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Research Article

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Sub-Acute Toxicity Study of a Type-I Pyrethroid Permethrin on Testis of *Mus Musculus*

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Abstract: Pesticide usage has witnessed a rise in the current years to overcome the glitches of food scarcity because of incessant increase in population. Regardless of its extensive use in agriculture and related fields, these should be properly and thoroughly investigated because of unexplored danger which they bring along. The present investigation deals about the effects of one such pesticide, Permethrin, belonging to the class of synthetic pyrethroids, having broad range application in the field of agriculture, as an insect repellent, in lice shampoos, in the treatment of scabies and numerous other commercial uses. Although few reports have documented its adverse effect on the vital organs in different experimental models, there still persist lacunae in literature about its effect on the male reproductive tissue. Hence, the present study was aimed to evaluate the effects of Permethrin on the male reproductive gonad of Swiss albino mice. Permethrin (130 mg/kg body weight) was administered orally for a period of 21 days after which the biochemical parameters in testis were assayed. The results showed that Permethrin administration lowered the gravimetric indices (body weight $p<0.002$, organ weight $p<0.001$) and decreased the levels of protein ($p<0.001$) and cholesterol ($p<0.001$), which is an important precursor in the formation of androgens. Essential enzymes like 3β and 17β hydroxysteroid dehydrogenase which are pivotal in the steroidogenesis pathway were also found to be depleted ($p<0.001$ and $p<0.002$ respectively). These findings were further confirmed by the histopathological analysis and indicated that Permethrin had negative effects on the male reproductive system and can be one of the contributing factors leading to infertility.

Keywords: Synthetic pyrethroids, Permethrin, Reproductive system, Testis, Infertility

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1. INTRODUCTION

With the growing evolution of chemicals in the field of agriculture at a rapid pace, the difficulties of feeding such a gigantic population across the globe is now achievable. Pyrethroids, which are a class of emerging pesticides and are a recent discovery, are gradually becoming widespread in agronomy; however, their impacts pertaining to the environment remains largely unknown. These were initially isolated from the Chrysanthemum species which possessed natural active pyrethrins. However, later, the synthetic versions were introduced due to their increased efficiency and these came to be known as Synthetic pyrethroids. These were derived in the early 1970's but have not been widely used until recently due to the recent reduction in organophosphate use enacted by the (EPA) Environmental Protection Agency.[1] . The development of synthetic pyrethroids has involved extensive chemical modification that has made these compounds less degradable. To name a few, cypermethrin (CM), cyfluthrin, permethrin and deltamethrin are increasingly being used in indoor pest control because of their high toxicity to insects and low toxicity to mammals.[2] Permethrin is an important synthetic type I pyrethroid which is commercially used for various purposes. It is used as an insecticide in food/feed yields, livestock and livestock housing, buildings, and for inhabitable areas as well as for treatment, application and inhibition of head lice and scabies.[3] It is also widely used in the control of ticks, especially the dog tick *Rhipicephalus sanguineus*.[4] This pyrethroid has gained immense popularity due to its myriad applications, its high photostability, high activity against insects, relatively low mammalian toxicity compared to other insecticide classes.[5] However, despite its presumed relative safety as an insecticide, recent reports suggest that low doses of permethrin (<120 mg/kg) cause mild neurological signs; higher doses (>120 mg/kg) in laboratory rats result in more severe peripheral and central nervous system clinical signs including hyperactivity, convulsions, paralysis, and even death.[6,7]. In addition, some laboratory animal experiments suggested that permethrin may adversely affect the liver, brain, and immune system.[8-10] Because of increased use of these compounds, concern of their possible non-target effects has increased.[11,12]It is therefore imperative to bridge this lacuna and fill this void by gauging the effect of the said pesticide on these tissues. The present investigation was therefore undertaken to evaluate the effect of oral administration of permethrin on the reproductive tissue (testis) of male albino mice for a duration of 21 days.

2. MATERIALS AND METHODS

2.1 Animals

Healthy, adult (10-11 weeks old), pathogen free, colony bred male albino mice *Mus musculus* of Swiss strain weighing between 30 and 40 gm, obtained from Cadila Heath Care and Pharmaceutical, Ahmedabad, Gujarat, India 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/GO/ReBi/S/99/CPCSEA, 1st December, 1999 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°C ± 2°C and exposed to 10-12 h of daylight 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*.[13]

2.2 Chemicals

Test chemical Permethrin (technical grade) of 95% purity was procured from Nanjing Essence fine chemicals, China. All the other chemicals used were procured from HiMedia Laboratories, India and Sigma Aldrich (UK). All the chemicals used were of analytical grade.

2.3 Experimental design

In the present investigation, the effect of Permethrin was carried out and compared to control and vehicle treated (Corn oil) animals as per the experimental protocol. Animals were treated using oral gavage. Moreover, control animals were provided with food and water *ad libitum*, whereas vehicle treated animals were given 0.2 ml corn oil. The duration of the treatment was of 21 days and the animals were necropsied on the 22nd day. Permethrin 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, an oral route of administration was selected for the treatment. Permethrin was administered via oral gavage dissolved in corn oil at a dose level of 130 mg /kg body weight (1/5th of LD_{50}). The doses were determined on the basis of LD_{50} of Permethrin in corn oil i.e. 650 mg/kg body weight.[14] The animals were divided into following groups (6 animals/group):

Sr. No.	Groups	Duration	Day of Necropsy
Group I	Control (Water and food *ad libitum*)	21 days	22nd of post treatment
Group II	Vehicle (Corn Oil)	21 days	22nd of post treatment
Group III	Treated (Permethrin)	21 days	22nd of post treatment

All the groups were treated for 21 days and were necropsied on the 22nd day. At the end of the experiment, animals were weighed and euthanized using light ether anesthesia.

2.4 Tissue collection

Collection of tissue was done following a proper protocol.[15]

After the treatment period of the experiment, all the animals from respective groups (Control, Vehicle and Pesticide treated) were necropsied and the tissue of interest (testes) was carefully dissected out and blotted free of blood. These were then weighed, processed and homogenates were prepared accordingly.

2.5 Parameters Studied

2.5.1 Body Weight And Organ Weight

The body weight of control, vehicle and treated groups of mice were recorded to the nearest milligram on a digital balance (Reptech).. Similarly, organ weight of the respective groups were also recorded to the nearest milligram on digital balance(Aczet).

2.5.2 Total Protein

Protein estimation was done using standard protocol of Lowry *et al.* (1951).[16] A known weight of tissue (100 mg fresh tissue) was homogenized in a definite volume of glass distilled water. In the sample tube 0.2 ml of tissue homogenate, 0.6 ml of distilled water and 4 ml of alkaline copper sulphate solution was taken. The contents of the tube were vortex mixed. In the blank tube instead of sample, 0.2 ml of distilled water was taken. The tubes were kept for incubation at room temperature for 15 minutes. Then 0.4 ml of Folin Ciocalteu phenol (diluted 1:1, Folin-phenol reagent : distilled water) was added to each tube which was thoroughly mixed. The tubes were allowed to stand at room temperature for 30 minutes. The optical density of blue colour developed was read at 540 nm on Systronics Digital Spectrophotometer 167.

2.5.3 Cholesterol

The levels of cholesterol in the testis of control, vehicle control and permethrin treated groups of mice were estimated by the method of Zlatkis *et al.* (1953).[17] The tissue (50 mg) was homogenized in 1 ml of glacial acetic acid, 0.2 ml of this homogenate was added to the test tubes containing 5 ml of working ferric chloride solution (1 ml of stock 10% FeCl3 solution diluted to 100 ml with glacial acetic acid). 0.2 ml of standard cholesterol (100 µg) was then added to the standard tube instead of homogenate. The standard and blank tubes contained the same volume of ferric chloride solution. 3 ml of concentrated sulphuric acid was then added to all the test tubes. After incubating the tubes for 20 minutes at room temperature, the optical density was read at 540 nm on Systronics Digital Spectrophotometer 167 against blank.

2.5.4 3β Hydroxysteroid Dehydrogenase (3β Hsd)

The testicular 3β hydroxysteroid dehydrogenase (3β HSD) activity was assayed by the method of Talalay (1962).[18] A known amount of tissue was homogenized in 0.02 M Phosphate buffer (pH 7.5) along with Triton X - 100 (2.5 mg/ml) (10:1 dilution). The homogenate was centrifuged at 8000 g for 30 minutes at 4° C. The supernatant was used for the assay. To 2 ml of 0.1 M sodium pyrophosphate buffer (pH 8.9), 0.2 ml of substrate (3mg epiandrosterone in 2ml of N, N-dimethylformamide) and 0.2 ml of homogenate were added. Then, 0.2 ml of nicotinamide adenine dinucleotide (NAD) and 0.4 ml of glass distilled water were added. Blank was prepared by adding 2.0 ml of phosphate buffer, 0.2 ml of substrate and 0.8 ml of distilled water. Final volume of assay was 3.0 ml. The reduction in absorbance of NAD was read at an interval of 15 seconds on a Systronics UV-visible spectrophotometer (Model 118). The enzyme activity was expressed as nanomoles of 5α-diol formed/mg protein/ hour.

2.5.5 17 B Hydroxysteroid Dehydrogenase (17 B Hsd)

The testicular 17β hydroxysteroid dehydrogenase (17β HSD) activity was assayed by the method of Talalay (1962).18 A known amount of tissue was homogenized in 0.02 M Phosphate buffer (pH 7.5) along with Triton x – 100 (2.5 mg/ml) (10:1 dilution). The homogenate was centrifuged at 8000 g for 30 minutes at 4°C. The supernatant was used for the assay. To 0.2 ml of 0.1 M sodium pyrophosphate buffer (pH 8.9), 0.2 ml of substrate (1.5 mg testosterone dissolved in 2.0 ml methyl alcohol) and 0.2 ml of homogenate were added. 0.2 ml of NAD and 0.4 ml of glass distilled water were added. Blank was prepared by adding 2.0 ml of phosphate buffer, 0.2 ml of substrate and 0.8 ml of distilled water. Final volume of assay was 3.0 ml. The reduction in absorbance of NAD was read at an interval of 15 seconds on a Systronics UV-visible spectrophotometer (Model 118). The enzyme activity was expressed as nanomoles of 5α- diol formed/mg protein/hour.

3. STATISTICAL ANALYSIS

For each parameter, a minimum of 6 replicates were done and the results were expressed as Mean ± Standard Error (S.E.). The data was statistically analyzed by Analysis of Variance (One way ordinary - ANOVA) by Graphpad Prism 8.0 software (version 8.0). Vehicle and Permethrin treated groups were compared with the control group.

3.1 *Histological Studies*

Histological studies were carried out by using the standard technique of haematoxylin and eosin staining. For light microscopic examination testis tissues from each group were dissected out, blotted free of blood and fixed in 10% formalin immediately after the necropsy. Fixation was carried out at room temperature for 18 hours, after which they were transferred to 70% alcohol. Several changes of 70% alcohols were given, there after tissues were dehydrated by passing through ascending grades of alcohol, cleared in xylene, embedded in paraffin wax (58 to 60°C M.P) and transverse sections (T.S) were cut at 5µm on a rotary microtome. These sections were stained with haematoxylin and eosin, dehydrated, cleared in xylene and mounted in DPX (Distyrene Plasticizer Xylene) as permanent slide. The photomicrographs of the relevant stained section slides were taken with the aid of a camera attached to a biological light microscope.[19]

4. RESULTS

The present investigation revealed non-significant changes in the control and vehicle groups of all the parameters studied.

4.1 Body Weight & Organ Weight

The present study revealed significant reduction ($p<0.005$) in the body weight of treated animals (Group III) when compared to control (Group I) after 21 days. Similarly, a highly significant decline ($p<0.001$) in the testes weight was noted in the treatment group (Group III) when compared to control (Group I) after 21 days of duration. However, non-significant changes in weight of testes were observed in vehicle control (Group II) when compared to control (Group I) (Table-1).

4.2 Total Protein

The existing study showed non-significant changes in the protein levels of the testis in the vehicle group (Group II) when compared to control (Group I), whereas a highly significant reduction ($p<0.001$) in the protein content was observed in the treated group (Group III) when compared to control (Group I) after the period of 21 days (Table-2).

4.3 Cholesterol

A highly significant decline ($p<0.001$) in the cholesterol content of the testis was witnessed in the treated animals (Group III) when compared to control (Group I) after 21 days of duration. However, the changes in the vehicle group (Group II) were found to be non-significant when compared to control (Group I) (Table-3).

In the present investigation a non-significant decrease was observed in the 3β HSD activity of the testis in the vehicle group (Group II), while, a highly significant decrease ($p<0.001$) was noted in the treated group (Group III) as compared to control (Group I) after the duration of 21 days (Table-4).

4.4 3 B Hydroxysteroid Dehydrogenase (3 B Hsd)

4.5 17 B Hydroxysteroid Dehydrogenase (17 B Hsd)

The existing study demonstrated non-significant alterations in the 17β HSD activity of the testis in the vehicle group (Group II), while, a significant decline ($p<0.005$) was noted in the treated group (Group III) as compared to control (Group I) after the duration of 21 days (Table-5).

Table 1 Body Weight (Gm) And Organ Weight (Mg) Of Control, Vehicle And Treated Mice After 21 Days.

Groups	Duration (21 Days)	
	Body Weight	Organ Weight (Testes)
Control (Group I)	37.00 ± 0.96	313.9 ± 4.79
Vehicle (Group II)	36.67 ± 0.84 ns	313.4 ± 5.06 ns
PER treated group (Group III)	32.17 ± 0.65**	235.7 ± 11.98***

*Values are mean ± S.E., *p<0.033, **p<0.002, ***p<0.001, NS=non-significant*

Table 2 Total protein concentration (mg/100 mg tissue weight) in testis of control, vehicle and treated mice after 21 days.

Groups	Duration (21 Days)
	Testis
Control (Group I)	8.766 ± 0.28
Vehicle (Group II)	8.756 ± 0.27 ns
PER treated group (Group III)	3.323 ± 0.14 ***

*Values are mean ± S.E., *p<0.033, **p<0.002, ***p<0.001, NS=non-significant*

Table 3 Cholesterol Content (Mg/100 Mg Tissue Weight) In Testis Of Control, Vehicle And Treated Mice After 21 Days.

Groups	Duration (21 Days)
	Testis
Control (Group I)	0.3867 ± 0.01
Vehicle (Group II)	0.3767 ± 0.01 ns
PER treated group (Group III)	0.2633 ± 0.008 ***

*Values are mean ± S.E., *p<0.033, **p<0.002, ***p<0.001, NS=non-significant*

Table 4 3 β hsd activity (mg protein/min) in testis of control, vehicle and treated mice after 21 days.

Groups	Duration (21 Days)
	Testis
Control (Group I)	0.2433 ± 0.007
Vehicle (Group II)	0.2367 ± 0.008 ns
PER treated group (Group III)	0.1917 ± 0.004***

*Values are mean ± S.E., *p<0.033, **p<0.002, ***p<0.001, NS=non-significant*

Table 5 17 B Hsd Activity (Mg Protein/Min) In Testis Of Control, Vehicle And Treated Mice After 21 Days.

Groups	Duration (21 Days)
	Testis
Control (Group I)	0.1700 ± 0.007
Vehicle (Group II)	0.1600 ± 0.008 ns
PER treated group (Group III)	0.1232 ± 0.009 **

*Values are mean ± S.E., *p<0.033, **p<0.002, ***p<0.001, NS=non-significant*

4.6 Histopathological Analysis

Histopathololological studies provide an important benchmark for identifying the action of a toxicant or contaminant by studying the structure of the test organ. The interpretation of the same helps in providing the new insights in the toxicity studies. Testicular sections of the control mice demonstrated normal histoarchitecture showing well-built seminiferous tubules with germinal epithelial cells and intact basement membrane as well as connective tissue. The lumen also possessed bundles of sperms in their different developmental stages. Leydig cells were also fully developed (Fig I & II). Similar results were found for the vehicle group showing intact structures with normal histology.

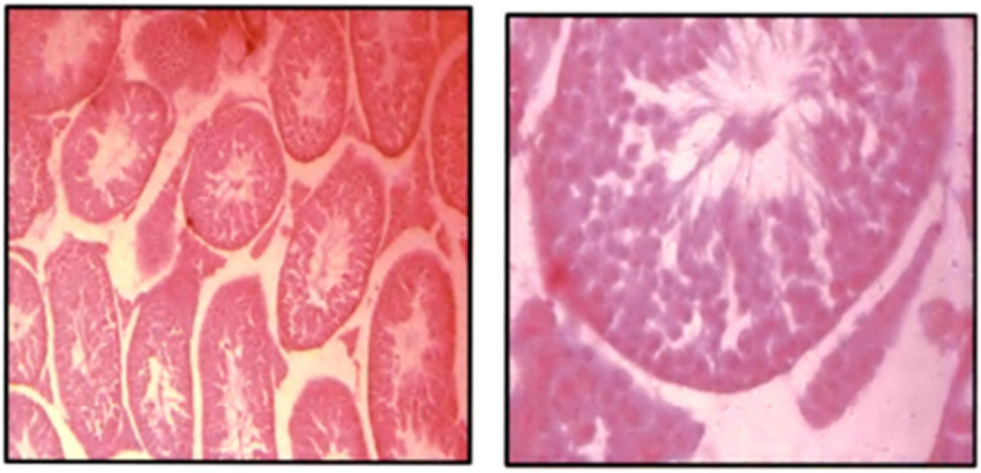

Fig: I & II Transverse sections (T.S) of testis of control mice (*Mus musculus*). (Haematoxylin-Eosin stain, 5µ section)

However, in the group of animals where Permethrin was administered at 130 mg/kg bw for 21 days, histology of the testis was comparable to the testis of control mice. The sections showed altered, damaged and distorted histoarchitecture. The tubular arrangement was highly deformed and showed degenerative variations in the germinal epithelium and severe necrosis in seminiferous tubules. Increase in the interstitial space, deficiency of germ cells in the seminiferous tubules and lack of mature spermatozoa were also observed at higher magnifications (Fig III & IV).

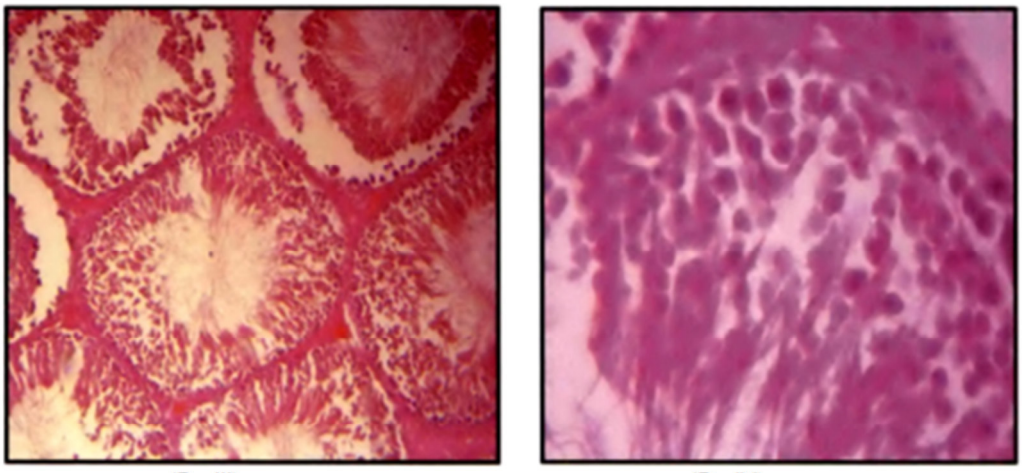

Fig III & IV Transverse sections (T.S) of testis of Permethrin treated mice (130 mg/kg body weight) for 21 days. (Haematoxylin-Eosin stain, 5µ section)

5. DISCUSSION

The present study revealed that administration of Permethrin (130 mg/kg body weight) to male mice for 21 days resulted in reproductive toxicity as confirmed by the biochemical assays and histopathological studies. Monitoring body weight during treatment provides an index of the general health status of the animals, and such information is important for the interpretation of reproductive effects[20] The present study demonstrated that oral administration of Permethrin to male Swiss albino mice caused a decrease in feed consumption. This hypophagia can be attributed to the decrease in metabolism, or inhibition of hunger resulting in a lack of appetite or anorexia.[21] Results are in agreement with the previous report which has also noticed reduced body weight in male rats after technical grade formulations of deltamethrin and cypermethrin.[22] Moreover, the weight of the male reproductive gonad *i.e.* Testes were also declined. The weight of testes is largely dependent on the mass of differentiated spermatogenic cells and reduction in weight of testes, as revealed in this study may be due to reduced tubule size, decreased number of germ cells, and elongated spermatids.[23] Our results were in agreement with Yousef (2010)[24] which reported that exposure to lambda cyhalothrin

for 16 weeks resulted in reduced testicular weight in male rabbits. Proteins which are building blocks in organisms are considered as an essential parameter to determine the physiological status of animal.[25] Along with reduced body weight and organ weight that was noted, simultaneous reduction in total protein was also recorded. Reduced food intake by animals after pyrethroid treatment results in utilization of amino acids for energy generation by catabolism of proteins through gluconeogenesis which leads to decreased protein content.[26] Similarly Moid *et al.* (2014)[27] also reported a decrease in the protein content of liver, spleen, and small intestine after treatment with technical grade deltamethrin after a duration of 45 days. Cholesterol is an important precursor which is involved in the synthesis of androgens and other steroidogenic hormones. In the present work significant upsurge was recorded in cholesterol content of the testis after 21 days of Permethrin exposure. According to Moid *et al.* (2014)[27] , elevated cholesterol level might be attributed to cholesterol accumulation or impaired turnover and may be due to decreased utilization of cholesterol under stress. Remai *et al.* (2008)[28] have also suggested that the elevation of cholesterol levels may be attributed to enhanced cholesterol and triglyceride synthesis and/or reduced cholesterol catabolism. The results are in accordance with the work of Uchendu *et al*. (2018)[29] who reported increased cholesterol content in Wistar rats after treatment with chlorpyrifos and deltamethrin simultaneously. 3β and 17β-hydroxysteroid dehydrogenase (HSD) are both essential enzymes active in testes and involved in the production of testosterone from the androstenedione. In the present investigation, animals administered with Permethrin revealed reduced activity of 3β and 17β hydroxysteroid dehydrogenase (HSD) in testis. Increase in cholesterol level and decrease in activities of 3β and 17β hydroxysteroid dehydrogenase (HSD) are suggestive of adverse effect of the said pesticide on testicular steroidogenesis, which further leads to changes in structure and functions of reproductive organs as obtained in the present study. Similar decline in the activity of 3β and 17 β HSD in testis was reported by Ksheerasagar and Kaliwal (2013)[30] by the use of Carbosulfan treatment for 20 and 30 days on albino mice. Aly and Azhar (2013)[31] also noted that oral administration of methoxychlor showed a significant reduction in 3β-HSD and 17β-HSD activities as compared to the corresponding control. Further, reduced levels of these dehydrogenases eventually can result in impaired reproductive functions in male leading to infertility.

6. CONCLUSION

The pilot study carried out in the present investigation demonstrated the toxicity of Permethrin on the male reproductive gonad, Testes, which is furthermore confirmed by the histopathological analysis. This suggests that pesticide exposure could be one of the trivial factors leading to infertility, as these are encountered on an everyday basis and on several magnitudes. Moreover, this demands further investigations to find effective ameliorative agents to combat the said toxicity and thereby prevent reproductive toxicity. Also, stringent laws should be introduced on the sale of these pesticides and their use should be properly monitored.

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8. AUTHORS CONTRIBUTION STATEMENT

Zeba Siddiqui designed, performed the experiments and analysed the data and Ketaki Desai helped her in the manuscript preparation.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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