Materials and Methods
MATERIALS AND METHODS

The following is a brief description of the history, mode of administration, therapeutic doses and side effects / toxicity, of the anti-cancer drugs used in the present study:

CISPLATIN

It is one of the platinum coordination complexes were first identified by Rosenberg and coworkers as cytotoxic agents in 1965. They observed that a current delivered between platinum electrodes produced inhibition of \(E. coli\) proliferation. The inhibitory effects on bacterial replication were later ascribed to the formation of inorganic platinum-containing compounds in the presence of ammonium and chloride ions (Rosenberg et al., 1965, 1967). \textit{cis}-Diamminedichloroplutonium (II) (Cisplatin) was the most active of these substances in experimental tumor systems and has proven to be of great clinical value (Rosenberg, 1973).

![Fig. 3: CISPLATIN (Structural Formula)]

Cisplatin has broad activity as an antineoplastic agent and the drug is especially useful in the treatment of epithelial malignancies. It has become
the foundation for curative regimens for advanced testicular cancer and has notable activity against ovarian cancer and cancers of the head and neck, bladder, esophagus and lung.

Cisplatin appears to enter cells by diffusion. The chloride atoms may be displaced directly by reaction with nucleophiles such as thiols: replacement of chloride by water yields a positively charged molecule and is probably responsible for formation of the activated species of the drug, which then reacts with nucleic acids and proteins.

Although it causes immunosuppression, stimulation of the host immune response against the tumor has been suggested as contributing to its antineoplastic action. Cisplatin is of value in the treatment of metastatic tumors of the testis, usually as a major component of combination chemotherapy regimens. It is also used in metastatic ovarian tumors and advanced bladder cancer and has been reported to be active against a wide range of other solid tumors.

Cisplatin is administered intravenously, not more frequently than every 3 to 4 weeks. It is usually given as a single dose of 50 to 120 mg/m² body-surface or in a dose of 15 to 20 mg/m² daily for 5 days. Cisplatin is well absorbed following intraperitoneal administration.
Prior to its use in humans, Cisplatin was tested in a variety of animal species. In dogs and monkeys, Cisplatin causes prompt and severe emesis, anorexia, abdominal tenderness and diarrhoea. The principal target organ for Cisplatin toxicity in animals is the kidney. This toxicity is manifested by reduced renal function, deranged serum electrolytes and pathological changes in the urine analysis. Dogs and monkeys show marrow hypoplasia with minimal changes in the peripheral blood following treatment with Cisplatin. Cisplatin is ototoxic in monkeys, rats and guinea pigs. Cisplatin is mutagenic in the microbial mutagenicity (Ames) assay and causes chromosomal aberrations in Chinese hamster bone marrow cells. The carcinogenic potential of Cisplatin has never been tested rigorously but it must be assumed that the drug can cause cancer. Cytotoxic agents such as Cisplatin, affect a wide variety of cells. The cytotoxic effects on non-cancer cells account for some of the drugs' toxicity cited above (Dollery, 1999)

Cisplatin is commonly administered in doses of 75-100 mg/sq.mt. The clinical toxicities include renal failure, severe bone marrow suppression and peripheral neuropathy. The ototoxicity caused by Cisplatin tends to be more frequent and severe with repeated doses. Marked nausea and vomiting occur in almost all patients. Electrolyte disturbances, including hypomagnesemia, hypocalcemia, hypokalemia and hypophosphatemia are common. Hyperuricemia, seizures, hemolytic anemia and cardiac
abnormalities have been reported.

ETOPOSIDE

It is derived from a natural product, Podophyllotoxin, extracted from the mandrake plant (mayapple: *Podophyllum peltatum*) was used as a folk remedy by the American Indians and early colonists for its emetic, cathartic and anthelmintic effects. Etoposide is a semisynthetic glycoside of the active principle, podophyllotoxin, showing significant therapeutic activity in several human neoplasms including pediatric leukemia, small cell carcinomas of the lung, testicular tumors, Hodgkin's disease and large cell lymphomas.

Fig. 4: ETOPOSIDE (Structural Formula)

It is given by slow intravenous infusion, as a solution in sodium chloride
(0.9%) or glucose (5%) injection in usual doses of 50 to 120 mg/sq.mt body surface daily for 5 days. The intravenous dose of Etoposide for testicular cancer in combination therapy is 50 – 100 mg/sq.mt, on alternate days for 3 doses.

Etoposide exposure results in cell cycle arrest during the G2 phase of the cell cycle, preventing cells from entering mitosis. Etoposide exerts its cytotoxic effect by inhibiting topoisomerase II, a nuclear enzyme that regulates the topology of DNA. Importantly, Etoposide does not inhibit the action of this enzyme per se, but stabilizes the enzyme-DNA complex after the DNA strand has been broken, halfway through the enzyme's catalytic cycle. Religation of the DNA strand is thereby prevented, resulting in double-strand DNA breaks. Subsequent cell death is typically by apoptosis.

The side effects like anemia, leukopenia and thrombocytopenia, secondary to bone marrow damage and degeneration of hepatocytes have been demonstrated in rats and monkeys. In dogs, mild but reversible impairment of renal and hepatic function has been demonstrated after chronic administration. Etoposide is mutagenic in cell culture and in mice. It crosses the placenta and has been shown to affect fetal development in rats at dose levels used clinically (Takahashi et al., 1996).
FLUTAMIDE

Flutamide is a non-steroidal compound reported to have anti-androgenic properties which appears to act by inhibiting the uptake and/or binding of androgens in target tissues. The usual dose by mouth is 250 mg three times daily.

The effects of single doses of Flutamide were observed in mice, rats, cats and guinea pigs after oral or intraperitoneal administration and in dogs after oral dosing. Daily doses were administered orally and ranged up to 180 mg/kg in rats, 100 mg/kg in dogs and 90 mg/kg in monkeys. In general, there was weight loss with anorexia in all species and vomiting in dogs and monkeys. Results of clinical laboratory determinations (hematology, blood chemistry, urinalysis) were generally insignificant and no consistent pattern of change was evident.

At necropsy, Flutamide-related changes in all species reflected the pharmacological action of the drug. Reductions in the size of prostate glands and seminal vesicles were observed and there was also evidence of reduction in testes size (rats and monkeys). Histological changes
Noble and coworkers (1958) observed granulocytopenia and bone marrow suppression in rats, effects that led to purification of an active alkaloid. Other investigations by Johnson and associates (cf:Hardman and Limbird, 1996), demonstrated neoplasm in mice. Fractionation of these extracts yielded four active dimeric alkaloids: Vinblastine, Vincristine, Vinleurosine and Vinrosidine. Two of these, Vinblastine and Vincristine, are important clinical agents for treatment of leukemias and testicular cancer.

Vincristine, like the other Vinca alkaloids, exerts its biological effects by binding specifically to the protein tubulin (Creasy, 1975). Tubulin normally polymerizes to form microtubules and in the presence of Vincristine, there is inhibition of the assembly of tubulin into microtubules with resultant dissolution of the mitotic spindle. Cell division is thus arrested in metaphase and in the absence of an intact mitotic spindle the chromosomes disperse throughout the cytoplasm or are aggregated together in unusual formations such as stars. The inability of chromosomes to segregate correctly leads to cell death. Both, normal and malignant cells exposed to Vinca alkaloids, undergo changes characteristic of apoptosis (Smets, 1994).

**Toxicology:** Standard toxicology studies are not normally carried out for cytotoxic drugs because of the toxicity inherent in their mechanism of
action in particular, to the hemopoietic stem cell system and gastrointestinal tract. Predicted toxic effects, therefore are bone marrow depression, dystrophic gastrointestinal mucosal changes, neurotoxicity and vomiting. In sensitive animal species, neurotoxicity is manifested by damage to myelin and axon degeneration (Bradley et al., 1970). Treatment over a period of seven months has been shown to decrease the number of neuronal microtubules in the peripheral nerves of cats (Todd et al., 1979).

Vincristine is teratogenic in the mouse, rat and monkey. The mutagenic tendency for Vincristine is unknown, although high concentrations will produce structural chromosomal aberrations and chromosomal breaks at doses of 2 mg/kg (Gebhart et al., 1969).

Vincristine sulphate is administered by intravenous injection and solutions containing 0.01 to 1 mg/ml in sodium chloride injection (0.9%) have been used. The adverse effects include neurotoxicity including constipation and alopecia. Intrathecal administration has proved fatal.

Experimental Protocol

Swiss albino mice (*Mus musculus* L.), 6-8 weeks old and weighing 23-27 gms were used for the study. Throughout the experiment, they were housed in individual cages, with food and water *ad libitum* and a 12hr
light/12hr dark schedule. The food provided was a commercial pelleted diet.

The experiments were done on 2 groups: control and experimental, including 6 mice each. The control group was given physiological saline. The mode of administration of saline was the same as that for the experimental group; oral for mice treated with Flutamide and intraperitoneal for mice treated with Cisplatin, Etoposide and Vincristine sulphate. The experimental mice were treated with the anti-cancer drugs at respective therapeutic doses and at 4 dose-levels as follows:

Table 2: PLAN OF EXPERIMENTS

<table>
<thead>
<tr>
<th>Name of the drug</th>
<th>Mode of Administration</th>
<th>Dosage</th>
<th>Duration of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>Intraperitoneal, mixed with 0.5ml saline and given as slow infusion for 30 mins.</td>
<td>4 dose-levels as mentioned in the tables, in the Results section.</td>
<td>Once a month</td>
</tr>
<tr>
<td>Flutamide</td>
<td>Oral, mixed with 0.5ml saline</td>
<td>- do -</td>
<td>Daily for 5 days for 1 week only</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Intraperitoneal, mixed with 0.5ml saline and given as slow infusion for 30 mins.</td>
<td>- do -</td>
<td>On alternate days for 1 week only</td>
</tr>
<tr>
<td>Vincristine sulphate</td>
<td>Intraperitoneal, mixed with 0.5ml saline and given as slow infusion for 30 mins.</td>
<td>- do -</td>
<td>Once a week for 4 weeks</td>
</tr>
</tbody>
</table>

After a recovery period of four weeks, the mice were sacrificed by decapitation and the following in vivo genotoxic and biochemical studies performed:
GENOTOXICITY STUDIES

A) Sperm-head morphology assay:

30 days after the treatment, control and treated animals were sacrificed by cervical dislocation. Cauda epididymis was dissected out and transferred to a petridish containing 0.5 ml of saline (0.9% NaCl). The epididymis was then cut into small pieces. The material was teased as much as possible and the cell suspension was transferred to a watch-glass. 2-3 drops of 1% aqueous eosin was added and kept for 30mins. A drop of suspension was taken on a clean slide and smeared. The slides were air-dried and mounted with DPX (Wyrobek and Bruce, 1975). 2000 sperms per animal were scored for the incidence of sperm-head abnormalities. The data was analyzed using ‘t’ test.

B) Micronucleus assay in peripheral blood erythrocytes:

Peripheral blood was collected from the tail tip of both control and treated animals and diluted with few drops of saturated trisodium citrate solution. The mixture was smeared on to clean dry slides and was fixed in methanol for five minutes. The slides were air-dried and stained with aqueous Giemsa for ten minutes and rinsed with distilled water and air-dried (MacGregor et al., 1980). 2500 peripheral erythrocytes per animal were screened for the presence of micronuclei. The data was analyzed statistically using ‘t’ test.
C) Chromosomal aberrations in bone marrow cells

The control and the experimental mice were injected intraperitoneally with Colchicine (0.025%). After two hours, the animals were sacrificed by cervical dislocation. The femur bones were dissected out and cleaned off from the tissues. One end of the bone was cut and the contents of the bone flushed with 2 ml of prewarmed hypotonic potassium chloride solution (0.75%). The cell suspension was incubated at 37 °C for 30mins. It was then centrifuged at 1000 r.p.m., for 8 mins. The supernatant was removed and the cell button was fixed in prechilled Carnoy’s fixative (methanol : acetic acid — 3 : 1 ). Three repeated washings were made and after the third washing, the cells were suspended in few drops of fixative and dropped on a clean, grease-free, prechilled slides. Air was blown over the slides and were warmed for few seconds. Later, the slides were stained with aqueous Giemsa for ten minutes and rinsed with doubled distilled water. The method followed was that of Preston et al., (1987).

The slides were screened for 100 well spread metaphase plates per animal. The difference in the frequencies of chromosomal aberrations between control and treated groups were tested for statistical significance by Chi-square ($X^2$) test.

BIOCHEMICAL STUDIES

A sample was homogenized in cold potassium phosphate buffer (pH
and was used for assay of enzyme activities and Thiobarbituric Acid Reactive Substances (TBARS). The homogenates were sonicated for 30s and centrifuged at 2°C and 3200g for 30 mins.

(i) **Catalase** (EC 1.11.1.6) activity was measured as described (Worthington, 1993). The final reaction mixture contained 50mM phosphate buffer (pH 7.0), 30mM of H₂O₂ and 2ml supernatant. Decrease in absorbance was measured spectrophotometrically, at 240nm after the addition of H₂O₂, for 3 mins.

(ii) **Glutathione reductase** (EC 1.6.4.1) assay was carried out by the method reported earlier (Goldberg and Spooner, 1983). The reaction mixture contained phosphate buffer (pH 7.2), NADPH and GSSG. The reaction was started by adding enzyme source and the decrease in absorbance due to the oxidation of NADPH was measured at 339nm.

(iii) **Thiobarbituric Acid Reactive Substances (TBARS):** Lipid peroxidation was determined by measuring the levels of malondialdehyde (MDA) using colorimetric reaction with thiobarbituric acid (TBA) by the method of Ohokawa et al., (1979). A total of 100µ l of the sample was mixed with 1% trichloroacetic acid (TCA) and 1% thiobarbituric acid. This mixture was heated for 45 mins at 95 °C. After cooling, the precipitate was removed by centrifugation at 3000 r.p.m for 20 mins. The absorbance
of the clear supernatant was determined at 532 nm. TBARS were quantified using the extinction coefficient $1.53 \times 10^5 \, \text{M}^{-1} \, \text{cm}^{-1}$.

The protein content was determined by Lowry's method (1951). Bovine serum albumin was used as the protein standard.