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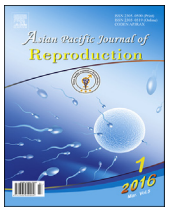

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Evaluation of Deltamethrin induced reproductive toxicity in male Swiss Albino mice

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ABSTRACT

Objective: To assess the adverse effect of Deltamethrin (DM) (technical grade) on reproductive organs and fertility indices of male Swiss albino mice, *Mus musculus*.
Methods: Forty male mice were divided into four experimental groups: control, vehicle control (peanut oil), high dose DM treated and low dose DM treated group. An oral dose of 3 mg/kg b.wt (low dose) and 6 mg/kg b.wt (high dose) of Deltamethrin was administered for a period of 45 days to male Swiss albino mice.
Results: DM caused a significant reduction in body and organ weights, sperm count, sperm motility percent, sperm viability, serum testosterone level, sialic acid content of cauda epididymis and fructose level of seminal vesicle. DM-treated groups also showed a significant decline in testicular 3β and 17β Hydroxysteroid Dehydrogenase (HSD) activities. Histological examinations revealed significant alterations in the testes of dosed groups.
Conclusion: Deltamethrin is a toxic pyrethroid pesticide that produced significant reproductive toxicity in treated male mice as revealed by the severely affected parameters and the altered gravimetric indices.

1. Introduction

Under the pretext of demographic growth with all its consequences, agricultural production resorts to the use of a varied and a large quantity of insecticides to improve the production and preservation of foodstuffs. Thus, the use of insecticides has increased rapidly and is now widespread to the lowest level of agricultural production. The increasing release of chemicals into the environment dictates attention to a better understanding of their toxicity in human and ecotoxicological effects. Several currently used pesticides are known to adversely impair reproductive competence of males under laboratory, field, clinical or occupational settings. Published studies have reported that pyrethroids can impair fertility, deteriorate semen quality, and cause testicular degeneration, male reproductive failure and malformations in the fetus of rodents following repeated exposure [1,2]. Synthetic pyrethroids are modified derivatives of pyrethins, natural substances obtained from flowers of pyrethrum species. Concerning to their high bio-efficacy at low concentrations, enhanced photo-stability and relatively low mammalian and avian toxicity, pyrethroid insecticides are widely used in agriculture, domestic and veterinary applications than other insecticides, particularly organochlorine, organophosphate and carbamate insecticides.

Deltamethrin [(R, S)] is a type-II pyrethroid synthetic insecticide, which has been widely used to control noxious insects in agriculture, forestry and horticulture. A number of studies have demonstrated genotoxic and tumorogenic effects of deltamethrin in mammalian and non-mammalian species [3]. During pyrethroid metabolism, reactive oxygen species (ROS) are generated and result in oxidative stress in intoxicated animals. In mammals, sperm plasma membranes have extremely high concentration of polyunsaturated fatty acids and insufficient antioxidant defenses; hence they are highly susceptible to lipid peroxidation. The production of ROS is a normal physiological event in various organs including the testis controlling sperm capacitation, acrosome reaction and sperm–oocyte fusion. However, overproduction of ROS can be harmful to sperm and subsequently to male fertility. Hence, the present study was carried out in order to assess the deleterious effect of technical

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grade Deltamethrin on various aspects of male reproduction to have an overall understanding of male infertility induced by this rampantly used pyrethroid. Open literature studies reveals several flaws including lack of appropriate control group, no data on the purity of test material, use of commercial mixtures containing Deltamethrin as a minor constituent, inadequate number of animals per group i.e. as low as only 3 animals per group, use of one dose level only, questionable route of administration (intraperitoneal or subcutaneous injection), no body and organ weight data, etc. A number of studies used commercial mixture containing Deltamethrin and other substances, such as Xylene, as the test material, making it impossible to attribute any effects to Deltamethrin or any other single component of the mixture since the controls received an entirely different vehicle. Keeping in view the above facts, present study has been designed accordingly so as to minimize the source of discrepancies in providing the evidence of reproductive toxicity of Deltamethrin.

2. Materials and methods

2.1. Animals & chemicals

Healthy, adult, pathogen free, colony bred male albino mice (*Mus musculus*) of Swiss strain weighing between 30 and 40 g obtained from IAEC recognized supplier were used for the experiments. The experimental protocol and the number of animals used for the experiments were mentioned in a detailed proposal and approval was obtained as per the guidelines of the institutional animal ethics committee, under registration No. 167/1999/CPCSEA from the Ministry of Social Justice and Empowerment, Government of India and Committee for the purpose of Control and Supervision of Experiments on Animals, Chennai, India. All the animals were acclimatized for seven days prior to the commencement of experiment. The animals were housed in an air-conditioned animal house at a temperature of 26 ± 20 °C and exposed to 10–12 h of day light and relative humidity of 40%–50%. Animals were randomized into control and treated groups and were caged separately. Standard chow (obtained from Amrut laboratory, Baroda, India) and water was provided *ad libitum*. Test chemical Deltamethrin (technical grade) of 98.11% purity was generously gifted from Meghmani Organics Limited, Ahmadabad (India). All the other chemicals used were procured from Himedia Laboratories, India and Sigma Aldrich (UK). All the chemicals used were of analytical grade.

2.2. Experimental design

Deltamethrin is considered to be readily absorbed when given orally as all pyrethroids are lipophilic; and absorption through gastrointestinal tract is higher than other routes. Hence, oral route of administration was selected for the treatments. Deltamethrin was administered via oral gavage dissolved in peanut oil at a dose level of 3 mg/kg body wt. (1/10th of LD_{50}) and 6 mg/kg body wt. (1/5th of LD_{50}). The doses were determined on the basis of LD_{50} of deltamethrin in peanut oil i.e. 30 mg/kg body weight [4].

Animals were divided into following groups (8 animals per group):

Group I: Control (given distilled water and food *ad libitum*); Group II (VC): Vehicle Control (given only peanut oil); Group III (HD): Deltamethrin HD treated (given 6 mg/kg body weight deltamethrin dissolved in peanut oil); Group IV (LD): Deltamethrin LD treated (given 3 mg/kg body weight deltamethrin dissolved in peanut oil).

All the groups were treated for 45 days and at the end of experiment, animals were weighed and sacrificed using light ether anesthesia.

2.3. Tissue collection

At the termination of experiment, animals were dissected and testis, cauda epididymis and seminal vesicle were dissected out carefully. Tissues were weighed, processed and homogenates were prepared accordingly.

2.4. Body and organ weight

The body weight of control and all treated groups of mice were recorded to the nearest milligram on a digital balance (Reptech). The animals were weighed before and at the end of each week prior to autopsy. Similarly, weights of organs were recorded to the nearest milligram on digital balance (Citizen, Japan).

2.5. Fructose

Fructose level was estimated in seminal vesicle of control and treated mice by the method of Foreman *et al.* [5]. The concentration of fructose was calculated using the regression formula obtained from the standard graph.

2.6. Sialic acid

Periodate resorcinol method was used for quantitative determination of free and glycosidically bound sialic acids [6]. The method involves oxidation of total sialic acid by treatment with periodic acid which forms a chromogen with resorcinol reagent. This chromogen is then extracted in an organic solvent and compared with standard at 630 nm.

2.7. Sperm count and sperm motility

Sperm count and motility in cauda epididymis of control and treated mice was determined using the Neubauer chamber of hemocytometer according to the method of Prasad *et al.* [7].

2.8. Sperm viability

Live: Dead ratio of cauda epididymal sperms was estimated by using the method of Talbot and Chacon [8].

2.9. Serum testosterone level

Serum testosterone levels were assayed using a solid phase enzyme immunoassay (ELISA) utilizing the competitive binding principle. Testosterone present in the sample competes with enzyme labeled testosterone for binding with anti testosterone antibody immobilized on the microwell surface. The amount of conjugate bound to the microwell surface decreased in proportion to the concentration of testosterone in

sample. The unbound sample and conjugate were removed by washing after which the color development reagents (substrates) were added. Upon exposure to the bound enzyme, a color change took place. The intensity of the color reflected the amount of bound enzyme testosterone conjugate and inversely proportional to the concentration of testosterone in sample within dynamic range of assay. After stopping the reaction the resulting color was measured using a Merck ELISA Reader at 450 nm. The testosterone concentration in the sample and concurrently run controls were determined from the standard curve.

2.10. 3β and 17β Hydroxysteroid Dehydrogenase (HSD)

The testicular 3β and 17β hydroxysteroid dehydrogenase (3β HSD) activity was assayed by the method of Talalay [9].

2.11. Histological studies

Histological studies were carried out using the standard technique of haematoxylene and eosin staining.

2.12. Statistical analysis

All the data are expressed as Mean ± SEM. Statistical analysis was performed using SPSS software package version 16.0 (USA). Comparison between groups was made by one-way analysis of variance (ANOVA) taking significance at $P < 0.05$ followed by Student's *t*-test taking significance at $^{**}P < 0.01$. Tukey's honestly significance difference (HSD) post hoc test was used for comparison among different treatment groups ($P < 0.05$).

3. Results

3.1. Body weight and organ weight

Terminal body weight of both high and low dose DM treated groups (Group III and Group IV) for 45 days exhibited significant reduction ($P < 0.01$) as compared to control mice (Table 1). Tissue weight of testis, cauda epididymis and seminal vesicle of both LD and HD Deltamethrin treated mice (Group III and Group IV) after 45 days recorded a significant fall ($P < 0.05$ or $P < 0.01$) as compared to control mice (Table 1).

3.2. Sperm parameters

A significant decline in caudal sperm count, sperm motility and sperm viability of LD ($P < 0.01$) and HD ($P < 0.01$) Deltamethrin treated mice (Group III and Group IV) was observed 45 days post-treatment as compared to control mice (Table 2). Serum testosterone level was significantly reduced by LD and HD (P both <0.01) treatment with DM (Group III and Group IV) at the end of experiment as compared to control animals (Table 2).

3.3. Sialic acid

Sialic acid content of cauda epididymis of 45 days treated mice was significantly reduced in LD and HD (P both <0.01) Deltamethrin treated groups (Group III and Group IV) as compared to control mice (Table 3).

Animals of both LD and HD Deltamethrin treated groups (Group III and Group IV) after 45 days revealed a significant decline ($P < 0.01$) in fructose level of seminal vesicle as compared to control group (Table 3).

3.4. 3β and 17β Hydroxysteroid Dehydrogenase (HSD)

Activities of testicular 3β and 17β HSD revealed a significant declining trend after 45 days treatment in LD and HD ($P < 0.01$) DM treated groups (Group III and Group IV) as compared to control (Table 3).

3.5. Histological observation of testis

Testicular sections of the control mice had normal histoarchitecture that consisted of uniform, well-organized seminiferous tubules with complete spermatogenesis and normal interstitial connective tissue. Seminiferous tubules revealed an intact epithelium with full complements of spermatogenic cells and different cellular associations. Lumens filled with mature

Table 1
Showing body and organ weight of control and treated mice after 45 days.

Groups	Body wt. (g)	Testis wt. (mg)	Cauda wt. (mg)	Seminal wt. (mg)
CON	43.20 ± 1.37	121.32 ± 1.43	14.11 ± 0.44	409.87 ± 8.03
OIL	44.52 ± 0.75	120.03 ± 1.46	14.09 ± 0.48	412.34 ± 6.83
LD	34.89 ± 0.90**	97.87 ± 1.37**	11.89 ± 0.40*	312.45 ± 6.82**
HD	28.87 ± 1.44**	85.66 ± 2.02**	10.23 ± 0.28**	245.66 ± 5.99**

Values are represented as Mean ± S.E., $^{**}P < 0.05$, $^{**}P < 0.01$.

Table 2
Sperm parameters and serum testosterone level of control and treated mice after 45 days.

Groups	Sperm count (10^6/mL)	Sperm motility (%)	Sperm viability (%)	Testosterone (ng/mL)
CON	41.75 ± 1.51	79.06 ± 2.49	72.23 ± 1.76	3.25 ± 0.03
OIL	40.11 ± 1.62	78.25 ± 0.97	71.85 ± 1.99	3.24 ± 0.03
LD	31.58 ± 2.03*	66.19 ± 3.13*	56.92 ± 3.39*	3.06 ± 0.03*
HD	27.05 ± 1.66**	62.73 ± 2.36**	53.43 ± 1.85**	2.95 ± 0.05**

Values are represented as Mean ± S.E., $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 3

Fructose content in seminal vesicle, sialic acid content in cauda epididymis, as well as 3β HSD & 17β HSD activity in testis of control and treated mice after 45 days.

Groups	Fructose (μg/mg tissue wt.)	Sialic acid (μg/mg tissue wt.)	3β HSD	17β HSD
CON	11.21 ± 0.29	5.75 ± 0.23	0.26 ± 0.02	0.210 ± 0.007
OIL	11.19 ± 0.42	5.74 ± 0.21	0.25 ± 0.01	0.200 ± 0.005
LD	9.69 ± 0.34*	4.85 ± 0.21**	0.18 ± 0.01*	0.160 ± 0.010*
HD	8.27 ± 0.42**	4.32 ± 0.20**	0.17 ± 0.01**	0.140 ± 0.005**

Values are represented as Mean ± S.E., $^{*}P < 0.05$, $^{**}P < 0.01$.

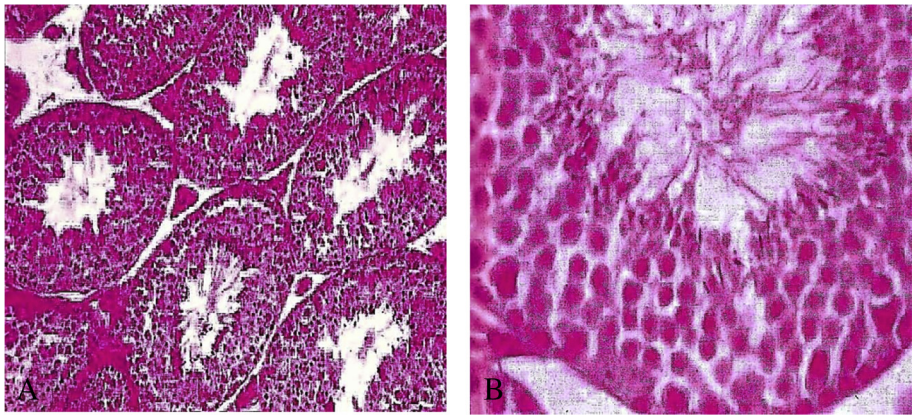

Figure 1. T.S of Testis of control mice showing normal arrangement of seminiferous tubules and normal spermatogenesis (A. 10×; B. 40×).

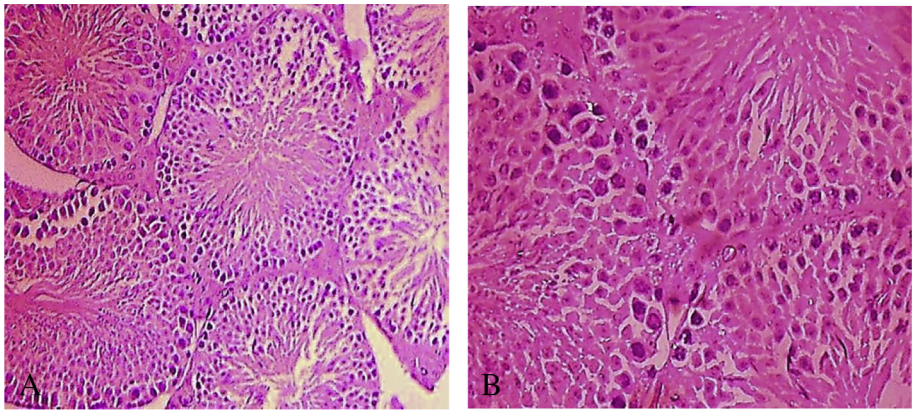

Figure 2. T.S of Testis of peanut oil treated mice showing normal testicular architecture, seminiferous tubules and basement membrane (A. 10×; B: 40×).

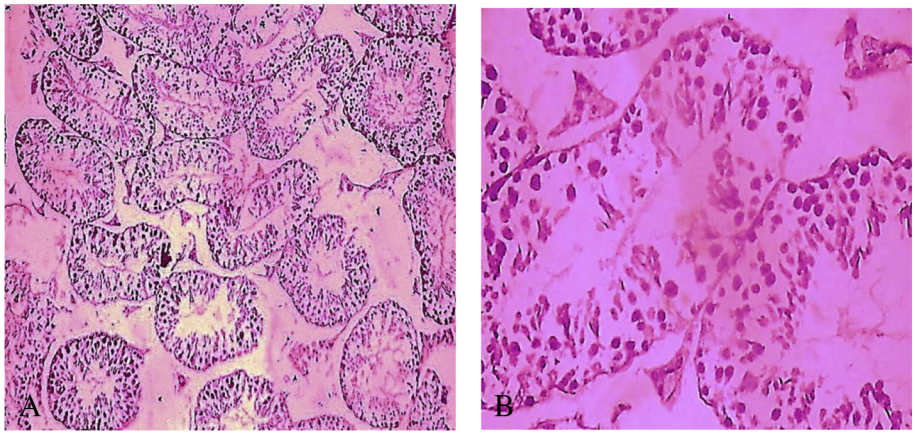

Figure 3. T.S of testis showing irregular and buckled basement membrane, sloughed and necrotic germinal epithelium, tubular deformation and degeneration, shrunken seminiferous tubules, increased luminal diameter, slight accumulation of oedamatous fluid in tubular, grossly reduced Leydig cell population and other alterations (10×; 40×).

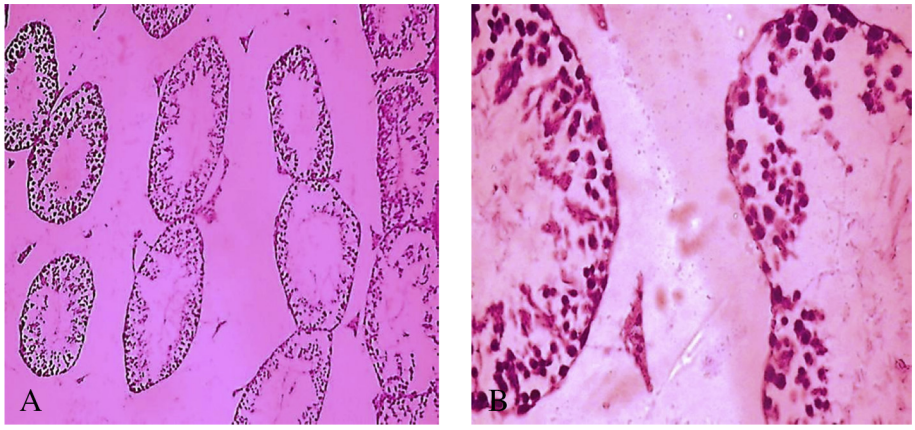

Figure 4. T.S of testis showing severely altered and damaged histoarchitecture. (A: 10×; B: 40×).

spermatozoa were also observed. Interstitial tissue had normal distribution of Leydig cells (Figure 1A). These observations can be seen with greater clarity at higher magnification of 40× (Figure 1B).

Peanut oil administered group revealed normal histology of Testis. No significant alterations were observed (Figure 2A) and at higher magnification (Figure 2B).

45 days LD (3 mg/kg body wt.) treated animals showed irregular and buckled basement membrane, sloughed and necrotic germinal epithelium, tubular deformation and degeneration, shrunken seminiferous tubules, mild accumulation of oedamatous fluid in tubules and grossly reduced Leydig cell population. Also, degenerative changes in tunica albuginea, necrosis in seminiferous tubule, vacuolization, exfoliation of spermatocytes, fewer number of spermatogenic cells, reduced population of mature spermatozoa, scattered spermatids and degeneration of interstitial and Leydig cells were evident (Figure 3A). The damage to the histo-architecture of testis at 45 days is clearly visualized at higher magnification with low dose of Deltamethrin (Figure 3B).

Further, when HD of Deltamethrin (6 mg/kg body wt.) was administered for 45 days severe alterations were observed in testicular architecture in the form of tubular deformation, disorganized, necrotic and degenerative changes in germinal epithelium, aspermatogenesis, hyalinization of luminal content, accumulation of oedamatous fluid in luminal space of tubules, vacuolar degeneration in tubules and severe necrosis in seminiferous tubules. Lack of germ cells in seminiferous tubules, vacuolization in Sertoli cells, absence of mature spermatozoa, degeneration of Leydig cells, widely increased interstitial space and vacuolization in interstitial spaces was also observed (Figure 4A) and at higher magnification of 40× (Figure 4B).

4. Discussion

Body weight and organ weight are an essential benchmark for the toxicological studies. In the present investigation significant reduction in terminal body weight in animals treated with both high and low dose of Deltamethrin was obtained after 45 days. Anorectic properties of Deltamethrin as well as poor feed conversion efficiency might be responsible for reduction in body weight. Other previous reports have also indicated a decline in body and organ weight due to administration of Deltamethrin and other pyrethroids in experimental animals [10,11]. The observed weight loss could also be attributed to reduced food intake i.e. loss of appetite in the treated groups. Reduced body weight might also be the consequence of direct cytotoxic effect of the pesticide on somatic cells or indirectly through the central nervous system which controls the feed and water intake and regulates the endocrine function [11].

Weight of reproductive organ is imperative for risk assessment in toxicological studies and testicular size is the best primary tool for assessment of spermatogenesis, since the tubules and germinal elements account for approximately 98% of the whole testicular mass [12]. Decline in testicular weight might also be due to decline in serum testosterone level. These results are in agreement with earlier findings where testicular weights of pups of dams treated by Deltamethrin at the dose of 4.0 mg/kg body weight from day 1 of pregnancy to day 21 of lactation, was found to be reduced [13]. Thus, the fall in the weight of the testis might be due to decreased number of germ cells, inhibition of spermatogenesis and decline in steroidogenic enzyme activities as observed in the present study. Reproductive damage was also confirmed by histological findings where loss of histoarchitecture, necrosis and atrophy of germ cells supported the reduction in organ weight.

Testicular steroidogenesis is regulated by two important dehydrogenases, 3β-HSD and 17β-HSD. Both these dehydrogenases are directly involved in biosynthesis of testosterone from pregnenolone as well as androstenedione. In the present work Deltamethrin treatment brought about a significant reduction in testicular dehydrogenases and serum testosterone level in exposed animals. It is suggested that decrease in the dehydrogenase activities might be associated with reduced testosterone secretion which in turn is the outcome of impaired steroidogenesis due to oxidative insult [14].

It is well known that androgens are the major regulators of growth, structure and functions of accessory sex organs. In the present study significant reductions in the accessory sex organs weights were recorded in Deltamethrin treated groups signifying inadequate levels of androgens. A large part of male reproductive system depends on testosterone. The process of spermatogenesis eventually ceases in the absence of this hormone. Reduction in testosterone dependent parameters by Deltamethrin might cause the so called “androgen deprived effect” to target organs by affecting testosterone synthesis in the testis [15]. Similar reduction in serum levels of testosterone, luteinizing hormone and follicle stimulating hormone was obtained in alpha-cypermethrin treated rats [16]. The decline in hormone levels was attributed to either

direct effect of toxicant on androgen biosynthesis pathway in testis or its effect on hypothalamus/anterior pituitary gland which might have indirectly affected the testis and sexual function [16]. Hence, the reduced testosterone might be responsible for the decreased sperm counts and motility and also morphological abnormality of testis in treated mice. It is also suggested in earlier studies that pyrethroid insecticides may cause mitochondrial membrane impairment in Leydig cells and disrupt testosterone biosynthesis by diminishing the delivery of cholesterol into the mitochondria and decreasing the conversion of cholesterol to pregnenolone in the cells, thus reducing subsequent testosterone production [17].

Measurement of fructose has been used in almost all laboratories of the world as a marker of the seminal vesicle function. In the present study a significant reduction in fructose content of seminal vesicle was observed in treated mice. Depletion of fructose content hampers the glycolytic metabolism of spermatozoa resulting in abnormal sperm functions, which ultimately leads to complete male sterility [18]. It is well known that the function of seminal vesicles is under androgen control and a direct association exists between serum testosterone, seminal fructose and spermatozoa motility/fertility [19]. Since fructose formation by the accessory glands is dependent on secretion of testosterone by the testis [20], the observed reduction in fructose suggests a corresponding decrease in testosterone secretion by pesticide treatment.

The synthesis and secretion of sialic acid is under androgen control. Alteration in sialic acid level in reproductive tissues indicate changes in the level of glycoprotein\ FSH and LH which is needed for normal functioning of gonads and accessory reproductive organs [21]. In the present study, sialic acid content of epididymis significantly decreased in both the dosed groups. This reduction possibly reflects the androgen and gonadotrophic deficiency eventually resulting into inhibition of spermatogenesis, loss of sperm motility and fertilizing capacity [22]. Moreover, structural integrity of acrosomal membrane depends on sialic acid and any alteration in its content might lead to structural and functional changes in sperm.

In correlation with the biochemical alterations, testicular damage was also observed in histology of testis, which revealed reduction in spermatogenic cells, exfoliation of spermatocytes and in some cases complete spermatogenic cells degeneration after Deltamethrin treatment. Several other anomalies such as severe deformity in seminiferous tubules, rupture of germinal epithelial layer surrounding the tubule, tubular atrophy, aspermatogenesis, vacuolization, cell necrosis along with apical sloughing, degeneration of spermatocytes and spermatids, hyalinization in intertubular tissues, Sertoli and Leydig cell degeneration and multilayered seminiferous epithelia with late spermatids lining the lumen of seminiferous tubules were also seen in the treated groups. Results of the present study corroborates to that of Rashid *et al*. (2012) who observed similar pathological changes in testis of deltamethrin treated mice [23]. Histopathological examination of testicular sections showed that apoptosis was confined to the basal germ cells, primary and secondary spermatocytes. Moreover, Sertoli cell vacuoles were also seen, which might be responsible for suppression of spermatogenesis. Further, it has been reported that vacuolization of the germinal and Sertoli cells might occur due to the dilation of smooth endoplasmic reticulum that possibly represents cellular permeability changes [24]. Sertoli cells are considered to be the supportive cells within the seminiferous tubule and have a key role in spermatogenesis. Hence, sloughing of germ cells point towards Sertoli cell damage due to microtubule impairment. These changes might be attributed to Deltamethrin induced lipid peroxidation and the reduction in testosterone hormone, since testosterone is required for the attachment of different generations of germ cells in seminiferous tubules. Therefore, low level of testosterone as observed in the present study might have led to detachment of germ cells from seminiferous epithelium leading to germ cell apoptosis and subsequent reproductive toxicity.

Based on the aforementioned data, we conclude that Deltamethrin is a toxic chemical pesticide that produced significant reproductive toxicity in treated male mice as revealed by the severely affected parameters and the altered gravimetric indices. Thus, confirming the toxic potential of these so called “safe to man” insecticides. Hence, rampant use of these pyrethroids should be regulated and monitored strictly especially when used for domestic purposes. Sensitive sub-groups of population like pregnant women and children should avoid any direct or indirect exposure as even low concentration of these insecticides can interfere with the normal physiology and overall well-being of the exposed organism. Moreover, proper measures should be taken by workers involved directly in manufacturing and application of this rampantly used insecticide. Injudicious and indiscriminate usage should be curbed and suitable alternatives should be employed wherever feasible.

Conflict of interest statement

The authors declare no conflict of interest related to employment, consultancies, stock ownerships, grants or other funding.

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