MATERIAL AND METHODS

Description of Test Material

Test substance is a combination of pesticides i.e., deltamethroin 1% +triazophos 35%. It was procured from local market of Vapi, available in under the name of Spark 36% EC manufactured by Aventis Crop Science Ltd. Gujarat India

Description of Test System

Animals

Healthy adult rats *(rattus norvegicus)* of Wistar strain obtained from Breeding Facility, Jai Research Foundation (JRF) were used in the experiment. The animals were received into the experimental room and were allowed to acclimatize for a minimum period of five days prior to treatment. During this period animals were checked for their good health and behaviour. Animals were identified using picric acid (1.2% W/V) colour marking on the body coat (HM = Head mark, BM = Body mark, TM = Tail mark, LM = Leg mark and NM = No mark). The appropriate labels were attached indicating group, dose, cage number and animal number.

Animal Husbandry

Animals were housed individually in polypropylene rat cage using autoclaved paddy husk as bedding material. The experimental animals were fed *ad libitum* Amrut brand rat pellet feed (manufactured by Pranav Agro Industries Limited, Pune, Maharashtra, India) with unlimited supply of clean drinking water in polypropylene bottles (capacity 300mL) filtered through Aquaguard water filtration system. The quality of feed was checked regularly and each batch was accompanied with a certificate of analysis of nutrient content. The quality of water was regularly monitored at Jai research Foundation. There were no contaminants in feed and water at the level, which can affect the results of the experiment. The temperature of the experimental room was maintained between 19 and 23°C and relative humidity measured was 60 to 70%. The light condition was maintained 12-hour light and 12 hour darkness.

Dose Selection

Following doses were selected based on the results of the acute oral toxicity (LD_{50}) study, which is described in **Chapter I.**

Group	Dose (mg/kg b.wt.)	N° Animals Used/Group	
		Male	Female
1	0	5	5
2	10	5	5
3	20	5	5
4	30	5	5

Treatment

Male

Male animals were dosed 70 days prior to mating (spermatogenesis period) and continued dosing till sacrifice.

Female

Female animals were dosed 14 days prior to treatment (at least two cycles), during mating, throughout gestation and lactation period.

Dosing

Oral intubation was performed **(Semler et al., 1992)** using an intubation cannula (Size: 16G × 5 cm) attached with a Boro-silicate hard glass syringe, which was graduated up to 5 mL. Required quantity of the test material (Spark 36% EC) was weighed using a calibrated analytical balance and was mixed with distilled water to provide a concentration of 3 mg/mL (high dose concentration). The mid and low dose solutions were prepared from high dose concentration by serial dilution to achieve the required concentration of 2 mg/mL and 1 mg/mL, respectively. The dose volume administered was 10-mL/kg body weights. The test solutions were prepared every day and were administered immediately. Individual dosage volume was calculated using initial body weight and subsequently after every week. During gestation and lactation period, body weight recorded at various intervals was considered for dosing. All the animals were starved for overnight prior to dosing and 3 - 4 hours post dosing (male were fasted throughout treatment and females during pre-mating).

Cohabitation

Treated male and female rats from the same dose group were allowed for cohabitation (1:1). Each morning, mated females were examined for the presence of cervical plug or sperm in vaginal smear **(Hafez, 1970).** The day when cervical plug was observed was considered as

day 0 of gestation/pregnancy. The pregnant females were isolated and housed individually. The day of parturition was taken as day 0 postpartum.

Litter Examination

Each litter was examined after parturition to establish the number and sex of pups born, stillbirth, live birth, runts and the presence of gross abnormalities, if any. Live pups were counted, sexed and weighed with dam within 24 hours of parturition (day 1 postpartum) and on day 4, 7, 14 and 21 postpartum.

Body Weight

Males

Male rats were weighed on the first day of dosing and weekly till termination of the experiment.

Females

Female rats were weighed on the first day of dosing, weekly (prior mating), during pregnancy (on days 0, 6, 14 and 20), and during lactation period (on days 1, 4, 7, 14 and 21).

Feed Consumption

Male

Feed consumption for male was measured every day throughout the experimental period and then average was calculated for weekly feed consumption.

Female

Feed consumption was measured during pre-mating (every day), during pregnancy (on days 0, 6, 14 and 20) and during lactation period (on days 1, 4, 7, 14 and 21). Average was calculated for weekly consumption (pre-mating).

Clinical Observation

During the study period, the clinical signs of toxicity including changes in fur, eye and mucous membrane, respiratory, circulatory, autonomous, central nervous system and urinogenital system were recorded daily

Blood Collection

Prior to sacrifice, blood was collected by orbital sinus puncture **(Riley, 1960)** under ether anesthesia of the animals. About 3mL of blood was collected in a clean centrifuge tube and serum was separated for analysis of biochemical parameters. A volume of 0.5mL of blood was collected in a vial containing EDTA for haematological analysis.

Haematological Parameters

Whole blood was used for the analysis. The blood collected in a clean vial containing EDTA was directly fed into a haematological analyzer (Sysmex K 1000) The procedure specified in the **Sysmex Operation's Manual (1988)** was followed. The results obtained in the form of printouts, e.g. white blood corpuscles (WBC), red blood corpuscles (RBC), haemoglobin (Hb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haematocrit (MCH), mean corpuscular haematocrit concentration (MCHC) and platelet Count (PLT) were recorded For determination of clotting time, blood was allowed to flow into 7.5 cm capillary tube and the time required for clotting was recorded manually

Serum Biochemical Parameter

The blood was collected in clean centrifuge tubes. The tubes were kept at room temperature for 30 minutes to clot. The blood was centrifuged at 2500rpm for 30 minutes after which the serum was separated and was transferred into dry clean sample cups using micropipette. The different parameters were analyzed using fully automatic analyzer, Hitachi 902 (Hitachi System Limited, Japan). The parameters studied were Glucose (GLU), alaline aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) alkaline phosphatase (ALP), cholinesterase (ChE), total protein (T.PRO), albumin (ALB), Cholesterol (CHO), Calcium (Ca) Cholinesterase (CHE). Sodium (Na) and Potassium (K) were analysed using flame photometer **(chemito 1020)** and chloride was analysed on Erba Chem 5 plus semiautomatic analyzer.

Sperm Anlysis

As soon as the animal were sacrificed (cervical dislocation), one of the caudal epididymis was excised and was transferred to a Petridish with Dulbecco's phosphate-buffered saline (DPBS) maintained at 37°C, analysed for the assessment of sperm motility epididymal sperm count and the remaining suspension for sperm morphology.

Solutions Preparation:

1. Dulbecco's Phosphate Buffered Saline (D-PBS)

Sodium chloride	:	8.0 g/L
Potassium chloride	:	0.20 g/L
Magnesium chloride	:	0.10 g/L
Calcium chloride	:	0.10 g/L
Sodium dihydrogen phosphate	:	1.15 g/L
Potassium dihydrogen phosphate	:	0.20 g/L

Dissolved Magnesium chloride and calcium chloride separately and add to the solution. Allowed the solution to remain at 37°C at 15 minutes.

2. Saline-merthiolate-Triton (SMT)

NaCl	:	0.9 % (9g/L)
Merthiolate	:	0.01% (100mg/L)
Triton X-100	.	0.05% (0.5mL/L)

Requisite quantities of both NaCl and merthiolate were added to approximately 75% of the final volume and stirred until it was dissolved. The triton X-100 was then added and stirred for one hour at room temperature. The final volume was then adjusted.

3. Eosin Y Stain (5%)

An amount of 10g of eosin Y (water soluble) was dissolved in 200mL of Distilled water at room temperature. Filtered through Whatman (No.1) filter paper and stored at room temperature.

Sperm Motility Count

A segment of distal cauda epididymus was removed and was placed in a test tube containing 2mL of DPBS (maintained at 36-38°C). Pierce sufficiently using a small stainless steel scissor. The test tube was placed in a water bath (36-38°C) to allow the sperm to disperse for 1 - 5 minutes then removed the test tube and gently mixed using Pasteur pipette. Diluted sample was loaded into both sides of the haemocytometer chamber avoiding flooding the center wall. A microscope fitted with a stage warmer (set at 37°C) was used to count the number of non-motile sperm in WBC counter (each comprise of 4 smaller squares) of haemocytometer. The haemocytometer was placed on to dry ice or clotting apparatus to cool the sample. This procedure rendered all sperm immotile. Haemocytometer was warmed up to room temperature and total number of sperm (minimum 200/sample) present in both WBC chamber were counted. Sperms exhibited any type of motion were classified as motile.

$$\text{Motility (\%)} = \frac{a - b}{a} \times 100$$

Where, "a" is the total number of sperm in the four corner squares and "b" is the number of non-motile sperm in the four corner squares **(Williams, 1993; Chinoy, et al., 1993).**

Epididymal Sperm Count

A quantity of 100 mg of the cauda epididymal tissue was taken in 2 mL of DPBS and maintained at 36 –38 °C. The cauda was shredded using forceps /scissor and the suspension was incubated at 36 -38°C Periodically, the petridish was swirled to facilitate the release of sperm. After incubation, suspension was mixed thoroughly then taken up to 0.5 mark in the WBC pipettes and was diluted up to 11 mark with 5% sodium bicarbonate ($NaHCO_3$), transferred the whole content in a fresh petridish and thereafter mixed well. The sodium bicarbonate acted as spermicide and killed spermatozoa, which facilitated count. Diluted sample was loaded into Haemocytometer chamber avoiding flooding the center wall. When the sample was settled in Haemocytometer, the counting grid of the Haemocytometer was focused under a microscope at 10x magnification. The objective of magnification was changed at 40x and the sperm present in WBC square (16 x 4 = 64 smaller square) were counted and recorded **(Williams, 1993).**

$$\text{Total Serm Concentration (Millions/ml)} = \frac{\text{Nx20x1000}}{\text{4 x 0.1}} \text{N x 50000}$$

Dilution factor	:	The dilution factor is 20
Area of one large corner square	:	(L x B) 1mm x 1mm = 1 sq. mm
Area counted	:	4 x 1 sq. mm
Depth of fluid	:	0.1mm (constant)
Haemocytometer factor	:	1 mL = 1000 cubic mm

(The multiplication factor is 50000)

Homogenisation Resistant Testicular Sperm Head Count:

One testis was weighed and required quantity (1g) was used to analyse homogenization resistant testicular sperm count as detailed below. Tunica albuginea was removed from one testis by making a shallow longitudinal incision, peeling with forceps and 1000 mg of parenchyma was weighed in petridish, and transferred to the blender by rinsing with 5 –10 mL of saline-merthiolate triton (SMT). The tissues were homogenized for 1 to 2 minutes at high speed and the homogenate was accredited to settle for few minutes, and allowed the froth to dissipate. The sample was made up to the required volume (50 mL) with SMT using measuring cylinder and stored in beaker/homogenized tubes, mixed the sample and taken aliquot to load both slides of the haemocytometer and waited for 5 minutes prior to count in microscope at 40x (RBC square). The same procedure was repeated with one more replication and was recorded **(Blazak et. al., 1993).**

The number of sperm heads per gram of tissue is calculated using the following formula :

$$\frac{\text{mean No of Sperm heads counted x squares factor x haemocytomet. factorr x (dilution factor)}}{\text{tissue weight (g)}}$$

Where the squares factor is 5 (counted as one-fifth of the central square), the haemocytometer factor is 10^4 (the volume of the central square is 0.0001 mL) and the dilution factor is the total volume of SMT, in milliliters.

Sperm Morphology

1-2 mL of the suspension was removed from the sample used for the motility count and made-up to 4 mL with DPBS 5 to 6 drops of 1 % Eosin Y were added and gently mixed the contents of test tube using pasture pipette and was allowed to stained and incubated at room temperature for 45 minutes. At the end of the staining period (45 minutes), re-suspended sperms were gently mixed with a Pasteur pipette. A smear was made following modification of the wedge method for preparing blood smear. 1-2 drops of the stained sperm suspension were placed 1 cm from one end or frosted end of a pre cleaned microscope slide (designated as sperm slide) lying on a flat surface. A second slide (designated as a pusher slide) was held in one hand with slide's edge gently touching across the width of the sperm slide. While, the slides were touching, the sperm slide was raised approximately at 45° angle with a rapid forward movement of the pusher slide, near frosted edge of sperm slide and the pusher slide was quickly moved down to the full length of sperm slide.

Slides were placed at 30-40°C to the horizontal angle on a tray whose surface was covered with absorbent paper to remove excess stained suspension. The slides were dried overnight and cover slipped later using a suitable mounting medium (DPX). A minimum of two hundred sperms per animal were examined at 40x magnification for morphological abnormalities e.g. such as no hook, excessive hook, pinhead, detached head, double head, coiled flagellum, bent flagellum, reduced hook, broken tail, blunt head, absence of middle piece, swollen middle piece **(Ron Filler, 1993).**

Organ Weight:

Absolute Weight of uterus, ovaries, testes, epididymis, spleen and thymus were recorded immediately after dissection for all animals euthanased at the end of the study period. Relative weight of these organs was calculated later. The organ was dissected out and was cleared from the adhering fat, and blotted free from the blood **(Paine, 1995).** The absolute weight was recorded to the nearest milligram using a calibrated balance.

Pathology

Gross Necropsy

At the end of experiment after blood collection, all the animals were euthanised by carbon dioxide asphyxiation and subjected to a complete necropsy. The animals were examined carefully for external abnormalities before the necropsy. The thoracic, abdominal and cranial cavities were cut open and through examinations of the organs were carried out to detect changes or abnormalities, if any. The gross changes in the internal organs were recorded.

Target organs e.g liver, kidney, spleen, thymus and lymph node and reproductive organs e g testes, seminal vesicle, coagulation gland prostate, epididymis, ovaries, uterus, vagina, cervix and mammary gland of the animals were collected at necropsy and preserved in 10% formalin (reproductive organs fixed in Bouin's solution) for processing and histopathological studies (reproductive organs fixed in Bouin's solution).

All the preserved organs were processed following suitable technique of washing by tap water, dehydration in ascending grades of isopropyl alcohol, clearing in xylene, embedding and blocking in paraffin wax (58 – 60°C melting point). Paraffin sections (4 to 5µm) were cut, processed and mounted on slides, stained by haematoxylin and eosin and examined microscopically **(Godkar, 1994).**

Data Evaluation

The data on the number of sperm positive animals in each group and number of animals which were found pregnant at term were compiled and the pregnancy rate was expressed in terms of percentage. The percentage of dead animals (mortality rate), percentage of sacrificed animals (survived) at term were evaluated. The maternal feed consumption was calculated for the gestations and lactation period. Similarly, the percent maternal body weight change during these gestation and lactation were also estimated from gestational and lactational body weights.

The male litter weights, female litter weights and the total litter weights for each pregnant female were calculated and the group means of these were compared with those of controls. The male to female (male/female) sex ratio was also calculated.

The following formulae were used for calculating these indices **(Thomas, 1996):**

$$\text{Male fertility index (\%)} = \frac{\text{No. of males impregnating female}}{\text{No. of male exposed to non pregnant females}} \times 100$$

$$\text{Female fertility index (\%)} = \frac{\text{No. of female conceiving}}{\text{No. of female exposed to male}} \times 100$$

$$\text{Gestation index (\%)} = \frac{\text{No. of animals with viable litters}}{\text{Total number of pregnant (positive sperm)}} \times 100$$

$$\text{Lactation index (\%)} = \frac{\text{No. of pups alive on day 21}}{\text{No. of pups alive on day 4}} \times 100$$

$$\text{Live birth index (\%)} = \frac{\text{No. of viable pups born}}{\text{Total number of pups born}} \times 100$$

$$\text{Survival index (\%)} = \frac{\text{No. of pups viable at lactation day}}{\text{No. of viable pups born}} \times 100$$

$$\text{Live litter size(\%)} = \frac{\text{No. of live offspring}}{\text{Pregnant females}} \times 100$$

$$\text{Dead litter size(\%)} = \frac{\text{No. of dead offspring}}{\text{Pregnant females}} \times 100$$

$$\text{Sex Ratio} = \frac{\text{No. of male litter}}{\text{No. of female litter}}$$

Statistical Analysis:

To calculate group mean and standard deviation with significance between the control and treated groups, raw data were analyzed using a window based statistical programme SPSS. Data were subjected to Bartlett's test to meet homogeneity of variance before conducting Analysis of Variance (ANOVA) and Dennett's multiple comparisons. Where the data did not meet the homogeneity of Variance, Students t-test was performed to calculate significance.

Significance was calculated at 1% as well as 5% level, and indicated in the summary table as follows:

↑ - Significantly higher than control ($p \leq 0.05$)
↓ - Significantly lower than control ($p \leq 0.05$)
↑↑ - Significantly higher than control ($p \leq 0.01$)
↓↓ - Significantly lower than control ($p \leq 0.01$)