilic pyrethroid compound and hence can be absorbed upon oral administration. Moreover, absorption of DTM *via* the gastrointestinal tract is more when compared with any alternative route. Consequently, the oral route was chosen for the DTM exposure in the present study. DTM was dissolved in peanut oil at a dose level of 3 mg/kg body weight and 6 mg/kg body weight which are 0.1 and 0.2 levels of LD₅₀ of DTM in peanut oil (*i.e.* 30 mg/kg)[27]. Male Wistar rats received DTM at 3 mg/kg and 6 mg/kg doses since PND 30 till PND 90 for observing the effects on steroidogenic biomarkers and testicular sperm maturation.

Gross body and individual organ weights of the animals displayed a significant reduction when compared with the control group rats in a similar manner to that of our previous study[28]. Possible reasons for this weight loss caused by the DTM might be attributed to the anorectic effect of DTM, thus animals exposed to the DTM might have experienced loss of appetite, which has a direct impact on their body weight. Alternatively, it might also be due to the indirect effects of DTM on the central nervous system, which normally regulates how much of the food and water an animal consumes[29,30]. Furthermore, we also identified the considerable loss in testicular weight in the treatment group rats (data not shown), which may be related to a decrease in testosterone hormone production levels. Desai *et al* showed a similar pattern of decreased testicular weights post DTM exposure through oral route in mice for 45 days and attributed the decreased spermatogenesis, decreased sperm count, or decreased testicular enzyme activity as the reasons[27].

Tissue sections of testis from rats treated with DTM displayed damaged epithelium and lumens devoid of sperm, in histopathological analysis. Sertoli cells showed further tubular shrinkage, cell necrosis, vacuolization with sloughing, and degraded spermatids and spermatocytes indicating moderate to severe tissue damage. A recent study revealed that DTM intoxication led to hyperchromatic germ cells and a reduction in mature sperm count, indicating severe necrosis and damage to the spermatogonial layers[31]. Efiowan Orlu also reported the degenerative changes in the testes, particularly affecting primary spermatocytes, leading to impaired spermatogenesis post 35 days of DTM oral exposure in Sprague Dawley rats[32]. The present study findings are consistent with the above reports and support the fact that DTM ability in

inducing histological alterations observed. Germ cell atrophy, necrosis and damage to histological architecture may be the causes of testicular weight loss recorded in the present study[33].

The 3 β -HSD and 17 β -HSD are the two key steroidogenic enzymes that participate in the steroidogenesis process, and were decreased significantly in DTM-treated rats when compared with both the control group rats. These two enzymes are crucial for the normal synthesis of testosterone and acts by converting the various precursor molecules required for steroidogenesis. Interestingly, these two steroidogenic enzyme levels were significantly diminished in the DTM exposed rats, which in turn had the direct effect on the testosterone production[34]. Current investigation found that DTM had a detrimental impact on both enzyme levels at the exposure concentrations that finally manifested a decline in serum testosterone levels inducing the ability of DTM to impair the steroidogenic pathway[35]. Our results are in agreement with the earlier studies conducted by Issam *et al* who demonstrated the impairment in the steroidogenesis as a result of pesticidal exposure, which eventually resulted in the impaired physiological mechanisms in the male reproductive system[29].

The testosterone has a major role in the male reproductive system development and is essential for spermatogenesis, particularly within the testis, where it is required for the completion of meiosis and the maintenance of spermatid attachment and release[36]. Decline in testosterone levels impacts spermatogenesis, as it is vital for the function of Sertoli cells, which support sperm development[37]. DTM early exposure in male rats may have deprived the androgens, and their testosterone-dependent activities ultimately impair the sperm. Observed morphological alterations in the sperm can be attributed to DTM early exposure and consequent effects on the steroidogenic enzyme levels and testosterone levels. Pesticide exposure has been associated with morphological anomalies in sperm, such as pinhead and short tails, which are indicative of compromised sperm quality[38]. A recent study by Uwamahoro *et al* showed the pesticide ability to mimic hormones and disrupt the endocrine system, leading to impaired sperm capacitation and morphological changes necessary for fertilization[39]. Furthermore, low doses of pesticides like dimethoate, glyphosate, and zineb have shown significant alterations in sperm morphology and plasma membrane integrity, along with hormonal imbalances[40]. Embryonic exposure to dimethoate and DTM in mice resulted in decreased testicular weight and impaired sperm parameters, including reduced density, mobility, and increased abnormal forms[41]. These reports corroborate the morphological abnormalities recorded in the present study.

In silico computational tools utilized to understand the affinity and interaction behaviour of DTM with 3 β -HSD enzyme produced interesting results. Further, molecular docking results for DTM are compared against the pregnenolone which is a bioligand for the 3 β -HSD enzyme[35] in the present study. Surprisingly, molecular

docking results revealed that pesticide DTM has made interactions with the 3β -HSD enzyme similar to the pregnenolone with high binding affinity. These results indicate the ability of DTM to compete with pregnenolone and thereby interfere with the natural conversion of pregnenolone to steroid hormone precursors which is essential for testosterone biosynthesis[42].

Molecular dynamics simulation data revealed that the complexes of DTM and 3β -HSD enzyme as well as pregnenolone and 3β -HSD enzyme are relatively stable during the 100 ns time. Moreover, MM-PBSA analysis results portrayed stronger van der Waals interactions (Δ VDWAALS) for DTM than those of pregnenolone with 3β -HSD enzyme indicating DTM has a better hydrophobic fit in the binding pocket than pregnenolone, possibly due to better shape complementarity and more favorable hydrophobic contacts. In contrast, coulombic (charge-based) interactions (Δ EEL) results showed that pregnenolone has a larger contribution to the binding from electrostatics than DTM indicating pregnenolone's binding to 3β -HSD enzyme may rely more on polar or charged interactions, possibly forming stronger hydrogen bonds or salt bridges with 3β -HSD enzyme. Overall, total free binding energy (Δ TOTAL) which quantifies the total binding free energy is almost identical for both ligands, with DTM having a slightly more favourable binding free energy. Furthermore, negative values of Δ TOTAL indicate binding of DTM with 3β -HSD enzyme is thermodynamically favourable. It is noteworthy that DTM binds slightly better than pregnenolone despite having less favourable electrostatic interactions. This is likely due to the stronger van der Waals contacts and better burial of hydrophobic surface area. These results are highly supporting the *in vivo* results of the present study. Thus, DTM exposure during early life stages can impair steroidogenic pathways and induce abnormalities in sperm health that in turn manifest adverse reproductive health outcomes in adult stage.

There are some limitations in the present study. This study lacks the experimental validation for the 3β -HSD enzyme levels estimation and also inhibitory action by DTM. Further, proteomics analysis studies comparing the levels of proteins involved in the steroidogenesis and sperm maturation might provide a comprehensive understanding on the adverse outcomes related to early exposure of DTM in animals during their adult stages. Hence, more studies are being warranted to explore the early life exposure to DTM and related pyrethroid pesticides along with the rigorous biomonitoring studies to aid the safety of the public and avoid possible adverse reproductive outcomes.

In conclusion, the current investigation showed that animals exposed to toxicants like DTM at their early stage of life *i.e.*, prepubertal stage can cause deleterious effects on reproductive

system and deteriorate testicular and post-testicular sperm maturation events. Exposure to insecticide, DTM, through oral route manifested hormonal disturbance and testicular and sperm morphological abnormalities. Present *in silico* and *in vivo* study results clearly reveal the mechanism behind the reduced testosterone production or levels in the DTM-exposed adult rats which might be due to its ability in disturbing the functions of 3β -HSD enzyme. Further mechanistic studies are warranted to elucidate the alterations in cellular microenvironment in the presence of DTM, and consequent adverse health outcomes linked to steroidogenesis.

Conflict of interest statement

The authors declared no conflicts of interest.

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Authors' contributions

Daveedu Thathapudi and Sainath Sri Bhashyam have designed and executed the *in vivo* study; Naresh Dumala, Shailendra Singh and Anil Kumar Adimulapu have designed and performed computational studies; all the authors are involved in the analysis and interpretation of the results and in the preparation of the manuscript.

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