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RESEARCH ARTICLE

EXTENDED ONE GENERATION REPRODUCTIVE TOXICITY STUDY OF THIAMETHOXAM TECHNICAL IN RATS

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ABSTRACT

Thiamethoxam, a second-generation neonicotinoid insecticide was assessed for its systemic and reproductive toxicity, developmental neurotoxicity and immunotoxicity through an extended one generation toxicity study (EOGRTS, OECD No. 443). Wistar rats were exposed to Thiamethoxam at doses of 0, 15, 50 and 150 mg/kg/day by oral intubation. Treatment of males was initiated 2-weeks before cohabitation and continued for 70 consecutive days whereas treatment of females began 2 weeks before cohabitation and continued until weaning of the F1 offspring. In view of reducing the number of animals, but without compromising on parameters to be assessed, cohort 1A was covered in the parental generation. Treatment with Thiamethoxam technical did not reveal any systemic/reproductive toxicity and abnormal changes in the fertility parameters of rats belonging to parental generation. However, males belonging to the F1 generation exhibited delay in the acquisition of balano-preputial separation and, decreased total and ambulatory counts at 50 and 150mg/kg/day. A decrease in the overall width of hippocampus was observed at 150mg/kg/day. The T-cell dependent antibody response (TDAR) functional assay exhibited the ability of Thiamethoxam to mount an antibody (IgM and/or IgG) response up to 150 mg/kg/day in the F1 generation. Based on findings, the No Observed Adverse Effect Level (NOAEL) of Thiamethoxam was 15 mg/kg/day for developmental and neurobehavioral end points and 150 mg/kg/day for reproductive toxicity.

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INTRODUCTION

The two-generation reproduction toxicity study (OECD Guideline for the Testing of Chemicals No. 416) is considered to be the best approach towards the assessment of reproductive toxicity. The design is complex and the number of animals required for the study are more. OECD has redacted the one-generation reproductive toxicity study design (OECD Guideline for the Testing of Chemicals No. 415) which was largely disfavored for regulatory purposes, since it does not cover the full reproductive cycle and was not updated in relation to the developing science (Nigel, Susanne *et al*. 2009). On the other hand, the extended one-generation reproductive toxicity study (EOGRTS) is a modular test method where breeding and assessment of reproductive toxicity is covered up to the F2 generation. Testing for developmental neurotoxicity (DNT) and developmental immunotoxicity (DIT) could also be added as distinct and independent modules (ECHA 2016; Fegert *et al* 2012; John *et al*. 2006). Neonicotinoids are a new class of insecticides with widespread use in veterinary medicine and crop production. The neonicotinoid insecticides include Imidacloprid, Acetamiprid, Dinotefuran, Thiamethoxam, and Clothianidin. Neonicotinoid compounds are highly specific for subtypes of nicotinic receptors that occur in insects. The neonicotinoids act on postsynaptic nicotinic receptors. These receptors are located entirely in the central nervous system of insects (Steve 2018). The toxicity of Thiamethoxam has previously been characterized in a series of toxicity studies (Shehata *et al*. 2010; Hassina *et al*. 2017; Pâmela *et al*. 2019). This extended one-generation reproductive toxicity study was carried out to evaluate the pre-natal and post-natal effects of Thiamethoxam on development, as well as the evaluation of systemic toxicity in pregnant and lactating females and the young and adult offspring. In addition, the study provides information about the effects of Thiamethoxam on the integrity and performance of the adult male and female reproductive systems including gonadal function, the estrous cycle, epididymal sperm maturation, mating behavior, conception, pregnancy, parturition, and lactation. The study was also designed to assess developmental neurotoxicity and immunotoxicity and the study also contributed in streamlining capabilities towards validation and execution of various procedures carried out in the study as required by OECD guideline 443.

MATERIALS AND METHODS

The methods followed were in line with the requirements as promulgated in OECD guideline 443. Technical-grade Thiamethoxam was used in the study. Animal usage was reviewed and approved by the Institutional Animal Ethics Committee (the Compendium of CPCSEA, 2018).

Male and female Wistar IGS Rat Crl: WI Outbred rats, approximately 9 weeks of age (Hylasco Bio-Technology (India) Pvt. Ltd., were acclimated to the laboratory (AAALAC NRC. 2011) for 7 days before study initiation. The animal room was maintained at 22°C ± 2°C. 60% ± 5% relative humidity, and 12/12- hour light/dark cycle. Food (Certified Lab Diet no. 5L79. PMI Nutrition International. USA) and drinking water were available ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee of Vimta labs. Ltd.

Housing, Water and Diet: Animals of same sex were housed in a clean, autoclaved polysulfone/polycarbonate cages (Length 425 mm x Breadth 266 mm x Height 185 mm) with stainless steel top grill having provision for holding pellet food and drinking water in polycarbonate bottles. Autoclaved corn cobs were used as the bedding material. Cages and water bottles were changed at least 2 times a week. The animals were fed with standard pellet laboratory animal diet (Krishna Valley Agro Tech LLP., Pune, India) *ad libitum*. Fresh potable drinking water processed through a reverse osmosis system was provided *ad libitum* to animals in water bottles *via* sipper tube. Feed, water and bedding material were analyzed for every batch for the presence of any microbial and chemical contaminants.

Study Design: Each parental group consisted of 10 males and 10 female Wistar rats. The test item was administered to parental male rats once daily beginning 2 weeks at 0, 15, 50 and 150 mg/kg/day before cohabitation and continued through mating followed by repeated exposure for 70 days (covering one spermatogenic cycle). In parental female rats, the test item was administered once daily at 0, 15, 50 and 150 mg/kg/day beginning 2 weeks before cohabitation and continued up to weaning of first filial (F1) offspring. Following 2 weeks of treatment, each female was cohabited with a single male from the same treatment group until confirmation of mating. Parental dams were sacrificed following weaning of F1 litters on lactation day (LD) 21. In view of reducing the number of animals, the parameters to be assessed in Cohort 1A was covered in the parental generation and hence a separate cohort 1A was not maintained. Second generation (F2) was excluded (Cohort 1B) as there were no triggers in parental generation. to carry out the same were observed. One male and one female per litter per dose group were randomly selected (10/sex/group) and allotted to Cohort 2A for continuation of treatment for developmental neurotoxicity testing in adults. Similarly, one male and one female per dam were allotted to Cohort 2B for developmental neurotoxicity assessment at weaning. F1 offspring (5 males and 5 females/group) were selected for Cohort 3 for developmental immunotoxicity and were treated from the day of weaning until PND 56. Schematic design of study is provided in Figure 1.

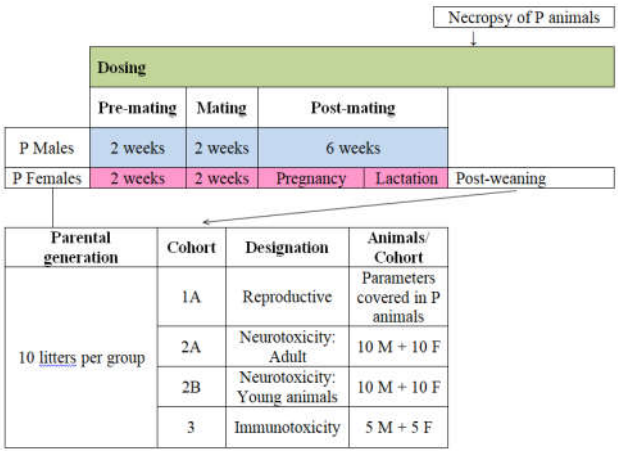

Figure 1. Experimental Design

Dose Administration – Parental Animals: Test item formulations were administered to male rats of specific groups (as mentioned in section 3.1) for 2 weeks prior to the mating, 2 weeks during mating and 6-weeks post mating period (to cover one complete spermatogenic cycle). Males were sacrificed after the 10-weeks treatment period. In females, test item formulations were administered to specific groups 2 weeks prior to mating (which covers 3-4 complete estrous cycles in order to detect any adverse effects on cyclicity). Treatment was continued during mating, throughout pregnancy and up to the weaning of the F1 offspring, after which parental females were sacrificed.

Dose Administration - F1 Animals

Cohort 2A: The test item formulations were administered to F1 offspring from weaning (PND 21) to PND 90. The animals were sacrificed on PND 91 ± 5 days.

Cohort 2B: Test item formulations were not administered to F1 offspring but were sacrificed upon weaning (LD 21).

Cohort 3: Test item formulations were administered to F1 offspring from weaning (PND 21) to PND 56 at the highest dose level of 150 mg/kg. The animals were sacrificed on PND 57.a concurrent vehicle control group was maintained. A positive control group was also maintained. Animals belonging to positive control were treated with cyclophosphamide at the dose level of 6 mg/kg throughout the treatment period. The antigen (KLH – Keyhole Limpet Hemocyanin) was used to immunize the animals selected for Cohort 3. KLH was dissolved in sterile water for injection to obtain the concentration of 10 mg/ml. Then subsequently KLH solution was prepared at 1 mg/mL and was used for dose administration.

Mating Procedure: Each female was placed with a single male from the same group one female to one male, until evidence of copulation was observed. All the mated females were maintained until they littered or for a maximum period of 25 days from the last day of cohabitation. Day '0' pregnancy was confirmed by the presence of sperm in the vaginal smear/vaginal plug. Subsequently, pregnant females were housed individually until weaning/sacrifice.

Clinical Observations: All animals were observed twice daily for signs of toxicity, morbidity, and mortality. P1 females were examined at the time of expected parturition for signs of dystocia, while dams were observed for abnormalities in nesting behavior, nursing, or failure to care for litters. Animals were observed weekly, for changes in skin and fur, eyes, mucous membrane, occurrence of secretions and excretions, autonomic activity, changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes and bizarre behavior.

Body Weights and Food Consumption: Individual animal body weights for all the groups of parental, Cohorts 2A and 3 were recorded once on the day of commencement of treatment respectively and at weekly intervals (± 2 days) thereafter. All dams were weighed on gestation days 0, 7, 14 and 20 and on lactation days 1, 4, 8, 14 and 21, and weights were recorded. Food consumption was measured at weekly intervals. Generally, feed consumption measurements were determined on the same day during which body weights were collected.

Estrous Cycle: Estrous cycle length and normality was evaluated daily by vaginal smears for all P females starting 2 weeks prior to mating.

Parturition: The duration of gestation was calculated from Day 0 of pregnancy to the day of parturition (gestation length).

Litters: At birth, all the pups (both dead and alive) in a litter from each dam were observed for any external deformities. The number of pups born (litter size), individual sex and body weight of male and female pups on Days 1 and 4 were recorded. The anogenital distance (AGD) of each pup was measured on PND 4 before standardization and pup body weight was measured. On Day 4 after birth, the size of each litter was adjusted by eliminating extra pups by random selection

to yield, as nearly as possible, 4 male and 4 female pups per litter. This adjustment was done only when the litter size was more than 8. Partial adjustment in selection of equal sex ratio was done (for example 5 males and 3 females) when the number of male or female pups is less than 4, per litter per sex. Adjustment was not made for litters of less than 8. After standardization of litter size to 8 pups, the pups were weighed individually on Days 8, 14 and 21 of lactation. The body weight of pups selected for Cohort 2A was also recorded at the time of vaginal patency or balano preputial separation and at termination. The number of nipples/areolae in male pups was recorded on PND 12. The pups were weaned on day 21 of lactation period. Fertility index for dams, sires as well as the pup survival index were calculated. The age of vaginal opening and preputial separation was determined for F1 weanlings selected for Cohort 2A. After standardization of the litters, remaining pups were sacrificed. No observations were carried out on the sacrificed pups.

Clinical Pathology: Clinical Pathology investigations were performed for all parental animals at termination. And standard hematology, clinical chemistry and coagulation parameters were measured.

Urinalysis: Urine was collected from animals using metabolic cages one day prior to scheduled necropsies from all animals.

Sperm Parameters: Sperm evaluation was carried out at termination for all the surviving parental males. At necropsy, right epididymis was collected for sperm count and the right epididymis was collected for evaluation of sperm motility and sperm morphology.

Neurobehavioral Examination: Neurobehavioral observations were carried out in animals belonging to Cohort 2A between PND 63 and PND 75. During home cage observations, animals in the cage were observed for body position and convulsions. Handling observations included palpebral closure, lacrimation, eye examination, piloerection and salivation. Open field observations were carried out for all animals in an open field arena under a clean absorbent paper. Animals were observed for 2 minutes for gait, mobility, arousal, respiration, tonic and clonic movements, stereotypic behavior, bizarre behavior, urination, defecation, vocalizations and rearing. Sensory reactivity and neuromuscular measurements were performed on all animals belonging to Cohort 2A between PND 63 and PND 75.

Sensory reactivity of all animals was assessed by subjecting them to approach response, touch response, click response, tail pinch response and pupil response measurements. Air righting reflex is carried out by holding the animals a supine position and dropping from a height of approximately 30 cm. The ease and uprightness of landing was recorded in a single score as normal or abnormal. The fore limb and hind limb grip strengths of the animals were measured. The fore and hind limb grip strength of each animal was measured 3 consecutive times and average values were calculated. To measure hind limb foot splay, the tarsal joint pad of each hind foot of the animals was marked with non-permanent non-toxic ink. The animals were suspended in a prone position and dropped from a height of approximately 30 cm on to a recording sheet. The distance between both the hind limb foot impressions while landing was measured. The procedure was repeated three times and the average was calculated. Motor activity (total and ambulatory activities) was evaluated in animals of Cohort 2A during PND 63 and PND 75 of treatment periods. Animals were monitored for 3 consecutive ten minutes' intervals allowing examination of exploratory and acclimatization activity levels. Stereotypic activity was calculated by subtracting ambulatory activity from total activity. An auditory startle test was performed in animals of Cohort 2A on PND 24 (± 1 day) using Responder-X instrument.

T-Cell Dependent Antibody Reaction: F1 offspring from Cohort 3 (5/sex/group) were evaluated for immune function using the T-Cell Dependent Antibody Reaction (TDAR) assay. On PNDs 45 and 50, F1 offspring were immunized with intravenous injections of KLH (300 µg/injection/rat). Five animals per sex from similarly aged satellite animals served as a concurrent positive control group (immunized with 6 mg/kg/day Cyclophosphamide (CP) by oral route. Blood was collected from individual rats on Days 48, and 56 under isoflurane anesthesia.

Gross Pathology, Organ Collection and Organ Weight: All surviving parental, Cohort 2A and Cohort 2B animals were euthanized by CO2 asphyxiation followed by exsanguination. The animals were subjected to detailed gross pathological observation during necropsy.

Histopathology

Histopathology of Parental Animals: Histopathology examination was carried out on the preserved organs of vehicle control and high dose group of parental animals. In addition, all gross lesions were examined microscopically.

Histopathology of Cohort 2A Animals: Perfusion fixation was done for Cohort 2A animals. Morphometric evaluation of brain was performed.

Brain Morphometry: Whole body perfusion fixation was performed for all animals belonging to Cohort 2A. The brain sections were examined under a microscope with a camera and imaging software (DP2-BSW). Histology images were captured at the desired section. Microscopic linear measurements were taken at representative locations to estimate the thickness of major layers, which included cerebral cortex and corpus callosum thickness and cerebellum thickness. Brain measurements for bilaterally symmetrical structures were taken, wherever feasible.

Histopathology of Cohort 2B Animals: Histopathological examination of the brain was carried out for vehicle control and high dose group animals. A 5-step grading system of minimum, mild, moderate, severe and marked was used to rank microscopic findings for comparison among groups.

RESULTS

Systemic Toxicity

General Observation / Detailed Clinical Examination: All animals survived the entire treatment period. There was no morbidity throughout the study period. All animals belonging to Thiamethoxam treated and control groups were found to be normal and free from any visible abnormalities throughout the study period. Detailed clinical examination did not reveal any changes in skin, fur, eyes, mucous membrane, occurrence of secretions and excretions, autonomic activity such as lacrimation, piloerection, pupil size, unusual respiratory pattern, changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes and bizarre behavior.

Body Weight and Feed consumption: In the EOGRTS study, systemic toxicity was assessed across life stages. Body mass did not differ markedly between treated and control group P1 generation males and females throughout the treatment period. Similarly, there were no treatment related changes in body weight in the F1 pups, F1 pubertal females and F1 males as compared to the control group (Figure 2). Food consumption was unaffected in all the animals belonging to different generations throughout the treatment/observation periods (data not shown).

Clinical Pathology: Parental animals treated at the dose levels of 15, 50 and 150 mg/kg of the test item did not reveal any changes in hematological (Figure 3), coagulation, clinical chemistry (Figure 4) and urinalysis parameters tested as compared to the vehicle control. Gross Pathology and Organ weights.

Figure 2a – Males

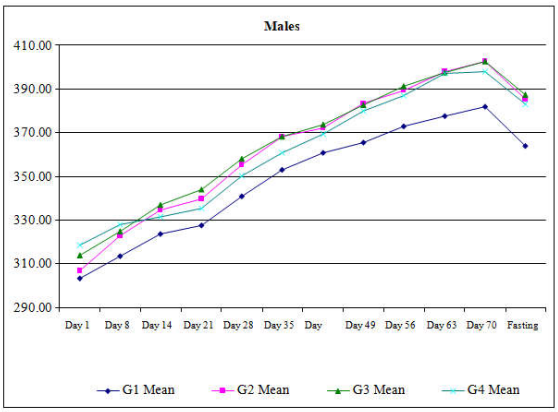

Figure 2b – Females

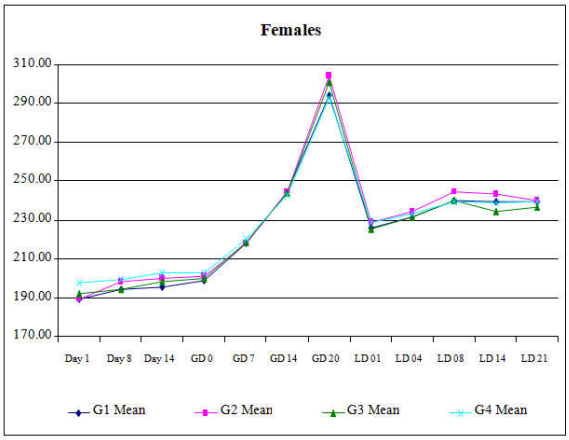

Figure 2. Summary of Body Weights (g)

Figure 3a: Hematology - Males

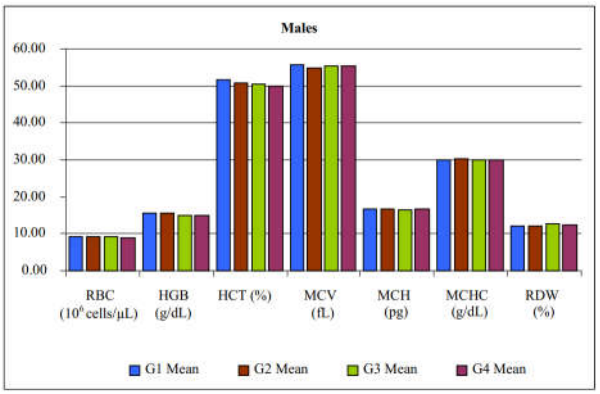

Figure 3b: Hematology – Males Continued

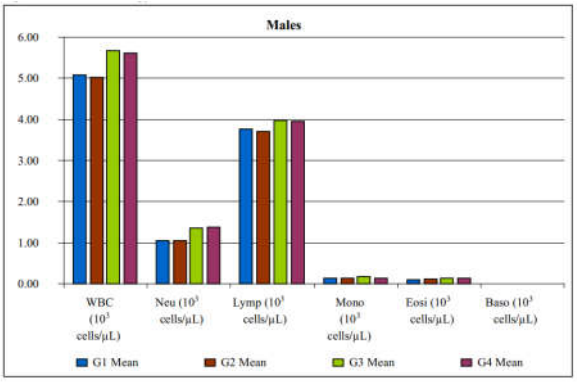

Figure 3c: Hematology – Males Continued

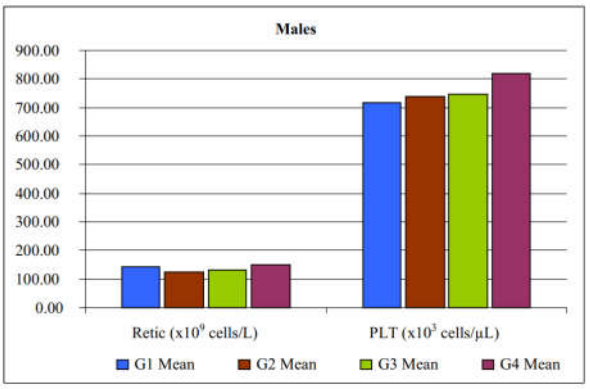

Figure 3d: Hematology – Females Continued

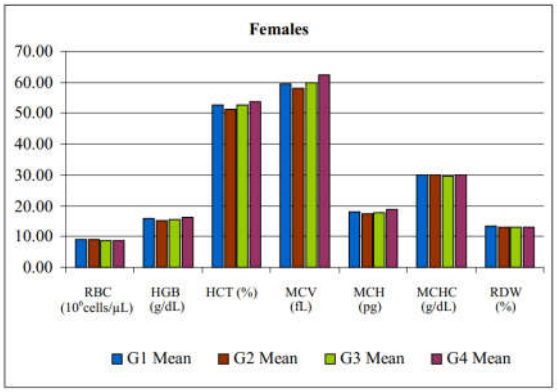

Figure 3e: Hematology – Females Continued

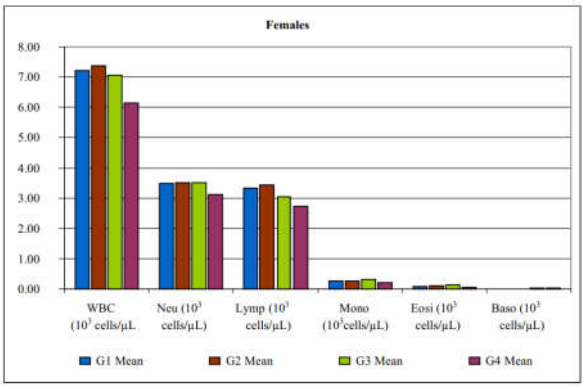

Figure 3f: Hematology – Females Continued

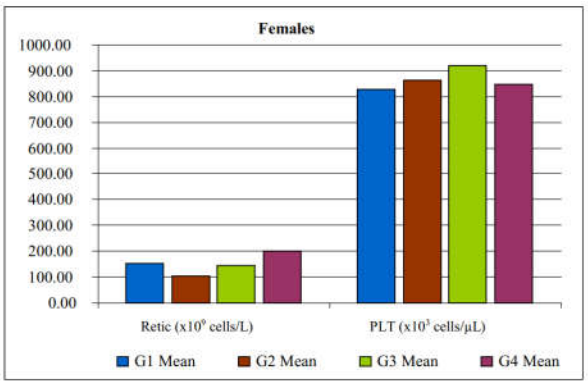

Figure 3. Summary of Hematology Values

No treatment related changes were observed in absolute and relative organ weights in treated group animals when compared to control. Gross pathological observations did not exhibit any lesions attributable to treatment. No spontaneous lesions were observed (Figure 5).

Figure 4a: Clinical Chemistry – Males

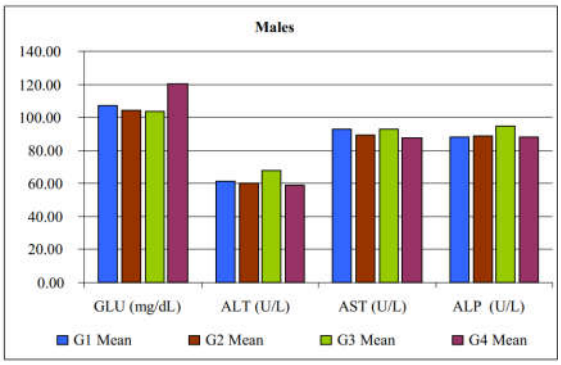

Figure 4b: Clinical Chemistry – Males

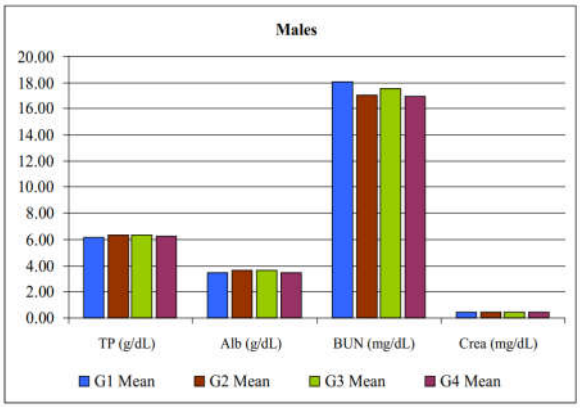

Figure 4c: Clinical Chemistry – Females

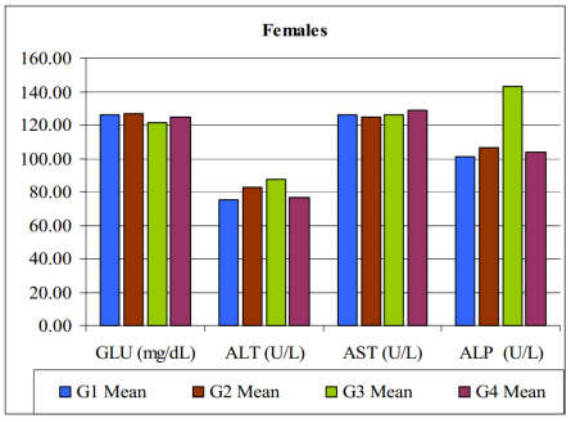

Figure 4d: Clinical Chemistry – Females

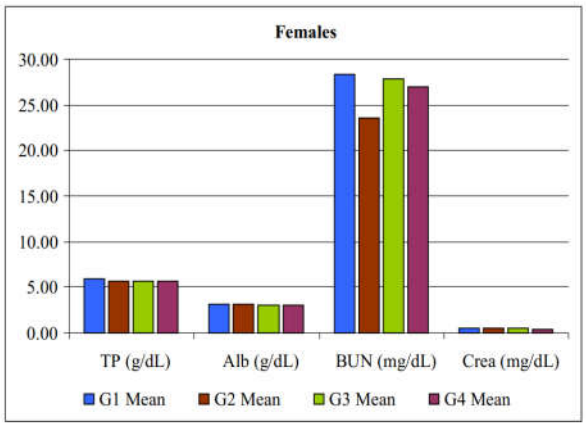

Figure 4. Summary of Clinical Chemistry Values

Figure 5a: Relative Organ Weight - Males

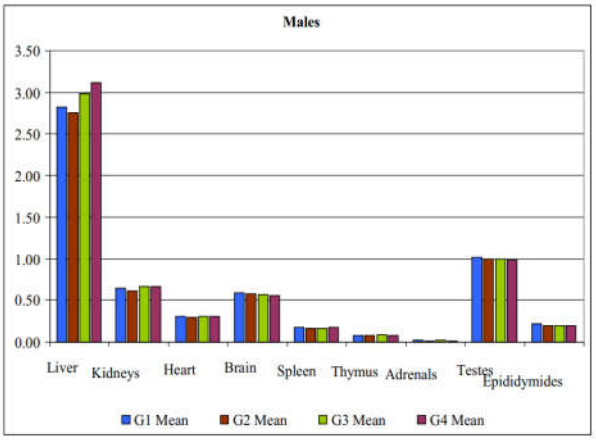

Figure 5b: Relative Organ Weight – Females

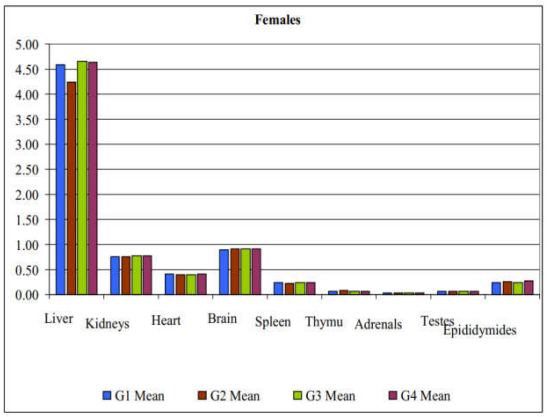

Figure 5. Summary of Relative Organ Weights (%)

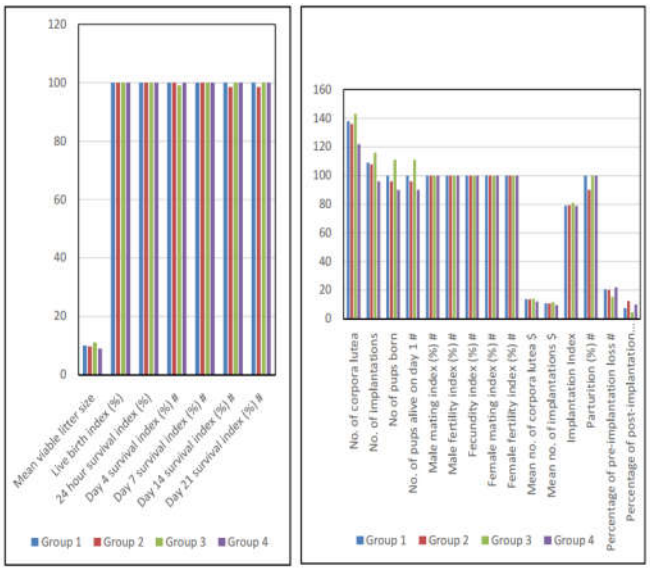

Figure 6. Summary of Survival Data of Pups and Fertility Indices

Histopathology: No histopathological findings attributable to treatment were observed in high dose (150 mg/kg) treated male and female animals.

F1 Generation: There was no mortality and morbidity throughout the study period. All animals from vehicle control and test item treated groups were found to be normal throughout study period. Body weights and feed consumption were comparable between treated and control groups.

Reproductive Toxicity

Parental Generation: There were no notable changes in the estrous cycle length between control and treatment groups. The mean weight (Figure 7) and number of male and female pups and the mean number of pups for combined sex were unaffected by the treatment at all the doses tested. No changes attributable to test item were detected in the AGD, body weight and ratio of AGD to the cube root of body weight of either sex. Areola/nipple retention in male pups was normal on PND 12.

Figure 7a: Mean Weight of Male pups

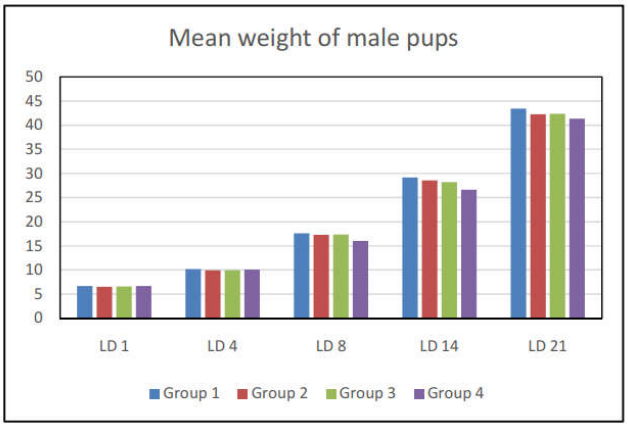

Figure 7b: Mean Weight of Female pups

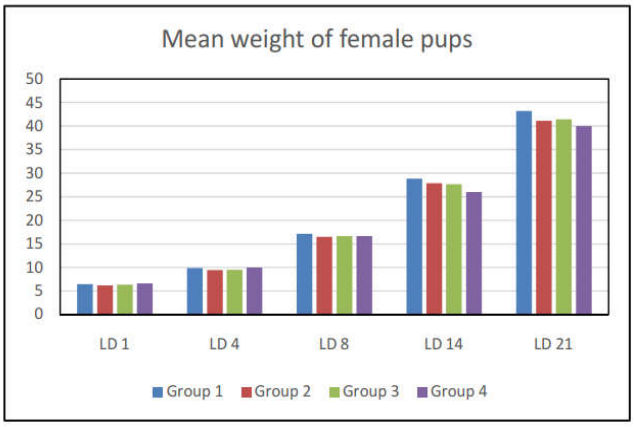

Figure 7c: Mean Weight of Male and Female pups

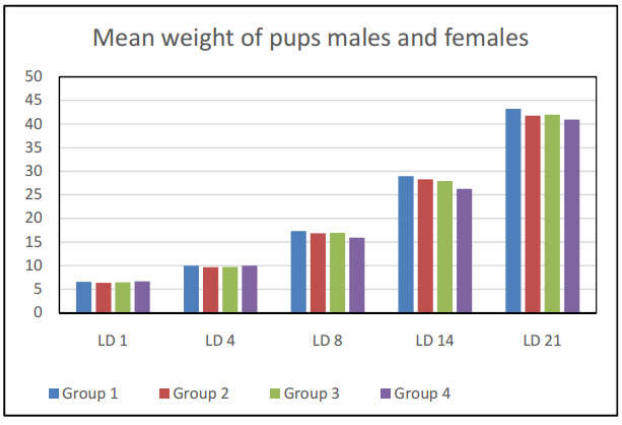

Figure 7. Summary of Mean Weight of Pups during Lactation Period

There were no external abnormalities in live pups in any of the groups. All pups survived up to LD 21 at all the tested doses (Figure 6). Test item had no treatment-related effects on the number of pregnancies, number littered and mean litter size. The mean pre-coital time and gestation length at all the tested doses were comparable to controls. Mean implantations and percentage of pre- and post-implantation loss were comparable to control (Table 1).

Table 1. Summary of Litter Data

Particulars	Group & Dose (mg/kg b.w.)			
	G1 (0)	G2 (15)	G3 (50)	G4 (150)
No. of pregnancies #	10	10	10	10
No. of dams dead	0	0	0	0
No. of littered #	10	9	10	10
No. of live litters #	10	10	10	10
Total no. of pups born	100	96	111	90
Mean litter size $	10.0	9.6	11.1	9.0
No. of pups dead on day 1 #	0	0	0	0
No. of live pups on Day 1 #	100	96	111	90

Key: # = Compared by 'Z' test, $ = Compared by 't' test

Cohort 2A

Vaginal Patency: Vaginal patency was evaluated for a visible break in the membranous sheath covering the vaginal orifice. The mean age at acquisition of vaginal patency were not affected by the treatment when compared to control (Figure 8).

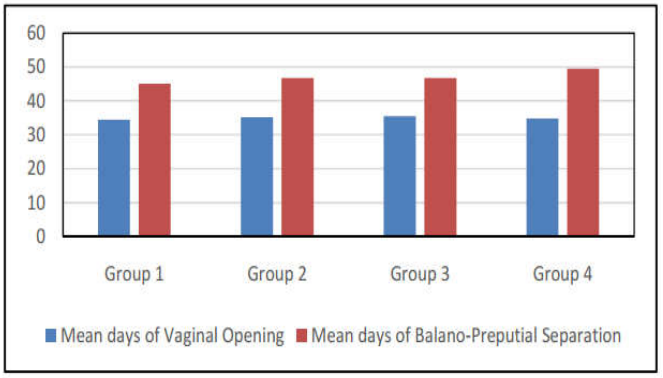

Figure 8. Vaginal Opening and Balano-Preputial Separation

Balano-preputial Separation: A delay in balano-preputial separation was observed in male pups by one day in G2, two days in G3 and three days in G4 group when compared to control (Figure 8). *Neurobehavioral Observations:* Home cage, handling and open field observations of treated animals were comparable to control group. All animals displayed normal gait throughout the treatment period. No clonic or tonic movements, stereotypic movements or bizarre behavior were observed during handling and open field observations throughout the study period. All animals of treated groups as well as the control groups displayed normal approach, tail pinch, touch, click and pupil responses. Air righting reflex of all the animals of both the control and treated groups were found to be normal. No alterations in the fore and hind limb grip strength were recorded in any of the treated group animals as compared to concurrent control groups. The hind limb foot splay of all treated groups was comparable to concurrent control groups. Administration of test item did not cause significant alterations in the total, ambulatory and stereotypic activities of treated rats as compared to control group animals. However, male rats displayed significant decrease in the total and ambulatory counts in mid and high dose as compared to control group animals.

Startle Response: Acoustic startle response at 65 to 120 db was decreased in high dose male animals when compared to control. However, in females, no treatment related changes were observed (Figure 9).

Gross Pathology: No gross pathological findings were observed in both sexes during terminal sacrifice.

Figure 9a: Acoustic Startle Response of Male pups

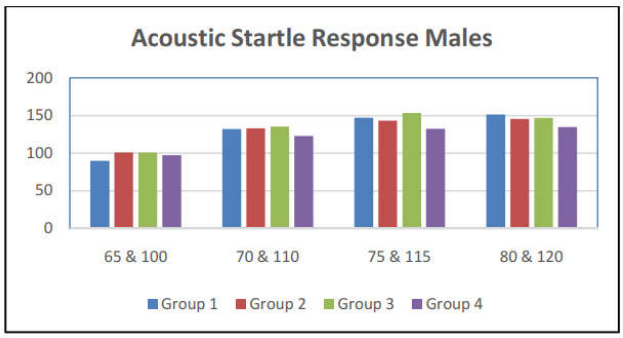

Figure 9b: Acoustic Startle Response of Female pups

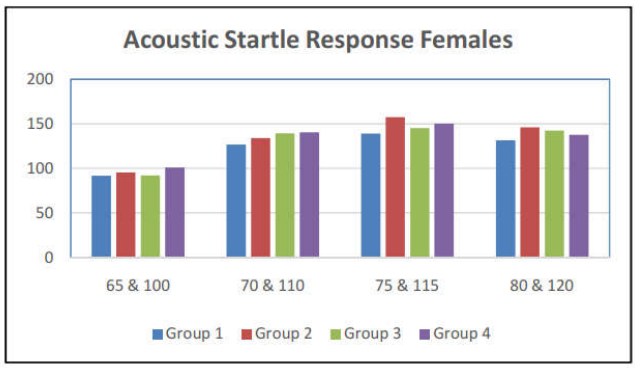

Figure 9. Acoustic Startle Response

Sperm Evaluation: There were no treatment related changes observed in sperm motility, sperm morphology or cauda epididymal sperm count (Figure 10 & Figure 11).

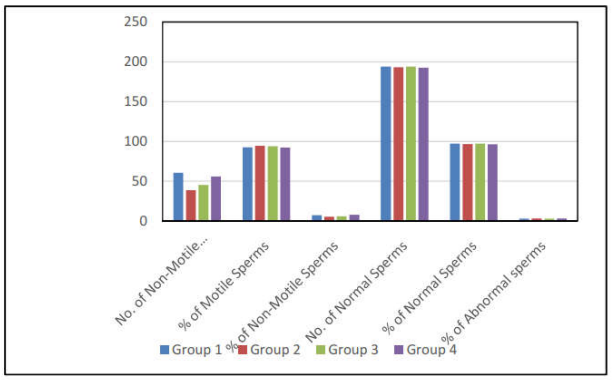

Figure 10. Summary of Epididymal Sperm Motility and Sperm Morphology

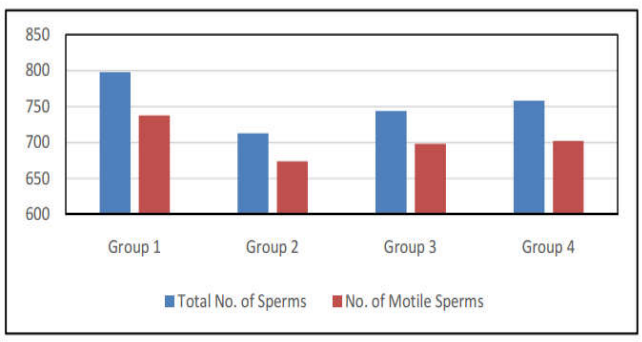

Figure 11. Summary of Epididymal Sperm Count

Histomorphometry: Histomorphometric analysis revealed a decrease in overall width of hippocampus of high dose treated adult animals (150 mg/kg) as compared to concurrent control animals. The overall width of all other regions (cerebral cortex motor, cerebral cortex sensory, corpus callosum and cerebellum) was comparable to control (Figure 12).

Figure 12a: Histomorphometry of Brain Tissue Findings – Males

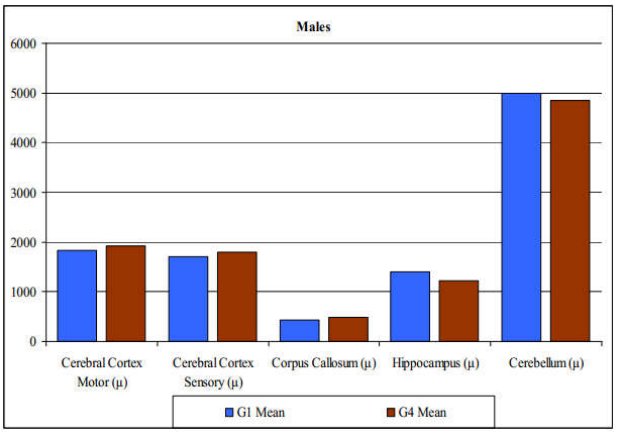

Figure 12b: Histomorphometry of Brain Tissue Findings – Females

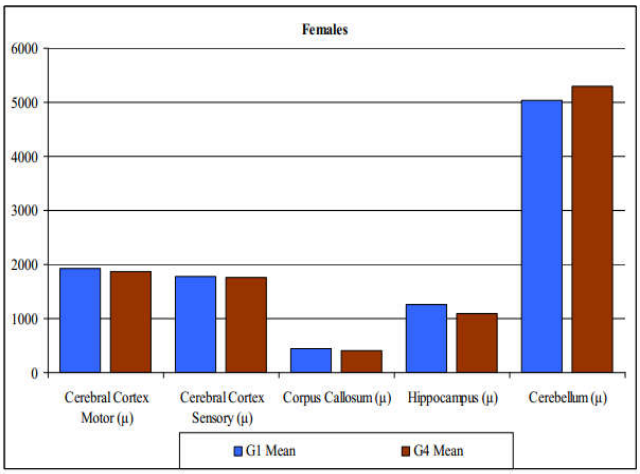

Figure 12.Histomorphometry of Brain Tissue Findings- (Cohort 2A)

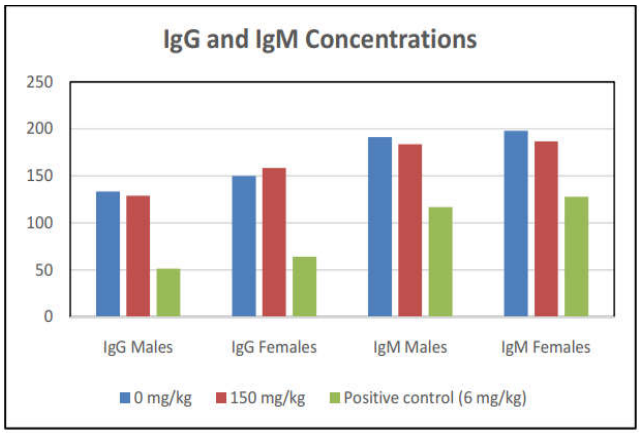

Figure 13. IgG and IgM Concentrations

Cohort 2B

Brain Weight: Absolute and relative organ weights of brain were comparable to vehicle control in both sexes.

Gross and Histopathology: Gross and histopathological observations did not reveal any lesions attributable to treatment in both sexes.

Cohort 3

Developmental Immunotoxicity: No Thiamethoxam related changes in the T-cell dependent antibody reaction (TDAR) were observed. Anti-KLH IgM and IgG development at the end of the dosing period (Day 56) was similar between control and Thiamethoxam treated animals (Figure 13).

DISCUSSION

Thiamethoxam was well tolerated in F0 (parental), F1 (first generation), and F2 (second generation) animals exposed at oral concentrations of 15, 50, and 150 mg/kg/day. Treatment with Thiamethoxam did not reveal any systemic/reproductive toxicity and abnormal changes in fertility parameters of rats belonging to parental generation. The T-cell dependent antibody response (TDAR) functional assay did not exhibit the ability to mount an antibody (IgM and/or IgG) response to treatment of Thiamethoxam up to 150 mg/kg in F1 generation. F1 generation males exhibited a delay in acquisition of balano-preputial separation, decreased total and ambulatory counts at 50 and 150 mg/kg/day along with a decreased overall width of hippocampus at 150 mg/kg/day. Based on the findings, the No Observed Adverse Effect Level (NOAEL) of Thiamethoxam was considered to be 15 mg/kg/day for development and neurobehavioral endpoints and 150 mg/kg/day for reproductive and immunotoxic endpoints. The study provided comparable toxicity findings to published findings and contributes supporting evidence for the validity of the procedure followed. The study provided empirical support for the effects of Thiamethoxam on the integrity and performance of the adult male and female reproductive systems including gonadal function, the estrous cycle, epididymal sperm maturation, mating behavior, conception, pregnancy, parturition, and lactation in addition to the developmental neurotoxicity and immunotoxicity endpoints and found to be a reliable methodology.

CONCLUSION

The present study adequately predicts the dose response relationship resulting from Thiamethoxam treatment and can potentially be applied to evaluate more comprehensive assessment of reproduction toxicity along with the guidance on the identification and exercise of triggers and waivers, in order to optimize the reduction and refinement possibilities.

Acknowledgements, avoiding identifying any of the authors prior to peer review

This is a note. The style name is Footnotes, but it can also be applied to endnotes.

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