MATERIALS AND METHODS

TEST CHEMICALS

Two different commercial insecticide formulations were used for the study. The first one was a combination insecticide (Ci) which constituted of chlorpyrifos (50%) and cypermethrin (5%), the second one was a biological insecticide, Spinosad (Sp).

Test Article 1

Commercial Name	:	*Anaconda 505* (55 EC)
Ingredients	:	Chlorpyrifos 50% EC + Cypermethrin 5%
Chemical name (IUPAC)	:	
1) Chlorpyrifos	:	O,O,-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate
2) Cypermethrin	:	(RS)-∝-cyano-3-phenoxybenzyl (1RS)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
Registration No.	:	CIR-27198/97/CHLOPYRIFOS+CYPERMETHRIN (EC)-923
Manufacture Licence No.	:	100/79
Batch No	:	AM 2908
Physical Appearance	:	pale yellow viscous liquid
Storage Conditions	:	Stored in original conditions as supplied by the manufacturer at room temperature
Manufactured by	:	AIMCO Pesticides Ltd; Mumbai

Test Article 2

Commercial Name	:	*Tracer* (Spinosad 45% SC)
Ingredients	:	Spinosyn A + Spinosyn D

Chemical name (IUPAC)	:	
1) Spinosyn A	:	(2*R*,3a*S*,5a*R*,5b*S*,9*S*,13*S*,14*R*,16a*S*,16b*R*)-2-(6-deoxy-2,3,4-tri-*O*-methyl-α-L-mannopyranosyloxy)-13-(4-dimethylamino-2,3,4,6-tetradeoxy-β-D-erythropyranosyloxy)-9-ethyl-2,3,3a,5a,5b,6,7,9,10,11,12,13,14,15,16a,16b-hexadecahydro-14-methyl-1*H*-*as*-indaceno[3,2-*d*]oxacyclododecine-7,15-dione
2) Spinosyn D	:	((2*R*,3a*S*,5a*R*,5b*S*,9*S*,13*S*,14*R*,16a*S*,16b*R*)-2-[(6-deoxy-2,3,4-tri-*O*-methyl-α-L-mannopyranosyl)oxy]-13-[[(2*R*,5*S*,6*R*)-5-(dimethylamino)tetrahydro-6-methyl-2*H*-pyran-2-yl]oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a,16b-tetradecahydro-14-methyl-1*H*-*as*-indaceno[3,2-*d*]oxacyclododecin-7,15-dione
Registration Number	:	CIR-09/2001(215) - SPINOSAD (SC)-01
Manufacture License No.	:	456 dt. 14/06/2002
Batch No	:	TE18688810
Physical Appearance	:	light grey- white liquid
Storage Conditions	:	Stored in original conditions as supplied by the manufacturer at room temperature
Manufactured by	:	Dow Agrosciences India Pvt. Ltd., Mumbai

EGG PROCUREMENT AND INCUBATION

Fertile RIR eggs were obtained from the Intensive Poultry Development Unit, Vadodara and refrigerated at 4^0C until use (stored not for more than four days). Eggs were wiped clean with povidone iodine and randomly allotted to control or treatment groups. Each egg was weighed, injected the appropriate substance and set to incubation in the incubator (Scientific equipment works, New Delhi) regulated to a temperature of 37.5±0.5^{0}C and 75-80% relative humidity, for 21 days or as per the requirement of the experiment. The eggs were manually turned over an angle of 180^0 for seven times a day until 3 days prior to hatch.

***IN OVO* INJECTIONS**

The eggs were injected through the air sac method as per **Blankenship *et al.* (2003)** on day '0' of incubation. The limits of the air space on the egg were marked with a pencil by viewing through a candler. The marked surface was then wiped with a 70% alcoholic swab. Using a sharp and sterile piercing tool a small hole was drilled at the centre of the air

chamber. Holding the egg horizontally, the appropriate dose was then injected through this hole by a sterile syringe with 36 gauge needle. The hole was immediately sealed with molten paraffin wax and transferred to the incubator. The above process of egg injection was carried out in a sterile laminar hood.

RANGE STUDY AND LD_{50}

A preliminary range study was conducted with different doses i.e. .001, 0.005, 0.01, 0.05, 0.1, 0.15, 0.2, 0.25, 0.5 and 0.8µg/egg of combination insecticide and 0.05, 0.1, 0.25, 0.5, 0.75, 1.5, 1.75, 2.0, 2.5 and 3.5 mg/egg of Spinosad. The injection volume was 50µl per egg. The dilutions were made in corn oil for the combination insecticide and 0.4% methyl cellulose for Spinosad. Twenty eggs were dosed for each group of insecticide dose as well as vehicle control.

On the 21st day of incubation, the hatchability and mortality were observed and the LD50 was calculated by probit analysis. Since the mortality in control was found to be more than 10%, the data were corrected using the Schneider-Orelli's (1947) formula:

$$\text{Corrected} = \frac{\%\ \text{responded} - \%\text{responded in control}}{100 - \text{responded in control}}$$

Based on hatchability and rate of development, the toxicity of these two compounds was estimated and three doses of insecticide, i.e. doses equivalent to $LD_{50}/10$, $LD_{50}/2$, and LD50 were chosen for further studies. Combination insecticide (Ci) was dosed in concentrations of 0.01, 0.05 and 0.1µg/egg while *Tracer* (Spinosad) was dosed in concentrations of 0.15, 0.75 and 1.5µg/egg. Controls were dosed with respective vehicles alone. Sham injections were given to check if there was any stress on the embryo due to the egg injections.

EFFECT ON EMBRYONIC GROWTH AND DEVELOPMENT

The weights of all the control and treated groups of eggs were recorded pre-incubation. After incubation, the eggs were candled on the 5th, 8th, 12th, and 18th day. The unfertile and dead embryos were culled out. The mortalities if noticed were recorded.

Simultaneously, six embryos from each group were removed from their eggs after 24, 48 and 72hr and observed under the microscope to check the growth and somite formation. Again on day 8 of incubation, few more embryos were collected and their wet weight was recorded and the crown and rump length of the embryo were measured. On the 21st day, the percent hatch and mortalities in all the groups were assessed. The hatchlings were weighed and their weight relative to the initial egg weight was calculated. The weight of hatchlings, their liver and brain were also recorded. The unfertile eggs were discarded whereas the dead ones were examined and classified to the various forms of developmental abnormalities.

BONE AND CARTILAGE STAINING

Chemicals and reagents:

Acetone

Ethanol

0.015% Alcian blue

0.005% Alizarin red

1% Potassium hydroxide (KOH)

Glycerol

Method:

For visualizing the skeletal development, the hatchlings as well as the unhatched /dead embryos of the various groups were collected on day 21 and stained for bone and cartilage as per **Lamb, (2003)**. The skin and viscera were removed and fixed in acetone for 2 days. Then rinsed in 96% ethanol for 1-2 hrs prior to staining and subsequently stained with the alcian blue and alizarin red stain for 4hr at 37^{0}C. The stained specimen were rinsed in 96% ethanol for 1hr, washed under running tap water and then cleared in 1% KOH solution until skeletons were visible. Then destained in graded sequence of glycerol and 1% KOH and finally stored in 100% glycerol.

HAEMATO-BIOCHEMICAL ANALYSIS

Blood samples were drawn from day old hatchlings by cardiac puncture using 2 ml disposable syringes and collected in EDTA rinsed or heparinised vials. After collection, the samples were used immediately or refrigerated and processed within 6hr.

For both **total red blood cell count** (TRBC) and **total white blood cell count** (TWBC), the whole blood was diluted using Natt and Herrick's solution (**Natt and Herrick, 1952**) in blood-cell dilution pipettes. The TWBC and TRBC were done with the help of a Neubauer haemocytometer, as per **Campbell, 1995**. The **differential white cell count** was determined by fixing and staining air-dried blood smears with Wright stain.

The **haemoglobin** (Hb) content was determined using the Drabkin's technique (**Drabkin, 1945; Henry, 1974**). 20µl of the blood sample was added to 5ml of the reagent containing potassium ferricyanide and potassium cyanide. Haemoglobin in the blood sample was thus oxidized to methaemoglobin and then converted to cyanmethaemoglobin. After three minutes of incubation the absorbance was read against distilled water at 540nm on a spectrophotometer.

Hb (gm/dl) = Abs. of test x F(36.7)
F = M.Wt. of Hb.

Packed cell volumes (PCV) were determined by centrifugation of whole blood in microhaematocrit tubes at 3,000*g* for 5 min. After centrifugation, the height of the red cell column was measured and compared to the total height of the column of whole blood using a PCV reader. The percentage of the total blood volume occupied by the red cell mass was recorded as the packed cell volume.

Mean cell volume (MCV), **mean corpuscular haemoglobin** (MCH) and **mean corpuscular haemoglobin concentration** (MCHC) were calculated using the following equations (**Campbell, 1995**):
MCV (μ^3) = (PCV/ RBC) 10
MCH (pg) = (Hb/RBC) 10
MCHC (%) = (Hb/PVC) 100

For the biochemical estimations, the blood was kept undisturbed in a refrigerator for a few minutes and was allowed to clot, then centrifuged at 3000rpm to collect the serum. The absorbance was read on spectrophotometer (Perkin Elmer, Lambda 25) when required.

Microassays when performed were carried out in 96 well microplates and read on an ELISA reader (Automated microplate reader ELx 800). The following tests were performed:

Glucose was estimated by the GOD/POD method (**Trinder, 1969**; **Henry *et al.*, 1974**). The substrate β-D-glucose present in the serum sample is oxidized by glucose oxidase to gluconic acid and hydrogen peroxidase is liberated. Hydrogen peroxidase thus produced is acted upon by peroxidase and oxygen is liberated. The liberated oxygen is transferred to chromogen system consisting of 4-aminoantipyrine and phenolic compound to produce red quinoneimine dye. The intensity of the colour is directly proportional to the concentration of the glucose and was measured photometrically at 505nm.

Serum albumin (Rodkey, 1965; Doumas *et al.*, 1971) was estimated by the BCG method. Albumin in the presence of bromocresol green at a slightly acid pH produces a colour change from yellow-green to blue-green. The spectra shift is directly proportional to the albumin present in the sample and was measured at 630nm.

Serum globulin was obtained by subtracting the value of serum albumin from the total serum protein.

Serum protein was estimated using the **Bradford's assay (Bradford, 1976)**. The assay is based on the principle that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm on binding to protein. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible colour change. A dilution series of protein standard (BSA) in sample buffer was prepared to cover a range from 100-1000µg/ml. 0.5ml of standard/sample/buffer was dispensed into appropriately labelled tubes. 5ml of Coomassie dye reagent (0.01% coomassie brilliant blue,. 4.7% ethanol, 8.5% phosphoric acid in distilled water) was added and vortexed immediately. After incubating for 10min, the absorbance was read at 595nm. The protein concentration of individual samples was determined by interpolation from the standard graph.

Alkaline phosphatase (ALP) was estimated by the method of **Kind and King, (1954)**. ALP catalyses the hydrolysis of p-nitrophenyl phosphate at pH 10.4 liberating p-nitrophenol and phosphate in the presence of magnesium ions. The rate of p-nitrophenol formation,

measured photometrically, is proportional to the catalytic concentration of alkaline phosphatase present in the sample. The reagent (buffered substrate to ALP) is added to the sample serum and the absorbance is read at 405nm wavelength successively after 30, 60, 90 and 120 seconds.

ALP activity (IU/L) = ΔA/min. X 2713 (calculated on the basis of molar extinction coefficient of p-nitrophenol and ratio of total assay volume to sample volume)

Alanine aminotransferase (ALT) or serum glutamate pyruvate transaminase (SGPT) was estimated by the **IFCC Method (1974)**. It is based on the principle that L- Alanine and α-ketoglutarate in the presence of ALT yield pyruvate and L-glutamate. Pyruvate is reduced by lactate dehydrogenase to yield lactate with the oxidation of NADH to NAD. The reaction is monitored by measurement of the decrease in absorbance of NADH at 340nm. The rate of reduction in the absorbance is proportional to ALT activity in the sample.

ALT activity (IU/L) = ΔA/min. X 3376 (millimolar extinction coefficient of NADH at 340nm)

Aspartate aminotransferase (AST) or serum glutamate oxaloacetate transaminase (SGOT) was determined by **IFCC Method (1986)**. AST reacts with L- Aspartate and α-ketoglutarate to form oxaloacetate and L-Glutamate. The oxaloacetate is reduced by malate dehydrogenase to yield L-malate with the oxidation of NADH to NAD. AST was estimated in the sample using the above principle by monitoring the decrease in absorbance of NADH at 340nm. The rate of reduction in absorbance is proportional to AST activity.

AST activity (IU/L) = ΔA/min. X 3376 (millimolar extinction coefficient of NADH at 340nm)

Blood Urea Nitrogen (BUN) was estimated by the enzymatic UV- kinetic initial rate method **(Gutmann and Bergmeyer, 1974; Sampson and Baird, 1979)**. The principle of the method is that, urea is hydrolyzed to ammonia and carbon dioxide by urease. Ammonia produced reacts with α-ketoglutarate to form glutamate in the presence of glutamate deydrogenase. NADH is oxidized to NAD^+ in this reaction, which is measured as decrease in absorbance at

340nm. The rate of decrease in absorbance is directly proportional to BUN concentration in the serum sample.

BUN concentration (mg/dl) = Δ absorbance of sample × factor

Factor = concentration of standard + Δ absorbance of standard

Cholinesterase Activity:

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were estimated in the plasma, liver and brain homogenates as per **Ellman (1961) and Gorun *et al.* (1978)**. The assay is carried out on the principle that, the substrates Acetylthiocholine iodide (AcTI) or Butyrylthiocholine iodide (BuTI) are hydrolyzed by AChE or BChE, respectively, producing carboxylic acid and thiocholine which reacts with the Ellman reagent (DTNB, dithionitrobenzoic acid) to form a yellow colour which is measured spectrophotometrically at 412nm. The rate of colour formation is proportional to the amount of either AChE or BChE.

Chemicals and reagents:

Sodium phosphate buffer (20mM, pH 7.6)

Acetylthiocholine iodide (40mM)

Butyrylthiocholine iodide (40mM)

Phosphate buffer (0.1 M, pH 7.6)

DTNB- phosphate – ethanol reagent (12.4mg DTNB dissolved in 96% ethanol, 80ml D/W, and 0.1M phosphate buffer)

Method

Whole brain homogenate was prepared in 20mM Sodium phosphate buffer. 200µl of homogenate was added to 200µl of 40mM acetylthiocholine iodide/ butyrylthiocholine iodide. Incubated at 37^0C for 30 min. Enzyme activity was stopped by addition of 3.6 ml of DTNB reagent. Absorbance was measured at 412 nm and the enzyme activity was calculated by using extinction co-efficient value of the chromophore as 13,600M^{-1} cm^{-1}.

$$\text{AChE or BChE activity (µmoles hydrolyzed/min/mg protein)} = \frac{0.1 \times OD \times 5}{0.420 \times 2}$$

IMMUNOTOXICITY

The control and pesticide treated hatchling groups were housed in separate pens. Layer starter mash and water were provided *ad libitum*. After one week the immunotoxicity was evaluated through the weights of thymus, spleen, bursa of Fabricius, bacterial clearance assay and NBT salt reduction test.

Lymphoid organ weight

Day old hatchlings were sacrificed and the thymus, spleen and bursa of Fabricius were excised, carefully blotted and weighed on a Sartorius balance (BS-223S). The relative of these organs were then calculated.

Bacterial Clearance Assay

Chemicals:

Escherichia coli inoculum
Nutrient broth (M 002, Himedia)
Nutrient Agar (M001, Himedia)
McConkey agar (58613, Qualigens)
Physiological Saline

Method

The assay was performed as per **Peterson *et al.* (1999)**. *Escherichia coli* were obtained from the microbiology department of the Baroda Medical College. A single colony was first taken into nutrient broth and cultured and then streaked onto nutrient slants. The culture was diluted in sterile physiological saline solution. The turbidity of the inoculum was standardized by measuring wavelength on a spectrophotometer, such that 0.2ml gives 2 x 10^5 CFU of *E.coli*. Inoculums of 0.2ml volumes were injected into the brachial vein of one week old chicks. Blood samples were drawn immediately and after 20, 40, and 60min post injection. After 110 min, the birds were euthanized; liver and spleens were removed aseptically and homogenized. All the samples thus collected were immediately cultured by pour plate on MacConkey agar. The bacterial load was quantified by plate count method on a colony counter.

NBT salt reduction test (Singh *et al.*, 1990)

NBT test was done on one week old chicks. Spleen was dissected and impression smears of spleen were taken on a glass slide and kept in petri dish. NBT salt solution was poured on the smear and incubated for 25min. Smears were examined for NBT positive macrophages having formazan deposit in their cytoplasm.

EARLY EMBRYONIC ESTIMATIONS

Further, to look into the early changes evoked by the pesticide treatment, the control and pesticide dosed embryos were collected on day eight of incubation and whole embryonic and/or brain homogenates were prepared and few biochemical estimations were made i.e. the acetylcholinesterase activity, NA^+- K^+ ATPase activity, DNA and RNA content were estimated.

Acetylcholinesterase activity

Chemicals and reagents:

Sodium phosphate buffer (20mM, pH 7.6)
Acetylthiocholine iodide (40mM)
Phosphate buffer (1mM, pH 8)
DTNB reagent (12.4mg DTNB dissolved in 96% ethanol, 80ml D/W, and 0.1M phosphate buffer)
1% Triton X 100

Method

Microplate method was adopted to assess the AChE enzyme activity in the embryonic homogenate (Ellman *et al.,* 1961; Lesser *et al.,* 2000; Steevens and Benson, 1999). Whole embryos were homogenized in 1mM phosphate buffer, pH 8 with 1% triton X 100. Homogenate was centrifuged in a cold centrifuge (Sorvall) at 8000rpm for 20min. Pellet was discarded and supernatant was utilized for the enzyme assay. 50µl of sample was diluted 1:1 in sample buffer. 100 µl of DTNB reagent was added to each well of the microplate. Enzyme reaction was initiated by addition of 50 µl of AcTI in buffer. The formation of DTNB thiol complex is measured in triplicates at 405nm on Automated Microplate Reader ELx 800 during 6 intervals of 10 min each. Activity was determined as micromoles acetylthiocholine iodide hydrolyzed per minute per milligram protein.

Protein Estimation

Protein concentration of the embryonic homogenate was determined by microanalysis modification of Bradford's method (1976) using Coomassie blue stain.

Chemicals and reagents

Protein Standard

BSA, 1mg/ml (diluted to make 0, 2, 4, 6, 10, 15 and 20 µg/ml BSA)

Bradford's reagent (0.01%w/v Coomassie Briliant Blue G-250, 4.75%w/v 95% ethanol, 8.5% w/v phosphoric acid made up to 100ml in d/w)

Method

The diluted protein standards and samples were pipetted individually into assigned wells of the 96-well microplate in volumes of 20µl each. 40 µl of Bradford's reagent was added to each well. Distilled water was added to all the wells to bring them to a final volume of 200 µl. The absorbance was read at 595nm on Microplate Reader (ELx 800). The absorbances of the samples were plotted against the standard graph to find the protein concentration in each sample.

Na^+- K^+ ATPase

Whole embryonic homogenates were analyzed for Na^+- K^+ ATPase activity (**Post and Sen, 1967**). From the rate of release of inorganic phosphate from ATP in the presence of Na^+, K^+, Mg^{2+} is subtracted the rate of release in the presence of Mg^{2+} and a cardiac glycoside inhibitor, oubain.

Chemicals and reagents

1.2M $HClO_4$ (containing 8% silicotungstinic acid)

60mM $NaMoO_4$

2.25M NaCl

Butyl acetate

Isopropyl alcohol (containing 0.06mM $CuCl_2.2H_2O$ and 0.27M H_2SO_4)

Reagent 1:

- 4.5mM Na_2ATP
- 5mM $MgCl_2$
- 0.75mM H_2Na_2 EDTA
- 30mM imidazole
- 30mM glycyl glycine

Reagent 2:

- 1.5M NaCl

0.3M KCl

Reagent 3:

2.5mM Oubain

Method:

The test was performed in three different sets of test tubes. In the first set, reagent 1, sample homogenate, reagent 2, and distilled water were added. In the second set, reagent 1, sample homogenate, reagent 3 and distilled were added. In the third set reagent 1, sample homogenate, and distilled water were added. The first and second sets of test tubes were incubated at 37^0C for 20min with shaking. The third was kept on ice. Reaction was stopped by returning heated tubes to ice bath. $HClO_4$ mixture, molybdate solution and butyl acetate were added sequentially and centrifuged for a minute. 2ml of the supernatant was added to isopropanol and then mercaptoethanol was added, contents were mixed and read at 625nm. The difference in the activity in the presence of Na^{2+}, K^+ and Mg^{2+} to that in the presence of Mg^{2+} and ouabain is expressed as micromoles of P_i released /min/mg of protein at 37 °C.

Quantification of Nucleic acids

Nucleic acid content was quantified as per **(Labarca and Paigen, 1980)** in the whole embryonic homogenates.

Total DNA Content

Chemicals and reagents

Digestion buffer

100 mM NaCl

10 mM Tris.Cl, pH 8

25 mM EDTA, pH 8

0.5% SDS

0.1 mg/ml proteinase K (added fresh when used)

25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol

25ml phenol

24ml chloroform

1ml isoamyl alcohol

7.5 M ammonium acetate

57.1g in 100ml d/w

Tris -EDTA buffer (TE), pH 8

10mM Tris.Cl

1mM EDTA

Embryos were weighed and homogenized in ice cold PBS and suspended in 1.2ml of digestion buffer per 100mg of tissue. Samples were then incubated with shaking at 50^0C for 12-18hrs in tightly capped tubes. A thorough extraction was done with an equal volume of phenol/chloroform/isoamyl alcohol and centrifuged for 10min. at 2000rpm. The aqueous top layer was transferred to a new tube and 7.5M ammonium acetate equivalent to half of its volume and 2 volumes of absolute ethanol were added. DNA which then precipitates into a stringy precipitate was recovered by centrifugation at 1700 x g for 2min. The pellet was rinsed with 70% ethanol. Then ethanol was decanted and the pellet was air dried. The pellet was resuspended in TE buffer until dissolved and read at 260, 280 and 230nm. TE buffer was used as blank. The A_{260}/A_{280} ratio and readings at A_{230} and A_{325} were used to estimate the purity of the nucleic acid sample.

The DNA content was estimated using the formula:

$$C\ (\mu g/ml) = \frac{A260}{0.020}$$

Total RNA Content

Sodium acetate buffer

100mM sodium acetate, pH 5.2

1mM EDTA

10% SDS

10g SDS in 100ml 2d/w

Sodium acetate saturated phenol

50g phenol

50ml sodium acetate buffer

Shake vigourously, let phases separate. Preheat to 60^0C before use

TE Buffer

10mM Tris.Cl

1mM EDTA

Embryos were homogenized in ice cold sodium acetate buffer keeping on ice. 0.05vol of 10% SDS and 1vol of 60^0C sodium acetate saturated phenol is added and immediately vortexed for 5-10 sec. The tubes were placed in 60^0C water bath for 1min. Then quickly cooled on ice and then centrifuged for 5min at 2000rpm. The aqueous upper phase was transferred to a new polypropylene tube. Equal volume of chloroform was added, mixed by inversion and centrifuged. Chloroform extraction was repeated. The aqueous layer was transferred to a new polypropylene tube and precipitated by adding 2.5vol of 95% ethanol and letting stand overnight at -20^0C. The pellets were air dried and resuspend in TE buffer, pH 8.0. The absorbance was read at 260 and 280nm. The A_{260}/A_{280} ratio should be 1.9 to 2.0 for highly purified RNA. The RNA content was estimated using the following formula:

$$\text{Single stranded RNA } C\ (\mu g/ml) = \frac{A260}{0.025}$$

CYTOTOXICITY

Nile Blue Sulphate Staining

Chemicals and reagents

Nile blue sulphate (1:50,000 in lactated ringer's solution)

Lactated Ringer's solution

- 0.6% Sodium chloride
- 0.25% Sodium lactate
- 0.04% Potassium chloride
- 0.027% Calcium chloride
- Distilled water
- PBS

Method

To investigate the cell death caused by the pesticide toxicity, the whole embryos at 24hr and 48 hrs were subjected to Nile blue sulphate staining (**Jeffs and Osmond, 1992**). Embryos

were dissected in lactated ringer's solution and incubated in 1: 50,000 Solution of NBS and lactated ringers solution at 37^0C for 30 min. Stained specimens were washed in lactated ringer's solution and immediately photographed.

Acridine orange and ethidium bromide staining

Chemicals and reagents

Acetone
Ethanol
Acridine orange
Ethidium bromide
PBS
Stock Solution
15mg acridine orange and 50mg ethidium bromide dissolved in 1ml 95% ethanol and 49 ml distilled water is added.

Method

Fluorescent staining method was used to view the embryonic cell death (**Ribble *et al.*, 2005; Natesan, *et al.*, 2006**). Thin transverse sections of the embryos (4-6μm) were taken on cryostat (IEC) and fixed on a clean microscope slides by using chilled acetone for 15 minutes. Then washed for three times in PBS (pH 7.5) and stain was added to cover the entire section. After 30 minutes of incubation, washed three times in PBS and then air dried. A drop of PBS was added to cover the stained section and slide was viewed under the fluorescent microscope (Leica DM2500) using appropriate filters and then photographed using the digital image grabber (Leica EC3 Camera). Acridine orange stains live cells and fluoresces green while ethidium bromide stains dead cells and fluoresces orange/red.

Scanning electron microscopy

Chemicals and reagents

Acetone
Ethanol
4% Paraformaldehyde
PBS

Method

Neural tube defects were studied through scanning electron microscopy. Embryos were fixed in 4% phosphate buffered paraformaldehyde at 4 °C for 2-12hrs. Dehydrated in increasing graded series of ethanol and finally transferred to 100% acetone. Critical point dried,

graded series of ethanol and finally transferred to 100% acetone. Critical point dried, mounted on Cambridge stubs and gold coated. Then observed and photographed under scanning electron microscope (JEOL, JSM-5600).

Immunohistochemical localization of neural crest cells and Sonic hedgehog protein

Chemicals and reagents

4% paraformaldehyde in PBS (PPBS)

PBS

Ethanol

1% H_2O_2

PBT (99.5ml PBS and 0.5ml Triton X 100)

Blocking reagent (10% heat inactivated goat serum in PBT and 1%BSA)

Anti-Shh (SHH-N, 5E1, DSHB, University of Iowa)

Anti-neural crest cell (20B4, DSHB, University of Iowa)

Incubating reagent (antisera 1:500 in PBS containing 0.1% BSA and 0.5% Triton X 100)

Secondary antibody (rabbit antimouse IgG-HRP, 105501, Banglore Genei), 1:500 in PBT containing 0.1% BSA

Streptavidin – HRP complex (105505, Bangalore genei)

DAB system (106038, Bangalore genei)

Method

The cryostat cross sections of the 48hr embryos (stage-12) of control (VC1 and VC2), Ci treated (0.05µg/egg) and Sp treated (0.75mg/egg) were fixed in 4% paraformaldehyde for 16 hr at 4^0c, rinsed in PBS (pH 7.4). Dehydrated in graded ethanol series and stored in 100% ethanol at -20^0c until use. The embryos were hydrated in ethanol series. The endogenous peroxidase activity was inactivated by 1% H_2O_2 in PBS. Antigen retrieval was performed by microwave heating for 10 min in citrate buffer solution (pH 6) to reveal antigens. Rinsed in PBT and blocked for 2hr at room temperature. The embryos/sections were incubated overnight at 4^0c with the antisera of Shh or neural crest cells. The primary antibodies were detected by secondary antibodies, followed by DAB colour detection. Mounted in 85% glycerol in PBT and photographed after observing under a microscope.

GENOTOXICITY

The genotoxic effects were studied trough the micronucleus test and comet assay.

Micronucleus test

Chemicals

May-Grunwald stain (0.25%)

Giemsa stain

Xylene

DPX

Distilled water

Method

The test was performed as per **Chaubey *et al.* (1993).** Blood samples were collected from one of the chorioallantoic blood vessel of 11 day old chick embryos and smears were prepared and stained using May-Grunwald and Giemsa stain. Rinsed in distilled water thrice (5 min each) and stained with diluted Giemsa (1:6 of the Giemsa stock: distilled water) for 10 min, and then rinsed in distilled water thoroughly. The slides were dried, cleared for 5 min in xylene and mounted in DPX.

The slides observed under the microscope at 1000x magnification. The identification of polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) was based on the criteria described by **Lucas and Jamroz (1961)** and **Wolf and Luepke (1997).**

The PCEs are round, large cells with round nuclei and stain blue, whereas the NCEs are oval in shape and stain pink. The frequencies of micronuclei in erythrocytes were detected under oil-immersion lens (100×) of a binocular microscope. Only cells with intact cellular and nuclear membranes were scored. Criteria previously described were used for scoring (**Saleh and Sarhan, 2007**). Briefly, the following criteria was followed:

(i) Micronuclei should be at least one-tenth in length and one-third in diameter of the main nucleus,

(ii) These should be on the same plane of focus,

(iii) Must have same colour, texture and refraction as that of main nucleus and

(iv) Must be clearly separated from the main nucleus.

For classifying all other abnormal cell types in the embryonic blood, the classification scheme of **Lucas and Jamroz (1961)** was followed.

Comet assay

Comet assay (**Malladi *et al.*, 2007**; **Singh et al., 1988**) was performed on the 11 day embryonic tissues i.e. blood, liver and brain.

Chemicals

10% Dimethylsulfoxide (DMSO) (CPW59, Qualigens)

Disodium EDTA - (RM1370, HiMedia)

Ethidium Bromide - Sigma (E-8751)

Phosphate Buffered Saline (PBS) (Ca^{2+}, Mg^{2+} free) - (TS1006, HiMedia)

2.5M Sodium Chloride (NaCl)

Sodium Hydroxide (NaOH) - BDH-Merck (89021)

Triton X-100 - (RM 845, HiMedia)

0.4M Tris.Hcl

Normal Melting Agarose (NMA) - (RM273, HiMedia)

Low Melting Point Agarose (LMPA) - Sigma (A9414)

Methanol

Method

The embryonic tissues (blood, liver and brain) of the different groups of treated and control embryos were isolated and kept in chilled PBS with 1% BSA solution. Six embryos were tested for each dose of the two pesticides and vehicle controls. Single cell suspensions of the liver and brain were made by mincing the tissues (**Tice *et al.*, 1991, 2000**) in mincing solution (HBSS containing 20 mM EDTA and 10% DMSO).

The viability of the cells was measured using Trypan blue dye exclusion method. Approximately, 2×10^5 cells/ml was used from each organ for the comet assay. The peripheral blood was collected in EDTA rinsed vials.

Frosted slides were uniformly layered with 1% hot agarose and air dried. The single cell suspensions of different samples (5 - 10µl each) were mixed with 75µl LMPA; while the blood samples were diluted in PBS and mixed with equal volumes of 1% LMPA and layered

uniformly over the agarose layered slide. After solidification, a third layer of agarose (LMPA) was added on to it. After solidification, the slides were kept in lysing buffer (2.5MNaCl, 100mM Na2-EDTA with freshly added 1% Triton-X 100 and 10% DMSO) for 1 h at 4 °C. After lyses, the slides were equilibrated for 20 min with alkaline electrophoresis buffer (300mM NaOH, 1mMNa2-EDTA, pH 13.0) in horizontal electrophoresis apparatus at room temperature. Electrophoresis was carried out for 30 min at 15V, 200mA using a compact power supply. After electrophoresis, the slides were washed gently in the neutralizing buffer (0.4M Tris–HCl, pH 7.5) to remove the alkali and detergents. Slides were then stained with Ethidium Bromide for 5 min and then dipped in chilled distilled water to remove excess stain. A cover slip was then placed over it and the slides were observed immediately under UV filter of the fluorescent microscope (Leica, 2500) and images were captured.

Fifty cells were captured at random from each slide. Analysis of **comets** was carried out by the software **TriTek Comet Score** (Version 1.5.0). The software quantifies:

(i) Comet length
(ii) Comet height
(iii) Comet area
(iv) Comet tail length
(v) Percentage of the DNA in the tail and
(vi) Tail moment.

The DNA damage was scored by comparative analysis of tail moment (product of percent DNA in comet tail and tail length) of the treated embryonic cells and the control embryonic cells.

Tail moment = %DNA in comet tail x tail length

STATISTICAL ANALYSIS

Raw data were processed and analyzed to give group means and standard error with significance. All the parameters characterized by continuous data were subjected to relevant statistical method (*viz.*, Bartlett's test, ANOVA, Dunnett's test or Student's t test) using

either GraphPad Prism version 5, GraphPad Software, San Diego California USA (**Motulsky, 1999**) or IBM SPSS Statistics 19.0.0.

The chemical used were of analytical grade and were purchased from SD fine, SRL, Qualigens , HiMedia, Mumbai and/or Sigma-Aldrich, USA.

Protocols for the present experiments were approved by IAEC (Institutional Animal Ethics Committee) according to CPCSEA, India (Committee for the Purpose of Control and Supervision of Experiments on Animals.