CHAPTER II

MATERIALS AND METHODS
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Animals and Environment

Healthy adult male rats (*Rattus norvegicus*) of Wistar strain received from animal house division, Jai Research Foundation, were used for the experiment. The animals were housed in an airconditioned animal house at a temperature of $23 \pm 2^\circ C$ and relative humidity of 30 - 70%. Twelve hour light and 12 h darkness were provided. The animals of different groups were housed in different polypropylene rat cages and a maximum of five animals per cage were maintained, with clean paddy husk as bedding material, covered with a stainless steel mesh. The animals were fed with standard rat pellet feed and pure drinking water was provided *ad libitum*.

Chemicals

The test chemical carbaryl + lindane 4 + 4 % granule (G) was procured from the local market. The test chemical was administered by oral route, in peanut oil as a vehicle.

The genetic, cellular and biochemical effects of carbaryl + lindane 4 + 4 % (G) in the vital organs of male Wistar rats was evaluated by a
set of experiments comprising acute, sub acute - repeated dosing, etc.

**ACUTE TOXICITY STUDY**

This is an initial step in establishing a dose regimen in subchronic and other studies and provides initial information on the mode of toxic action of a substance.

**Principle**

The test substance is administered orally, to several groups of experimental animals, single dose being used per group. The dose selection was based on the results of a range finding study. Subsequently observations for toxic effects and mortality were made. Animals which died during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed by necropsy.

**LD₅₀**

It is a statistically derived single dose of a substance that can be expected to cause death of 50 % of treated animals when administered through the oral route. The LD₅₀ value is expressed as mg/kg body weight.
Procedure

For a compound of unknown LD$_{50}$ initially a range finding study is to be carried out. Based on the mortality, a full study or main study was performed.

Range finding

Two animals were used per group and a broad range of doses ranging from 2000 to 10000 mg/kg body weight was taken for testing.

Main study

Based on the results of the range finding study 4 dose groups and a control group comprising 5 animals each were used. The doses selected were 4250, 4590, 4957 and 5353 mg/kg body weight. Pharmacotoxic symptoms were observed following treatment and the animals were kept on observation for a period of 14 days. The surviving animals were necropsied. The mortality data were subjected to Finney's probit analysis for arriving at the LD$_{50}$ value (Finney, 1971).

SUB CHRONIC STUDY

The study is designed to determine the adverse effects of carbaryl + lindane (4 + 4 % G) occurring as a result of repeated daily oral
dosing to experimental animals. The experimental protocol was shown in Table-I

<table>
<thead>
<tr>
<th>Group &amp; dose</th>
<th>No. of animals</th>
<th>Treatment days</th>
<th>Sacrificed on day</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Control (Peanut oil)</td>
<td>10</td>
<td>60</td>
<td>61</td>
</tr>
<tr>
<td>II Carbaryl + lindane (440 mg/kg b. wt.)</td>
<td>10</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td>III Carbaryl + lindane (440 mg/kg b. wt.)</td>
<td>10</td>
<td>60</td>
<td>61</td>
</tr>
<tr>
<td>IV Control recovery (Peanut oil)</td>
<td>10</td>
<td>60</td>
<td>91</td>
</tr>
<tr>
<td>V Treated recovery (Carbaryl + lindane 440 mg/kg b. wt.)</td>
<td>10</td>
<td>60</td>
<td>91</td>
</tr>
</tbody>
</table>

The animals were observed daily for any pharmacotoxic symptoms and their weekly body weight was also recorded on an Atco animal weighing balance. The weekly feed leftover and feed input were recorded. This data were used to calculate the feed consumption by using the following formula:

\[
\text{Feed consumption} = \frac{\text{feed input} - \text{feed leftover}}{\text{No. of animals}}
\]

Prior to sacrifice, blood was collected by orbital sinus puncture (Riley, 1960) after etherising the animal. A volume of 3 ml blood was collected in a clean centrifuge tube for the separation of serum and subsequent biochemical analysis. A volume of 0.5 ml blood was collected in a vial containing EDTA for haematological analysis as well as cholinesterase estimation, and a drop of blood was placed
on a clean glass slide and smear was prepared for differential leucocyte count. After blood collection the animals were euthanised by an overdose of chloroform and dissected. The gross changes in the internal organs were recorded. The vital organs such as brain, liver, heart, kidney and testis were removed for various biochemical estimations as well as histological studies after weighing.

**ORGAN WEIGHTS**

The organs were dissected out, cleared off from the adhering fat and blotted free of blood. The absolute weights of brain, liver, kidney, heart and testis were recorded to the nearest milligram on a Conweigh balance.

**HAEMATOLOGICAL PARAMETERS**

Whole blood was used for the analysis. The blood collected in a clean vial containing EDTA was directly fed into a fully automatic (Sysmex K 1000) haematological analyser. The procedure as mentioned in Sysmex operators manual (1988) was followed. The results of parameters obtained in the form of printouts were of white blood corpuscles (WBC), red blood corpuscles (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haematocrit (MCH), mean corpuscular haematocrit concentration (MCHC) and platelet count (PLT).
Differential leucocyte count (DLC)

This is an important blood test to find the pattern of variation in the proportion of different types of leucocytes in the blood. DLC read together with total leucocyte count (TLC) gives an indication about nature of response of the body to an injurious agent.

Principle

Leishman’s stain contains methylene blue and Eosin in acetone-free methanol base, which also serves as smear fixative. In this stained blood smear the WBCs (leucocytes) with their purple-stained nucleus and pink cytoplasm appear brightly stained and stand out against RBCs (Erythrocytes), which are stained pale and show rouleaux formation. The tiny platelets are seen as scattered clusters of small pinkish particles.

Procedure

The method described by Jain (1986) was followed. Clean, dry and grease free slides were taken and a drop of blood was placed on it. A smear was prepared by pulling it with another slide at an angle, dried to room temperature and stained with Leishman’s stain. After one minute it was diluted with distilled water and allowed to stand for 10 minutes, followed by washing in tap water. Slides were allowed to air dry and observed under 100X oil immersion of a
Nikon microscope. Cells (100) were observed and different types of leucocytes were expressed as their percentage.

SERUM BIOCHEMICAL PARAMETERS

The blood collected in clean centrifuge tube was kept at the room temperature for 30 minutes to clot. This was centrifuged at 3000 rpm for 10 minutes after which the serum was separated and transferred into an Ependoff tube using a clean dry pasteur pipette. The serum was stored in refrigerator for further analysis.

The parameters were analyzed using semi automatic serum analyzer ERBA chem - 5 plus. Serum glucose was analyzed immediately after separation of the serum. The other parameters studied were bile urea nitrogen, creatinine, total bilirubin, aspartate amino transferase (GOT), alanine amino transferase (GPT), gamma glutamate transaminase, total protein, albumin, chloride, calcium, phosphorus and cholesterol.

Glucose

Glucose was estimated by Trinder’s glucose oxidase and peroxidase method (Trinder, 1969). It involves two step reactions involving glucose oxidase and hydrogen peroxidase respectively. In the first step glucose is converted to gluconic acid and hydrogen peroxide by glucose oxidase and in the second step hydrogen peroxide with the reaction of 4 aminoantipyrine is converted to a red
dye by peroxidase which will give a pink colour with the absorbance of 510 nm. 5 µl of the sample was added to 500 µl of reagent and incubated for 15 minutes at 37°C and absorbance measured at 510 nm.

The reagents are composed of:-

**Reagent I**

Glucose oxidase (20,000 IU/L), Peroxidase (3250 IU/L), 4-aminoantipyrine (0.52 mmol/L), 4-hydroxy benzoic acid (10 mmol/L) and phosphate buffer (110 mmol/L).

The reagents were reconstituted at room temperature by dissolving the contents of each vial using deionised water free from contaminants. The samples were mixed well with the reagents and incubated for 15 minutes at 37°C. The absorbance of standards and each sample against reagent blank were read at 510 nm and expressed as mg/dL.

**Calculation**

\[
\text{Glucose (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard standard (mg/dL)}} \times \text{Concentration of standard (mg/dL)}
\]
Blood Urea Nitrogen (BUN)

The methodology of Talke and Schubert (1965) was followed. Urea in the presence of water is converted to ammonium and carbon dioxide by the action of urease. The ammonium reacts with alpha ketoglutarate and NADH in the presence of glutamate dehydrogenase to produce L-glutamate and NAD.

The reagents include 2-oxoglutarate (7.5 mM/L), NADH (0.32 mM/L), urease (8.000 IU/L), GLDH (1.000 IU/L), ADP(1.2 mM/L) and Tris buffer pH 7.9 ± 0.1 at 25°C (100 mM/L). A volume 10 µl of the sample was mixed with 500 µl of the reconstituted reagent and aspirated and the absorbance was read against blank at 340 nm.

Calculation

\[
Urea (mg/dl) = \frac{\Delta A \text{ of sample}}{\Delta A \text{ of standard}} \times \text{Concentration of standard (mg/dl)}
\]

\[
\Delta A = A_1 - A_2
\]

Creatinine

The modified Jaffe reaction was followed (Jendrassik and Grof, 1938). Creatinine reacts with alkaline picrate to produce a reddish colour (the Jaffe reaction). Specificity of the assay has been improved by the introduction of an initial rate method, cephalosporin antibiotics are still major interferants. The orange-yellow colour
formed is directly proportional to creatinine concentration and was measured photometrically at 510 nm.

The reagents comprised of picric acid (25.8 mMol/L), sodium hydroxide (95 mMol/L) and creatinine standard (2 mg/dl). Equal volumes of picric acid and base were mixed and allowed to stand for 15 minutes before use. 50 µl of the sample was taken and mixed with 500 µl of the reagent and the absorbance read against blank at 510 nm.

**Calculation**

\[
\text{Creatinine (mg/dl)} = \frac{\Delta A \text{ of sample}}{\Delta A \text{ of standard}} \times \text{Concentration of standard (mg/dL)}
\]

\[\Delta A = \text{Final absorbance} - \text{Initial absorbance}\]

**Total bilirubin**

The method of Walter and Gerarde (1970) with dimethyl sulfoxide (DMSO) as accelerator was followed. Total bilirubin reacts with diazotised sulphanilic acid to form a coloured azobilirubin in strongly alkaline or acidic solution. In this method DMSO is used as accelerator or solvent. Conjugated and solubilised unconjugated bilirubin reacts with diazotised sulphanilic acid to produce an acid azobilirubin. Absorbance of azobilirubin was measured at 546 nm and is directly proportional to bilirubin concentrations.
Reagent A comprised surfactant (2.0 %) and HCL (300 mmol/L).
Reagent B comprised sulphanilic acid (100 mMol/L) and HCL (300 mMol/L).
Reagent C comprised sodium nitrite (72 mMol/L).

50 µl of the sample was added to 500 µl of the reagent and incubated in dark at room temperature for 10 minutes and the absorbance was read against blank at 546 nm.

**Calculations**

\[
\text{T.Bilirubin (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard of standard (mg/dL)}} \times \text{Concentration}
\]

**Aspartate aminotransferase (GOT) [E. C. 2.6.1.1]**

The methodology of International Federation of Clinical Chemistry (IFCC, 1980) was adopted.

L-Aspartate in the presence of aspartate amino transferase combines with 2-oxalo glutarate to form oxaloacetate and L-glutamate. The oxaloacetate then combines with NADH in the presence of malate dehydrogenase to produce malate and NAD. The sample pyruvate then combines with NADH in the presence of lactate dehydrogenase to form L-lactate and NAD.
The reagents used were 2-oxoglutarate (12 mMol/L), L-aspartate (200 mMol/L), MDH (≥ 545 U/L), LDH (≥ 909 U/L), NADH (≥ 0.18 mMol/L), Tris buffer (pH 7.8 ± 0.1 at 25°C) (80 mMol/L) and EDTA (5.0 mMol/L).

50 µl of the sample was taken and mixed with 500 µl of the reagent and the absorbance was read against blank at 340 nm.

Activity of AST (IU/L) = Abs/min x 1768 (factor)

Alanine aminotransferase (GPT) [E. C. 2.6.1.2]

The methodology of International Federation of clinical chemistry (IFCC, 1956) was adopted. L-alanine combines with 2-oxoglutarate in the presence of alanine aminotransferase to produce pyruvate and L-glutamate. Pyruvate with NADH in the presence of lactate dehydrogenase gives rise to L-lactate and NAD.

The reagents used were L-alanine (400 mMol/L), NADH (0.18 mMol/L), LDH (1820 IU/L), 2-oxoglutarate (12 mMol/L) and Tris buffer (pH 7.5 ± 0.1 at 25°C).

50 µl of the sample was taken and mixed with 500 µl of the reagent and the absorbance was read against blank at 340 nm.
Calculation

Activity of ALT (IU/L) = Abs/min x 1768 (factor)

Gamma Glutamyl Transferase (GGT) [E. C. 2.3.2.2]

This method is based on the work of Szasz (1969). The glutamyl moiety is enzymatically transferred from Gamma-glutamyl-p-nitroanilide to glycylglycine releasing p-nitroaniline. Since p-nitroaniline absorbs at 405 nm, the activity of GGT can be monitored by the increase in absorbance.

The reagents used were Gamma-glutamyl-p-nitroanilide (4 mM), glycylglycine (130 mM) and Tris buffer (100 mM).

25 µl of the sample was taken and mixed with 500 µl of the reagent and the absorbance was read against blank at 405 nm.

Calculation

Activity of GGT at 37°C (IU/L) = (Δ A 405/µν) x Factor (2121)

Total protein

The peptide bonds of protein react with copper in alkaline solution to form a blue-violet complex (Biuret reaction). Each copper ion complexes with 5 or 6 peptide bonds (Tietz, 1986). Tartarate was
added as a stabilizer while iodide was used to prevent auto reaction of the alkaline copper complex. 10 µl of the sample was added to 500 µl of reagent, incubated at 37°C for 10 minutes and absorbance was read at 540 nm.

The reagents used were Copper II sulphate (19 mMol/L), Potassium sodium tartarate (43 mMol/L), Potassium iodide (30 mMol/L), Sodium hydroxide (600 mMol/L) and total protein standard (6.0 gm/dl).

Calculation

\[
T. \text{Protein (g/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard of standard (g/dl)}} \times \text{Concentration of standard (g/dl)}
\]

Albumin

The methodology of Doumas et al. (1972) was followed. Albumin binds with Bromocresol Green (BCG) at pH 4.2 causing a shift in absorbance of the yellow BCG dye.

The reagents used were Bromocresol Green (0.08 mMol/L), succinate buffer (pH 4.2 ± 0.1 at 25°C) (50 mMol/L), sodium azide (1.50 mMol/L), surfactant and albumin standard (3.6 g/dl).

5 µl of the sample was added to 500 µl of reagent, and absorbance was read against blank at 630 nm.
Calculation

\[
\text{Albumin (g/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard}
\]

Chloride

Methodology of Zall et al. (1956) was followed. When chloride is mixed with a solution of undissociated mercuric thiocyanate, the chloride preferentially combines with mercury forming mercuric chloride. The thiocyanate released combines with ferric ions present in the solution forming strongly coloured ferric thiocyanate at 480 nm. 5 μl of the sample was mixed with 500 μl of the reagent and incubated at 37°C for 1 minute and absorbance was read at 492 nm. The Reagent I comprises of mercuric thiocyanate (1.96 mMol/L), Ferric Nitrate (45.5 mMol/L), Nitric Acid (53.7 mMol/L), Mercuric Nitrate (105 mMol/L) and Methanol (1822 mMol/L).

Calculation

\[
\text{Chloride (mEq/L)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Conc. of standard (mEq/L)}
\]
Calcium

Methodology is based on the metallochromogen Arsenazo method (Beeler and Catrou, 1983). Arsenazo III combines with calcium ions at pH 6.5 to form a coloured chromophore, the absorbance of which is measured at 650 nm and is proportional to calcium concentration. Arsenazo III has a high affinity ($K^0 = 1 \times 10^{-7}$) for calcium ions and shows no interferance from other cations normally present in serum, plasma or urine.

10 µl of the sample was mixed with 500 µl of reagent and the absorbance was read at 630 nm.

Reagent I: Arsenazo III (0.20 mMol/L) and Imidazole buffer (pH 6.5 ± 0.1)

Calculation

\[ \text{Calcium (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Conc. of standard} \]

Inorganic phosphorus

The modified methodology of Wang et al. (1983) was followed. Inorganic phosphorus combines with ammonium molybdate in the presence of strong acids to form phosphomolybdate. The formation of the unreduced phosphomolybdate was measured at 340 nm.
Reagents used were ammonium molybdate (0.43 mM), sulphuric acid (213 mM), surfactant and inorganic phosphorus standard. 10 μl of the sample was mixed in 500 μl of reagent, incubated at 37°C for 5 minutes and read at 340 nm.

Calculation

\[
\text{Phosphorus (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard}
\]

Cholinesterase

Principle

This method is based on the hydrolysis of acetyl thiocholine or other thiocholine esters by cholinesterase. The hydrolysis yields thiocholine which is allowed to react with the thiol reagent 5,5'-Dithiobis 2 nitro benzoic acid. This gives at alkaline pH a yellow product which can be determined spectrophotometrically.

\[
\begin{align*}
\text{Acetyl thiocholine} & \rightarrow \text{Thiocholine + Acetate} \\
\text{Thiocholine + dithionitro} & \rightarrow \text{yellow colour benzoate (DTNB)}
\end{align*}
\]
Reagents

1. DTNB solution.
   Dissolve 10 mg of 5,5-dithiobis-2-nitro benzoic acid in 50 ml of 0.9% sodium chloride solution and add 50 ml of 1/15M Sorensen’s phosphate buffer pH 8.0.

2. Acetyl thiocholine iodide
   Dissolve 75 mg of acetyl thiocholine iodide in 50 ml of distilled water. This solution must always be freshly prepared and should preferably be kept in an ice bath during work.

3. Eserine salicylate
   Dissolve 50 mg of eserine salicylate in 50 ml of distilled water. This solution must always be freshly prepared.

4. Sorensen’s phosphate buffer pH 8.0 (ratio 2:48)
   Dissolve 0.454 g of potassium dihydrogen phosphate in 50 ml distilled water and 4.732 g of disodium hydrogen orthophosphate in 500 ml distilled water.

Procedure

Whole blood [E.C. 3.1.1.7] and plasma [E.C. 3.1.1.8]

The method described by Voss and Sachsse (1969) was followed. A volume of 30 μl blood was added to 10 ml DTNB buffer solution. From this 4 ml was pipetted out for estimating AchE in whole
blood. To this separate 4 ml aliquot ATI (1 ml) was added and incubated at 30°C for 10 minutes followed by addition of 2 drops of eserine solution in order to stop the reaction. The tubes were centrifuged at 3000 rpm for 10 minutes and the OD of the supernatant was read at 420 nm against blank in Spectronic 20.

For estimating the AchE in plasma, the remaining 6 ml of DTNB buffer with blood was centrifuged for 10 minutes at 3000 rpm and 4 ml of supernatant was taken separately. To this ATI (1 ml) was added and incubated at 30°C for 10 minutes. Eserine solution (2 drops) was added to stop the reaction and the OD was read at 420 nm against blank in Spectronic 20. The blank tubes contained 4 ml DTNB, 1 ml ATI and 2 drops of eserine solution.

**Calculations**

0.1 μ moles of glutathione (one - SH group) present in 5 ml caused an absorbance of = 0.212.

\[
\mu \text{ moles of -SH/thiocholine formed in } 0.4V \text{ of sample/ 10 minutes} = \frac{\text{OD of sample} \times 0.1}{0.212}
\]

\[V = \text{volume of blood}\]

0.4V = the actual volume (μl) of blood present in the 4 ml aliquot.
To express the values as μ moles of - SH/thiocholine produced / ml / minute

\[
\text{OD of sample} \times 0.1 \times 1000 = \frac{0.212 \times 0.4V \times 10}{0.212 \times 0.4V \times 10}
\]

(1000 to convert μl to ml, 10 to convert to minutes)

**Brain cholinesterase [E.C. 3.1.1.7]**

Fresh brain tissue was weighed (50 mg) and transferred to mortar and pestle kept in ice bath. 0.9% Nacl (5 ml) was added and homogenized in ice bath. The homogenate was centrifuged at 3000 rpm for 10 minutes. From the supernatant 0.5 ml was taken and made up to 25 ml using freshly prepared DTNB (10 mg DTNB in 100 ml Sorensen’s phosphate buffer pH 8.0). From this solution 4 ml was pipetted out in to 2 different tubes. To one of the tubes 2 drops of eserine solution was added and incubated for 10 minutes at 30°C. The solution in the tube containing the eserine was used for zeroing the colorimeter and its yellow colour is due to a reduction of DTNB by certain substances in the brain homogenate and to non enzymatic hydrolysis of the substrate. The AchE activity is measured at 420 nm after zeroing.

Reagents and calculations are same as mentioned above.
Testosterone assay

Testosterone concentration in serum was estimated by “solid-phase RIA” procedure using kits based on the techniques of Peterson and Swerdlof (1979) and Odell and Franchimont (1993). In this type of assay the antibody is immobilised to the wall of a polypropylene tube (antibody coated tube). Competition of $^{125}$I-labeled hormone in the sample for binding sites on the antibody is allowed for a fixed time, to reach an equilibrium. At the end of incubation the tubes are decanted to separate the antibody bound and free radio-labeled antigen.

The assay was carried out in duplicates, with total counts, non-specific binding, maximum binding, standards and serum sample tubes.

1) Plain 12 x 75 mm polypropylene tubes, in duplicates, were used for total and non-specific binding.

2) Testosterone antibody coated tubes were used for maximum binding, standards and serum samples.

After mixing the reagents and samples, the tubes were vortexed (except total count tube) and then incubated for 3 hours at 37 °C. At the end of incubation, the tubes were thoroughly decanted, dried and counted in a microprocessor based LKB gamma counter for 1 minute.

Testosterone concentration in the sample was calculated from a logit log representation of the calibration curve and expressed as ng/ml.
Estimation of Protein

The protein content in liver, kidney and testis of control, treated and recovery group of animals were estimated following the method of Lowry et al. (1951).

Principle

The protein containing sample when treated with phenol reagent of Folin-Ciocalteau, a deep blue colour develops. This colour development is due to two reactions occurring simultaneously i.e. the reaction of alkaline copper sulphate solution with peptide bonds and the reduction of phosphomolybdic and phosphotungstic acids by the aromatic amino acids present in the protein. The blue colour developed is quantitatively proportional to the total protein in the medium which was measured colorimetrically.

Reagents

1) Sodium hydroxide, (NaOH), 0.1 N (w/v)
0.4 g of sodium hydroxide was dissolved in 100 ml of distilled water.
2) Sodium carbonate, 2% (w/v) Reagent A
   2 g of sodium carbonate was dissolved in 100 ml of 0.1 N sodium hydroxide.

3) Sodium-potassium tartarate 1.35% (w/v)
   1.35 g of sodium-potassium tartarate was dissolved in 100 ml of distilled water.

4) Copper sulphate, 0.5% (w/v) Reagent B
   5 mg of copper sulphate was dissolved in 1 ml of 1.35% sodium – Potassium tartarate just priority use.

5) Alkaline copper reagent (v/v) Reagent C
   This was prepared by mixing 50 ml of reagent A with 1 ml of reagent B, just before use.

6) Folin-Ciocalteau phenol reagent 1N (v/v)
   Commercially available 2N Folin-Phenol reagent was diluted to 1 N solution with distilled water.

7) Bovine serum albumin standard (w/v)
   12.5 mg of bovine serum albumin (BSA) was dissolved in 50 ml of 0.1 N of sodium hydroxide.

Procedure

To the sample tubes containing 0.2 ml of tissue homogenate, 0.6 ml of distilled water and 4 ml of alkaline copper sulphate solution [a mixture of 100 ml of alkaline Na - K tartarate (0.1 N NaOH, 2% sodium carbonate, 0.01% Na - K tartarate) and 2 ml of 0.5% copper sulphate] were added. In the blank tube, instead of the sample, 0.2 ml of physiological saline was taken. The tubes were kept for
incubation at 37°C for 20 minutes. Then 0.4 ml of Folin Phenol (one part of Folin - Ciocalteu solution (2N) diluted with 2 parts of distilled water) was added to each tube and was mixed thoroughly. Then the tubes were allowed to stand at room temperature for 20 minutes. The optical density was read at 540 nm on a spectrophotometer against the blank. The calculations were done using standardized regression formula:

\[
X = 649.119 \, Y + 3.106
\]

Conc. of protein = \( \frac{X \times \text{Dilution}}{\text{Tissue weight} \times \text{Aliquot volume}} \times 100 \)

Where \( Y \) = O.D. of the sample

\( X \) = Conc. of the sample.

The concentration was expressed as mg protein / 100 mg of tissue weight.

**Estimation of Cholesterol**

The level of cholesterol in testis of control, treated and recovery group animals was assayed by the method of Pearson *et al.* (1953), using Paratoluene sulphonyl acid as the coloring reagent.
**Principle**

Cholesterol reacts at room temperature with acetic anhydride and concentrated sulfuric acid and forms a brownish red compound with paratoluene sulphonic acid.

To the test tube containing 5 ml of colouring reagent mixture (4.0 gms of paratoluene sulphonic acid dissolved in 100 ml of a mixture of acetic anhydride and glacial acetic acid in a ratio of 3 : 2), 0.2 ml of homogenate prepared in glacial acetic acid was added, followed by 1 ml of concentrated sulphuric acid. The standard contained 0.2 ml of standard cholesterol solution (0.1% w/v in acetic acid) instead of the sample, whereas the blank contained 0.2 ml of glacial acetic acid. After colour development, the optical density was used at 620 nm on a Spectrophotometer.

Cholesterol concentration was calculated as follows:

\[
\frac{\text{O.D. of sample} \times \text{Concentration of standard (\mu g)}}{\text{O.D. of standard} \times 1000 \times \text{Aliquot volume}} \times 100 \times 5
\]

where, 5 = dilution factor.

The concentration was expressed as mg cholesterol / 100 mg of tissue weight.
Estimation of alkaline phosphatase (ALP : E.C. 3.1.3.1)

The alkaline phosphatase was estimated by the method mentioned by Sood (1990). The enzyme alkaline phosphatase hydrolyses the substrate p-nitrophenyl phosphate into inorganic phosphate and p-nitrophenol. The quantity of p-nitrophenol released under standardised conditions was measured at 410 nm.

One ml of mixture of equal volume of alkaline buffer and substrate was pipetted in a test tube and incubated at 37°C for 5 minutes and then, 0.2 ml of the homogenous tissue was added in sample tubes and 0.2 ml of distilled water to the blank test tube. After incubation period of 30 minutes, 10 ml of 0.02N NaOH was added in all the test tubes. Absorbance was read at 410 nm. To this, 0.1ml of concentrated HCl was added to all the test tubes. The optical density was read again and the second reading was subtracted from the first one for calculating the enzyme activity. This removed bilirubin contamination and turbidity.

The enzyme activity was expressed as μ moles p.nitrophenol released/30 min./100 mg tissue weight.
Calculations

The enzyme activity was calculated as follows:

\[
\text{ALP activity} = \frac{\text{O.D of the sample} \times \text{dilution} \times \text{Conversion factor} \times 100}{\text{Aliquot Vol} \times \text{Tissue weight}}
\]

Conversion factor = 1.88

Units are expressed as \( \mu \) moles of p.nitrophenol released/30 min./100 mg tissue weight.

Estimation of acid phosphatase (ACP: E.C. 3.1.3.2)

Acid phosphatase activity was estimated by the method of Bessey et al. (1946). Acid phosphatase, orthophosphoric monoesterase phosphohydrolase catalyses the hydrolysis of p.nitrophenyl phosphate at pH 4.8, liberating p.nitrophenol and inorganic phosphate. The liberated p.nitrophenol combines with NaOH to form a yellow coloured complex, which is measured and directly proportional to the acid phosphatase activity.

A known amount of tissue was homogenised in desired amount of distilled water. The assay system consisted of 0.2 ml of homogenate, 0.6 ml of substrate solution (16.5 mg of p-nitrophenyl phosphate dissolved in 10 ml of citrate buffer). The blank tubes were run with 0.2 ml of distilled water. All the tubes were kept for 30 minutes at 37°C. Then 4ml of NaOH was added to all the tubes to
terminate the reaction. The activity of ACPase was measured at 420 nm wavelength, on a Spectronic 20 Colorimeter and calculated according to the following formula:

\[
\frac{\text{O.D of sample} \times \text{Conversion factor} \times \text{Dilution} \times 100}{\text{Tissue weight} \times \text{Aliquot volume}}
\]

Conversion factor = 0.741

The ACPase enzyme activity was expressed as \( \mu \) moles of \( p \)-nitrophenol released /100 mg tissue weight / 30 minutes.

REPRODUCTIVE TOXICITY STUDIES

Fertility test

The fertility rate of control, treated and recovery groups of animals were assessed according to the methodology of WHO protocol MB – 50 (1983) and Imahie et al. (1995). Male rats were paired for mating with pre-estrous or estrous female rats at the ratio of 1 : 2 after 60 days treatment with carbaryl + lindane (4 + 4% G). The cyclacity of the female rats was determined by vaginal smear. The vagina was aspirated with normal saline. The aspirated fluid was smeared onto a clean slide and observed under the microscope. Intromission was observed by a sperm positive smear and the day was designated as the day one of the onset of pregnancy. All the gestating females were allowed to carry their pups to term. Fertile
males were those which caused pregnancy and in whose case a healthy litter was born. Failure to give birth to a healthy litter was considered as a parameter to indicate fertility arrest. The copulatory index (Cl) and the fertility index (FI) were calculated as follows:

\[
Cl = \frac{\text{No. of animals with successful copulation}}{\text{No. of paired animals}} \times 100
\]

\[
FI = \frac{\text{No. of pregnant animals}}{\text{No. of animals with successful copulation}} \times 100
\]

**Preparation of the sperm suspension**

To obtain sperm suspension for the experimental analysis, the cauda epididymides were teased and flushed gently into normal saline (0.87%) immediately after the autopsy to release sperms.

**Sperm motility**

The cauda epididymal sperm motility of the experimental animals was assessed according to the method of Prasad et al. (1972). Freshly prepared sperm suspension was diluted suitably with physiological saline as mentioned above and observed after placing a drop on a Neubauer chamber under low magnification (10X). The quantitative motility was determined by counting motile and total number of spermatozoa in 20 separate random fields. The
percentage of motile spermatozoa was calculated from the mean of per cent motility of all the fields counted.

**Sperm count**

The cauda epididymal sperm count was carried out according to the method of Prasad *et al.* (1972) using the Neubauer Chamber of a Haemocytometer. Freshly prepared sperm suspension in normal saline (100 mg/2 ml) was well mixed and diluted (1 : 20) with a spermicide (5% NaHCO₃) in a WBC micropipette. The diluted sample was mixed thoroughly and a drop was placed onto the haemocytometer and covered gently with a cover slip. Spermatozoa were then counted in 64 sub-squares of the white blood cell counting regions.

Sperm concentration was calculated as

\[
X = \frac{N \times \text{Dilution} \times 1000}{\text{Volume of 64 sub-squares}}
\]

Where, \(N\) = total number of sperms counted in 64 sub-squares.

Dilution = 20 times.

Volume of 64 sub-squares = \(\frac{1}{4} \times \frac{1}{4} \times \frac{1}{10} \times 64 = 0.4 \text{ cu. mm.}\)

\[
X = \frac{N \times 20 \times 1000}{0.4}
\]

The sperm count was expressed as million spermatozoa per ml.
Sperm Viability (Membrane Integrity)

The percentage of the live spermatozoa was determined using 1% trypan blue (supravital stain) as described by the method of Talbot and Chacon (1981).

An aliquot of 0.2 ml of sperm suspension was incubated with 0.2 ml of 0.1% trypan blue stain prepared in (0.87%) physiological saline. A drop of the suspension was placed on the Neubauer Chamber under a cover slip and allowed to settle for one minute. Observation were carried out under 40X magnification of a Nikon binocular microscope. The number of stained / total number of spermatozoa were scored in 10-20 separate visual fields. The live viable spermatozoa remained unstained while dead sperm took up the supravital stain. This technique makes it possible to differentiate motile but live spermatozoa from motile non-viable ones. In each sample, the percentage of live/dead (non-viable) spermatozoa was calculated as follows:

\[
\% \text{ viable} = \frac{\text{Total number of viable sperm observed}}{\text{Total number of sperm observed}} \times 100
\]

HISTOLOGY

Tissues for histological examination require appropriate processing, so that they are imparted optimum hardness for sectioning with a microtome. The method described by Godkar (1994), which is a
standard technique of haematoxyline and eosin staining was followed for histology. Brain, liver, kidney, and testis were dissected out, blotted free of blood and fixed in alcoholic Bouin’s fixative immediately after the autopsy. Fixation was carried out at room temperature for 18 hours, after which the tissues were transferred to 70% alcohol. A pinch of lithium carbonate was added to remove excess of picric acid. Several changes of 70% alcohol were given until the yellow colour disappeared from the tissue. The tissues were then dehydrated by passing through descending grades of alcohol, cleared in xylene, embedded in paraffin wax (58 - 60°C M.P) and transverse sections were cut at 5µm on a rotary microtome (Lieca). These sections were stained in Ehrlich’s haematoxyline - eosin (sprit soluble), dehydrated, cleared in xylene and mounted in DPX for photography.

GENOTOXIC STUDIES

For the assessment of genotoxic potential of carbaryl + lindane (4 + 4% G), the end points selected were i) Chromosomal aberrations ii) Micronucleus test and iii) Sister chromatid exchanges in bone marrow cells of rats treated for 30, 60 and 90 days. The results were compared with concurrent negative (vehicle) and positive (substance known to induce genotoxicity) controls.
Experimental design

Animals

Young healthy animals were selected with a body weight of around 50g and reached about 150g by the end of 90 days treatment. Young animals were selected for the genotoxicity studies since the bone marrow would not contain high lipid content which would affect the quality of slides. The experimental groups were shown in Table II.

<table>
<thead>
<tr>
<th>Group and Treatment</th>
<th>No. of Animals</th>
<th>No. of Days treated</th>
<th>Sacrificed on day</th>
<th>Experimental end point @</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Control)</td>
<td>30</td>
<td>90</td>
<td>91</td>
<td>CA/MN/SCE</td>
</tr>
<tr>
<td>II (440 mg/kg b. wt.)</td>
<td>30</td>
<td>30</td>
<td>31</td>
<td>CA/MN/SCE</td>
</tr>
<tr>
<td>III (440 mg/kg b. wt.)</td>
<td>30</td>
<td>60</td>
<td>61</td>
<td>CA/MN/SCE</td>
</tr>
<tr>
<td>IV (440 mg/kg b. wt.)</td>
<td>30</td>
<td>90</td>
<td>91</td>
<td>CA/MN/SCE</td>
</tr>
<tr>
<td>V (PC. MMC 4 mg/kg b. wt. IP)</td>
<td>30</td>
<td>01</td>
<td>02</td>
<td>CA/MN/SCE</td>
</tr>
</tbody>
</table>

@ =10 animals each used for CA, MN & SCE., CA = chromosomal aberration., MN = micronucleus assay., SCE = Sister Chromatid Exchange., PC = Positive control, MMC = Mitomycin C., IP = Intraperitonial.

Chromosomal aberrations

In the evaluation of the toxic character of a substance, determination of chromosomal aberration induction potentiality is usually a vital step. The experiment is mainly based on the determination of percent aberrant bone marrow cells.
Principle

Bone marrow contains large numbers of dividing cells which are suitable for chromosome analysis. Cell division is arrested \textit{in vivo} by colchicine at the metaphase. The cells were then collected and allowed to swell by hypotonic treatment with potassium chloride. The cells in metaphase are fixed on slides following carnoy’s fixative treatment. Slides were stained with Giemsa in phosphate buffer (pH 7.2). A minimum of 100 metaphase spreads per animal were analyzed for various kinds of damages.

Preparation of reagents

i) Phosphate (Sorensen) buffer pH 6.8
Solution A: 6.8 g of potassium dihydrogen orthophosphate was dissolved in 500 ml of distilled water.
Solution B: 1g sodium hydroxide was dissolved in 250 ml of distilled water.
For preparing 100 ml of buffer, 50 ml solution A, 22.4 ml Solution B and 27.6 ml of distilled water were mixed thoroughly.

ii) Phosphate buffered saline (pH 7.4)
8.0 g of sodium chloride, 2.8 g of di sodium orthophosphate and 0.19 g of potassium hydrogen orthophosphate were dissolved in triple distilled water and made upto 1 liter. The
pH of prepared solution was adjusted to pH 7.4 and is stored at 4°C.

iii) 0.075 M potassium chloride
5.5912 g of potassium chloride was weighed and dissolved in 1000 ml of distilled water. This was freshly prepared every time before use.

iv) Colchicine
40 mg of colchicine was dissolved in 100 ml of sterile, triple distilled water, and administered at a dose level of 4.0 mg/kg b. wt.

v) Carnoy's Fixative
Freshly prepared before use. Methanol and Glacial acetic acid were mixed in the ratio of 3:1 and stored at 4°C.

vi) Giemsa solution (Stock)
Giemsa powder (3.80 g), 250 ml methanol and 250 ml glycerin was titurated with pestle and mortar, adding alcohol and glycerin in small quantities. This was transferred to a flask and was allowed to stand for 24h with occasional shaking. It was filtered into a bottle, labelled and stored.

vii) Preparation of Giemsa stain (Working solution)
To 5ml of Giemsa stock 95 ml of phosphate buffer was added and mixed well to get 5% Giemsa solution.
Procedure

The method described by Evans (1984) was followed with slight modifications. After 24h at the end of the treatment period, rats of both control and treated groups were injected with colchicine (4 mg/kg b. wt) intraperitoneally and sacrificed 2h later by cervical dislocation. Both femurs were quickly excised, muscle was cleaned away from the bone and the epicondyle tips were cut. The bone marrow was expelled by aspiration with 5 ml of phosphate buffer saline (PBS) through 22 gauge needle. The cell suspension was mixed vigorously to assure dissociation of the cells and avoid clumping followed by centrifugation at 2000 rpm for 10 minutes. The supernatant was removed by using a pasteur pipette leaving about 0.5 ml of PBS above the cell pellet. The pellet was resuspended in the remaining volume and 8 ml hypotonic solution of (0.075 M) potassium chloride was added to the residual cell pellet and mixed thoroughly. The cells were incubated in water bath maintained at 37°C for 20 min. Freshly prepared freezer chilled Carnoy’s fixative was added and the resuspended cells were refrigerated at 4°C for 12h. Prior to refrigeration 2 washes of fixative were given in order to clear the debris. The cells were centrifuged on the following day for 10 minutes at 2000 rpm, and the supernatant was discarded leaving about 0.5 ml of fixative along with the cell pellet.
Slide Preparation

The slides were stored overnight in refrigerator in a beaker containing Methanol and Acetic acid in the ratio 1:1. A few (2-3) drops of the cell suspension was dropped on the slide from a distance and placed on hot plate. The slides were observed under a low power phase contrast objective for proper density and cell spreading. Two slides per animal was prepared. Air dried slides were stained with Giemsa (5%) for 3 minutes and again dried. The slides were made permanent by mounting a cover slip with DPX.

Scoring Chromosomal Aberrations

A minimum number of 500 cells were counted in different fields of slide per animal to determine the mitotic index by using the following formula

\[
\text{Mitotic index} = \frac{\text{No. of metaphases}}{\text{No. of cells counted}} \times 100
\]

A minimum of 100 consecutive metaphase were scored under 100X oil immersion objective. Aberrations are divided into chromatid type and chromosome type. The former involving only one chromatid, the latter involving both chromatids at identical sites. Chromatid type aberrations like gaps, breaks, chromatid exchanges and chromosome type aberrations like chromosome gaps and breaks, ring, dicentric etc. were scored.
Micronucleus test

Micronucleus assay is a technique for demonstrating genotoxicity of chemicals in vivo in bone marrow cells. The method is a simple and rapid measure of chromosomal damage in interphase stage of cell division.

Principle

The chromatin fragments which may be produced by the clastogenic agents or spindle poisons lag behind during anaphase due to chromosomal breakage and spindle malformation and are not included into the nucleus of the daughter cells. These small fragments of chromatin give rise to micronuclei which are present in the cytoplasm of the daughter cells.

Reagents

i) Fetal Calf Serum
ii) Giemsa stain (stock and working solution)
Preparations are same as mentioned above

Procedure

The test method as described by Schmid (1976) and modified by Salamone and Heddle (1983) was followed. Control and treated animals were sacrificed by cervical dislocation at different time
points after treatment with carbaryl + lindane (4 + 4 % G). Both femoral bones were dissected out and cleared from adherent tissue. The epicondyle tips were cut and the marrow content was flushed into a centrifuge tube, along with 3 ml of fetal calf serum, using a 1 ml syringe and 22 gauge needle. The contents of the tubes were mixed thoroughly to dissociate cell clumps, centrifuged for 10 minutes at 2000 rpm and the supernatant discarded and cell pellet with 0.5 ml fetal calf serum was retained. Cells were then resuspended and a drop was placed on a clean slide using a pasteur pipette and a smear was prepared. Two slides were prepared from each animal. These were allowed to air dry, fixed in methanol and stained the following day with 5 % Giemsa stain for 15 minutes. Excess stain was removed from the slides by successive rinsing in distilled water for about 1 minute. Glass cover slips were attached to the dried slide preparations by mounting in a mixture of DPX and xylene.

**Analysis of slides**

The slides were coded prior to scoring, to avoid bias. Initial screening was done under low power objective inorder to select area with good staining and proper morphology of erythrocytes which are generally present towards the end of the smear. A total of 1000 polychromatric erythrocytes (PCE) from each prepared slides were scored to calculate frequency of micronucleus. The corresponding number of normochromatric erythrocytes (NCE), with and without micronuclei were also recorded under 100X oil immersion.
Sister Chromatid Exchanges (SCEs)

Principle

The basic technique involves labeling the cells for two replicate cycles with Brdu so that at metaphase, the two chromatids of a chromosome are uni- and bi-filarly substituted with Brdu. When such chromosomes are examined for their fluorescence patterns (with Hoechst 33258), the bifilarly substituted chromatid fluoresces dully while the other one is brightly fluorescing.

Reagents

1) Brdu pellet
   Mix 50 mg gum acatia powder, 50 mg activated charcoal powder and 50 mg Budr using carboxy methyl cellulose solution and make it into a pellet. Air dry in a dark place and store it in dark.

2) Hoechst stock
   1 mg vial was dissolved in 1 ml distilled water

3) Hoechst working solution
   0.1 ml of stock was added to 9.9 ml Sorensen’s buffer.
4) Sorensen’s buffer
2.55 gm disodium hydrogen orthophosphate and 1.75 gm potassium dihydrogen orthophosphate were dissolved in 500 ml distilled water.

5) 2X SSC
0.88 gm sodium citrate and 1.75 gm sodium chloride were dissolved in 100 ml distilled water.

Procedure

Twenty hours from the last treatment the animals were implanted subcutaneously with Brdu pellet (Allen et al., 1978) after anaesthetising. A slow but constant release of Brdu from this tablet occurs to the blood stream of animals and results in a satisfactory Brdu substitution for 2 cycles in dividing cells. The animals were sacrificed at the end of 18h period and bone marrow cells aspirated and processed for metaphase preparation as described under chromosomal aberrations.

Staining procedure

A modified method of Goto et al. (1975) was followed. Few drops of Hoechst working solution was added on the slide and kept in darkness for 15 minutes. This was followed by exposure to UV light for 1 hour and treated with 2X SSC for 20 minutes at 60°C in a
water bath. Finally the slides were stained with 2% Giemsa in Sorenson’s buffer for 5 minutes and rinsed in distilled water.

**Analysis of slides**

The number of cells in M1, M2 and M3 stage of cell cycle was recorded. From these data the cell cycle proliferative index (CCPI) or replicative index was calculated (Lamberti *et al.*, 1983) by using the following formula:

\[
CCPI = \frac{1M_1 + 2M_2 + 3M_3}{100}
\]

This was followed by scoring SCE’s per metaphase under 100X oil immersion. The SCE per chromosome was also computed.

**Analysis of data**

The mitotic index and percent aberrations in the case of chromosomal aberration study; P/N ratio and percent micronucleated erythrocytes in the case of micronucleus assay; CCPI, SCE/metaphase as well as SCE/chromosome in the case of Sister chromatid exchange were subjected to students t-test.

For all the above parameters 8-10 replicates were done and analysed using modified Students ‘t’ test (Gad and Weil, 1994). A value of P < 0.05 was considered to be significant.