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Melatonin Ameliorates 2,4-Dichlorophenoxyacetic Acid Induced Testicular Steroidogenesis Upset in Mice: An *In Vivo* and *In Silico* Study

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Abstract

2,4-Dichlorophenoxyacetic acid (2,4-D) is used as a selective herbicide and associated with a variety of toxicities in mammals. In contrast, melatonin is an antioxidant that promotes the elimination of free radicals. In the present study, the protective effects of melatonin (10 mg/kg body weight) against 2,4-D (low, mid, and high dose-16.5, 33.0, and 66.0 mg/kg body weight) induced testicular steroidogenesis alteration were examined using *in vivo* and *in silico* models. Doses of 2,4-D and melatonin were administered orally for 28 days. The evaluated parameters were body weight, total protein, markers for male reproductive function, and steroidogenesis i.e. testis weight, total lipid, cholesterol, testosterone, 3 beta-hydroxysteroid dehydrogenase, 17 beta-hydroxysteroid dehydrogenase, total sperm count, sperm motility, and sperm viability along with the histopathology of the testis. The statistical significant value was considered at $p<0.05$. Molecular docking study was performed for interaction of 2,4-D and melatonin with steroid binding proteins. *In vivo* results revealed that 2,4-D treatment showed a significant dose-dependent alteration in above all studied parameters. No significant auto-recovery was observed in the withdrawal study, on the contrarily, the altered parameters were normalized and comparable to control when melatonin was given alone and in combination with 2,4-D. *In silico* results also demonstrated that the binding affinity of melatonin with steroid binding proteins is higher than 2,4-D. Collectively, these *in vivo* and *in silico* findings indicated that 2,4-D induced testicular toxicity accompanied by steroidogenesis upset and can be reduced by melatonin significantly by interacting directly and strongly with studied molecular markers.

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1. Introduction

One of the most important tasks faced by modern agriculture is to feed a growing human population. Since the agricultural land is limited, it becomes necessary to increase the efficiency of its production. To achieve this purpose, fertilizers and plant protection products known collectively as pesticides are used. Their types are herbicides, fungicides, insecticides, and many others[1]. 2,4-Dichlorophenoxyacetic acid (2,4-D) is in the phenoxyacetic acid class of compounds and has been used as an herbicide/pesticide world widely since the 1940s. There are over 1500 herbicide/pesticide products containing 2,4-D as the main ingredient. Due to the inadvertent use of potentially dangerous pesticides, humans and other organisms are highly vulnerable to their exposure via soil, food, water, and animal-derived oil or fat products incorporated into processed feeds[2-4]. The incidence of male fertility decline has increased alarmingly since the 1980s as a result of multi-factorial events including environmental pollution. The number of experimental studies on the effect of agrochemicals on the reproductive system has grown in recent years and some possible mechanisms of toxicity have been suggested. The most widely accepted effect is pesticide-induced hormonal disruption[5] concomitantly with alterations in the antioxidant defense system causing huge disturbances in steroidogenesis, spermatogenesis, and exhibition of sexual behaviour[6–8]. It was previously reported that 2,4-D exposure increased the percentage of dead and abnormal sperm along with impaired sperm motility, decreased serum testosterone levels, and testicular weight in studied organisms[9–15].

In contradiction to 2,4-D perilous activity, there are many therapeutic and herbal remedies available like melatonin, vitamin C, curcumin, etc., pertaining capacity of reducing toxicity, through their antioxidant properties. Amongst them, melatonin, N-acetyl-5-methoxytryptamine, is a pleiotropic hormone mainly released from the pineal gland[16]. Numerous reports documented its antioxidant, anticancer, and free radical scavenging properties[17]. Melatonin can regulate several physiological functions i) from well-known sleep/wake cycles and modulation of the circadian rhythms to neural development and protection and ii) regulation of the immune system and endocrine functions[16,17]. Melatonin acts as a local modulator of the endocrine activity in Leydig cells whereas in Sertoli cells melatonin influences cellular proliferation and energy metabolism, and consequently, these suggest its role as a key player in the regulation of steroidogenesis[18]. However, its effect on testicular steroidogenesis against 2,4-D has remained unexplained. Therefore, the present study was aimed to investigate the potential of melatonin in therapeutic use against 2,4-D administered testicular toxicity accompanied by steroidogenesis upset using *in vivo* and *in silico* models. Furthermore, the molecular docking simulation technique was also evaluated for the exploration of the possible binding modes of receptors with selected ligands which reveals binding energetics and molecular interactions.

2. Materials and Methods

2.1 Chemicals

The herbicide, 2,4-D (CAS No. 94-75-7; Purity: ≥ 98.0%) was obtained from Loba Chemie, Mumbai, India. Melatonin (CAS No. 73-31-4; Purity: ≥ 99.0%) was obtained from HiMedia Laboratories, Mumbai, India. All the other chemicals (AR grade) utilized in different assays were procured from Sigma Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany), and HiMedia Laboratories (Mumbai, India).

2.2 Experimental Animals

Healthy, adult male albino mice of the Swiss strain (*Mus musculus*) obtained from Zydus Cadila Pharmaceutical, Ahmedabad, Gujarat, India were used for all experiments. Mice were kept in the Animal House of Zoology Department of Gujarat University, Ahmedabad, India under controlled conditions (temperature 22 ± 3°C, 12 hours light/dark cycle, and relative humidity 50–55%). They were fed with certified pelleted rodent feed supplied by Amrut Feeds, Pranav Agro Industries Ltd., Pune, India and water *ad libitum*. All the experimental protocols were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg-GU/IAEC/02/2017/CPCSEA), New Delhi, India. Mice were handled according to the guidelines published by the Indian National Science Academy, New Delhi, India[19].

Table 1. Experimental groups

Groups	Treatment and Dose	Duration (days)	Day of Autopsy
I	Control (CN)	28 days	29th day
II	Melatonin (MT) alone (10 mg/kg body weight)	28 days	29th day
III	2,4-D Low Dose (LD) (16.5 mg/kg body weight)	28 days	29th day
IV	2,4-D Mid Dose (MD) (33 mg/kg body weight)	28 days	29th day
V	2,4-D High Dose (HD) (66 mg/kg body weight)	28 days	29th day
VI	2,4-D High Dose (HD) (66 mg/kg body weight) + Withdrawal (WD)	28 days + 28 days	57th day
VII	2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight)	28 days	29th day

2.3 Dose Selection

Different doses of 2,4-D were selected based on its LD_{50} value (330 mg/kg body weight)[20]. Mice of the 2,4-D treated groups received three different doses of 2,4-D which are 1/20, 1/10, and 1/5 of LD_{50} value (low, mid, and high dose-16.5, 33.0, and 66.0 mg/kg body weight) respectively. For the amelioration of 2,4-D induced toxicity dosage of melatonin (10 mg/kg body weight) was selected based on the study of Upadhyaya and co-workers[21,22].

2.4 Treatment

As shown in Table 1, forty-two mice were randomly divided into seven groups with six mice in each group. Mice were weighed before commencement and at the end of the experiment. Group I served as control and received an equivalent amount of sterile distilled water used as a vehicle; Group II (antidote control) was given administration of melatonin alone (10 mg/kg body weight), mice of groups III, IV, and V received three different doses of 2,4-D (low, mid, and high dose-16.5, 33.0, and 66.0 mg/kg body weight) respectively, however, mice of Group VI received a high dose of 2,4-D (66 mg/kg body weight) followed by 28-day withdrawal (auto-recovery) period. Group VII mice were administered a 2,4-D high dose (66 mg/kg body weight) along with melatonin (10 mg/kg body weight). The mode of administration in all the groups was oral by force-feeding. The volume of administration (0.2 ml/mice/day) in all the groups was adjusted throughout the experiment. Group I-V and Group VII mice received 28-day treatment and were sacrificed on the 29th day whereas in Group VI mice received treatment for 28 days and then remained untreated for the next 28 days and sacrificed on the 57th day. For sacrification of the mice, the cervical dislocation method was opted. In each group of mice, the testis and cauda epididymis were dissected out carefully and blotted free of blood. The testis was utilized for estimation of testis weight, biochemical parameters viz. total protein, total lipid, cholesterol, 3 beta-hydroxysteroid dehydrogenase, 17 beta-hydroxysteroid dehydrogenase, and histopathology whereas the cauda epididymis was utilized for assessment of total sperm count, sperm motility, and sperm viability. Blood samples were collected by cardiac puncture in non-anticoagulant added tubes, allowed to clot for 20–30 min, and centrifuged at 1000 rpm for 10 min at 4 °C. The non-haemolysed serum samples obtained were stored at −20 °C and used for the analysis of the serum testosterone level.

2.5 Tissue Biochemical Parameters

The levels of total protein[23], total lipid[24], and cholesterol[25] were determined by the standard protocols. Activities

of 3 beta-hydroxysteroid dehydrogenase[26] and 17 beta-hydroxysteroid dehydrogenase[26] were assayed by standard methods.

2.6 Serum Testosterone Level

The serum sample of mice was estimated for the testosterone level by the chemiluminescence immunoassay (CLIA) method using an Advia Centaur XP (Siemens) Immunoassay system.

2.7 Total Sperm Count, Sperm Motility, and Sperm Viability

Cauda epididymal sperm count and motility were determined according to the protocol given in the WHO laboratory manual[27]. Cauda epididymal sperm viability (live: dead ratio) was analyzed using 0.1% trypan blue stain according to the procedure described by Talbot and Chacon[28].

2.8 Histopathology

Histopathological studies were carried out using the standard technique of hematoxylin and eosin staining[29]. The fresh pieces of the testis were fixed for 18 hours in alcoholic Bouin's fixative. The tissue was dehydrated by passing through ascending grades of alcohol, cleared in xylene, and embedded in paraffin wax. About 5 µm thick sections were cut on a rotary microtome and stained with Ehrlich's hematoxylin and eosin (alcohol soluble), dehydrated in alcohol, cleared in xylene, mounted in DPX, and examined microscopically.

2.9 Percentage Amelioration

Percentage amelioration in all studied parameters was calculated by using the following formula:

$$\text{Percentage Amelioration (\%)} = \frac{\text{Prooxidant Group-Respective Antioxidant Group}}{\text{Prooxidant Group-Control}} \times 100$$

2.10 Statistical Analysis

All the data were expressed as mean±standard error (SE). Statistical analysis was performed using GraphPad Prism software version 7.04. The data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test except in the case of body weight where two-way analysis of variance (ANOVA) was performed. The level of significance was accepted at $p<0.05$.

2.11 Molecular Docking

To identify the plausible mechanism of 2,4-D and melatonin, blind molecular docking was performed using Autodock Vina[30] version 1.1.2. This analysis provided the interaction and structural insight to 2,4-D and melatonin compounds. The chemical structures of 2,4-D (CID: 1486) and melatonin (CID: 896) were downloaded from NCBI PubChem database[31] using compound identifiers respectively. The three-dimensional structures of testosterone (PDB code: 2AM9)[32], 3 beta/17 beta-hydroxysteroid dehydrogenase (PDB code: 1HXH)[33], and 7 beta-hydroxysteroid dehydrogenase (PDB code: 5FYD)[34] having resolutions 1.64 Å, 1.22 Å, and 1.60 Å were retrieved from the RCSB (Research Collaboratory for Structural Bioinformatics) protein data bank[35]. The binding affinity and the mode were analyzed using Discovery Studio Visualizer[36] version 16.1.0.15350.

The molecular docking experiment resulted in top 10 conformations. Amongst them, the best binding structures were taken into the consideration according to most negative Gibbs free energy (-ΔG) of the protein-ligand complex and type of interaction. Moreover, the interaction profiles have been evaluated to understand the mechanism of better binding affinity based on van der Waals, conventional hydrogen bond, alkyl, Pi-alkyl, unfavourable donor–donor, unfavourable acceptor–acceptor, Pi–Pi T-shaped, carbon-hydrogen bond, Pi–anion, and Pi– Sigma interactions to anticipate the interacting atoms with the respective selected receptors.

3. Results

3.1 Effects of 2,4-D and/or Melatonin on Clinical Indications of Mice

No treatment related clinical signs were observed in the control (Group I) and antidote control (Group II) mice. The administration of 2,4-D in mice caused a reduction in feed intake, dullness, lethargy, and body hair loss in a dose-dependent manner (Group III-VI) as compared to control (Group I). No auto-recovery was observed in the withdrawal study (Group VI) whereas the co-administration of melatonin with the 2,4-D high dose

(Group VII) showed normal clinical symptoms when compared with the 2,4-D high dose administered (Group V) mice. Treatment related mortality was absent in all the treated mice (Groups II-VII). No other abnormal clinical symptoms were noted in all the studied mice (Group I-VII).

3.2 Effects of 2,4-D and/or Melatonin on Body Weight of Mice

The present study revealed that no significant change in the body weight was observed after 28-day treatment of sterile distilled water in control mice (Group I). Similar findings were also observed for antidote control (Group II) after 28 days of treatment and when compared to the commencement of the experiment (0-Day) as well as the control (Group I). The 28 days administration of 2,4-D caused a significant dose-dependent reduction (low dose-$p<0.05$; mid dose-$p<0.001$; high dose-$p<0.0001$) in the body weight (Group III-V) as compared to the control (Group I). Similar 2,4-D dose-dependent decrement (low dose-$p<0.01$; mid dose-$p<0.001$; high dose-$p<0.0001$) was noted (Group III-VI) when compared to the commencement of the experiment of the same group. Mice of 28-day withdrawal study (Group VI) after 28-day treatment of 2,4-D high dose showed a highly significant difference ($p<0.0001$) in body weight as compared to the commencement of the experiment and no significant auto-recovery was observed, on the other hand, after the co-administration of melatonin with a 2,4-D high dose (Group VII), the body weight was normalized significantly ($p<0.0001$) with percentage amelioration 81.19% as compared to alone 2,4-D high dose treated (Group V) mice. Moreover, it (Group VII) showed no significant difference when compared to the commencement of the experiment (Figure 1).

3.3 Effects of 2,4-D and/or Melatonin on Testis Weight of Mice

No significant change was observed in testis weight of antidote control (Group II) and 2,4-D low dose administered mice (Group III) whereas significant 2,4-D dose-dependent decrement (Group IV and V) was observed (mid dose-$p<0.01$; high dose-$p<0.0001$) in mice as compared to control (Group I). No significant auto-recovery was noted in the withdrawal study (Group VI) on the contrary melatonin supplemented with a high dose of 2,4-D (Group VII) showed significant recovery ($p<0.0001$) with percentage amelioration 92.10% as compared to the alone 2,4-D high dose administered mice (Group V) (Figure 2).

3.4 Effects of 2,4-D and/or Melatonin on Total Protein, Total Lipid, and Cholesterol Levels in the Testis of Mice

The administration of 2,4-D (Group III-V) caused a significant dose-dependent decrease in the levels of total protein, total lipid, and cholesterol (low dose-$p<0.05$; mid dose-$p<0.001$; high dose-$p<0.0001$) whereas melatonin alone treatment (Group II) showed no significant change in mice in all three parameters as compared to control (Group I). No significant reversal effects were observed in the withdrawal study (Group VI) of the above three parameters, on the other hand, significant ameliorative effects ($p<0.0001$) in all three parameters with high percentage amelioration (total protein-80.58%; total lipid-85.87%; cholesterol-83.65%) were noted in mice when melatonin was co-administered with a high dose of 2,4-D (Group VII) as compared to alone 2,4-D high dose treated mice (Group V) (Figure 3).

3.5 Effects of 2,4-D and/or Melatonin on Serum Testosterone Level, 3 beta-Hydroxysteroid Dehydrogenase, and 17 beta-Hydroxysteroid Dehydrogenase Activities in the Testis of Mice

2,4-D treatment (Group III-V) led to the significant dose-dependent decrement in testosterone level (low dose-$p<0.05$; mid dose-$p<0.01$; high dose-$p<0.0001$) as well as in the activities of 3 beta-hydroxysteroid dehydrogenase (low dose-$p<0.01$; mid dose-$p<0.001$; high dose-$p<0.0001$) and 17 beta-hydroxysteroid dehydrogenase (low dose-$p<0.05$; mid dose-$p<0.001$; high dose-$p<0.0001$) whereas melatonin alone administered mice (Group II) showed no significant alteration in all three parameters as compared to control (Group I). Mice showed no significant auto-recovery in withdrawal investigation (Group VI) of the above three parameters whereas significant mitigative effects (testosterone-$p<0.001$; 3 beta-hydroxysteroid dehydrogenase-$p<0.0001$; 17

beta-hydroxysteroid dehydrogenase-p<0.0001) with percentage amelioration (testosterone-81.10%; 3 beta-hydroxysteroid dehydrogenase-78.50%; 17 beta-hydroxysteroid dehydrogenase-82.28%) were observed in mice when melatonin was treated with a high dose of 2,4-D (Group VII) as compared to alone 2,4-D high dose administered mice (Group V) (Figure 4).

3.6 Effects of 2,4-D and/or Melatonin on Total Sperm Count, Sperm Motility, and Sperm Viability in the Cauda Epididymis of Mice

Mice treated with 2,4-D (Group III-V) caused a significant dose-dependent reduction in the total sperm count (low dose-p<0.01; mid dose-p<0.001; high dose-p<0.0001), sperm motility (low dose-p<0.05; mid dose-p<0.01; high dose-p<0.0001), and sperm viability (low dose-p<0.05; mid dose-p<0.01; high dose-p<0.0001) whereas melatonin alone (Group II) administered mice showed no significant change in all three parameters as compared to control (Group I). Mice showed no significant reversal effects in withdrawal evaluation (Group VI) of the above three parameters whereas significant recovery (total sperm count-p<0.0001; sperm motility-p<0.001; sperm viability-p<0.001) with percentage amelioration (total sperm count-85.50%; sperm motility-81.57%; sperm viability-80.35%) was noted in mice when melatonin was co-administered with a high dose of 2,4-D (Group VII) as compared to alone 2,4-D high dose treated mice (Group V) (Figure 5).

3.7 Effects of 2,4-D and/or Melatonin on Testicular Histopathology of Mice

Histology of the testis (Figure 6: A-G) of control (Group I; Figure 6A) mice exhibited normal histoarchitecture evident by the normal organization of the germ cells and distinctly arranged Sertoli cell in seminiferous tubules. The Leydig cells are observed normally in the intertubular interstitium with a distinct nucleus. Melatonin alone (Group II; Figure 6B) and 2,4-D low dose (Group III; Figure 6C) administered mice showed normal histoarchitecture as observed in the control (Group I; Figure 6A). In contradiction, 2,4-D mid dose (Group IV; Figure 6D) and high dose (Group V; Figure 6E) treatment caused various dose-dependent alterations in mice testis. In mid dose treated mice, the seminiferous tubules showed multinucleated giant spermatids (marked with arrow: Group IV; Figure 6D) and increased space between seminiferous tubules whereas high dose treated mice showed atrophy and appearance of tubules characterized by the absence of almost all germ cells in affected tubules as well as tubules lined only by Sertoli cells (marked with arrow: Group V; Figure 6E). Along with that, extensive degeneration of the spermatocytes and germinal epithelium was observed. Mild degeneration of the spermatocytes and Leydig cells followed by decreased sperms in the lumen were also observed (Group V; Figure 6E). Almost similar findings were observed portrayed by few tubules showing multinucleated giant spermatids and atrophy of tubules (marked with arrow: Group VI; Figure 6F) suggesting no auto-recovery in withdrawal study (Group VI) whereas melatonin supplemented with a high dose of 2,4-D (Group VII; Figure 6G) showed recovery evident by normal histoarchitecture of seminiferous tubules and spermatogenic cells as compared to the alone 2,4-D high dose administered mice (Group V; Figure 6E).

3.8 Molecular Docking of 2,4-D and Melatonin with Steroid Binding Proteins [Testosterone (2AM9), 3 beta/17 beta-Hydroxysteroid Dehydrogenase (1HXH), and 7 beta-Hydroxysteroid Dehydrogenase (5FYD)]

The binding affinities of ligands 2,4-D and melatonin towards the receptor proteins 2AM9, 1HXH, and 5FYD are investigated in detail. The binding energy, hydrogen bond, and contacting receptor residues of the interactions are listed in Table 2 and shown in Figures 7 and 8: A-C.

3.8.1 Hydrogen Bond and Binding Energy

The binding affinity of the ligand-protein complex is based on the hydrogen bond and the binding energy -ΔG which is indicating more spontaneous interaction in the actual system.

The docking complex of 2,4-D with 2AM9, 1HXH, and 5FYD showed binding affinity (2AM9: -5.6, 1HXH: -4.7; and 5FYD: -6.0) kcal mol^{-1}. These complexes possess hydrogen bonds (2AM9: 1; 1HXH: 3; and 5FYD: 2) with residues (2AM9: Thr 877; 1HXH: Asp 200, Lys 366; and

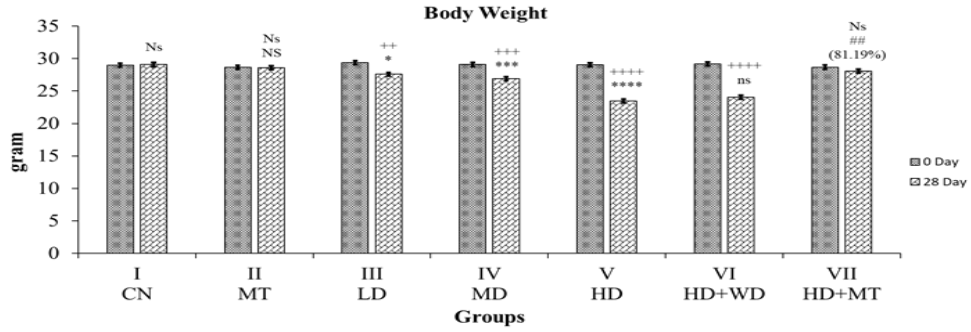


Figure 1. Effects of 2,4-D and/or melatonin on body weight of mice.

Values are Mean ± S.E.; Group-I = Control (CN); Group II = Melatonin (MT) (10 mg/kg body weight); Group III = 2,4-D Low Dose (LD) (16.5 mg/kg body weight); Group IV = 2,4-D Mid Dose (MD) (33 mg/kg body weight); Group V = 2,4-D High Dose (HD) (66 mg/kg body weight); Group VI = 2,4-D High Dose (HD) (66 mg/kg body weight) – 28 Days Exposure + 28 Days Withdrawal (WD); Group VII = 2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight). Ns = Non significant; + = p<0.05; ++ = p<0.01; +++ = p<0.001; ++++ = p<0.0001 when body weight after 28 days of treatment were compared with commencement of experiment (0 Day) in each group; Values in parenthesis are percent amelioration after 28 days melatonin administration along with 2,4-D high dose.

NS = Non Significant; * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 when 28 days treated mice (groups II to V) were compared with 28 days sterile distilled water administered control mice (group I); ns = non significant; # = p<0.001; ## = p<0.0001; when 28 days- withdrawal (group VI) or melatonin along with 2,4-D high dose treatment (group VII) were compared with 28 days high dose of 2,4-D treatment (group V).

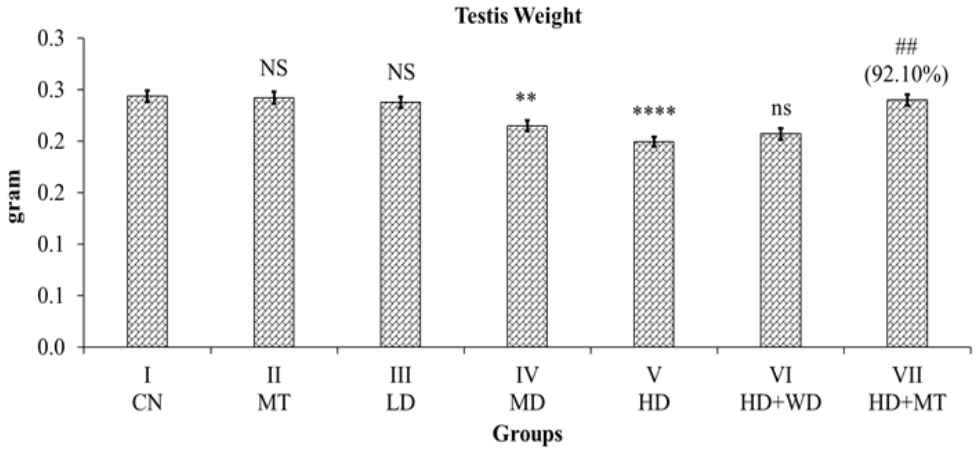


Figure 2. Effects of 2,4-D and/or melatonin on testis weight of mice.

Values are Mean ± S.E.; Group-I = Control (CN); Group II = Melatonin (MT) (10 mg/kg body weight); Group III = 2,4-D Low Dose (LD) (16.5 mg/kg body weight); Group IV = 2,4-D Mid Dose (MD) (33 mg/kg body weight); Group V = 2,4-D High Dose (HD) (66 mg/kg body weight); Group VI = 2,4-D High Dose (HD) (66 mg/kg body weight) – 28 Days Exposure + 28 Days Withdrawal (WD); Group VII = 2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight). Values in parenthesis are percent amelioration after melatonin administration along with 2,4-D high dose.

NS = Non Significant; * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 when groups II to V were compared with control (group I);

ns = non significant; # = p<0.001; ## = p<0.0001; when groups VI and VII were compared with high dose of 2,4-D treatment (group V).

5FYD: Thr 17, Ala 16). Each docking complex of 2,4-D with 2AM9, 1HXH, and 5FYD owns one hydrogen bond per residue mentioned respectively except two hydrogen bonds in the case of Lys 366 residue (Figure 7B; 1HXH-

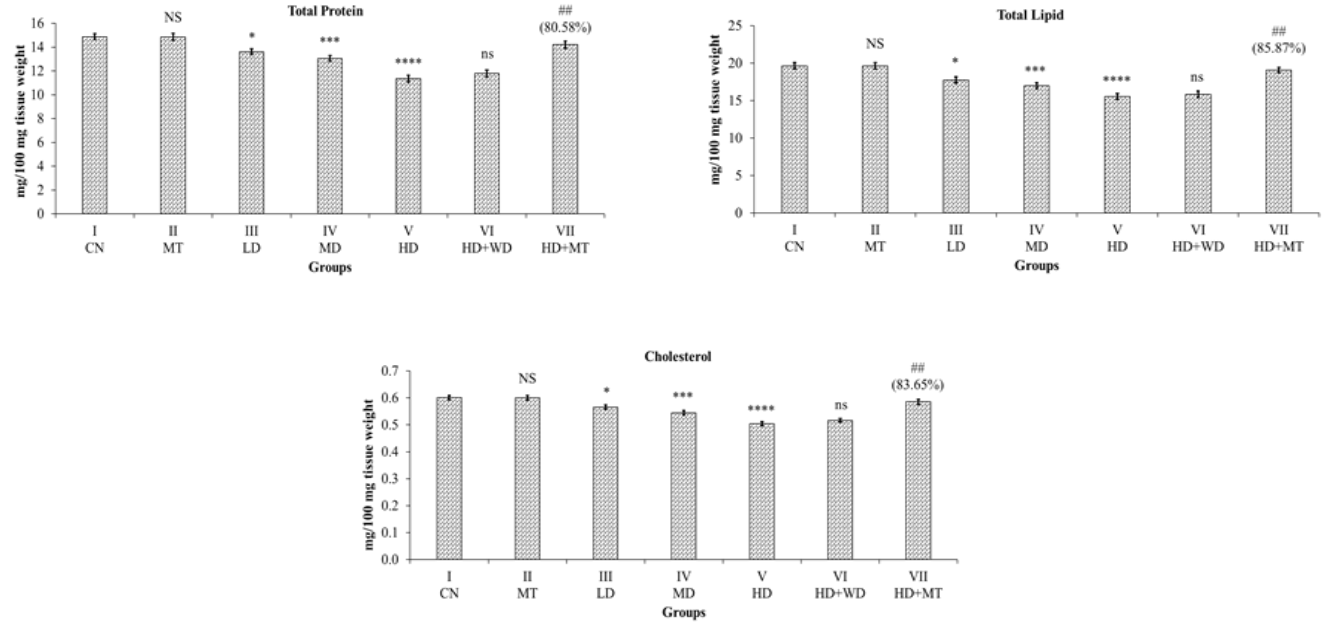


Figure 3. Effects of 2,4-D and/or melatonin on total protein, total lipid, and cholesterol levels in the testis of mice.

Values are Mean ± S.E.; Group-I = Control (CN); Group II = Melatonin (MT) (10 mg/kg body weight); Group III = 2,4-D Low Dose (LD) (16.5 mg/kg body weight); Group IV = 2,4-D Mid Dose (MD) (33 mg/kg body weight); Group V = 2,4-D High Dose (HD) (66 mg/kg body weight); Group VI = 2,4-D High Dose (HD) (66 mg/kg body weight) – 28 Days Exposure + 28 Days Withdrawal (WD); Group VII = 2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight).

Values in parenthesis are percent amelioration after melatonin administration along with 2,4-D high dose. NS = Non Significant; * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 when groups II to V were compared with control (group I); ns = non significant; # = p<0.001; ## = p<0.0001; when groups VI and VII were compared with high dose of 2,4-D treatment (group V).

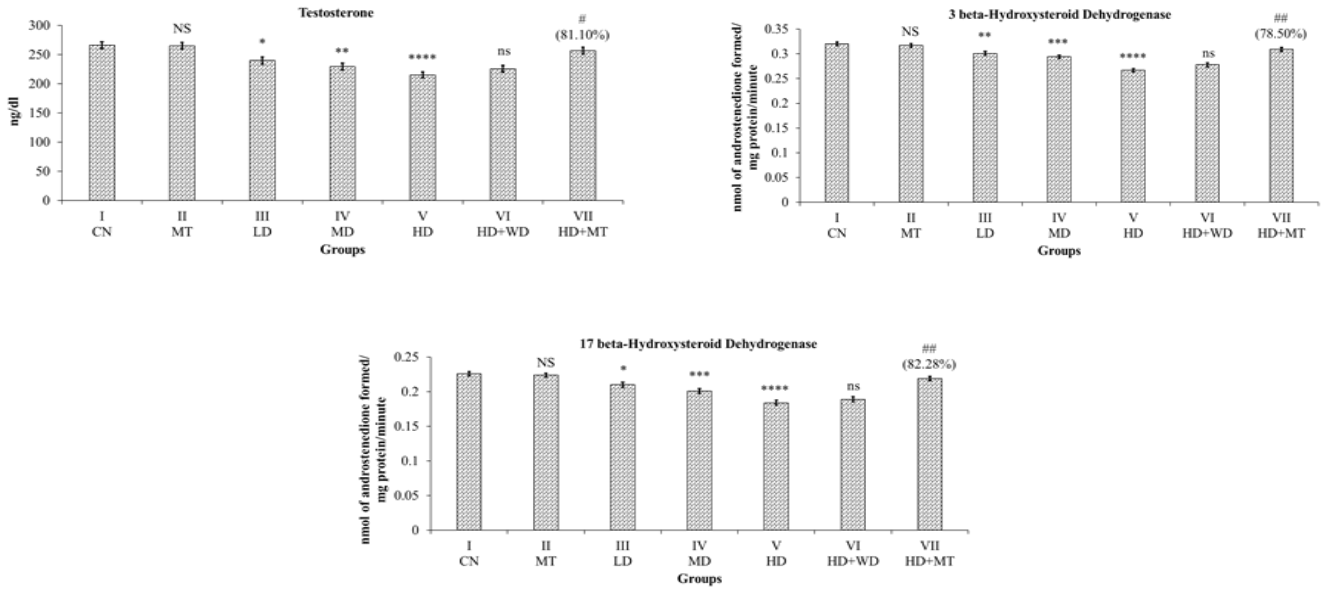


Figure 4. Effects of 2,4-D and/or melatonin on serum testosterone level, 3 beta-hydroxysteroid dehydrogenase, and 17 beta-hydroxysteroid dehydrogenase activities in the testis of mice.

Values are Mean ± S.E.; Group-I = Control (CN); Group II = Melatonin (MT) (10 mg/kg body weight); Group III = 2,4-D Low Dose (LD) (16.5 mg/kg body weight); Group IV = 2,4-D Mid Dose (MD) (33 mg/kg body weight); Group V = 2,4-D High Dose (HD) (66 mg/kg body weight); Group VI = 2,4-D High Dose (HD) (66 mg/kg body weight) – 28 Days Exposure + 28 Days Withdrawal (WD); Group VII = 2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight).

Values in parenthesis are percent amelioration after melatonin administration along with 2,4-D high dose. NS = Non Significant; * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 when groups II to V were compared with control (group I); ns = non significant; # = p<0.001; ## = p<0.0001; when groups VI and VII were compared with high dose of 2,4-D treatment (group V).

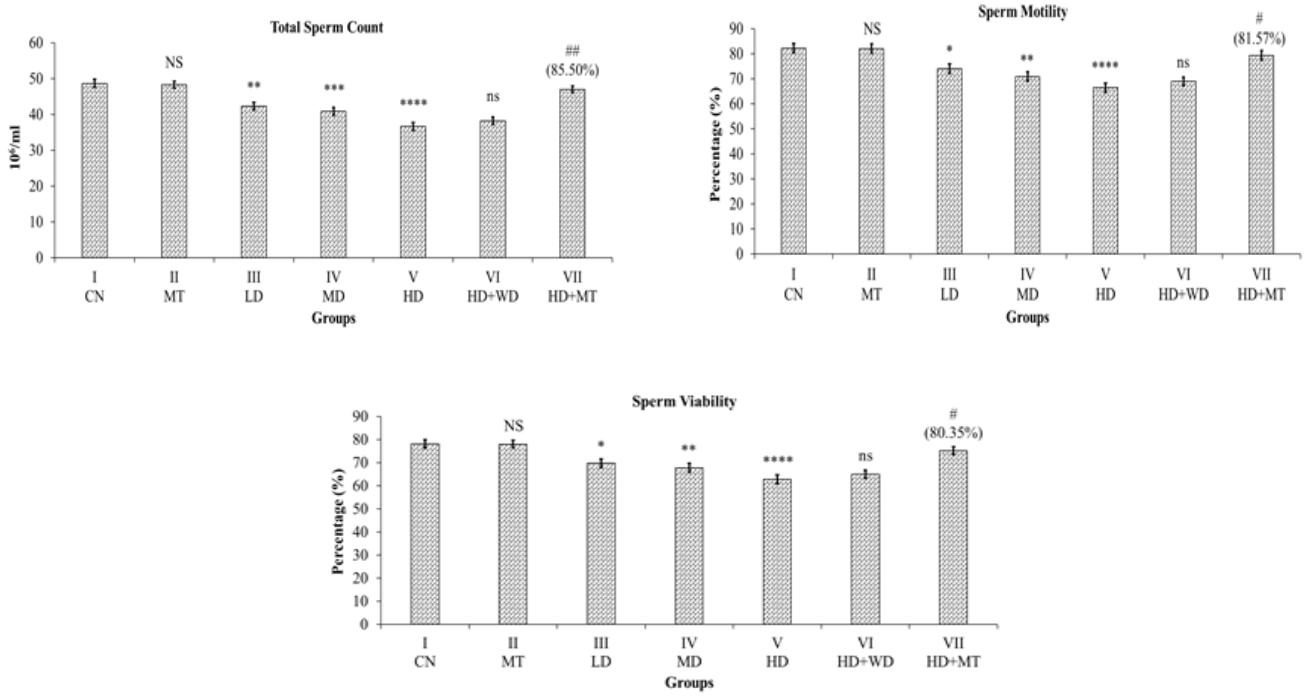


Figure 5. Effects of 2,4-D and/or melatonin on total sperm count, sperm motility, and sperm viability in the cauda epididymis of mice.

Values are Mean ± S.E.; Group-I = Control (CN); Group II = Melatonin (MT) (10 mg/kg body weight); Group III = 2,4-D Low Dose (LD) (16.5 mg/kg body weight); Group IV = 2,4-D Mid Dose (MD) (33 mg/kg body weight); Group V = 2,4-D High Dose (HD) (66 mg/kg body weight); Group VI = 2,4-D High Dose (HD) (66 mg/kg body weight) – 28 Days Exposure + 28 Days Withdrawal (WD); Group VII = 2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight).

Values in parenthesis are percent amelioration after melatonin administration along with 2,4-D high dose. NS = Non Significant; * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 when groups II to V were compared with control (group I); ns = non significant; # = p<0.001; ## = p<0.0001; when groups VI and VII were compared with high dose of 2,4-D treatment (group V).

2,4-D). Along with this, other weak interactions are shown in respective protein-ligand complexes (Table 2; Figure 7: A-C).

The docking complex of melatonin with 2AM9, 1HXH, and 5FYD showed binding affinity (2AM9: -7.0; 1HXH: -5.7; and 5FYD: -6.9) kcal mol^{-1}. These complexes involve hydrogen bonds (2AM9: 2; 1HXH: 2; and 5FYD: 3) with residues (2AM9: Asn 705, Thr 877; 1HXH: Asp 200, Lys 366; and 5FYD: Asn 153, Gly 204, Met 257). Each docking complex of melatonin with 2AM9, 1HXH, and 5FYD owns one hydrogen bond per residue mentioned respectively. Furthermore, other interactions are shown in respective protein-ligand complexes (Table 2; Figure 8: A-C).

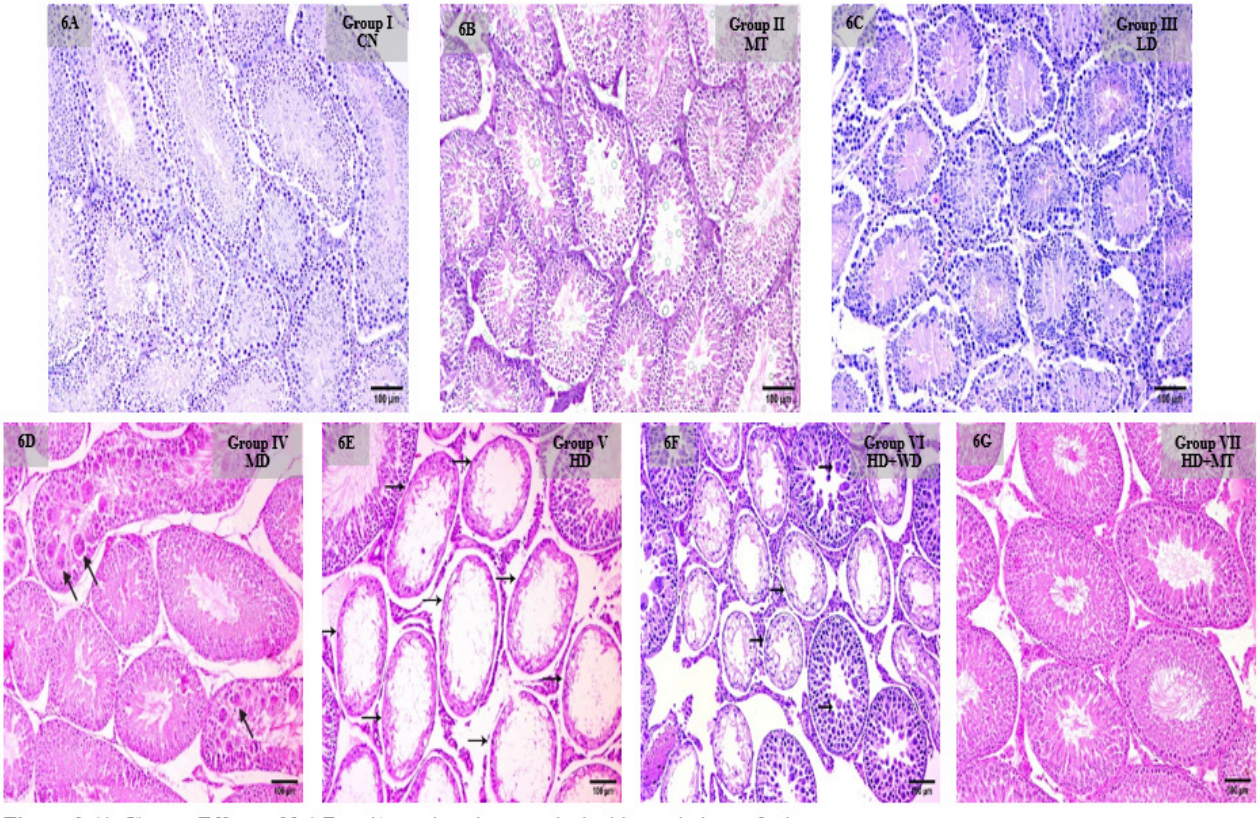


Figure 6. (A-G) Effects of 2,4-D and/or melatonin on testicular histopathology of mice. (Transverse section with hematoxylin and eosin stain, Magnification: 100 X, A-G).

A: Group-I = Control (CN); B: Group II = Melatonin (MT) (10 mg/kg body weight); C: Group III = 2,4-D Low Dose (LD) (16.5 mg/kg body weight); D: Group IV = 2,4-D Mid Dose (MD) (33 mg/kg body weight); E: Group V = 2,4-D High Dose (HD) (66 mg/kg body weight); F: Group VI = 2,4-D High Dose (HD) (66 mg/kg body weight) – 28 Days Exposure + 28 Days Withdrawal (WD); G: Group VII = 2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight).

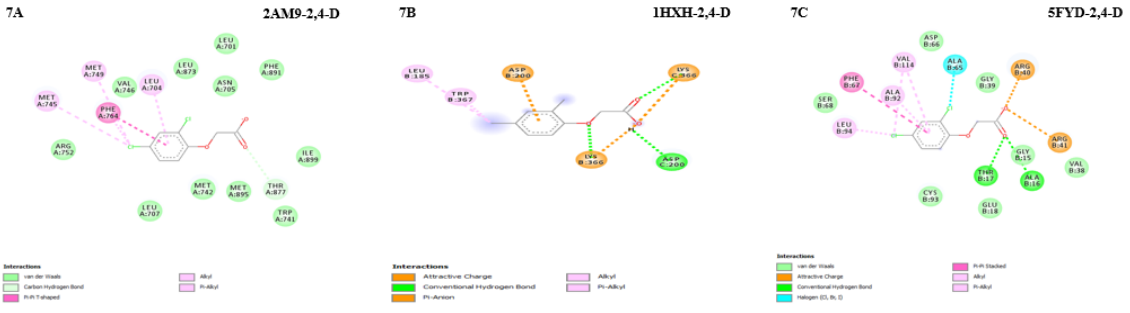


Figure 7. (A-C) Binding modes of 2,4-D with the crystal structure of proteins (A-C). Receptor–ligand interactions with 2D representation of 2,4-D.

Table 2. Molecular docking result analysis: binding energy, hydrogen bond, and contacting receptor residues

Proteins	Ligands	Binding Energy (kcal mol^{-1})	Hydrogen bond	Contacting Receptor Residues
Testosterone (2AM9)	2,4-D	-5.6	Thr 877	Thr 877, Leu 873, Leu 701, Asn 705, Phe 891, ILE 899, Trp 741, Met 895, Met 742, Leu 707, Arg 752, Met 745, Phe 764, Met 749, Val 746, Leu 704
	Melatonin (MT)	-7.0	Asn 705, Thr 877	Asn 705, Thr 877, Leu 707, Arg 752, Gln 711, Met 745, Met 749, Phe 764, Leu 704, Met 787, Val 746, Met 780, Leu 873, Leu 701, Met 895, Leu 880, Phe 876, Met 742
3 beta /17 beta-hydroxysteroid dehydrogenase (1HXH)	2,4-D	-4.7	Asp 200, Lys 366	Asp 200, Lys 366, Trp 367, Leu 185
	Melatonin (MT)	-5.7	Asp 200, Lys 366	Asp 200, Lys 366, Gln 183, Met 385, Leu 185, Gly 197, Trp 367, Ile 201,
7 beta-hydroxysteroid dehydrogenase (5FYD)	2,4-D	-6.0	Thr 17, Ala 16	Thr 17, Ala 16, Asp 66, Ala 65, Gly 39, Arg 40, Arg 41, Gly 15, Val 38, Glu 18, Cys 93, Leu 94, Ser 68, Phe 67, Ala 92, Val 114
	Melatonin (MT)	-6.9	Asn 153, Gly 204, Met 257	Asn 153, Gly 204, Met 257, Trp 152, Pro 151, Phe 97, Gly 258, Met 208, Leu 198, Leu 195, Tyr 156, Ser 196

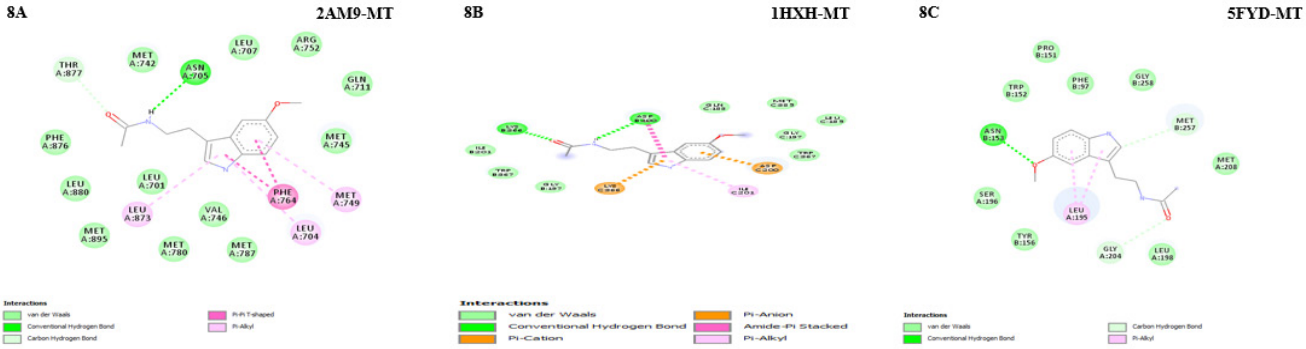


Figure 8. (A-C) Binding modes of melatonin (MT) with the crystal structure of proteins (A-C). Receptor–ligand interactions with 2D representation of melatonin.
A: Testosterone (2AM9); **B:** 3 beta/17 beta-Hydroxysteroid Dehydrogenase (1HXH); **C:** 7 beta-Hydroxysteroid Dehydrogenase (5FYD).

Amongst both the ligand interaction with respective proteins, melatonin showed more binding affinity in terms of more numbers of hydrogen bonds and low free negative energy. These observations are significant and might be the probable cause for the highest affinity of melatonin to the 2AM9, 1HXH, and 5FYD complexes.

4. Discussion

The increasing application of 2,4-D in the environment has caused considerable concern since years[2-4]. Several studies attempted to explore the adverse effects of 2,4-D as well as other herbicides and pesticides on the endocrine system of the different organisms[5-15], but still, there is a paucity of

data regarding its correlation with steroidogenesis. In that context, the present study revealed that 2,4-D alters the testicular steroidogenesis pathway in adult albino male mice of Swiss strain. The administration of 2,4-D showed abnormal clinical symptoms also evident by a significant dose-dependent reduction in total body and testis weight of mice. Our findings are in line with the most earlier studies which showed severe symptoms of toxicity[37], decrement in body weight[9,12,22,37–39], and testis weight[9,12] of 2,4-D exposed rats suggesting decrement in food intake, malabsorption of nutrients from the gastrointestinal tract, and impaired feed conversion efficiency. It is also well established that reproductive function is impaired if food intake is not adequate. Past investigation had shown that starvation and protein-calorie malnutrition cause atrophy of parts of the rat male reproductive system[40]. In our experiment, 2,4-D treatment exhibited significant dose-dependent decrement in the testicular total protein levels of mice. Similar results were also obtained by other authors in serum of rats[39,41] as well as in kidney of mice[22] after 2,4-D and Desormone lourd intoxication in support of our study. These reduced protein levels are associated with changes in circulating androgen and gonadotrophin levels and subsequent disruption of spermatogenesis[40].

The protein and lipid composition of membranes differ among cellular organelles, depending on their specific functions[42]. With 65% to 80% of the total cellular cholesterol located in the plasma membrane, comprising 20% to 25% of the total lipids present, cholesterol and lipid play a significant role in the structure and function of the plasma membrane[43]. Joshi and co-workers[12] reported the elevated levels of testicular total protein and cholesterol in rats indicating their accumulation by non-utilization in the systems. In contrast, the present study showed a significant decline in levels of testicular cholesterol, as well as total lipid in a dose-dependent manner after 2,4-D treatment in mice, maybe due to decreased lipid droplets and cholesterol contents in Leydig cells of mice[15] which showed an agreement with our findings. Depleted cholesterol and lipid contents in the present study are characterized by a wide range of herbicide induced clinical effects, including impaired cellular membrane function and steroid production, resulting in developmental and behavioral abnormalities[44]. Disruption of cholesterol homeostasis has been implicated in some human pathologies, including prostate cancer, in which androgen signaling is a principal factor as well as in dementia, diabetes, and Alzheimer's disease[45].

Steroidogenesis is the complex multienzyme process by which cholesterol is converted to biologically active steroid hormones[42,46]. Although the majority of cholesterol for sex steroid production is synthesized in the gonads and adrenal gland[44]. Testosterone, which is the principal androgen of male gonads[47] showed a significant dose-dependent reduction in the serum of 2,4-D induced mice in the present experiment. Various authors had demonstrated a similar decrement in the serum testosterone of rats after 2,4-D[9,12,14,15] as well as another pesticide[8] intoxication which supports the results observed in the present study. The reduction in serum testosterone level could be due to the decreased androgen biosynthesis by Leydig cells accompanied by decreased germ cells and elongated spermatids[15]. The androgenic status also depends on the activities of 3 beta-hydroxysteroid dehydrogenase and 17 beta-hydroxysteroid dehydrogenase enzymes which catalyze key steps in the androgenic pathway and are under the influence of environmental pollutants[8]. Our study noted a significant dose-dependent decrement in testicular activities of both these enzymes in 2,4-D treated mice. Similar findings were obtained previously in pesticide intoxicated rats in support of the present investigation signifying interference with the testosterone synthesis as well as incompetency in sex steroid formation[8].

Semen parameters are performed as a measurement of sperm production, testicular function, and male fertility[10]. Low sperm counts, percent sperm mortality, and viability as well as a high percentage of abnormal spermatozoa have been associated with reduced fertility[9]. In the present experiment, 2,4-D administration in mice caused a significant dose-dependent reduction in cauda epididymal total sperm count, percent sperm motility, and viability. Tan and co-workers[10] reported 2,4-D exposure on human sperm led to adverse effects on percent sperm motility with no change in percent viability whereas earlier studies of 2,4-D exposure in rats[9,11,12] and embryonic chickens[13] showed an agreement with our results. 2,4-D reduced spermatogenic potential by reducing the number of Sertoli cells[48]. Gonadotropins are essential for normal sperm production, growth, development, and maturation of testis and cauda epididymis. The suppression of gonadotropins may decrease sperm density in testis and

cauda epididymis. In addition, the reduction in sperm count, percent sperm motility, and viability indicate that high levels of herbicide have adverse effects on cell membrane[9,10,48].

Histopathology is an important tool of early-stage organ toxicity diagnosis due to chemical exposure. 2,4-D treatment caused various dose-dependent alterations in testicular histopathology of mice in the present study. The most common histopathological testicular impressions observed in the various literature[9,11,12,14,15] in 2,4-D induced mice or rats are the immature histological appearance of the male testis, manifested by marked reductions in spermatogenesis, moderate tubular atrophy, and increased incidences of spermatic giant cells. These previous observations showed consistency with our present results. There could be various possible causes of these 2,4-D induced alterations: i) Compromised genetic material in the head of sperm or perhaps because of an abnormal chromosome complement[11,49]. ii) Disruption of the blood-testis barrier as a consequence of the herbicide accumulation in the testis by disturbing the junction between Sertoli cells and germ cells[12,50]. iii) Reduction of testosterone production by Leydig cells because it is well known that testosterone affects and controls the physiological functions of Sertoli cells[9,15,49]. iv) Generation of ROS in the reproductive tracts[9,14,51] as also evident by severe pathological lesions observed in the present study.

All the parameters of the withdrawal investigation were unchanged compared to a high dose of 2,4-D suggesting an accumulation of the chemical into the physiological system[52,53] attributed to its pro-inflammatory action[54] and hence, confirming the strong need of a mitigating agent. Melatonin is a widespread molecule among living organisms involved in multiple biological, hormonal, and physiological processes at the cellular, tissue, and organic levels. It is well-known for its ability to cross the blood-brain barrier, and renowned antioxidant effects, acting as a free radical scavenger, up-regulating antioxidant enzymes, reducing mitochondrial electron leakage, and interfering with pro-inflammatory signaling pathways[55]. Hence to evaluate the possible role of melatonin against 2,4-D induced testicular toxicity as well as steroidogenesis upset, it was used as an antidote in the present study. The severe clinical symptoms and altered gravimetric indices (body and testis weight) along with total protein levels were found to be normalized significantly in the present study evident by melatonin co-administration with a high dose of 2,4-D as compared to 2,4-D high dose alone treatment. Dietary melatonin is absorbed best in the gastrointestinal tract and transported into the bloodstream, the ingestion of medicinal and food plants by mammals as a source of melatonin may be conceived as a key step in serum melatonin modulation and, consequently, health promotion[55].

Along with proteins, oxidative stress is damaging to DNA, lipids, and many more vital macromolecules[6,56,57]. The altered levels of total lipid and cholesterol along with the abnormal histopathology were significantly ameliorated when melatonin was co-administered along with a high dose of 2,4-D as compared to 2,4-D high dose alone treatment in the present investigation. Melatonin protects the testis from lipotoxicity through the activation of sirtuin 1 (SIRT1), which alleviates oxidative stress, endoplasmic reticulum stress, mitochondrial dysfunction, and DNA damage[58]. Melatonin improves the cholesterol level by interacting directly with cholesterol and by giving rise to fluidizing effect on monolayers comprising only lipid molecules[59]. Melatonin is a powerful antioxidant with a particular role in the protection of cell membrane as well as nuclear and mitochondrial DNA by limiting free radical formation against oxidative injuries in the tissues[56] as evident by our histopathological findings.

Besides its antioxidant properties and protective nature against toxicity, melatonin is involved in the function of the male reproductive system[18]. In the present study, the altered testosterone level, as well as activities of 3 beta-hydroxysteroid dehydrogenase and 17 beta-hydroxysteroid dehydrogenase enzymes had shown significant recovery noted by melatonin co-administration with a high dose of 2,4-D as compared to 2,4-D high dose alone treatment. Melatonin regulates androgen secretion through a melatonin membrane receptor in Leydig cells[60]. The binding of phosphorylated cAMP-response element-binding protein (CREB) to the cAMP response element of the StAR promoter accelerates steroid synthesis[60]. The previous report had indicated that the increasing androgens may be primarily due to the stimulatory effect of melatonin on the steroidogenic enzymes such as 3 beta-hydroxysteroid dehydrogenase and 17 beta-hydroxysteroid dehydrogenase[61].

Leydig cells synthesize and secrete testosterone, and are regulated by Sertoli cells[62]. In our study, the altered values of total sperm count, percent motility, and viability were found to be normalized significantly

when melatonin was co-administered along with a high dose of 2,4-D as compared to 2,4-D high dose alone treatment. Melatonin also affects Sertoli cell growth and proliferation as it upregulates the expression of spermatogenesis-related genes including cyclin D1, cyclin E, platelet-derived growth factor subunit A, desert hedgehog signaling molecule, occludin, and claudin in bovine Sertoli cells. These genes may interfere in spermatogenesis[63]. In addition, melatonin significantly increases inhibin B, considered a marker of Sertoli cell damage. Together, melatonin has multiple transduction pathways for modulating the Sertoli cell physiology and influencing spermatogenesis[62,63].

In silico data of the present study showed that melatonin was bound to testosterone, 3 beta/17 beta-hydroxysteroid dehydrogenase, and 7 beta-hydroxysteroid dehydrogenase with better affinity compared to 2,4-D in terms of docking score, hydrogen bond, and contacting receptor residues. Previously, neuroprotective effects of melatonin against ethanol-induced neurodegeneration were observed in the computational approach attributed to the antioxidant efficacy of melatonin[64]. However, to the best of our knowledge, this *in silico* study is the first evidence signifying interaction of 2,4-D and melatonin with testosterone, 3 beta/17 beta-hydroxysteroid dehydrogenase, and 7 beta-hydroxysteroid dehydrogenase. Along with that, this *in vivo* study also retained novelty related to the combined effects of 2,4-D and melatonin on testis histoarchitecture and biochemical evaluation with special reference to steroidogenesis.

5. Conclusion

2,4-D has been increasingly popular for agricultural, industrial, as well as domestic uses. Indiscriminate and excessive use of it may exert a serious destructive impact on the reproductive system by acting on steroidogenesis as well as the endocrine system in vertebrates. Melatonin has excellent antioxidant properties in testis cell environments due to its ability to prevent oxidative damage to lipids and proteins. It also appears to block the pro-inflammatory action of 2,4-D and can efficiently restore the histological condition of the testis. The molecular docking study reveals the same plausible mechanism which supports the *in vivo* study. In nutshell, our *in vivo* and *in silico* experimental data proved melatonin as a promising therapeutic agent in testis against 2,4-D induced impairment in the male reproductive system evident by its protective impact on steroidogenesis as well as on the components of the antioxidant defense system.

6. Conflict of Interest

The authors declare that there is no conflict of interest.

7. Acknowledgement

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